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THE ROLE OF EXBD IN TONB-DEPENDENT ENERGY TRANSDUCTION IN *ESCHERICHIA COLI*

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ABSTRACT

In *Escherichia coli* K12, the TonB system serves to couple the electrochemical gradient of the cytoplasmic membrane, protonmotive force (pmf), to high affinity TonB-gated outer membrane transporters, promoting active transport of iron-siderophore complexes and vitamin B_{12} across the outer membrane. Iron is essential for *E. coli* but bioavailable iron is scarce in the environment, making this a critical process in the competition for survival of these cells. In other Gram negative bacterial species, TonB energizes transport of heme, maltodextrin, sucrose, and nickel. The precise mechanism of TonB-dependent energy coupling is unknown and studies aim to elucidate the details involved in this process. In the cytoplasmic membrane, TonB is thought to form a hetero-oligomeric complex with two other integral membrane proteins, ExbB and ExbD. ExbB and ExbD appear to harness the pmf to energize TonB. TonB spans the periplasm, retaining cytoplasmic membrane association, directly contacts OM transporters, and transmits energy for release of ligands into the periplasm. ExbD and TonB each have a single transmembrane domain (TMD), with the majority of each protein occupying the periplasm, while ExbB has three transmembrane domains with the majority of soluble domains in the cytoplasm. TonB is a conformationally dynamic protein, and ExbD is proposed to direct the conformational changes of TonB.

This study addressed the role of ExbD in TonB-dependent energy transduction and provides evidence to support the proposed function of ExbD in modulating TonB conformation. Formaldehyde crosslinking was used to identify ExbD-specific interactions *in vivo*. ExbD crosslinked into a homodimer and ExbB-ExbD or TonB-ExbD heterodimers. The TonB-ExbD heterodimer was dependent on the presence of ExbB, supporting the predicted formation of a multimeric energy transduction complex. The first mechanistic role for pmf was identified, where pmf is required for specific periplasmic domain interaction that allows crosslinking of

ExbD to TonB. The importance of wild-type TMDs of ExbD and TonB for this interaction suggests a role for signaling from the TMD to the periplasmic domain. Pmf-dependent conformational changes in the TonB system were further examined with limited proteolysis studies. ExbD conformation was demonstrated to be responsive to changes in pmf. Pmf appears to be a toggle switch for TonB and ExbD conformational changes. ExbD was also shown to determine the ability of TonB to change conformation in response to pmf, with distinct roles of the ExbD TMD and periplasmic domain. A model is proposed for three stages leading to energization of TonB, emphasizing the importance of the ExbD periplasmic domain for TonB to progress to subsequent stages. The importance of the ExbD periplasmic domain was also apparent in further studies addressing specific regions of ExbD important in its function, using a 10-residue deletion scanning analysis. Functional division of the ExbD periplasmic domain is proposed, with residues 42-61 important in response to pmf and residues 62-141 important for interaction with TonB. The conformation of the region between residues 92-121 was especially important in supporting multiple ExbD protein-protein interactions. Subsequent studies focused on this region with cysteine scanning and disulfide crosslinking. Multiple sites of homodimeric interaction *in vivo* were identified. Specific interaction with TonB was observed through many of the same sites. Studies of TonB-ExbD interaction were extended to 45 individual cysteine substitutions in the TonB carboxy terminus. Three of six ExbD cys substitutions examined in combination with these TonB substitutions formed multiple disulfide-linked heterodimeric interactions. A wild-type ExbD TMD and ExbB were important for disulfide-linked heterodimer but not homodimer formation. A model is presented where the default configuration of the ExbD periplasmic domain is dimeric with transition from localized homodimeric interactions to heterodimeric interactions with TonB, mediated by the ExbD TMD. Specific interaction sites on TonB further suggested interaction with ExbD may resolve TonB homodimeric interfaces and configure the TonB periplasmic domain for productive interaction with OM transporters. This

work has provided significant insight into the functional role of ExbD in TonB energization and expanded knowledge of both the mechanisms and sequence of events involved in the cycle of TonB-dependent energy transduction.

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CHAPTER 1

INTRODUCTION TO TONB-DEPENDENT TRANSPORT

Chapter 1

Introduction

Transport and the Gram negative bacterial cell envelope

Nutrient acquisition by Gram negative bacteria is influenced by factors such as bioavailability, the nature of nutrients such as size and charge, and the importance of the nutrient to survival of the organism. Characteristics of the Gram negative bacterial cell envelope determine the uptake of nutrients and compounds from the environment into the cell. The cell envelope consists of two concentric membranes, an asymmetric lipid bilayer outer membrane (OM) and phospholipid bilayer cytoplasmic membrane (CM). The OM and CM are separated by an aqueous periplasmic space, which contains a thin layer of crosslinked glycan strands forming the structurally important peptidoglycan layer (Fig. 1-1). Both the physical properties of and proteins embedded in the CM and OM define their functional roles. The majority of integral membrane proteins are located in the CM. CM proteins include those involved in transport, secretion, and chemotactic response. The CM is uniquely "energized," serving as the location for energy generation by the creation and maintenance of an electrochemical potential, the protonmotive force (pmf) and use of this potential by CM proteins to generate ATP. The OM contains limited types of proteins. Lipoproteins, mostly with functions unknown, are embedded in the phospholipid inner leaflet of the OM, and β-barrel proteins span the OM as determinants of permeability. The OM, as the barrier between the cell and its environment, regulates passage of environmental compounds into the periplasm. The external leaflet of the OM contains tightly packed lipopolysaccharide (LPS), which presents an effective barrier to diffusion of hydrophobic molecules (Silhavy *et al.*, 2010, Nikaido, 2003). While the OM is a necessary defense, affording protection from variable and potentially unfavorable environments, the protective nature of the

OM presents a dilemma for the uptake of certain required nutrients due to the requisite limitations to traversing such a barrier (Postle, 1990). OM permeability for passive diffusion of nutrients is limited to small hydrophilic molecules, less than 600 Da, which can diffuse through OM porin proteins, or substrates of specific OM channels. Many nutrients diffuse into the periplasm in this manner, but bioavailability and native characteristics exclude some important nutrients, such as iron, from passage across the outer membrane barrier in this manner. These nutrients are acquired by TonB-dependent active transport, which is facilitated by specific, high-affinity OM transporters and the CM proteins TonB, ExbB, and ExbD (Fig. 1-1) (Silhavy *et al.*, 2010, Nikaido, 2003). ExbB and ExbD are thought harness the pmf to energize TonB, which transmits this energy to OM TGTs for the active translocation of ligands into the periplasm [recently reviewed in (Braun, 2009, Kuehl & Crosa, 2010, Noinaj *et al.*, 2010, Krewulak & Vogel, 2011)]. Additional TonB-dependent ligands include vitamin B_{12}, heme, maltodextrin, sucrose, and nickel (Blanvillain *et al.*, 2007, Lohmiller *et al.*, 2008, Schauer *et al.*, 2007, Cescau *et al.*, 2007, Schauer *et al.*, 2008).

TonB-dependent active transport

Iron is essential for biological processes, such as electron transport, amino acid synthesis, and DNA synthesis, of *Escherichia coli* and other Gram negative bacteria. Bacteria typically require iron concentrations of 10^{-6}-10^{-7}M (Schaible & Kaufmann, 2004). However, while iron is an abundant element, bioavailable iron is scarce. In aerobic, neutral environments, iron is present mainly as insoluble (10^{-18}M at pH 7.0) ferric hydroxides (Andrews *et al.*, 2003). In host organisms, where free iron (FeII) catalyzes formation of toxic hydroxyl radicals, iron is tightly sequestered by host proteins, such as lactoferrin, transferrin, or ferritin (Schaible & Kaufmann, 2004). Ability to scavenge iron is a known determinant of bacterial pathogenicity (Williams,

1979, Crosa, 1980, Linggood & Ingram, 1982, Carniel *et al.*, 1987, Genco *et al.*, 1991). Understanding the mechanism of TonB-dependent iron transport can facilitate targeting proteins of the TonB system with antimicrobials.

Competitive scavenging of iron from their native environments is aided by the bacterial secretion of siderophores, such as enterochelin produced by *E. coli*, which function as high-affinity ferric iron chelators (Schaible & Kaufmann, 2004, Wandersman & Delepelaire, 2004, Nairz *et al.*, 2010). Ferric-siderophores are too large to passively diffuse through porin proteins and are instead bound with high-affinity by specific OM transporters, called TonB-gated transporters (TGTs), for active transport into the periplasm. All known TGTs have a conserved structure of a 22-stranded β-barrel, occluded by a globular domain termed the plug (Noinaj *et al*., 2010). The amino-terminal region of the plug domain contains a semi-conserved sequence spanning seven amino acids called the TonB box (Coulton *et al.*, 1986, Heller & Kadner, 1985, Lundrigan & Kadner, 1986, Pressler *et al.*, 1988, Sauer *et al.*, 1987, Nikaido, 2003, Noinaj *et al*., 2010). Deletion or mutation of the TonB box prevents transport, but not binding, of ligands (Gudmundsdottir *et al.*, 1989, Schoffler & Braun, 1989, Kadner, 1990, Endriss *et al.*, 2003). The TonB box was initially suggested as a direct site of interaction with TonB by the observation that TGT TonB box mutations are suppressed by mutations in the TonB periplasmic domain (Schoffler & Braun, 1989, Bell *et al.*, 1990, Günter & Braun, 1990, Heller *et al.*, 1988). Direct interactions were later demonstrated (Cadieux & Kadner, 1999, Cadieux *et al.*, 2000, Ogierman & Braun, 2003). Bactericidal agents such as colicins and bacteriophage have parasitized the TonB system to enter and ultimately kill the cells (Cascales *et al.*, 2007, Rabsch *et al.*, 2007, Braun, 2009). Colicins B, D, M, and Ia also contain a TonB box (Köck *et al.*, 1987, Schramm *et al.*, 1988, Roos *et al.*, 1989). As observed with TGTs, mutations in the TonB boxes of colicins B, D, and M that prevent their translocation across the OM are also suppressed by mutations in the TonB periplasmic domain (Mende & Braun, 1990, Pilsl *et al.*, 1993, Mora *et al.*, 2005).

Active transport of ligands through TGTs lacks a direct energy source at the OM, due to lack of ATP in the periplasm and free diffusion of ions preventing formation of an electrochemical gradient. Transport is energized by the CM protonmotive force (pmf) (Bradbeer, 1993), requiring a means for inter-membrane energy coupling. This need is fulfilled by the cytoplasmic membrane proteins, TonB (239 amino acids), ExbB (244 amino acids), and ExbD (141 amino acids), which couple energy derived from the pmf to OM TGTs for active transport of TonB-dependent substrates into the periplasm. All three proteins are thought to form a heteroligomeric complex in the CM. Heterodimeric complexes of TonB-ExbB have been directly observed after formaldehyde crosslinking *in vivo* (Skare *et al.*, 1993). Current methods, however, have not trapped a TonB/ExbB/ExbD complex. The cellular stoichiometry of TonB/ExbB/ExbD is 1:7:2, but the stoichiometry within an energy transduction complex is unknown (Higgs *et al.*, 2002). The energy transduction process and the precise mechanism by which TonB transmits energy to TGTs are unknown.

The inter-membrane energy coupling protein TonB

The majority of work on the TonB system has focused on TonB, with *tonB* strains first isolated based on resistance to bacteriophage T one (Anderson, 1946). TonB is required for the active transport of ferric-siderophores, vitamin B_{12}, and bactericidal agents such as group B colicins, which include colicins B, D, Ia, and M, and bacteriophage ϕ80, T1, or T5, each of which parasitizes TonB-dependent receptors for entry into the periplasm (Frost & Rosenberg, 1975, Hantke & Braun, 1975, Davies & Reeves, 1975, Bassford *et al.*, 1976, Hancock & Braun). TonB is anchored in the CM by a single TMD, formed by an uncleaved hydrophobic region similar to a signal sequence (Postle & Skare, 1988, Hannavy *et al.*, 1990, Roof *et al.*, 1991). This amino-terminal region of TonB is important for TonB export, function, and interaction with ExbB

(Karlsson *et al.*, 1993b, Jaskula *et al.*, 1994). The TonB TMD retains its CM association throughout the TonB energy transduction cycle, based on function of full-length TonB stably fused at its cytoplasmic N-terminus to the homodimerization domain of the ToxR protein (Gresock *et al.*, 2011). Only one TMD residue of TonB, His20, has functionally essential side chain properties. His20 is the only TonB TMD residue that is inactive when substituted with Ala (Larsen *et al.*, 2007). The only other side chain at TMD position 20 that supports TonB function is Asn. Unlike His, Asn is not protonatable, ruling out a direct role for TonB His20 as part of a proton pathway (Swayne & Postle, 2011). TonB has two soluble domains, consisting of a short cytoplasmic domain and the majority of TonB in the periplasm (Roof *et al*., 1991) (Fig. 1-2). The TonB periplasmic domain contains two distinct regions, a proline rich region from amino acids 66-102 and the carboxy terminus spanning residues 103-239. It has been suggested that the proline rich region could serve a structural role, allowing TonB to span the periplasm to contact OM proteins (Evans *et al.*, 1986). The proline rich region does not have an essential role, however, since this region can be deleted, and TonB still functions *in vivo* (Larsen *et al.*, 1993, Seliger *et al.*, 2001). The TonB carboxy terminus is important in its interactions with OM TGTs. Residues 192-239 are apparently important in this interaction, as TonB truncated at residue 191 does not crosslink with the OM TGT FepA, but it is unknown if the deleted region is involved in direct interaction with FepA (Larsen *et al.*, 1997). To date, direct interaction has only been demonstrated between TonB residues 160-164 and the TonB box of OM TGTs (Cadieux & Kadner, 1999, Cadieux *et al*., 2000, Ogierman & Braun, 2003). However, TonB with a deletion of residues 158-162 is still active, suggesting other regions must be involved in interactions that support TonB function. Any larger deletions centered on TonB Q160 are inactive, suggesting the conformation of this region is important (Vakharia-Rao *et al.*, 2007). It appears to be the conformation of the TonB periplasmic domain, and not individual residues, that define TonB

function. In fact no individual residue in the TonB carboxy terminus, from 150-239, is essential for function (Postle *et al.*, 2010).

TonB is a conformationally dynamic protein. The periplasmic domain of TonB changes conformation in response to changes in pmf, such that a unique protease resistant TonB fragment is detected in spheroplasts only when pmf is collapsed. These changes are dependent on the presence of ExbB, ExbD, and a wild-type TonB TMD (Larsen *et al.*, 1994, Larsen *et al.*, 1999, Held & Postle, 2002). These same conditions are also important for detection of three conformations of homodimeric TonB. When individual cys substitutions are introduced in the TonB carboxy terminus, six substitutions each form three conformations of disulfide-linked homodimers that migrate at different apparent molecular masses on non-reducing SDS-polyacrylamide gels (Ghosh & Postle, 2005). Crystal structures have been solved of homodimers of periplasmic domain fragments of TonB, but these structures do not represent the TonB homodimers observed *in vivo* (Chang *et al.*, 2001, Kodding *et al.*, 2005, Postle *et al*., 2010).

Energy harvesting proteins ExbB and ExbD

Exb strains were first identified for excretion of an inhibitor of colicin B (Guterman, 1973). Inhibition was attributed to enterochelin, which is hypersecreted by strains with mutations in the *exb* locus and competes for a common receptor, FepA, with colicin B (Gutermann & Dann, 1973, Pugsley & Reeves, 1977). Iron starvation of *exbB* strains resulted in the hypersecretion of enterochelin (Pugsley & Reeves, 1976a). Unlike mutations in *tonB*, which eliminate TonB-dependent active transport, mutations in *exb* result in reduced transport of iron-siderophores such as ferric-enterochelin or ferrichrome and partial sensitivity to group B colicins (Pugsley & Reeves, 1976b, Hantke & Zimmerman, 1981, Eick-Helmerich & Braun, 1989). The residual activity of *exbB* strains is due to cross-talk between ExbB/D and paralogues TolQ/R,

where TolQ and TolR can partially substitute for ExbB and ExbD function, respectively. Strains with mutations in both *exbB* and *tolQ* are completely tolerant to group B colicins, and sensitivity can be restored by overexpression of TolQ/R (Braun, 1989, Braun & Herrmann, 1993). The functional roles of ExbB and ExbD are essential for TonB-dependent energy transduction, with the TonB protein itself irreplaceable in its role.

E*xbB* and *exbD* are transcribed as an operon (Ahmer *et al.*, 1995). Early studies on the role of ExbB in TonB-dependent energy transduction used *exbB* mutations such as a Tn*10* insertion at the 5' end (Hantke & Zimmerman, 1981). This was later shown to have polar effects on *exbD* expression, where only about 4% of an $ExbD_{1-121}$-PhoA fusion was detected from chromosomal *exbD*::Tn*phoA* in the presence of *exbB*::Tn*10* compared to *exbB*+ under iron deplete conditions (Ahmer *et al.*, 1995). Some studies acknowledged that phenotypes could not exclude potential effects on *exbD* expression (Larsen *et al.*, 1994), but phenotypes were primarily ascribed to ExbB functions based on these studies. It was later determined that strains individually lacking ExbB or ExbD had identical effects on TonB—loss of activity, proteolytic instability, inability to conformationally respond to pmf, and increased association with OM proteins. ExbB and ExbD functions are interdependent (Ahmer *et al.*, 1995, Held & Postle, 2002).

Defining a role for ExbD

ExbD has an identical topology to TonB, with a single TMD (amino acids 23-43), an amino-terminal cytoplasmic domain (amino acids 1-22) and carboxy-terminal periplasmic domain (amino acids 44-141) (Kampfenkel & Braun, 1992). This is distinct from ExbB, which has 3 TMDs and large regions in the cytoplasm (Kampfenkel & Braun, 1993b, Karlsson *et al.*, 1993a) (Fig. 1-2). The shared topological features of TonB and ExbD and the known

conformational dynamics of TonB, contribute to the hypothesis that ExbD regulates or directs the conformational changes of the TonB periplasmic domain (Ghosh & Postle, 2005, Larsen *et al*., 2007). Between all three proteins, pmf-dependent signaling from ExbB, to ExbD, to TonB has been proposed (Ghosh & Postle, 2005). Recently, evidence for signaling from the cytoplasmic domain of ExbB to the periplasmic domains of ExbD and TonB was presented (Jana *et al*., 2011).

In 2007, the nuclear magnetic resonance (NMR) structure of the isolated ExbD periplasmic domain (residues 44-141) was solved. The ExbD periplasmic domain was prone to extensive aggregation *in vitro*, with 4-7 copies of the domain forming a homomultimer with 1mM protein at pH 7.0. The pH of the periplasm of *E. coli* is equal to the external pH (Wilks & Slonczewski, 2007), so the native ExbD periplasmic domain would typically be in a neutral pH environment under optimum growth conditions. A monomeric state suitable for structure determination, however, was only obtained with low protein concentration (0.2mM) and low pH (3.0) (Garcia-Herrero *et al*., 2007). Under these conditions, the ExbD periplasmic domain formed a region of defined tertiary structure (residues 64-133), flanked by flexible N-terminal (residues 44-63) and C-terminal (residues 134-141) tails (Fig. 1-3). The structured region consists of a 5-stranded β-sheet, with 2 α-helices on one face. Similarities were observed between the ExbD periplasmic domain structure and the C-terminal lobe of Type-III periplasmic binding proteins (PBPs) (Garcia-Herrero *et al*., 2007). PBPs bind siderophores in the periplasm for subsequent trafficking to CM ATP-binding cassette transporters [recently reviewed in (Chu & Vogel, 2011)]. The ability of the ExbD periplasmic domain to bind siderophores was not examined. Siderophore binding by ExbD was considered unlikely since the periplasmic domain represented only half of the binding pocket of PBPs (Garcia-Herrero *et al*., 2007). A homodimeric state, however, which could potentially provide the equivalent to a second PBP lobe, was not considered, and the biological relevance of any similarities between the structured region of ExbD and a single lobe of this class of PBPs is unknown. This study also examined the potential for *in vitro* interactions

between the ExbD periplasmic domain and fragments of the TonB periplasmic domain, including two synthetic peptides spanning the TonB proline-rich domain (Glu-Pro residues 70-83 and Lys-Pro 84-102) and the TonB carboxy terminus (residues 103-239). At pH 3.0, the only interaction detected was weak interaction between the ExbD periplasmic domain and the TonB Glu-Pro peptide, with the majority of interaction detected in the flexible ExbD carboxy-terminal tail. No significant interactions were detected at pH 7.0. It was concluded that interactions *in vivo* must occur primarily through the TMDs of the TonB /ExbB/ExbD complex (Garcia-Herrero *et al*., 2007).

Prior to the generation of ExbD-specific antibodies (Higgs *et al*., 2002), ExbD-specific interactions *in vivo* were studied using ExbD containing an N-terminal T7 epitope tag and detection with T7 epitope-specific antibodies. The crosslinking agent formaldehyde is membrane permeable and creates a methylene bridge between reactive residues. Under short treatment times (20 minutes), formaldehyde is primarily reactive with the N terminus of a protein or the side chain of lysine. Reactivity is also observed with arginine, tryptophan, and cysteine (Toews *et al.*, 2008, Toews *et al*., 2010). T7 epitope tagged ExbD forms formaldehyde crosslinked homodimers and homotrimers *in vivo*. Prior to studies presented here, there was no direct evidence of hetero-oligomeric ExbD-specific complexes *in vivo*. *In vitro* work, however, suggested the likely-hood of such interactions. TonB and ExbD individually bind and co-elute with ExbB containing a His_6 tag at its C terminus that was bound to a Ni-NTA agarose column. Binding is specific to ExbB-His_6 as no binding of TonB nor ExbD was observed when columns were loaded with the PBP His_{10}-FhuD or an unnamed cytoplasmic protein (Braun *et al.*, 1996).

Only one prior mutagenesis study has focused on ExbD regions important for function, identifying two point mutations that inactivate ExbD. L132Q in the ExbD periplasmic domain was isolated as a spontaneous mutant and renders ExbD inactive. D25N in the ExbD TMD was constructed to investigate the functional importance of the only charged residue in the ExbD

TMD (Fig. 1-2). Plasmid-encoded ExbD D25N was concluded inactive based on its failure to complement a strain with chromosomal *exbD*(L132Q), lacking native ExbD activity. Overexpressed ExbD D25N was also dominant in a strain expressing wild-type ExbD (Braun *et al*., 1996). ExbD is an exception to the positive inside rule of membrane protein topology, with an amino-terminal net charge of -5, compared to no net charge C terminal to the TMD (Hennessey & Broome-Smith, 1994). The net charge of the cytoplasmic domain, however, is not critical for function as ExbDΔ4-15, which has a cytoplasmic domain with no net charge, retains activity (Braun *et al*., 1996).

Comparison to the Mot and Tol protein systems

ExbB and ExbD are paralogues of MotA and MotB, proteins which serve as the stator of the flagellar motor, and TolQ and TolR, proteins (Fig. 1-4), along with TolA, involved in maintaining the integrity of the outer membrane (Eick-Helmerich & Braun, 1989, Cascales *et al.*, 2001). TolA, however, shares no homology with TonB (Webster, 1991). The TonB, Tol, and Mot systems all function using the energy of the CM pmf, and based on direct studies with MotA (Blair & Berg, 1990), ExbB/D, TolQ/R, and MotA/B complexes are hypothesized to form proton conducting channels. ExbD, TolR, and MotB share the greatest homology between their TMDs, and are thought to have similar mechanisms in harnessing the pmf (Kampfenkel & Braun, 1993a, Cascales *et al*., 2001, Kojima & Blair, 2001, Zhai *et al.*, 2003). All three proteins have a highly conserved TMD Asp residue, thought to be involved in response of these proteins to pmf (Zhou *et al.*, 1998b, Cascales *et al*., 2001). The periplasmic domains of ExbD and TolR are more similar to each other than to the MotB periplasmic domain (Cascales *et al*., 2001).

A precise role for TolR is unknown. *In vivo*, overexpressed TolR can be crosslinked with formaldehyde into heterodimers with TolA or TolQ and homodimers, with the periplasmic

domain important for the latter two interactions. A potential TolR homodimer crosslinked to TolQ is also detected (Derouiche *et al.*, 1995, Journet *et al.*, 1999). TolR is also thought to interact directly with the amino terminus of colicin A, based on a formaldehyde crosslinked interaction if both TolR and a TolA fragment are overexpressed in the periplasm (Journet *et al.*, 2001). The biological relevance of this interaction is unknown, and the overexpression of the proteins could have increased the incidence of artifactual associations. TolR is important for pmf-dependent conformational changes of TolA, based on protease accessibility studies (Germon *et al.*, 2001). TolR itself is also conformationally dynamic. Cys-substituted TolR TMDs form disulfide-linked homodimers *in vivo*, where the highest level of homodimer formation involved substitutions on opposite faces of the TMD. Homodimer formation through these sites inhibited TolR activity. Low levels of homodimers were observed on a helix face between these two positions, and the presence of an inactivating D23A mutation prevented formation of these low level homodimers. Results suggested the TolR TMD undergoes functionally important rotation (Zhang *et al.*, 2009). The TolR periplasmic domain is conformationally responsive to changes in pmf, exhibiting increased accessibility of introduced cysteines to labeling with the thiol-specific probe MPB after collapse of pmf or mutation of TMD residue Asp23 (Goemaere *et al.*, 2007b). It is proposed that these changes are related to direct contact of the TolR periplasmic domain with TolQ and a potential role in regulating ion conductivity or opening of the putative TolQR proton channel. However, there is currently no direct evidence of such an interaction, and this hypothesis is based primarily on suppressors of TolQ TM helix 3 mutations isolated in the periplasmic domain of TolR (Lazzaroni *et al.*, 1995, Goemaere *et al.*, 2007a).

Unlike ExbD and TolR, a potential function for MotB was readily proposed primarily based on its topology. To date, it is proposed that MotB serves to secure the MotA/B stator to the cell wall (Chun & Parkinson, 1988). MotB is predicted to interact directly with the peptidoglycan layer, based on sequence conservation within its periplasmic domain, including a predicted α-

helical peptidoglycan-binding motif, with OmpA and other peptidoglycan-associated proteins such as the lipoprotein Pal (De Mot & Vanderleyden, 1994, Koebnik, 1995). While inactivating mutations in or near the putative MotB peptidoglycan binding domain indicate the importance of this region in MotB function (Blair *et al.*, 1991, Togashi *et al.*, 1997, Muramoto & Macnab, 1998), direct interaction between MotB and peptidoglycan has yet to be demonstrated. One study reported isolation of Pal (Peptidoglycan-associated lipoprotein) but neither MotB nor an exported fragment of the carboxy terminus of MotB, residues 78-309, in peptidoglycan-associated fractions (Kojima *et al.*, 2008). Besides the predicted peptidoglycan-binding region, much of the MotB periplasmic domain is not essential for function, and as many as 60 consecutive residues can be deleted while retaining MotB function (Muramoto & Macnab, 1998, Van Way *et al.*, 2000). One additional region of the MotB periplasmic domain, however, has been assigned a potential role. Residues 52-65 are thought to serve to "plug" the MotA/B proton channel, preventing undesired opening of a channel not associated with the flagellar motor (Hosking *et al.*, 2006, Van Way *et al*., 2000). The sequence of this region is not conserved in ExbD or TolR (Goemaere *et al*., 2007b). A construct of green fluorescent protein fused to MotB, GFP-MotB, showed dynamic association/dissociation of GFP-MotB with the flagellar motor, with GFP-MotB expressed at native MotB levels, supporting the idea that MotB is not a statically anchored protein (Leake *et al.*, 2006). The MotB periplasmic domain is also thought not to associate with peptidoglycan until incorporated with the rotor, perhaps triggered by electrostatic interactions between the cytoplasmic loop of MotA and rotor component FliG (Zhou *et al.*, 1998a, Van Way *et al*., 2000). Interaction of MotB with MotA occurs through the TMDs of each protein (Braun *et al.*, 2004). MotB also forms homodimers through its TMD and periplasmic domain. Periplasmic domain dimerization is important for homodimerization of the MotB TMDs (Braun & Blair, 2001, Kojima *et al.*, 2009). While the exact role is unknown, homodimerization is a common trait for ExbD, TolR, and MotB.

This study examined the functional role of ExbD in the TonB system of *Escherichia coli*, providing evidence to support a hypothesized role for ExbD in directing conformational changes of the TonB periplasmic domain, and developing a model where ExbD directly responds to pmf to elicit conformational changes in TonB. Chapter 2 defines ExbD-specific interactions *in vivo*, with the identification of ExbD homodimers and heterodimers with ExbB and TonB. Importantly, this work identified the first mechanistic role of pmf in the TonB system as promoting interaction between the periplasmic domains of ExbD and TonB. The TMDs of both ExbD and TonB were demonstrated to be important for this interaction, implicating signaling from the TMD to the periplasmic domain of these proteins. A model is presented for pmf-dependent assembly or conformational changes of TonB and ExbD periplasmic domain. Chapter 3 further defines ExbD-TonB interactions, with results suggesting the occurrence of initial pmf-independent interactions, defined by specific conformations of an amino-terminal portion of the TonB periplasmic domain and the carboxy terminus of ExbD. ExbD L132 and TonB H20 were important for this initial interaction. Three stages are proposed in the process of energizing TonB defined by differences in associations of the TonB and ExbD periplasmic domains. Novel evidence is presented that ExbD conformation changes in response to changes in pmf, and TMD residue Asp25 is important for this change. Response of ExbD to pmf is proposed to result in the observed conformational changes of TonB, perhaps directing this change. Pmf toggles ExbD and TonB conformations. Chapter 4 used a 10-residue deletion scanning approach to identify regions of ExbD important for function. Only the extreme amino terminus of ExbD was expendable. Results presented support functional division of the ExbD periplasmic domain, with the amino-terminal region important in response to pmf and the carboxy terminus important for proper interaction with TonB. A periplasmic subdomain was identified from residues 92-121 where conformation of this region was particularly important in supporting protein-protein interactions of ExbD. Chapter 5 further explores the region of ExbD from residues 92-121. Cys scanning and

disulfide crosslinking were used to demonstrate a direct role for portions of this region in homodimerization of ExbD. Sites of homodimer formation mapped to opposite ends of the ExbD periplasmic domain NMR structure, and results suggest a dynamic ExbD periplasmic domain. ExbD was shown to be active as a homodimer, but not all sites of interaction represented stable homodimeric interfaces. Dimerization through a face of a predicted α-helix inhibited ExbD activity, indicating the importance of conformational flexibility for this region. The majority of substitutions mediating ExbD homodimer formation also formed heterodimers with TonB periplasmic domain residue A150C. ExbD TMD residue Asp25 was important for ExbD-TonB heterodimer formation but not homodimer formation. A model is presented for localized homodimeric to heterodimeric transitions of the ExbD periplasmic domain. Chapter 5 examines specific interactions of the ExbD and TonB periplasmic domains, using six ExbD cys substitutions in combination with 45 TonB cys substitutions. Three of the six ExbD substitutions formed significant heterodimers mapping to 4 regions of TonB. ExbD Asp25 was important for heterodimer formation. Regions of TonB involved in these interactions are thought to be important in conformational recognition of OM TGTs. Chapter 6 discusses how this apparent complex interplay of interactions may fit into a model of the TonB energy transduction cycle. Questions raised by this work are addressed and potential lines of investigation are proposed.

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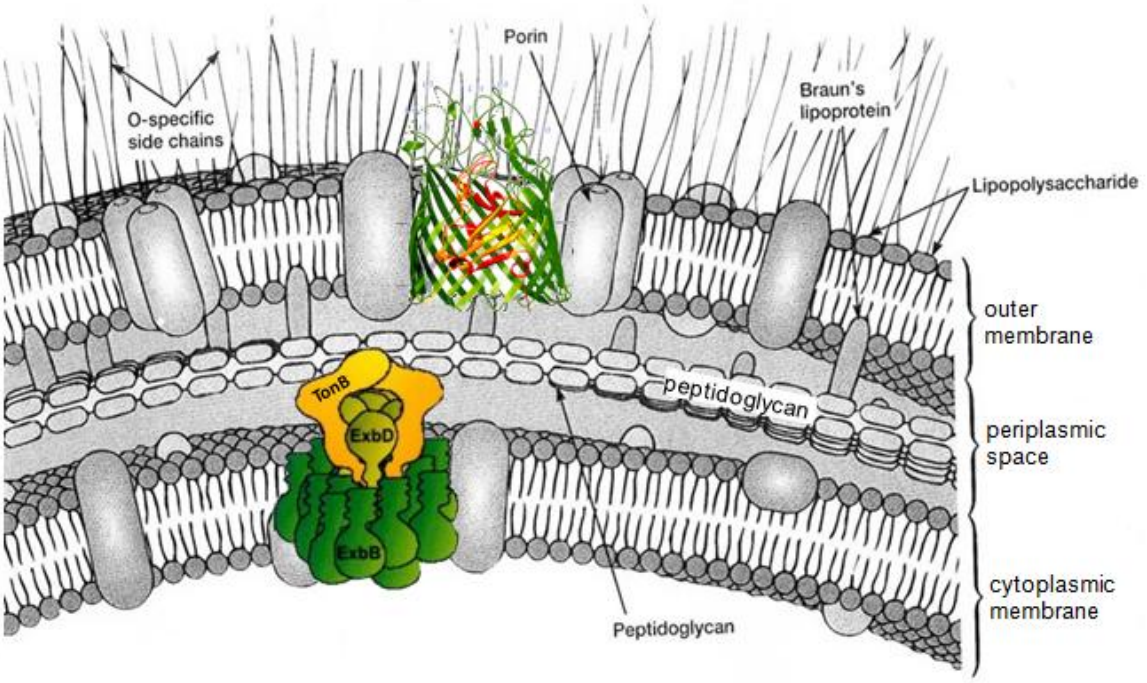

Figure 1-1. Organization of the Gram negative bacterial cell envelope. A cartoon illustrates the concentric membranes of the cell envelope and associated proteins. Cytoplasmic membrane and outer membrane components of the TonB system are depicted in color. A putative hetero-oligomeric TonB, ExbB, and ExbD complex is located in the cytoplasmic membrane. The TonB-gated transporter FepA is located in the outer membrane and is the transporter for ferric-enterochelin. This illustration is not drawn to scale. The cell envelope illustration is adapted from Prescott *et al*, 1996. The crystal structure of FepA is from Buchanan *et al*., 1999.

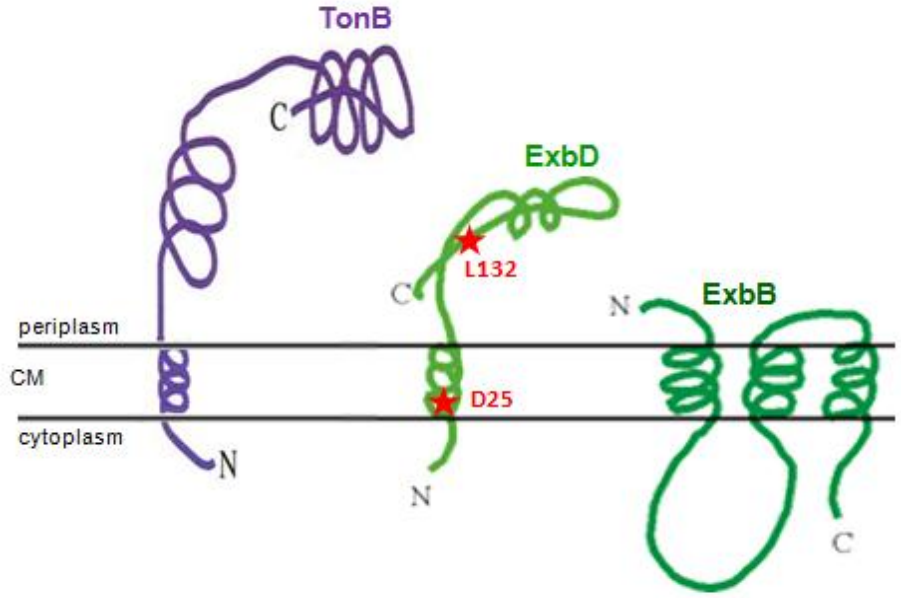

Figure 1-2. Topology of CM proteins in the TonB system and location of functionally important ExbD residues. Illustrations are not drawn to scale and are not intended to reflect tertiary structure. Stars indicate topological location of ExbD Asp25 and Leu132. D25N or L132Q render ExbD inactive (Braun *et al*., 1996). CM = cytoplasmic membrane. C or N indicate the carboxy or amino terminus, respectively, of each protein.

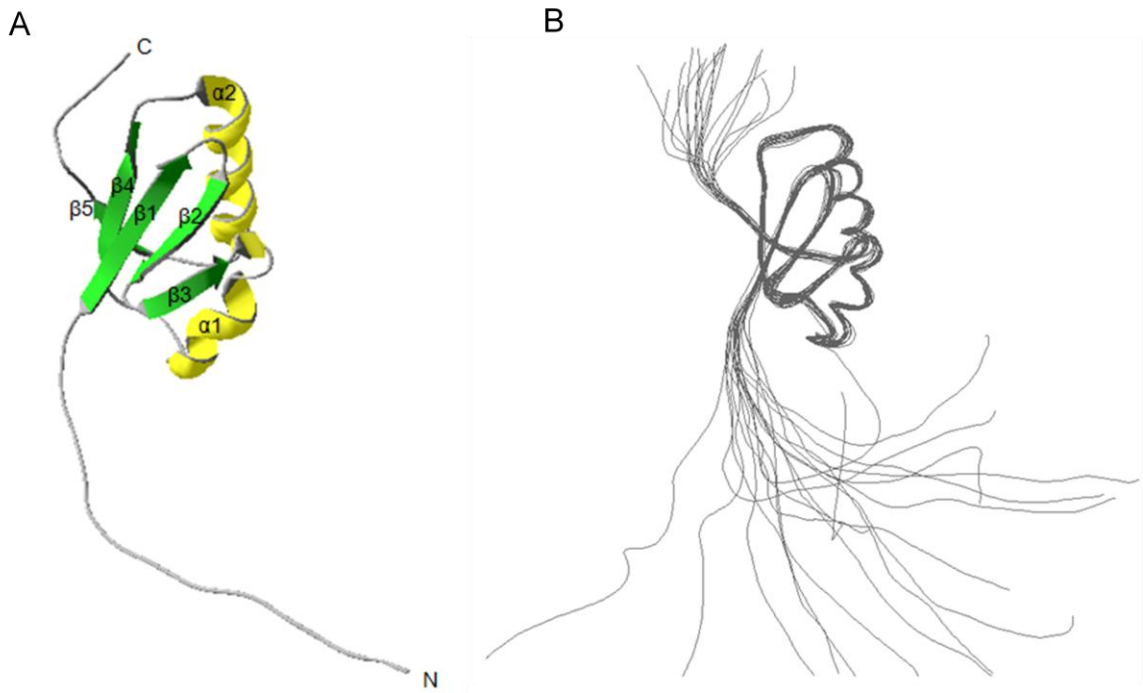

Figure 1-3. Solution structure of the ExbD periplasmic domain (residues 43-141). The NMR structure of the isolated domain (pdb code, 2pfu) is shown. A, Ribbon diagram showing one model colored by secondary structural elements. α-helices and β-strands are numbered by order from amino (N) to carboxy (C) terminus. B, The flexibility of the N- and C-terminal tails of the structure is shown by depiction of all 19 models. Some flexibility is also evident in the loop between β1 and β2. The images were generated using Swiss-PdbViewer (http://www.expasy.org/spdbv/, (Guex & Peitsch, 1997).

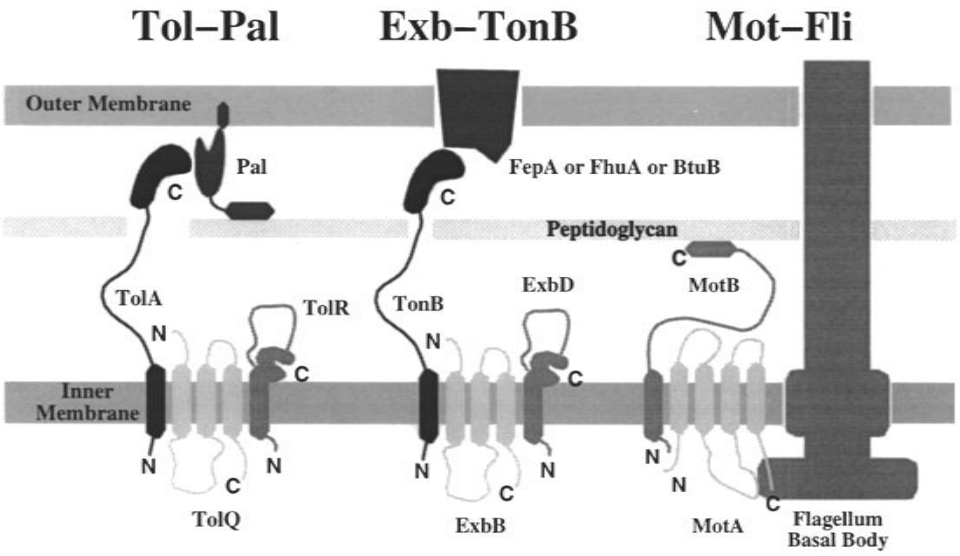

Figure 1-4. Three systems use energy of the pmf for distinct functions. ExbD is similar to TolR and MotB. ExbB shares similarities with TolQ and MotA. Reprinted with permissions from Cascales 2001.

CHAPTER 2

CYTOPLASMIC MEMBRANE PROTONMOTIVE FORCE ENERGIZES PERIPLASMIC INTERACTIONS BETWEEN EXBD AND TONB

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Chapter 2

Cytoplasmic Membrane Protonmotive Force Energizes Periplasmic Interactions between ExbD and TonB

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KEYWORDS: *Escherichia coli*, ExbD, TonB, transmembrane domains, protein conformation, iron transport.

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Summary

The TonB system of *Escherichia coli* (TonB/ExbB/ExbD) transduces the protonmotive force (pmf) of the cytoplasmic membrane to drive active transport by high affinity outer membrane transporters. In this study, chromosomally encoded ExbD formed formaldehyde-linked complexes with TonB, ExbB, and itself (homodimers) *in vivo*. Pmf was required for detectable crosslinking between TonB-ExbD periplasmic domains. Consistent with that observation, the presence of inactivating transmembrane domain mutations ExbD(D25N) or TonB(H20A) also prevented efficient formaldehyde crosslinking between ExbD and TonB. A specific site of periplasmic interaction occurred between ExbD(A92C) and TonB(A150C) and required functional transmembrane domains in both proteins. Conversely, neither TonB, ExbB, nor pmf were required for ExbD dimer formation. These data suggest two possible models where either dynamic complex formation occurred through transmembrane domains or the transmembrane domains of ExbD and TonB configure their respective periplasmic domains. Analysis of T7-tagged ExbD with anti-ExbD antibodies revealed that a T7 tag was responsible both for our previous failure to detect T7-ExbD-ExbB and T7-ExbD-TonB formaldehyde-linked complexes and for the concomitant artifactual appearance of T7-ExbD trimers.

Introduction

The Gram-negative bacterial cell envelope serves the dual roles of protective barrier for the cell and a gateway for the entry of essential elements and nutrients. The cell envelope consists of the concentric cytoplasmic (CM) and outer (OM) membranes, separated by an aqueous periplasmic space. The OM protects the cell from hydrophobic antibiotics, degradative enzymes, and detergents while allowing small (< 600 Da) hydrophilic nutrients entry by passive diffusion through OM porin proteins (Nikaido, 2003). Nutrients that are too large, too scarce or too important to pass through porins—iron siderophore complexes, vitamin B12, nickel, sucrose and possibly sulfate—are actively transported across the OM. Energy for transport across the unenergized OM is supplied by the protonmotive force (pmf) of the CM, with the TonB system acting as the energy-coupling agent between the two membranes (recently reviewed in (Postle and Larsen, 2007)). In the presence of protonophores such as dinitrophenol (DNP) or carbonylcyanide m-chlorophenylhydrazone (CCCP), ligands can bind to the outer membrane transporters but are not transported across the outer membrane. The conformation of TonB changes depending on whether pmf is present or absent (Larsen *et al.*, 1999). The pmf-dependent mechanisms of this system, however, remain largely unknown.

The TonB system of *Escherichia coli* consists of a complex of the CM proteins TonB, ExbB, and ExbD. ExbD is topologically similar to TonB, with each containing a single transmembrane domain (TMD) and the majority of the soluble domain occupying the periplasm (Hannavy *et al.*, 1990; Kampfenkel and Braun, 1992; Roof *et al.*, 1991). In contrast, ExbB has three transmembrane domains, with the majority of its soluble domains occupying the cytoplasm (Kampfenkel and Braun, 1993). TonB is known to form homodimers in the CM, and both ExbB and ExbD form homomultimers *in vivo* (Ghosh and Postle, 2005; Higgs *et al.*, 1998; Sauter *et al.*, 2003). The cellular ratio of TonB:ExbB:ExbD is 1:7:2, but the stoichiometry within an energy

transduction complex is unknown (Held and Postle, 2002; Higgs *et al.*, 2002b). Paralogues of ExbB and ExbD have been proposed to form complexes with a 4:2 MotA:MotB or 6-4:2 TolQ:TolR stoichiometry (Braun *et al.*, 2004; Kojima and Blair, 2004; Cascales *et al*., 2001).

ExbD is an essential component of the TonB system, required for TonB activity (Brinkman and Larsen, 2008). Little is known about the precise role of ExbD, though it was recently proposed to have a chaperone-like function in regulating the dynamics of TonB conformation (Ghosh and Postle, 2005; Larsen *et al.*, 2007). Only two essential residues, aspartate 25 in the TMD and periplasmic residue leucine 132, have been identified, such that D25N or L132Q substitutions render ExbD inactive (Braun *et al.*, 1996). The functional significance of these residues, however, remains obscure. The conserved corresponding TMD residues in TolR and MotB, D23 and D32, are also essential for activity within their respective systems. It has been proposed that these essential acidic residues are part of proton pathways through the putative TolQR or MotAB proton channels (Cascales *et al.*, 2001; Zhou *et al.*, 1998).

The sole functionally significant side chain in the TonB TMD is histidine 20. The remainder of the TonB TMD residues can be replaced by alanine without significant effect (Larsen *et al.*, 2007). The TonB amino terminal TMD serves as a signal anchor, a means by which TonB dimerizes in vivo, a means of contact with ExbB, and to regulate the conformation of the TonB carboxy terminus (Ghosh and Postle, 2005; Jaskula *et al.*, 1994; Karlsson *et al.*, 1993; Larsen *et al.*, 1994; Larsen *et al.*, 1999; Larsen and Postle, 2001; Larsen *et al.*, 2007; Postle and Skare, 1988; Sauter *et al.*, 2003; Skare *et al.*, 1989).

Although many details remain unclear, current data suggest a mechanism for energy transduction whereby ExbB/ExbD harvest the pmf and transmit it to TonB, allowing the TonB carboxy terminus to transduce energy to a ligand-loaded OM transporter. Following the energy transduction event, TonB is recycled back to an energizable state by ExbB/ExbD, undergoing conformational changes both prior to and following interaction with OM transporters (Ghosh and

Postle, 2005; Larsen *et al.*, 1999; Postle and Kadner, 2003). These conformations require a functional TonB transmembrane domain, the pmf, and ExbB/ExbD.

In this study, we demonstrated for the first time that pmf was required for the interaction of ExbD and TonB periplasmic domains trapped by formaldehyde crosslinking. Consistent with that, transmembrane domain mutations proposed to be on the proton pathway across the cytoplasmic membrane in either ExbD or TonB prevented formaldehyde and disulfide-directed crosslinking of the periplasmic domains. ExbD also efficiently crosslinked to ExbB and formed homo-dimers, but not homo-trimers *in vivo*. Our earlier study showed that T7-tag-ExbD formed crosslinked homo-dimers and homo-trimers, but did not crosslink to TonB or ExbB (Higgs *et al.*, 1998). We show here that those artifactual results were due to the presence of the T7 tag at the ExbD amino terminus.

Results

Wild-type ExbD crosslinks to ExbB and TonB and forms homodimers in vivo

To examine *in vivo* interactions of wild-type, chromosomally-encoded ExbD with itself or other proteins, whole cells were treated with monomeric formaldehyde, processed for immunoblot analysis, and characterized using polyclonal anti-ExbD antibody (Higgs *et al.*, 2002a). Along with the ExbD monomer, migrating at an apparent molecular mass of 15 kDa, three higher molecular mass complexes, at approximately 30, 41, and 52 kDa, were detected (Fig. 1, wild-type lane). An active plasmid-encoded ExbD size variant, ExbDΔ2-11, which lacked ten residues in the cytoplasmic domain, was used to determine which, if any, of the complexes represented ExbD homo-multimers. This size variant migrated with an apparent molecular mass of 13 kDa. Accordingly, for the formaldehyde crosslinking profile of ExbDΔ2-11, complexes

containing homomultimers of ExbDΔ2-11 were expected to show a shift in migration equal to a multiple of this difference. The 30 kDa complex obtained with wild-type ExbD was replaced by a 26 kDa complex when the ExbDΔ2-11 size variant was crosslinked (Fig. 2-1). This shift of twice the difference in monomeric masses identified the 30 kDa complex as a homodimer of ExbD. Both of the remaining complexes showed a shift in migration of approximately 2 kDa, suggesting each contained monomeric ExbD in complex with other proteins. Previous work using T7 epitope tag-specific antibody had demonstrated the ability of a T7 epitope-tagged ExbD to form homodimers and homotrimers *in vivo* (Higgs *et al.*, 1998). This work confirmed the ability of wild-type ExbD to form homodimers *in vivo*. The ExbD trimer previously observed with T7 epitope-tagged ExbD at ~48 kDa was not observed for wild-type, chromosomally-encoded ExbD or plasmid-encoded ExbD expressed near chromosomal levels. An explanation for this discrepancy will be addressed below.

Other proteins likely to interact with ExbD include TonB and ExbB. Based on a theoretical mass of 41.6 kDa for an ExbB-ExbD heterodimer, the 41 kDa ExbD-specific complex had the potential to be a complex between ExbB and ExbD. When ExbD was expressed in a strain lacking ExbB, both the 41 kDa and 52 kDa complexes were absent, demonstrating the dependence of both of these complexes on the presence of ExbB (Fig. 2-2).

To determine if either complex contained ExbB protein, *exbBD* cells (KP1392) expressing wild-type ExbD and ExbB fused to the fluorescent protein Venus were crosslinked with formaldehyde. When expressed at normal chromosomal levels, ExbB-Venus (52.3 kDa) was approximately 90% active compared to wild-type ExbB (Bulathsinghala and Postle, unpublished results). The presence of ExbB-Venus resulted in the loss of the 41 kDa ExbD-specific complex and the appearance of two novel higher molecular mass complexes at ~68 kDa and ~84 kDa. The 68 kDa complex corresponded to the theoretical mass of an ExbD-(ExbB-Venus) complex (Fig. 2-3). The 41 kDa complex therefore represented a complex between wild-type ExbB and ExbD.

While previous work identified the potential for ExbB-ExbD interaction through *in vitro* binding of ExbD to ExbB (Braun *et al.*, 1996), this is the first direct evidence of *in vivo* ExbB-ExbD complex formation. A less intense band migrating slightly above the ExbB-ExbD complex, at approximately 44 kDa, was also dependent on the presence of ExbB. However, a corresponding band was not detected for wt ExbD in the presence of ExbB-Venus and its identity remains unknown. The identity of the 84 kDa complex observed for ExbD in experiments with ExbB-Venus also was not determined. The 52 kDa complex was still detected in the presence of ExbB-Venus, indicating it did not contain ExbB. But based on its absence in the *exbB* strain, it clearly required ExbB for its assembly (Fig. 2-2, 3).

The mass of the 52 kDa ExbD-containing complex closely matched the predicted mass of a complex between ExbD (15.5 kDa) and TonB (with a calculated molecular mass of 26 kDa, but an apparent molecular mass of 36 kDa in SDS polyacrylamide gels) (Eick-Helmerich and Braun, 1989; Postle and Reznikoff, 1979). To determine if the 52 kDa complex was dependent on the presence of TonB, the formaldehyde crosslinking profile of ExbD was examined in a strain lacking TonB (KP1503). In this strain, the 52 kDa ExbD-specific complex was not detected (Fig. 2-4, A), suggesting this complex consisted of a heterodimer of TonB and ExbD. Using anti-TonB antibody, a TonB-ExbD complex was also identified by the absence of the 52 kDa complex in a strain lacking ExbD (RA1045) (Fig. 2-4, B). Taken together these data indicated that the 52 kDa complex detected with ExbD-specific antibody was a TonB-ExbD complex that required ExbB to form. A TonB-ExbD-ExbB complex was not detected, most likely due to inefficiency of trimolecular crosslinking.

Formaldehyde-specific crosslinks between TonB and ExbD almost certainly occurred between their periplasmic domains rather than their transmembrane domains. Formaldehyde crosslinking is initiated by formation of methylol derivatives at 1° amino groups or 1° thiol groups which then undergo a condensation to form a Schiff-base that can subsequently crosslink

to a variety of amino acids (Means and Feeney, 1971; Metz *et al.*, 2004). However, under the conditions of rapid in vivo crosslinking with formaldehyde, the spectrum of subsequent interactions is limited primarily to lysyl, tryptophanyl, and cysteinyl residues (Toews *et al.*, 2008). Given the sequences of the ExbD and TonB cytoplasmic and transmembrane domains and their identical topologies, the only likely crosslink would form between the amino terminus of ExbD and Trp 11 in TonB; however, deletion of Trp 11 does not prevent ExbD-TonB crosslinking (data not shown). In contrast, the periplasmic domains of each protein contain numerous crosslinkable residues; the TonB periplasmic domain has 1 tryptophanyl and 18 lysyl residues and the ExbD periplasmic domain has 10 lysyl residues. The predicted pIs of the periplasmic domains [TonB residues 33-239 (9.6) and ExbD residues 44-141 (5.3)] are also consistent with their interactions.

Pmf is required for in vivo formation of TonB-ExbD formaldehyde crosslinks

The role of ExbB in formation of TonB-ExbD crosslinks suggested that proposed proton translocation through ExbB might also be important (Braun and Herrmann, 2004). Although the pmf is clearly required for TonB-dependent transport across the OM, there is little known about its mechanistic role (Bradbeer, 1993). To determine if pmf was required for any ExbD interactions, we treated cells with various amounts of DNP and with CCCP prior to and during formaldehyde crosslinking (Fig. 2-5). In the presence of the protonophores, TonB-ExbD crosslinks were undetectable while levels of ExbD dimer and ExbB-ExbD crosslinks remained unchanged. A similar decrease in TonB-ExbD crosslinking was also observed with anti-TonB antibodies with no other detectable changes to the normal crosslinking profile other than slight increases in the TonB-FepA and TonB-Lpp complexes (data not shown), consistent with the fact that these complexes occur with unenergized TonB (Ghosh and Postle, 2005). Comparison with

control immunoblots indicated that monomeric TonB and ExbD levels were unaffected by protonophore treatments.

A D25N substitution in the ExbD transmembrane domain disrupts TonB-ExbD periplasmic domain interaction

The transmembrane domain of ExbD might be on the proton translocation pathway. Asp 25, a residue in the transmembrane domain of ExbD, is essential for ExbD activity (Braun *et al.*, 1996). We confirmed that D25N inactivates ExbD and also showed that ExbD(D25A) is inactive (Table 2-1), which ruled out the possibility that D25N was inactive due to steric hindrance. The D25N substitution also did not prevent proper localization of ExbD to the cytoplasmic membrane (Fig. 2-6).

Interestingly, the (D25N) or (D25A) substitutions in the ExbD transmembrane domain both prevented formaldehyde crosslinking to TonB, as detected by either anti-ExbD or anti-TonB immunoblots (Fig. 2-7 and data not shown). The presence of ExbD(D25N)-ExbB complexes also confirmed that ExbD(D25N) was localized properly to the cytoplasmic membrane (Fig. 2-7). ExbD(D25N) also formed homodimers, although the apparent molecular mass of the complex was slightly less than that observed for the wild-type ExbD dimer, even though ExbD(D25N) monomer has an apparent molecular mass similar to wild type. The identity of the ExbD(D25N) dimer was confirmed using size variants (data not shown). An unidentified complex containing ExbD(D25N) migrated slightly above the dimer and was more abundant compared to wild-type ExbD. The increased intensity for this band was also observed for the crosslinking of ExbD in the absence of TonB (Fig. 2-4, A), suggesting that it increases when ExbD does not interact with TonB.

The H20A substitution in the TonB transmembrane domain also disrupts ExbD-TonB interaction

A complementary approach was used to examine the effect of a TonB transmembrane domain mutation on TonB-ExbD complex formation. Histidine 20 in TonB is the only functionally important side chain in its transmembrane domain, with a H20A substitution leading to inactivity of chromosomally encoded TonB in all assays (Larsen *et al.*, 1999; Larsen *et al.*, 2007), (Table 2-1). Like ExbD (D25N), the TonB(H20A) was properly localized to the CM. (Fig. 2-6). The formation of a formaldehyde-crosslinked complex between TonB and ExbB was unaffected by the H20A substitution, also confirming that TonB(H20A) was correctly assembled in the cytoplasmic membrane. Like ExbD(D25N), the TonB(H20A) mutation eliminated detection of the TonB-ExbD formaldehyde-crosslinked complex by either anti-TonB or anti-ExbD antibodies (Fig. 2-8). The formaldehyde crosslinked complexes of TonB(H20A) with Lpp and FepA increased in intensity relative to wild-type TonB, consistent with previous observations that those complexes arise from inactive TonB and do not require a functional TonB TMD to form (Ghosh and Postle, 2005; Jaskula *et al*., 1994). In an attempt to determine whether TonB(H20) and ExbD(D25) form a salt bridge, TonB(H20D) and ExbD(D25H) were constructed. Each was individually inactive, and they were inactive (and present at chromosomal levels) when co-expressed—a negative, and thus uninterpretable, result (Table 2-1).

T7-tagged ExbD crosslinks artifactually

As noted above, we had previously observed that T7-tagged ExbD complemented an *exbD* mutation and could be formaldehyde-crosslinked into dimers and trimers, but did not detectably crosslink to TonB or ExbB (Higgs *et al.*, 1998). To determine the source of this difference with chromosomally encoded ExbD, the formaldehyde crosslinking of T7-ExbD was

revisited, this time using ExbD-specific antibody for detection. T7-ExbD was recloned into propionate and arabinose expression vectors, expressed by induction to chromosomal levels or overexpressed, crosslinked in vivo with formaldehyde, and analyzed by immunoblot with ExbD- or T7-epitope-tag-specific antibodies.

In contrast to the previous results, the formaldehyde crosslinked T7-ExbD detected by anti-ExbD antisera matched the profile of wild-type ExbD when expressed at chromosomal levels (Fig. 2-9A). This difference was explained, however, by comparison to identical samples detected with T7 epitope tag-specific antibody, which unexpectedly revealed that the vast majority of the T7-ExbD had been proteolytically processed to remove the T7-tag. Thus the normal ExbD crosslinking profile of the "T7-ExbD" originated from ExbD lacking the T7 tag. In this experiment the level of intact T7-ExbD was so low as to make detection of formaldehyde cross-linked complexes impossible.

To detect formaldehyde crosslinked complexes specific to the tagged population of ExbD, T7-ExbD was overexpressed from the arabinose promoter, crosslinked with formaldehyde and detected with anti-T7-tag antibody. Like the 1998 study that this replicated, the anti-T7 crosslinking profile contained complexes at the molecular masses of a T7-ExbD dimer (33.4 kDa) and trimer (50.1 kDa), and did not contain TonB-ExbD or ExbB-ExbD complexes (Fig. 2-9B and data not shown). This crosslinking result was not due to overexpression, since overexpressed ExbD had the same crosslinking profile as chromosomally expressed ExbD (data not shown).

Taken together, these results indicated that 1) 1998 immunoblots with T7 tag-specific antibody were detecting only the minor subpopulation of ExbD protein that retained the T7 epitope tag and 2) that the artifactual formation of formaldehyde crosslinked trimeric T7-ExbD and lack of TonB-ExbD and ExbB-ExbD complexes was due to the presence of the T7 tag and did not reflect the normal behavior of ExbD. Even though the activity of T7-ExbD could not be

determined against a background containing a preponderance of full-length active ExbD, based on its abnormal crosslinking behavior the T7-ExbD was almost certainly inactive.

Active cysteine substitutions ExbD(A92C) and TonB(C18G, A150C) demonstrate specific TonB-ExbD periplasmic domain contact in vivo.

While the periplasmic domain of ExbD was identified as the site of formaldehyde-mediated crosslinking to TonB, we did not identify specific residues through which it occurred. To begin to map regions of interaction between ExbD and TonB, cysteine substitutions were engineered in their respective periplasmic domains, and the existence of disulfide-linked heterodimers was monitored on non-reducing SDS-polyacrylamide gels. ExbD has no native cysteinyl residues whereas TonB carries a single cysteinyl residue at position 18. As an example of this approach, ExbD(A92C) and TonB(C18G, A150C), which were fully active when expressed at near chromosomal levels (Ollis, Kastead, and Postle, unpublished results), were analyzed. The appearance of identical novel complexes at 52 kDa on immunoblots developed with either TonB- or ExbD-specific antibodies indicated that the periplasmic domains of these two proteins were indeed interacting *in vivo* (Fig. 2-10). The 52 kDa complex was specific to the presence of the introduced cysteine in each protein (data not shown).

To determine if the 52 kDa disulfide-linked complex represented a biologically relevant interaction, the effect of ExbD(D25N) or TonB(H20A) transmembrane domain substitutions was also examined using either anti-TonB or anti-ExbD antibodies (Fig. 2-10 A, B respectively). The inactivating H20A substitution in the transmembrane domain of TonB(C18G, A150) essentially eliminated TonB-ExbD disulfide-linked complex formation detected with either antibody. The inactivating D25N substitution likewise prevented TonB-ExbD complex formation. Coexpression of the inactive mutants did not restore detection of a disulfide-linked complex. Taken together

these results indicate that interactions of the TonB and ExbD periplasmic domains require activities attributable to their transmembrane domains. It was not possible to assess the effects of protonophores on formation of disulfide crosslinks because 1) disulfide crosslinks are pre-existing in the population and 2) synthesis of the two proteins in a pulse requires pmf for their export to the periplasm.

Similar to the formation of ExbD dimers through formaldehyde crosslinking, ExbD(A92C) formed disulfide-linked dimers through its periplasmic domain (Fig. 2-8, B). ExbD(D25N, A92C) monomer ran, if anything, slightly slower than the ExbD(A92C) monomer. Interestingly, the D25N transmembrane domain substitution resulted in an apparently smaller molecular mass, suggesting that a novel conformational change had been trapped.

Discussion

The TonB/ExbB/ExbD proteins of *E. coli* couple the cytoplasmic membrane ion electrochemical potential (most likely a proton potential) to active transport of iron-siderophore and vitamin B12 nutrients across the outer membrane. In other Gram-negative bacteria, the TonB system energizes outer membrane transport of iron-binding proteins, sucrose, Ni(II), and potentially sulfate, suggesting that it serves as the general means by which the limiting porosity of the outer membrane can be overcome (Blanvillain *et al.*, 2007; Cescau *et al.*, 2007; Schauer *et al.*, 2007; Tralau *et al.*, 2007). TonB undergoes cyclic energization, transduction of that energy to a TonB-gated transporter, and recharging to allow re-energization (Fischer *et al.*, 1989; Larsen *et al.*, 1999). ExbB and ExbD appear to have roles in both harvesting the protonmotive force, allowing TonB to then transduce this energy to TonB-gated transporters, and in recycling TonB after it has transduced energy. If TonB is not energized, it is not recycled (Larsen *et al.*, 1999; Letain and Postle, 1997). ExbD has been proposed to chaperone the conformation of the TonB

carboxy terminus and is specifically involved in the recycling of TonB following energy transduction (Brinkman and Larsen, 2008; Larsen *et al.*, 2007). Its role in energization of TonB has not been directly determined.

A new role for the pmf in TonB-dependent energy transduction

Our results here indicate for the first time a definitive role for the cytoplasmic membrane pmf in promoting functionally important interaction of TonB and ExbD through their periplasmic domains. First, two different protonophores that collapse the proton gradient of the cytoplasmic membrane prevent formation of ExbD-TonB formaldehyde crosslinks in vivo. Second, the ExbD(D25N) transmembrane domain mutation, which inactivates ExbD, also prevents ExbD-TonB formaldehyde crosslinks and disulfide-directed crosslinks between their periplasmic domains. The ExbD(D25N) mutation occurs at a conserved residue that is equally important in ExbD paralogues TolR and MotA, considered to be on the proton pathway, and responsible for conformational changes in those proteins (Cascales *et al.*, 2001; Goemaere *et al.*, 2007; Kojima and Blair, 2001). Third, the TonB(H20A) transmembrane domain mutation, which inactivates TonB, also prevents ExbD-TonB formaldehyde crosslinks and disulfide crosslinks between their periplasmic domains. The His 20 is conserved among most TonB genes and also conserved in the analogous TolA protein of the Tol system (Germon *et al.*, 1998). His20 is required for pmf-dependent conformational changes in the TonB carboxy terminus and is the sole functionally significant side-chain in the entire transmembrane domain (Larsen *et al.*, 1999; Larsen *et al.*, 2007). Fourth, the L132Q mutation in the periplasmic domain of ExbD knocks out ExbD function (Braun et al., 1996). ExbD(L132Q) does not crosslink in vivo to TonB although it can still crosslink into dimers and crosslink to ExbB, indicating that the periplasmic interaction

between TonB and ExbD is a functionally important one (data not shown). These data support the idea that ExbD manages the conformational changes in the carboxy terminus of TonB.

We previously observed that the TonB energy transduction cycle is functionally divided into events that occur prior to energy transduction and those that occur following energy transduction, by performing the experiments in an *aroB* strain that cannot synthesize enterochelin or any of its precursors. In the absence of ligand, TonB does not transduce energy to the TonB-gated transporter FepA, thus interrupting the cycle (Larsen *et al.*, 1999). Because the ExbD-TonB crosslinked complex (as well as the ExbD dimer and ExbD-ExbB complex described below) was detected equally well in a wild-type or *aroB* strain, it indicated that the TonB-ExbD interaction detected by formaldehyde crosslinking occurred prior to the energy transduction step (data not shown). This was also consistent with the requirement for pmf and intact transmembrane domains, and indicated that ExbD plays a role in the energization step on the front half of the energy transduction cycle. Since a role for ExbD in recycling TonB has been identified, ExbD appears to play critical roles both before and after energy transduction by TonB.

Two models for the TonB-ExbD interaction

These results suggest two possible models for TonB-ExbD interaction. In the first model, the TonB-ExbD complex is formed dynamically, and only in response to the presence of the pmf. Thus the pmf would be responsible for allowing TonB and ExbD transmembrane domains to move close enough for interactions between their periplasmic domains to be captured through crosslinking. There is evidence to support the idea of dynamic complexes: the ratios of the total numbers per cell for ExbB and ExbD proteins are, at 7:2, significantly higher than the ratios of the total numbers per active complex for paralogues MotA:MotB, TolQ:TolR, or PomA:PomB at 4:2 (Cascales *et al.*, 2001; Guihard *et al.*, 1994; Kojima and Blair, 2004; Sato and Homma, 2000).

It thus may be that the TonB/ExbB/ExbD complex is in equilibrium with pools of uncomplexed ExbB, assembling in response to cellular signals to transduce energy. Consistent with that idea, it has been recently shown that MotB moves in and out of the flagellar rotor complex (Leake *et al.*, 2006).

In the second model, the stably assembled transmembrane domains of TonB and ExbD in association with ExbB would be somehow responsible for directly transmitting conformational information to their periplasmic domains. The TonB transmembrane domain is known to play a role in regulating the conformation of its carboxy terminus (Ghosh and Postle, 2005; Larsen *et al.*, 1999; Larsen *et al.*, 2007). Because the TonB amino terminus and carboxy terminus are separated by a non-essential proline-rich region, it seems unlikely that the regulation occurs via a proton-wire (Larsen *et al.*, 1993; Seliger *et al.*, 2001). However, different types of transmembrane helix motions have been proposed to propagate conformational changes to adjacent domains including a piston motion between helices, pivoting of helices and rotation of helices (Matthews *et al.*, 2006). For the ExbD paralogue, MotB, Asp32 is required for conformational changes in MotA, the ExbB paralogue (Kojima and Blair, 2001). It will be important to distinguish between the two models.

The nature of ExbD dimerization

ExbD dimerization occurred in the absence of ExbB and in the presence of the ExbD(D25N) substitution believed to render ExbD unresponsive to pmf, consistent with previous observations that ExbD(D25N) is dominant negative (Braun *et al.*, 1996). Also consistent with these observations, formaldehyde crosslinking of ExbD dimers did not require pmf. In spite of the fact that the ExbD transmembrane domains appear to interact closely, the formaldehyde-specific ExbD dimers were mediated through the periplasmic domain and almost certainly

required interaction of many residues. Indeed, deletion of the periplasmic domain of ExbD(D25N) relieved its dominant negativity (Braun *et al.*, 1996). Perhaps ExbD(D25N) is blocked in the ability to transition from homodimeric periplasmic domain interactions to functionally important heterodimeric interactions with TonB. If so, deletion of periplasmic domain residues, which eliminated the dominant negative effect of ExbD(D25N), would then have freed the periplasmic domain of wild-type ExbD to transition to its normal interactions.

At least one of the residues in the dimerization region was A92, which when substituted with a cysteinyl residue, was capable of trapping a disulfide-linked ExbD dimer. A92C was also a residue through which ExbD contacted the periplasmic domain of TonB at residue A150C. It may be that the ExbD dimer was maintained through its transmembrane domain while the periplasmic domain cycled between interactions with another ExbD periplasmic domain or a TonB periplasmic domain. In our hands, ToxR-ExbD fusion proteins can activate a *ctx*::*cat* fusion (Russ and Engelman, 1999), indicating that the transmembrane domains of ExbD are sufficiently close that that they allow functional dimerization of ToxR, whether or not ExbB is present (Vakharia-Rao and Postle, unpublished results). Movement of the dimeric ExbD transmembrane domains relative to one another could drive changes in interactions between periplasmic domains. Rotation of dimeric paralogue TolR transmembrane helices relative to each other has been documented recently (Zhang *et al.*, 2009). The physiological role of the ExbD dimer is currently unknown.

In contrast to results seen here, in the Tol system formaldehyde crosslinking of TonB paralogue TolA with ExbD paralogue TolR is not pmf-dependent; however, TolA interaction with lipoprotein Pal is (Cascales *et al.*, 2000). TolA-TolR interaction is mediated through the last 25 amino acids of TolR (Journet *et al.*, 1999). Structural changes in the periplasmic carboxy terminus of TolR are, however, dependent on the presence of pmf and residues in the predicted TolQR ion pathway, including TolR Asp23, the residue analogous to ExbD Asp25 (Goemaere *et*

al., 2007). The differences in pmf-dependent interaction partners may reflect the divergent functions identified for the periplasmic domains of ExbD and TolR (Brinkman and Larsen, 2008).

Comparison of in vitro and in vivo structural predictions

The importance of TonB and ExbD transmembrane domains in determining the conformations and interactions of their periplasmic domains is underscored by comparison to the structures of the soluble periplasmic domains of these two proteins lacking their transmembrane domains (Chang *et al.*, 2001; Garcia-Herrero *et al.*, 2007; Kodding *et al.*, 2005; Peacock *et al.*, 2005). In the recently solved NMR structure of the ExbD soluble domain 5-7 copies of the ExbD monomer formed a multimeric complex at pH 7.0. Residue A92, through which ExbD can efficiently form dimers *in vivo*, was far from this multimeric interface (Fig. 2-11). Additionally in that paper, no significant interactions between purified ExbD and TonB periplasmic domains were detected *in vitro*, leading the authors to conclude that the functional interactions between TonB and ExbD likely occurred primarily through their transmembrane domains *in vivo*. In contrast, we propose here that the lack of detectable interaction *in vitro* was almost certainly due to the absence of the transmembrane domains of TonB and ExbD as well as ExbB and the pmf.

With respect to the TonB transmembrane domain, the *in vivo* data on full-length TonB also diverge from major aspects of the solved structures for the periplasmic carboxy terminus of TonB protein. In particular, while the 5 aromatic residues of the carboxy terminus are buried in the crystal and NMR structures, the *in vivo* data indicate that they are surface exposed, accessible for homodimeric interactions, and virtually the only functionally important residues in the carboxy terminus [(Ghosh and Postle, 2004; Ghosh and Postle, 2005); Kastead and Postle, unpublished observations]. A mutant TonB transmembrane domain that inactivates TonB also prevents TonB homodimer formation through cysteine substitutions at the aromatic residues

(Ghosh and Postle, 2005). Thus for both ExbD and TonB, the structural results obtained *in vitro without transmembrane domains and access to the pmf* are significantly different than those obtained *in vivo where all needed components are present*. It is thus not clear if the structures of the soluble domains of ExbD and TonB represent in vivo conformations.

Artifactual crosslinking results arising from the use of a T7 epitope tag

The acquisition of anti-ExbD antibodies allowed the characterization of wild-type ExbD interactions at chromosomally encoded levels (Higgs *et al.*, 2002a). ExbD could be crosslinked by formaldehyde to itself (homo-dimers), to ExbB, and to TonB. This was the first time that crosslinks to either ExbB or TonB had been observed *in vivo*. Consistent with the requirement for pmf, TonB-ExbD crosslinks did not form in the absence of ExbB. Both TonB and ExbD are proteolytically unstable in the absence of ExbB [(Fischer *et al.*, 1989) and data not shown]. ExbB has three transmembrane domains and appears to be the glue that holds the complex together since when expressed in the absence of TonB or ExbD, ExbB is proteolytically stable [(Fischer *et al.*, 1989) and Higgs and Postle, unpublished results].

Before anti-ExbD antibodies were available, we had characterized plasmid-encoded ExbD tagged with a T7-epitope and observed only ExbD dimers and trimers *in vivo* (Higgs *et al.*, 1998). The data presented here show that the ability to detect artifactual ExbD trimers, as well as the artifactual *inability* to detect interactions with ExbB and TonB, was due to the presence of the T7-tag. The fact that the T7-ExbD could complement an *exbD* mutation was meant to provide confidence in the results from an overexpressed epitope tagged protein study. Instead, the observed complementation was almost certainly due to proteolytic cleavage of the majority of the T7 tag, leaving behind full-length ExbD. Thus we have no evidence that the ExbD trimers are biologically relevant. Without the use of anti-ExbD antibodies, for which absence the T7-tag was

originally meant to compensate, these discrepancies would not have been apparent. These results provide a direct and important demonstration of the hazards involved in relying on interpretations of data from tagged or fused proteins.

In summary, TonB and ExbD interact through their periplasmic domains in vivo, with that interaction guided by not-well-understood aspects of their transmembrane domains. The study of the periplasmic domains of TonB and ExbD *in vitro* has greatly enhanced our knowledge of what their structures and behaviors are in the absence of transmembrane domains and protonmotive force, and provided an important basis for comparison with *in vivo* results. Could it be that the *in vitro* structures of soluble domains of TonB and ExbD represent a default non-energized state of these proteins and that the protonmotive force is somehow used to perturb these conformations? As one of the central questions in membrane protein signal transduction biology, it will be important to understand how transmembrane domains regulate conformations and interactions of their soluble domains.

Experimental Procedures

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 2-2. KP1484 was constructed by P1*vir* transduction of Δ*tonB, P14*::kan from KP1477 into GM1. KP1503 was constructed by P1*vir* transduction of Δ*tonB, P14*::kan from KP1484 into KP1038. KP1509 was constructed by P1*vir* transduction of Δ*tonB, P14*::kan from KP1484 into RA1045. To create pKP660, the *exbB, exbD* operon was amplified by polymerase chain reaction (PCR) and cloned into the SmaI site of plasmid pBAD24.

pKP1186 was constructed by extra-long PCR on pKP999, using forward and reverse primers each encoding one half of the T7 epitope tag, placed at the extreme amino-terminus of

ExbD. The PCR products were recircularized and ligated, joining the halves of the T7 tag sequence. The correct T7 epitope tagged ExbD was confirmed by DNA sequencing of the *T7-exbD* gene. pKP1195 was constructed by digestion of pKP1186 and pBAD24 with NcoI. Fragments were separated by gel electrophoresis. The 4542 bp fragment of pBAD24 and 539 bp fragment of pKP1186 were purified by gel extraction and ligated together after treatment of the vector fragment with Antarctic Phosphatase (New England Biolabs). Proper orientation of the insert was verified by FspI digestion. The correct T7 epitope tagged ExbD in pBAD24 was confirmed by DNA sequencing.

pKP761 was constructed by in-frame deletion of ten *exbD* codons using extra-long PCR, as previously described (Higgs *et al.*, 1998). The resulting construct, ExbD(Δ2-11), was determined to be active by standard Fe transport and spot titer assays performed as previously described (data not shown) (Larsen *et al.*, 2003; Postle, 2007). To construct pKP999 and pKP1000, forward and reverse primers were designed to amplify the last 22 codons of *exbB* through the stop codon of *exbD* from a pKP660 or pKP880 template, respectively, introducing flanking NcoI sites. The PCR-amplified, NcoI digested fragment was cloned into the unique NcoI site in pPro24. Proper orientation was determined by FspI digestion. Sequences of the *exbB* segment and *exbD* gene were confirmed by DNA sequencing.

TonB and ExbD single residue substitutions are derivatives of pKP325 and pKP999, respectively, unless otherwise stated. pKP879 and pKP945 are derivatives of pKP568. pKP1049 is a derivative of pKP1000. Substitutions were generated using 30-cycle extra-long PCR using *Pfu* Ultra Hotstart DNA Polymerase from Stratagene or Phusion Hotstart DNA Polymerase from Finnzymes. 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. Substitutions were verified by DNA sequencing to avoid unintended base changes.

pKP944 was constructed by directional cloning. First, using a pKP660 template, KpnI and XhoI sites were introduced to the 3' end of *exbB*, adding 7 residues (Ala, Gly, Thr, Gly, Gly Leu, Glu) before the stop codon, creating pKP930. The gene encoding the fluorescent GFP derivative Venus was amplified from a pET21a background, introducing a 5' KpnI site and 3' XhoI site. The KpnI, XhoI *venus* fragment was ligated in frame into the corresponding sites in the *exbB* gene of pKP930 to create pKP944. The resulting ExbB-Venus fusion has three introduced residues, Ala Gly Thr, linking the cytoplasmic carboxy terminal domain of ExbB to Venus. Sequences of *exbB* and *exbD* genes were confirmed by DNA sequencing to rule out unintended base changes. To construct pKP1031, plasmids pKP879 and pKP945 were digested with BstEII, resulting in 2 fragments for each. Fragments were separated by gel electrophoresis. The large fragment of pKP945 and small fragment of pKP879 were purified by gel extraction and ligated together after treatment of the large fragment with antarctic phosphatase (New England Biolabs). Proper orientation was determined by BamHI digestion. All DNA sequencing occurred at The Pennsylvania State University Nucleic Acid Facility, University Park, PA.

Media and culture conditions

Luria-Bertani (LB), tryptone (T), and M9 minimal salts were prepared as previously described (Miller, 1972). Liquid cultures, agar plates, and T-top agar were supplemented with 34 μg ml^{-1} chloramphenicol and/or 100 μg ml^{-1} ampicillin and plasmid-specific levels of L-arabinose and/or sodium propionate, pH 8, as needed for expression of TonB and ExbD proteins from plasmids. M9 salts were supplemented with 0.5% glycerol (w/v), 0.4 μg ml^{-1} thiamine, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.2% casamino acids (w/v), 40 μg ml^{-1} tryptophan, and 1.85 μM $FeCl_3$. Cultures were grown with aeration at 37°C.

Spot titer activity assays

Assays were performed essentially as previously described (Larsen *et al.*, 2003; Postle, 2007).

Sucrose density gradient fractionation

Mid -exponential phase cultures were grown in M9 medium as described above, harvested, lysed by French pressure cell and fractionated on a 25%-56% (w/w) sucrose gradient as described previously (Letain and Postle, 1997).

In vivo *formaldehyde crosslinking*

Saturated overnight cultures were subcultured 1:100 into M9 minimal media (above) supplemented arabinose and/or propionate concentrations as needed to achieve chromosomal levels of plasmid expression, and at mid -exponential phase treated with formaldehyde as previously described (Higgs *et al.*, 1998). Crosslinked complexes were detected by immunoblotting with ExbD-specific polyclonal antibodies(Higgs *et al.*, 2002a), TonB-specific monoclonal antibodies (Larsen *et al.*, 1996), or T7-epitope tag-specific monoclonal antibodies (Novagen). For crosslinking in the presence of protonophores, 1, 5, or 10mM 2, 4 dinitrophenol (DNP) or 50μM carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) were added following resuspension of cell pellets in phosphate buffer. An equal volume of dimethyl sulfoxide (DMSO) was added to wild-type samples as a solvent control. Cells were incubated 5 min at 37°C. Formaldehyde was then added and procedure continued as referenced above.

In vivo disulfide crosslinking assay

Saturated overnight cultures of strains carrying plasmids encoding combinations of TonB and ExbD cysteine substitutions were subcultured 1:100 in T broth containing chloramphenicol

and ampicillin and supplemented with L-arabinose and sodium propionate, pH 8, as described below. Cultures were harvested in mid-exponential phase and precipitated with trichloroacetic acid (TCA). Cell pellets were resuspended in non-reducing Laemmli sample buffer containing 50mM iodoacetamide, as previously described (Ghosh and Postle, 2005). Samples were resolved on 13% non-reducing SDS-polyacrylamide gels and evaluated by immunoblot analysis. Levels of inducers for coexpression of the TonB and ExbD cysteine variants were as follows:

pKP1000, pKP945 = 1mM sodium propionate, 0.0005% (w/v) L-arabinose;

pKP1000, pKP1031 = 0.5mM sodium propionate, 0.0005% (w/v) L-arabinose;

pKP1049, pKP945 = 0.5mM sodium propionate, 0.0003% (w/v) L-arabinose;

pKP1049, pKP1031 = 0.3mM sodium propionate, 0.0003% (w/v) L-arabinose

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Figure Legends

Fig. 2-1. Wild-type ExbD forms homo-dimers *in vivo*. Strains expressing chromosomally encoded (W3110) or plasmid-encoded wild-type ExbD (RA1017/pKP660), and ExbD(Δ2-11) (RA1017/pKP761) were crosslinked with formaldehyde as described in Materials and Methods. Plasmids encoding ExbD also encoded wild-type ExbB. Levels of L-arabinose for induction were 0.0002% (w/v) for pKP660 and 0.001% for pKP761. Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted. ExbD was visualized with ExbD-specific polyclonal antibodies. Positions of molecular mass standards are indicated on the left. Identities or apparent molecular masses of ExbD-specific crosslinked complexes and the ExbD monomer are indicated on the right.

Fig. 2-2. The 41 and 52 kDa ExbD-specific complexes are dependent on the presence of ExbB. Strains expressing chromosomally-encoded (W3110) or plasmid-encoded wild-type ExbD (pKP999) in ExbB+ (RA1045) or ExbB- (RA1017) backgrounds were crosslinked with formaldehyde as described in Materials and Methods. pKP999 was induced with 3mM sodium propionate, pH 8 in RA1045 and 20mM sodium propionate, pH 8 in RA1017. Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted. Approximately 40% more of the ExbB- sample (right lane) was loaded to achieve ExbD monomer levels of equal intensity to wild-type. ExbD was visualized with ExbD-specific polyclonal antibodies. "+" or "-" indicates the presence or absence, respectively, of ExbB in the sample resolved in the lane below the symbol. Positions of molecular mass standards are indicated on the left. Identities of ExbD-specific crosslinked complexes and monomers are indicated on the right. (*) indicates an unidentified complex.

Fig. 2-3. The 41 kDa complex contains one ExbD and one ExbB. Strains expressing chromosomally-encoded (GM1) or plasmid-encoded (KP1392/pKP660) ExbB and ExbB-Venus fusion protein (KP1392/pKP944) were crosslinked with formaldehyde as described in Materials and Methods. All plasmids also encoded wild-type ExbD. Proteins were expressed using two different percentages of arabinose, as indicated above each lane. ExbB monomer levels were determined from culture samples that were TCA precipitated immediately after harvesting. Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted. ExbD and ExbB were visualized with ExbD- or ExbB-specific polyclonal antibodies, respectively. Positions of molecular mass standards are indicated on the left. Identities of ExbD-specific crosslinked complexes and the ExbD monomer are indicated on the right. (*) indicates an unidentified complex.

Fig. 2-4. The 52 kDa complex contains one ExbD and one TonB. Strains expressing chromosomally-encoded (GM1) or plasmid-encoded wild-type ExbD in the presence (KP1392/pKP660) or absence (KP1503/pKP660) of TonB were crosslinked with formaldehyde as described in Materials and Methods. Plasmids encoding ExbD also encoded wild-type ExbB and were induced with 0.0004% (w/v) L-arabinose. Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted. A. ExbD visualized with ExbD-specific polyclonal antibodies. B. TonB visualized with TonB-specific monoclonal antibodies. Positions of molecular mass standards are indicated on the left. Identities of ExbD- or TonB-specific crosslinked complexes and monomers are indicated on the right. Light exposures for comparison of monomer levels are present at the bottom of each figure. (*) indicates an unidentified complex.

Fig. 2-5. Pmf regulates TonB-ExbD complex formation. Strains expressing chromosomally-encoded (W3110) or plasmid-encoded (RA1045/pKP999) ExbD were crosslinked with

formaldehyde in the presence of protonophores that collapse the pmf as described in Materials and Methods. The expanding triangle above the +DNP lanes indicates the presence of 1, 5, or 10 mM DNP. +CCCP indicates the presence of 50 μM CCCP. Solvent only (DMSO) was added to samples lacking protonophore. Plasmid-encoded ExbD was expressed with 3 mM sodium propionate, pH 8. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted. ExbD was visualized with ExbD-specific polyclonal antibodies. Positions of molecular mass standards are indicated on the left. Identities of ExbD-specific crosslinked complexes and monomers are indicated on the right. Light exposures for comparison of monomer levels are present at the bottom of each figure. TonB monomer levels were visualized with TonB-specific monoclonal antibodies.

Fig. 2-6. ExbD(D25N) and TonB(H20A) fractionate identically to the wild-type forms of each protein. Strains expressing chromosomally-encoded ExbD and TonB (W3110), ExbD(D25N) (RA1021/pKP1064), and TonB(H20A) (KP1344/pKP381) were fractionated using sucrose density gradient fractionation, as described in Materials and Methods. No inducer was needed for ExbD(D25N). TonB(H20A) was induced with .00025% (w/v) arabinose. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted. ExbD and TonB were visualized with ExbD-specific polyclonal or TonB-specific monoclonal antibodies, respectively.

Fig. 2-7. ExbD(D25N) does not crosslink to TonB *in vivo*. Strains expressing chromosomally-encoded ExbD (W3110) and ExbD(D25N) (RA1045/pKP1064) were crosslinked with formaldehyde as described in Materials and Methods. ExbD(D25N) was induced with 0.05mM sodium propionate, pH 8. Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted. ExbD was visualized with ExbD-specific polyclonal antibodies. To verify that TonB levels were unchanged, TonB monomer was visualized with TonB-specific monoclonal

antibodies. Positions of molecular mass standards are indicated on the left. Identities of ExbD-specific crosslinked complexes and the ExbD monomer are indicated on the right. (*) indicates an unidentified complex.

Fig. 2-8. TonB(H20A) does not crosslink to ExbD *in vivo*. Strains expressing chromosomally-encoded wildtype TonB (W3110) and TonB(H20A) (KP1344/pKP381) were crosslinked using formaldehyde. L-arabinose at a final concentration of 0.001% (wt/vol) was used to induce pKP381. Samples were resolved on an 11% SDS-polyacrylamide gel and analyzed using immunobloting with ExbD-specific polyclonal antibodies and TonB-specific monoclonal antibodies. Positions of molecular mass standards are indicated on the left. Identities of crosslinked complexes and the protein monomers are indicated on the right.

Fig. 2-9. ExbD with an amino terminal T7-epitope tag crosslinks artifactually. Strains expressing chromosomally encoded ExbD (W3110), plasmid-encoded wild-type ExbD (RA1045/pKP999), and T7-epitope tagged ExbD (RA1045/pKP1186 or RA1045/pKP1195 for overexpression) were crosslinked with formaldehyde as described in Materials and Methods. Plasmid-encoded ExbD was expressed with 3mM sodium propionate, pH 8. T7-ExbD was induced with two different concentrations of sodium propionate for pKP1186 (A) or two different percentages of arabinose for overexpression from pKP1195 (B) as indicated above each lane. Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted. ExbD was visualized with ExbD-specific polyclonal antibodies or T7 epitope tag-specific monoclonal antibodies. Positions of molecular mass standards are indicated on the side. Identities of ExbD-specific crosslinked complexes and monomers are indicated in the middle. (*) indicates a non-specific cross-reactive band.

Fig. 2-10. Active TonB and ExbD cysteine substitutions form specific periplasmic domain contacts. Strains expressing wild-type ExbD and TonB (W3110), ExbD(A92C) with TonB(C18G, A150) [KP1509/pKP1000, pKP945], ExbD(A92C) with TonB(C18G, H20A, A150C) [KP1509/pKP1000, pKP1031], ExbD(D25N, A92C) with TonB(C18G, A150C) [KP1509/pKP1049, pKP945] were processed in non-reducing sample buffer containing iodoacetamide as described in Materials and Methods. Samples were resolved on a 13% non-reducing SDS-polyacrylamide gel and immunoblotted. TonB (A) or ExbD (B) was visualized with TonB-specific monoclonal antibodies or ExbD-specific polyclonal antibodies. Positions of molecular mass standards are indicated on the left. Lanes 3 through 6 contained strain KP1509 expressing derivatives of ExbD(A92C) coexpressed with TonB(C18G, A150C). Derivatives contained (+) or lacked (-) the residue substitutions listed to the right.

Fig. 2-11. ExbD A92 is distantly located from the proposed multimeric interface of ExbD. The NMR structure of the carboxy-terminal domain (amino acids 44-141) of ExbD is shown (pdb code: 2pfu). The proposed multimeric interface (amino acids 104-116) (Garcia-Herrero *et al.*, 2007) is highlighted in blue. Residue A92 is highlighted in red. ExbD(A92C) spontaneously formed dimers through this residue *in vivo* (Fig.2-10) .

Table 2-1: Spot titer assay results

Strain	Phenotype	Sensitivity[a]			
		Colicin B	Colicin Ia	Colicin M	ϕ80
W3110	WT	8,8,8	7,7,7	6,6,6	8,8,8
RA1045	ExbD-,TolQR-	T,T,T	T,T,T	T,T,T	T,T,T
KP1509	ExbD- TonB-	T,T,T	T,T,T	T,T,T	T,T,T
KP1344/pKP381	TonB(H20A)	T,T,T	T,T,T	T,T,T	T,T,T
KP1344/pKP1054	TonB(H20D)	T,T,T	T,T,T	T,T,T	T,T,T
RA1045/pKP1055	ExbD(D25H)	T,T,T	T,T,T	T,T,T	T,T,T
KP1509/pKP1054/ pKP1055	ExbD(D25H)/ TonB(H20D)	T,T,T	T,T,T	T,T,T	T,T,T
RA1045/pKP999	ExbD	8,8,7	7,7,7	6,6,6	8,8,8
RA1045/pKP1064	ExbD(D25N)	T,T,T	T,T,T	T,T,T	T,T,T
RA1045/pKP1191	ExbD(D25A)	T,T,T	T,T,T	T,T,T	T,T,T

[a]TonB system activity of strains expressing variants of ExbD and TonB was evaluated using spot titre assays. Cultures with the proteins expressed to near-chromosomal levels (verified through Western blot – not shown) were plated on T-plates and spotted with fivefold serial dilutions of colicins and 10-fold serial dilutions of bacteriophage ϕ80. Values were recorded as the reciprocal of the highest dilution at which clearing of the bacterial lawn was evident after 18 h of incubation at 37°C. ‘T’ indicates tolerance (no sensitivity).

Table 2-2. Strains and Plasmids used in this study.

Strain or Plasmid	Genotype or Phenotype	Reference
Strains		
W3110	F⁻ IN*(rrnD-rrnE)1*	(Hill and Harnish, 1981)
GM1	*ara*, Δ*(pro-lac)*, *thi, F' pro lac*	(Sun and Webster, 1987)
KP1038	GM1 *exbB*::*Tn10*, *tolQ(am)*	
KP1344	W3110 *tonB*::*blaM*	(Larsen *et al.*, 1999)
KP1392	GM1 *exbB*::*Tn10*, *tolQ(am)*, *recA*::*cat*	(Held and Postle, 2002)
KP1477	W3110 Δ*tonB*::*kan*	(Devanathan and Postle, 2007)
KP1484	GM1 Δ*tonB*::*kan*	Present study
KP1503	GM1 *exbB*::*Tn10*, *tolQ(am)*, Δ*tonB*::*kan*	Present study
KP1509	W3110 Δ*exbD*, Δ*tolQR*, Δ*tonB*::*kan*	Present study
RA1017	W3110 Δ*exbBD*::*kan*, Δ*tolQRA*	(Larsen *et al.*, 2007)
RA1021	W3110 Δ*exbD*	Ray Larsen
RA1045	W3110 Δ*exbD*, Δ*tolQR*	(Brinkman and Larsen, 2008)
Plasmids		
pKP325	pBAD-regulated TonB	(Larsen *et al.*, 1999)
pKP381	TonB(H20A)	(Larsen *et al.*, 2007)
pKP568	TonB(C18G)	(Ghosh and Postle, 2005)
pKP879	TonB(C18G, H20A)	Present study
pKP945	TonB(C18G, A150C)	Present study
pKP1054	TonB(H20D)	Present study
pKP1031	TonB(C18G, H20A, A150C)	Present study
pBAD24	L-arabinose-inducible, pBR322 *ori*	(Guzman *et al.*, 1995)
pKP660	pBAD24 expressing *exbBD* from the pBAD promoter	Present study
pKP761	ExbB, ExbDΔ2-11	Present study
pKP880	ExbB, ExbD(A92C)	Present study
pKP930	ExbB with 7 residue C-terminal insertion	Present study
pKP944	ExbB-Venus, ExbD	Present study
pET21a-Venus	Venus fluorescent protein	(Anderson and Yang, 2008)
pKP1186	pPro24-(T7-ExbD)	Present study
pKP1195	pBAD24-(T7-ExbD)	Present study
pPro24	propionate-inducible, pBR322 *ori*	(Lee and Keasling, 2005)
pKP999	pPro24 expressing *exbD*	Present study

pKP1000	ExbD(A92C)	Present study
pKP1049	ExbD(D25N, A92C)	Present study
pKP1055	ExbD(D25H)	Present study
pKP1064	ExbD(D25N)	Present study
pKP1191	ExbD(D25A)	Present study

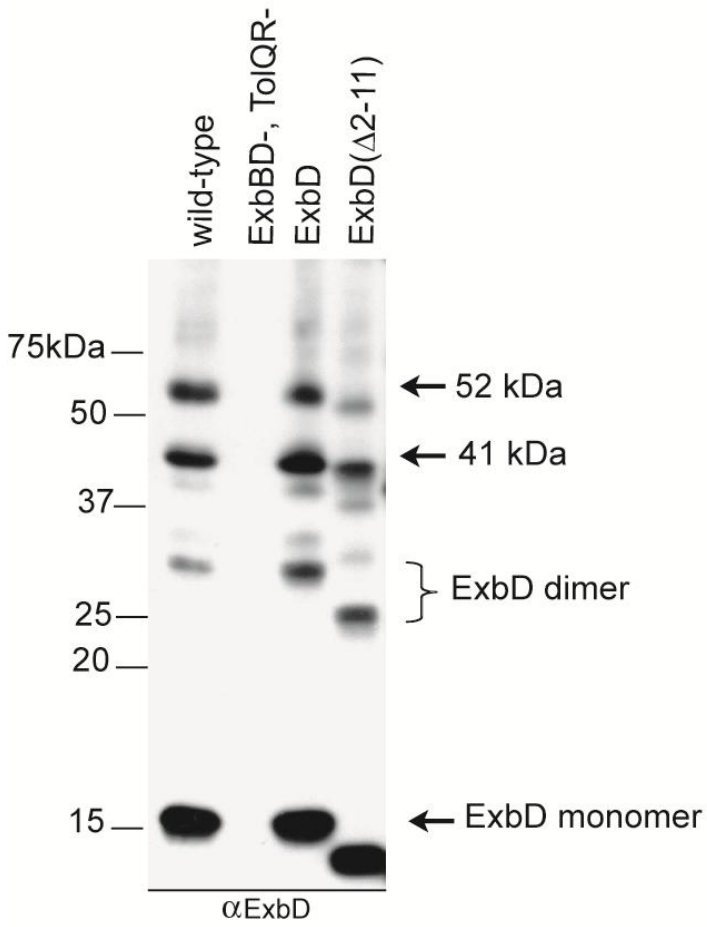
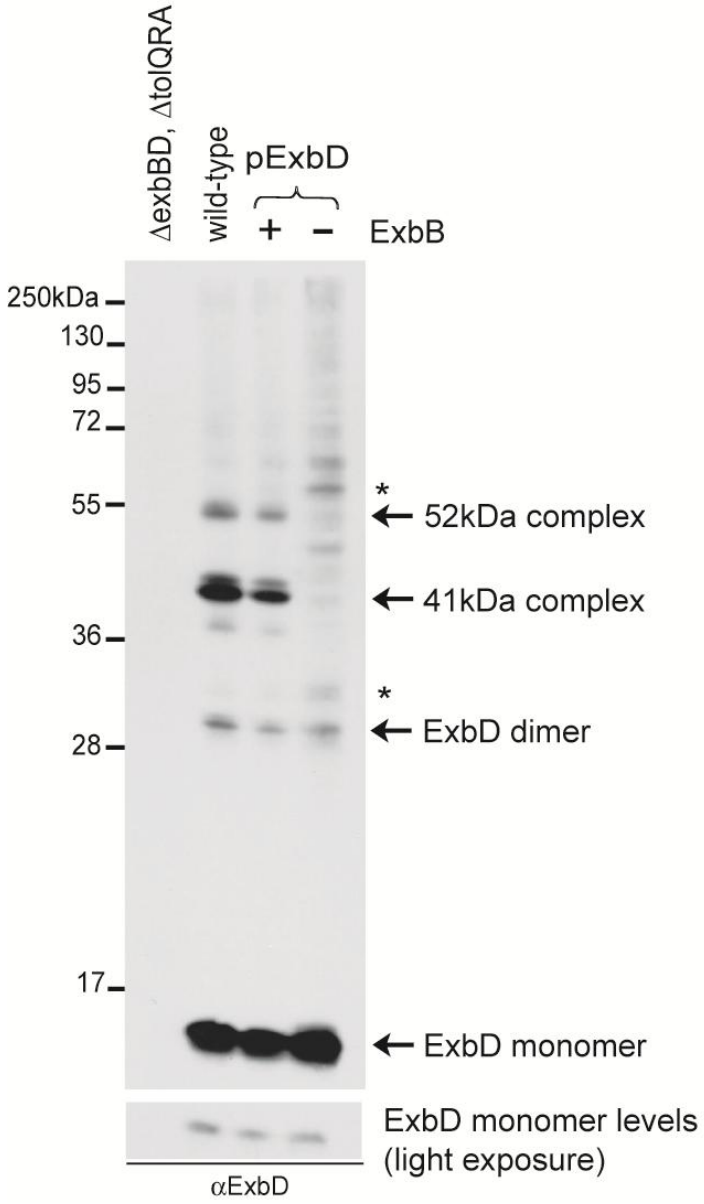


Figure 2-1, Ollis *et al*.



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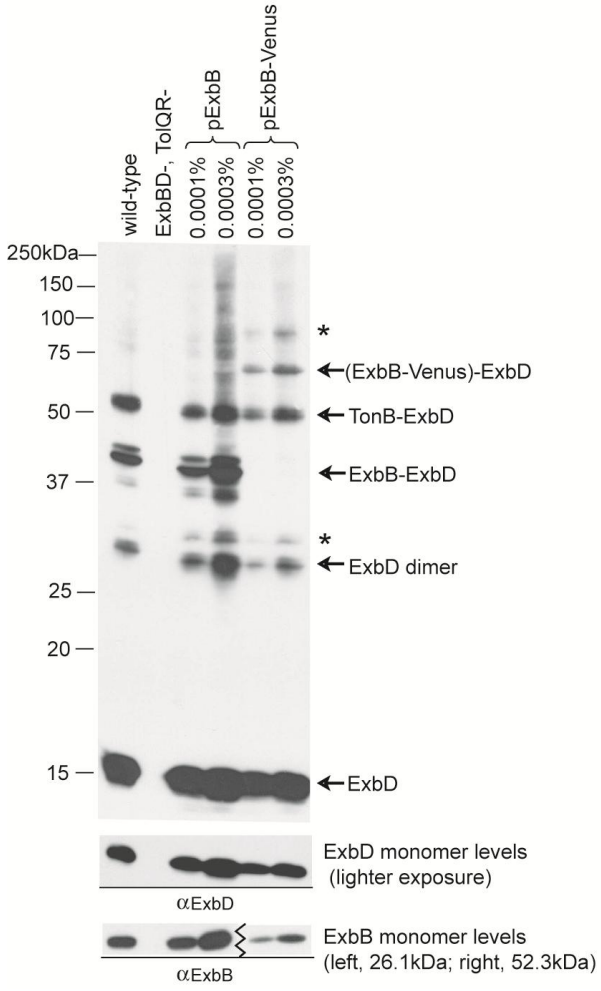

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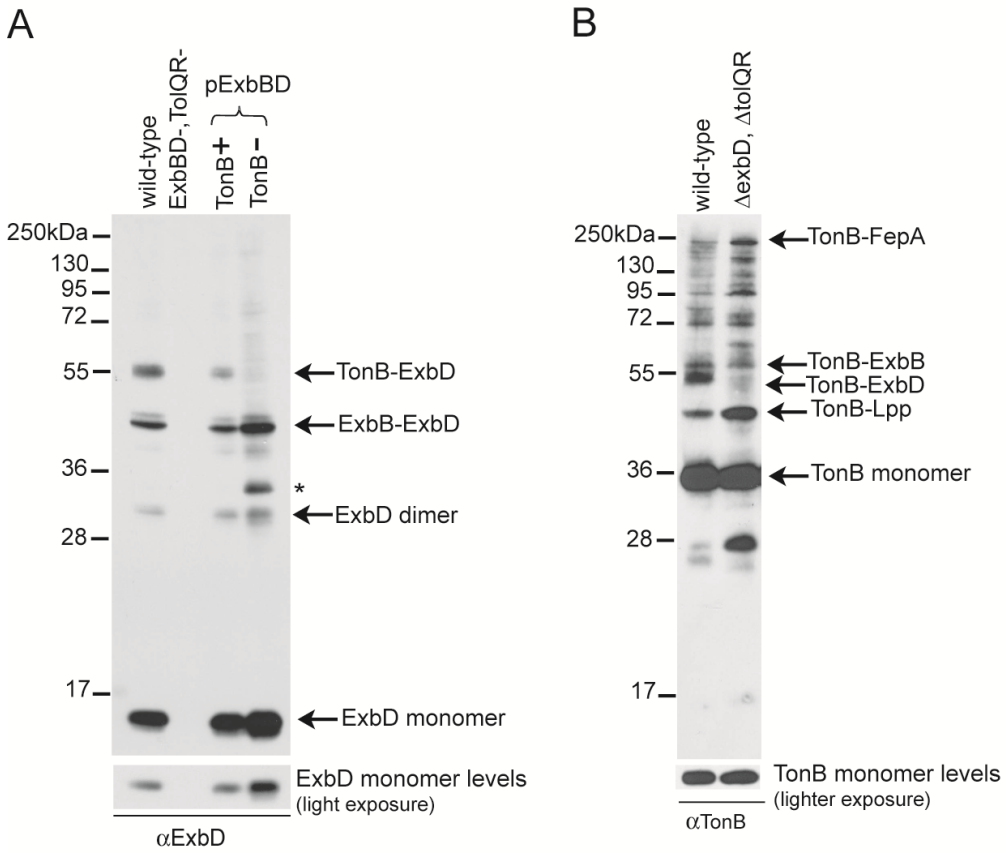

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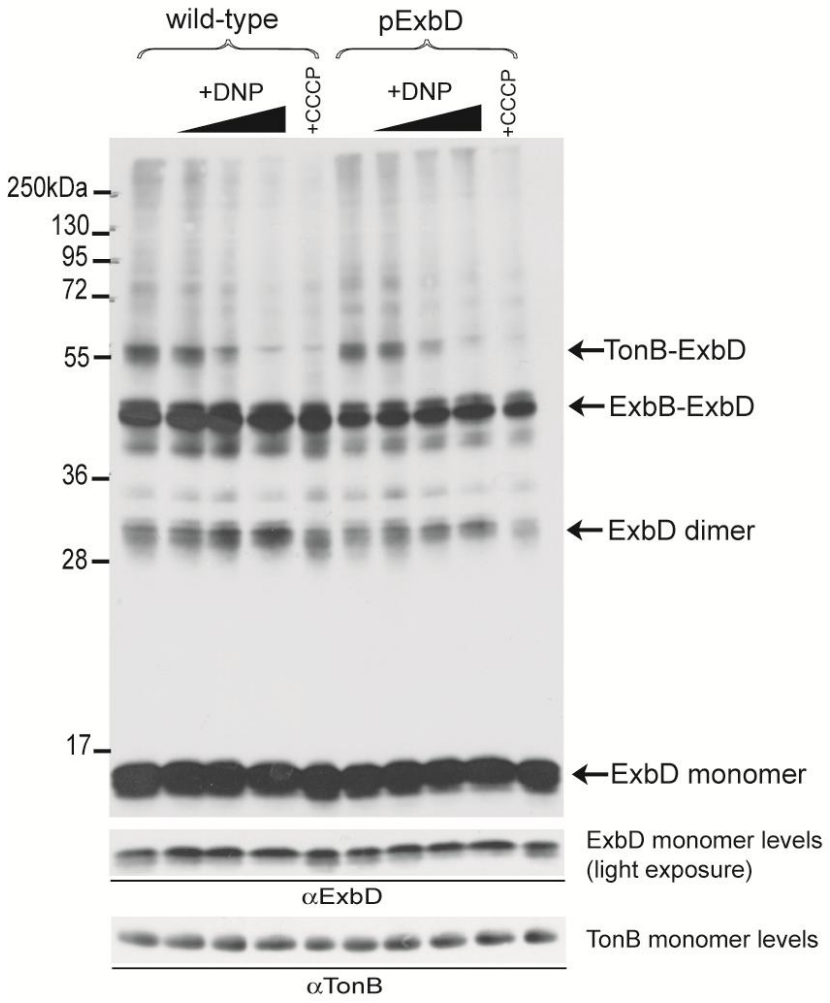

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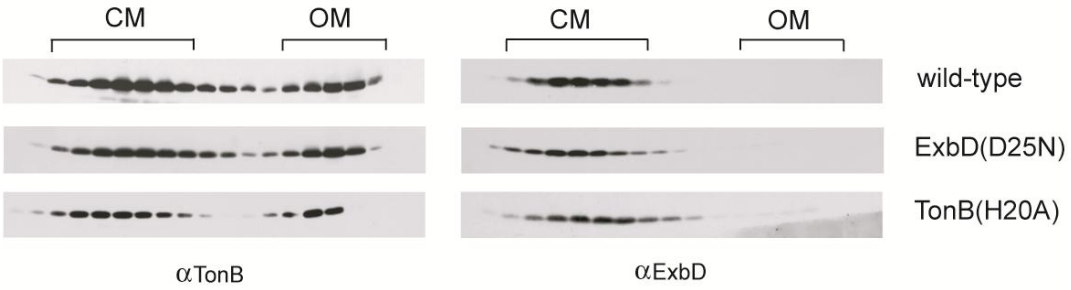

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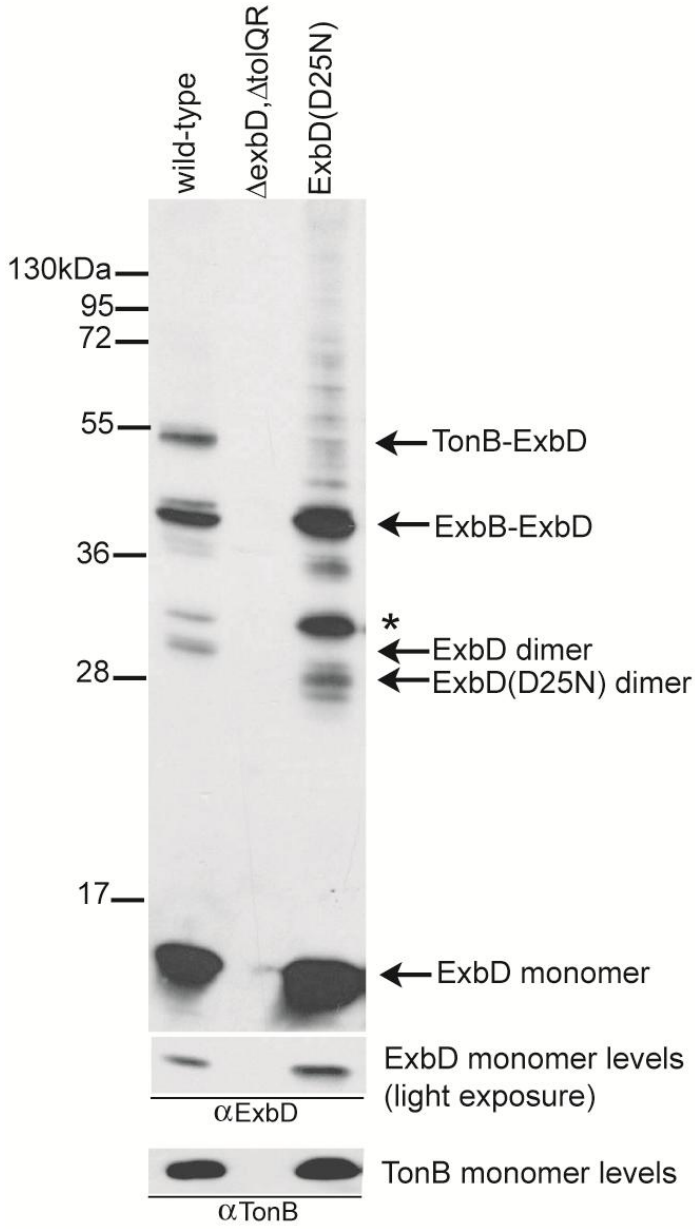

Figure 2-7, Ollis *et al*.

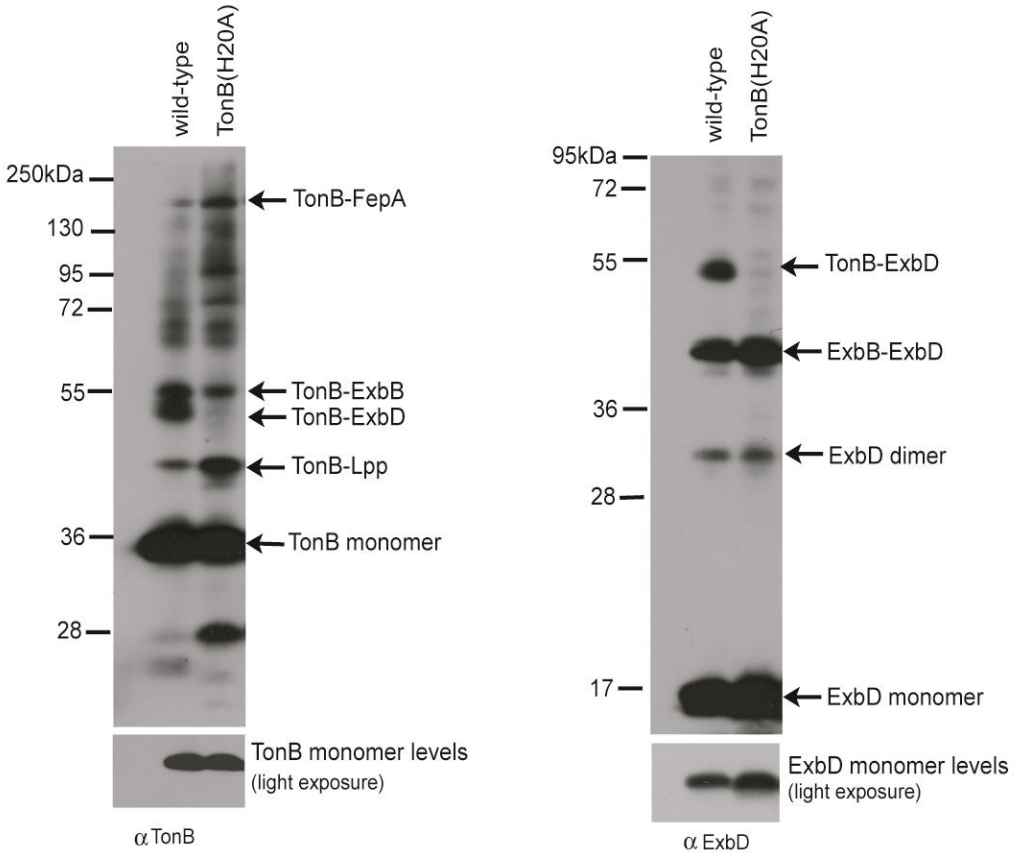

Figure 2-8, Ollis *et al*.

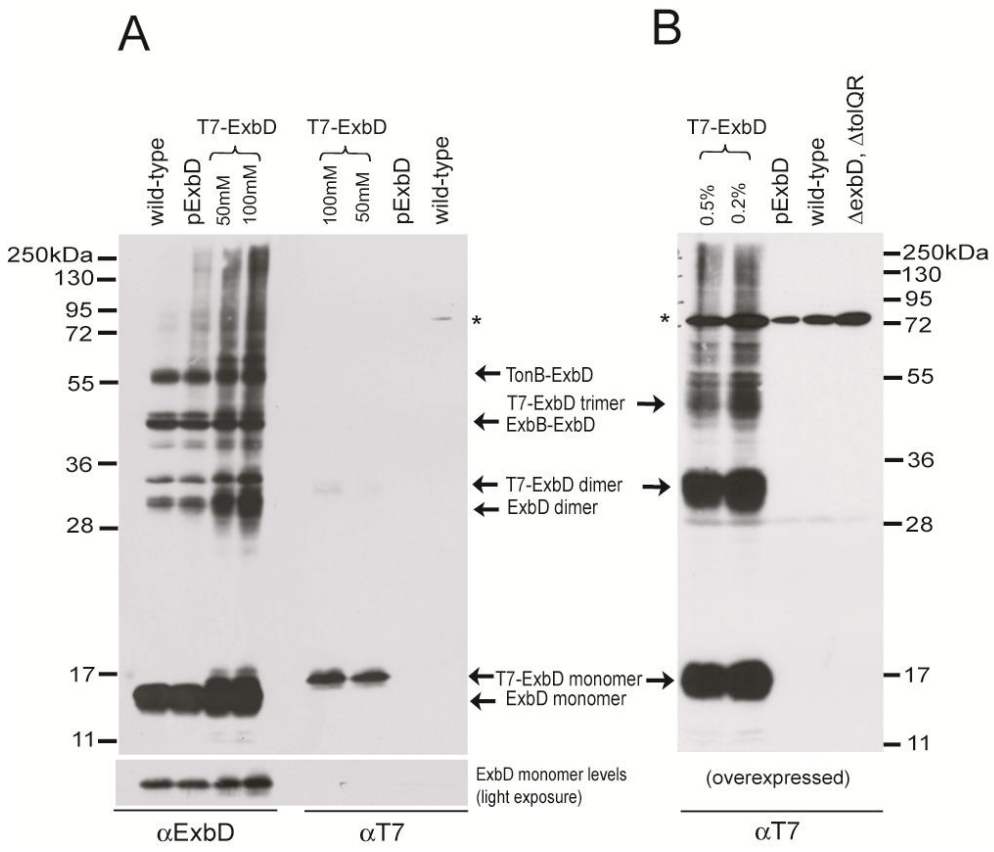

Figure 2-9, Ollis *et al*.

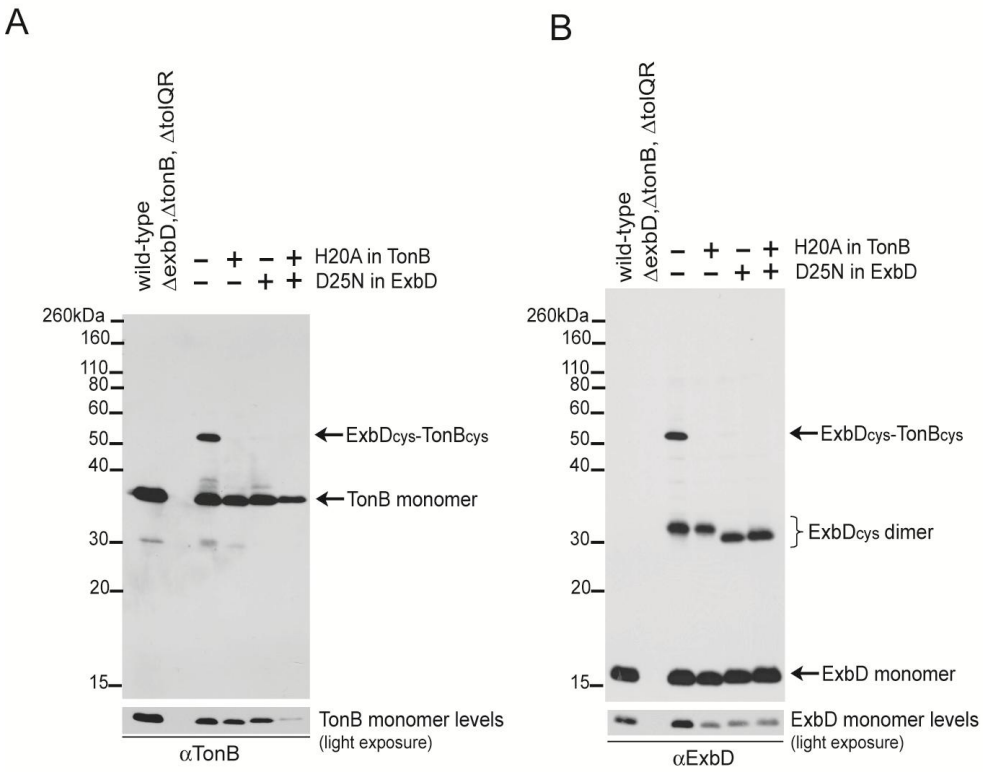

Figure 2-10, Ollis *et al*.

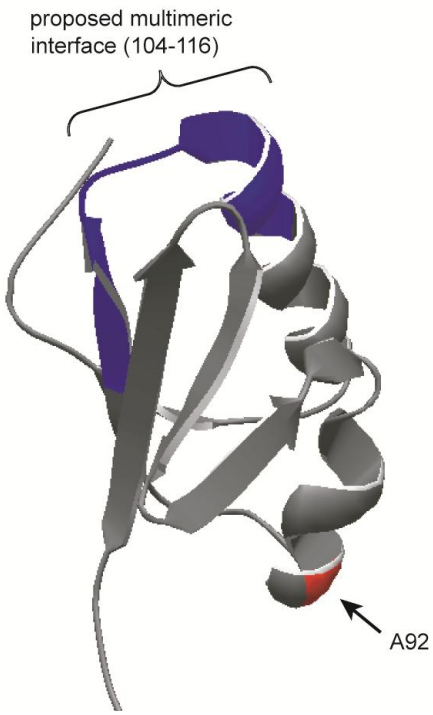

Figure 2-11, Ollis *et al*.

CHAPTER 3

EXBD MUTANTS DEFINE INITIAL STAGES IN TONB ENERGIZATION

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Chapter 3

ExbD Mutants Define Initial Stages in TonB Energization

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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; TMD, transmembrane domain

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 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 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. 3-1A, 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. 3-1A, 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. 3-1A, 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. 3-1A, lane 6 and 1B, lane 4). When CCCP was washed away and pmf restored, TonB again became completely sensitive to proteinase K (Fig. 3-1B, lane 5), like TonB in spheroplasts (Fig. 3-1A, 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. 3-1B, 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. 3-1B, 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.

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. 3-2, 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. 3-2, 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. 3-2, 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. 3-2, 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. 3-3A, sph, lane 5). However, at the 2 min time point, a low level of the proteinase K resistant fragment was detected (Fig. 3-3A, 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.

However, when pmf was collapsed, stable accumulation of the TonB proteinase K resistant conformation was observed across the time-course (Fig. 3-3A, 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. 3-3A, 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. 3-3B, 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. 3-3A, sph, lane 5 and Fig. 3-3B, 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. 3-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. 3-3B, 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. 3-3A, lane 7). Taken together these results suggested that 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. 3-3A, 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. 3-3B, 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. 3-3A lane 7 and 3B lanes 11 and 14). Because this ~28 kDa fragment was also present in

the Δ*exbD* (Fig. 3-3B, 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. 3-3A, sph, lane 7), with the degree of formation equal to a wild-type strain after collapse of pmf (compare to Fig. 3-3A, 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. 3-3A, 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. 3-3A, 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. 3-1 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. 3-1B, lane 5) to the full length of ExbD becoming resistant to proteinase K when pmf was collapsed (Fig. 3-1B, lane 4). As seen for TonB, these pmf-dependent conformational changes of ExbD were reversible (Fig. 3-1B, 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. 3-4A, sph, lanes 2 through 5). Like TonB, after collapse of the pmf, ExbD remained stalled in the proteinase K resistant conformation (Fig. 3-4A, 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. 3-4A, 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. 3-4B, lane 6), ExbD L132Q (Fig. 3-4B, lane 12), and TonB H20A (Fig. 3-4B, 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. 3-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.

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. 3-5, 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. 3-3B, 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. 3-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. 3-5, 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. 3-3A, 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. 3-5, 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. 3-3A, ExbD sph and Fig. 3-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. 3-1B, 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 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 3-1. 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 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].

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Figure Legends

Fig. 3-1. 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 a proteinase K resistant fragment of TonB.

Fig. 3-2. 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 a proteinase K resistant fragment of TonB. "**" indicates an unidentified crosslinked complex of the TonB proteinase K resistant fragment.

Fig. 3-3. 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.

Fig. 3-4. 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.

Fig. 3-5. 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.

Table 3-1. Strains and plasmids used in this study.

Strain or Plasmid	Genotype or Phenotype	Reference
Strains		
W3110	F^- IN*(rrnD-rrnE)1*	[50]
RA1045	W3110, Δ*exbD*, Δ*tolQR*	[33]
KP1344	W3110 *tonB, P14*::*blaM*	[25]
Plasmids		
pPro24	sodium propionate (2-methyl citrate)-inducible, pBR322 *ori*	[51]
pKP381	TonB H20A	[27]
pKP999	*exbD* in pPro24	[32]
pKP1064	ExbD D25N	[32]
pKP1333	ExbD L132Q	Present study

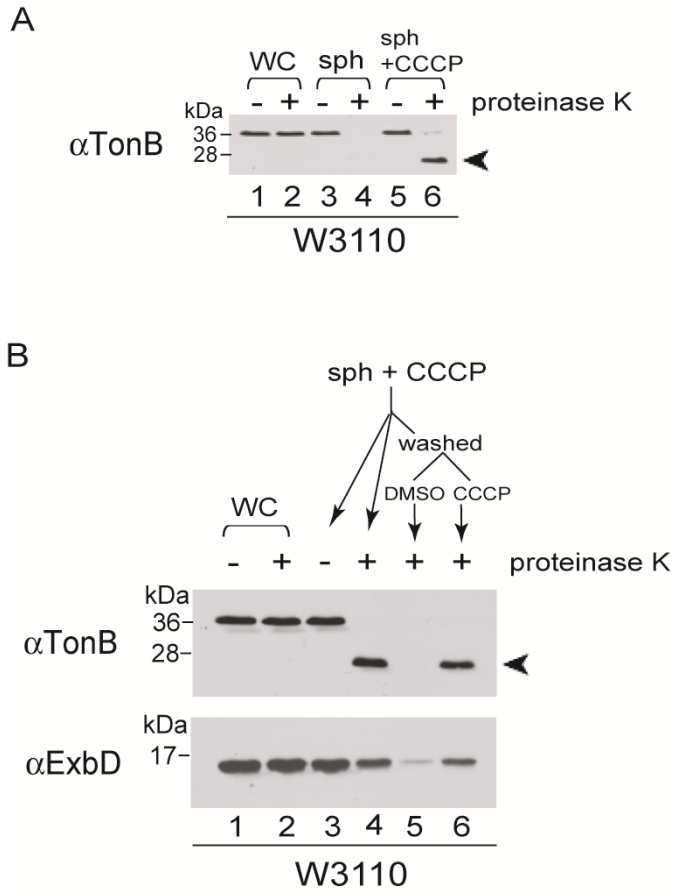

Figure 3-1, Ollis and Postle

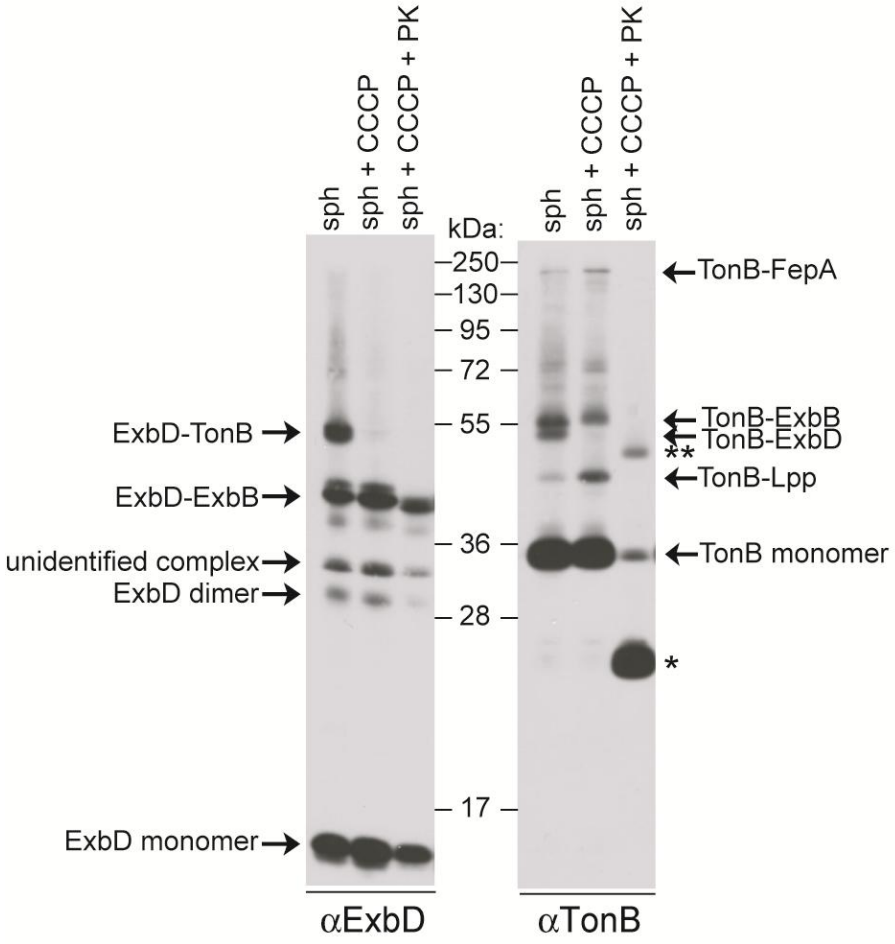

Figure 3-2, Ollis and Postle

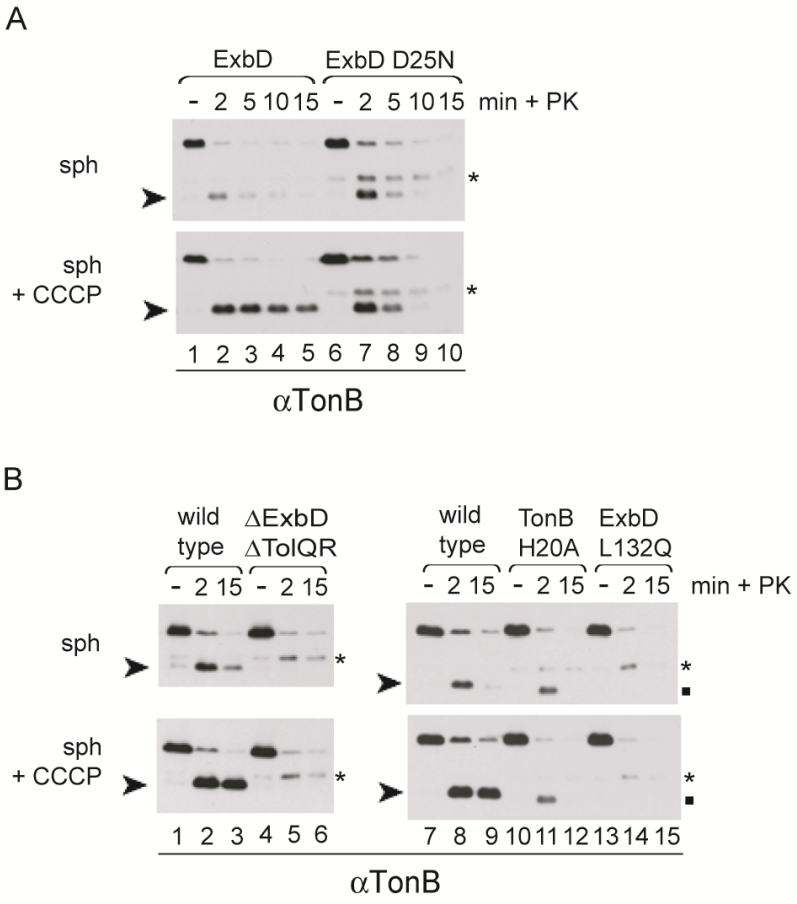

Figure 3-3, Ollis and Postle

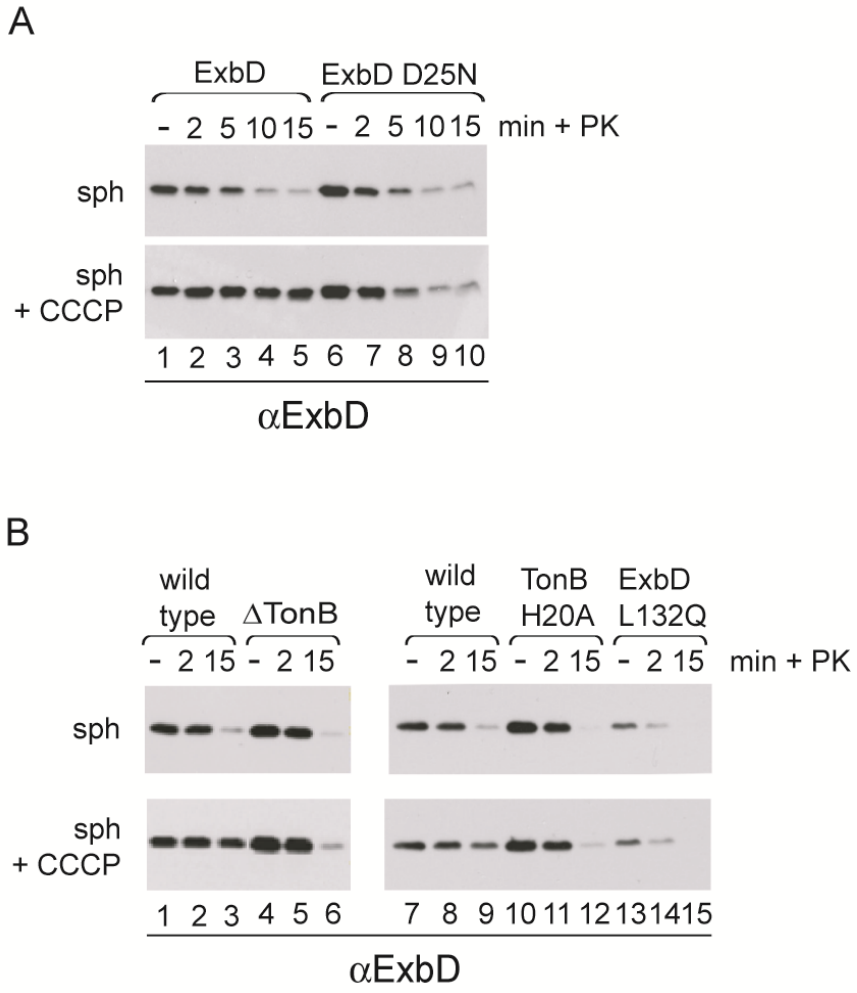

Figure 3-4, Ollis and Postle

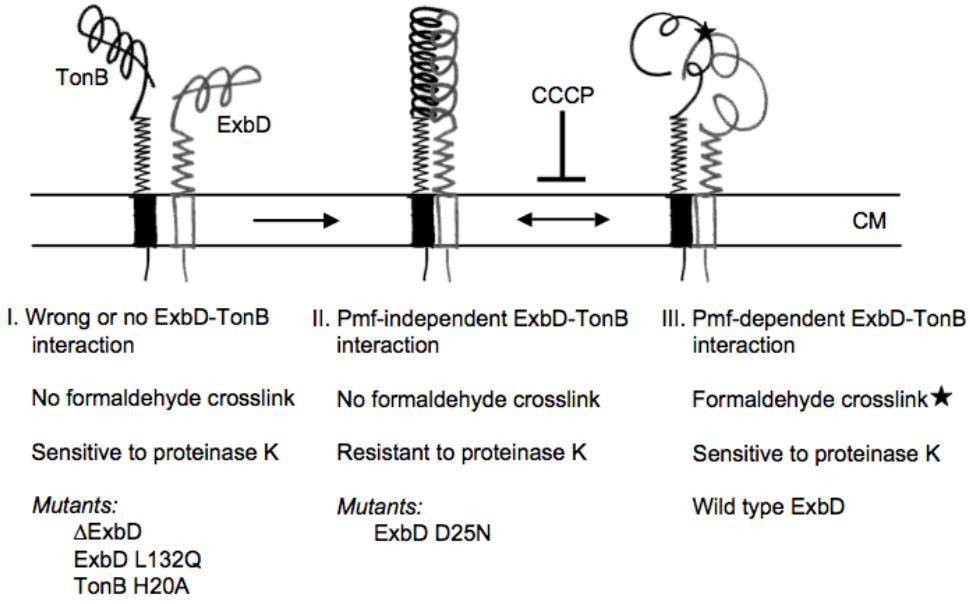

Figure 3-5, Ollis and Postle

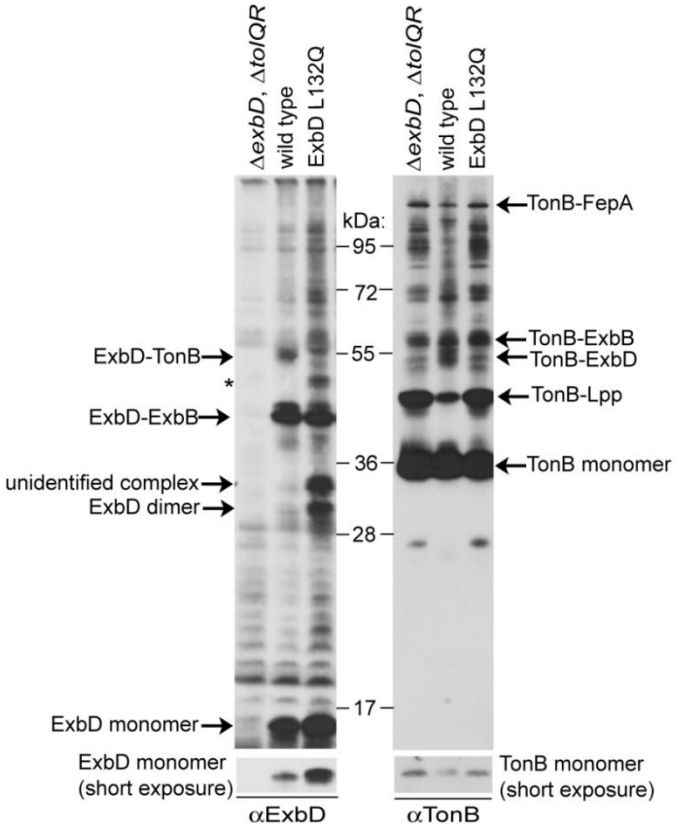

Figure 3-S1. ExbD L132Q does not form a formaldehyde crosslinked complex with TonB. A Δ*exbD*, Δ*tolQR* strain (RA1045), a strain expressing wild-type chromosomally-encoded ExbD (W3110), and a Δ*exbD*, Δ*tolQR* strain expressing plasmid-encoded ExbD L132Q (RA1045/pKP1333) were crosslinked with formaldehyde as previously described (Higgs *et al*., 1998). Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted with ExbD-specific polyclonal antibodies or TonB-specific monoclonal antibodies. ExbD specific complexes are identified on the left. TonB-specific complexes are identified on the right. Positions of molecular mass standards are indicated in the center. "*" indicates an unidentified complex.

CHAPTER 4

FUNCTIONAL DIVISION OF THE EXBD PERIPLASMIC DOMAIN IN ENERGIZATION OF TONB

This chapter is in preparation for submission. AAO is the first author of this work. AK constructed the initial set of ExbD 10-residue deletions. The format of the following chapter is slightly different than the preceding chapters to accommodate publication guidelines.

Chapter 4

Functional Division of the ExbD Periplasmic Domain in Energization of TonB

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Abstract

The TonB system of Gram negative bacteria energizes active transport of diverse nutrients through high-affinity TonB-gated outer membrane (OM) transporters (TGTs) using energy derived from the cytoplasmic membrane (CM) protonmotive force (pmf). CM proteins ExbB and ExbD harness the energy to energize TonB, which directly contacts and transmits this energy to ligand-loaded TGTs. The periplasmic domain of ExbD appears to transition from pmf-independent to pmf-dependent interactions with TonB, catalyzing the conformational changes of TonB. A ten-residue deletion scanning analysis showed that all but the extreme amino terminus of ExbD were indispensable for function. Distinct roles for the amino and carboxy terminus regions of ExbD were evident. Residues 42-61 were essential for the conformational response of both ExbD and TonB to pmf. ExbD residues 62-141 were required for proper assembly with the periplasmic domain of TonB.

Introduction

In Gram negative bacteria, specific high-affinity TonB-gated transporters (TGTs) bind large, scarce, and essential nutrients for active transport across an unenergized outer membrane (OM). The TonB system, with a complex of TonB, ExbB, and ExbD in the cytoplasmic membrane (CM), couples energy derived from the CM protonmotive force (pmf) to TGTs, energizing active transport of nutrients into the periplasm. TonB-dependent substrates include iron-siderophore complexes, vitamin B_{12}, heme, maltodextrin, sucrose, and nickel [1; 2; 3; 4; 5]. The precise mechanism by which TonB transmits energy to TGTs is unknown. ExbB and ExbD are thought to harness the energy of the pmf to energize TonB. TonB maintains its amino-terminal association with the CM, spans the periplasm to directly contact TGTs through its

periplasmic domain, transmits energy when ligand is bound, and is recycled to initiate the cycle again [6; 7; 8; 9; 10]. The TonB periplasmic domain is dynamic—three conformations have been identified *in vivo* [11]. Conformation of the TonB periplasmic domain changes in response to pmf. These conformational changes require ExbB, ExbD, pmf, and a wild-type TonB TMD [7; 12]. His20 is the only TonB TMD residue that cannot be functionally substituted with ala [13]. The TonB TMD, however, is almost certainly not involved directly in response to pmf, as His20 can be functionally substituted with non-protonatable asn. Thus His20 is proposed to have a structural role in this system [14]. In contrast to the TMD, no single residue of the TonB periplasmic domain is essential for TonB function. In fact, large regions can be deleted, and TonB retains function [15; 16; 17].

ExbD (141 amino acids) has an identical topology to TonB (239 amino acids), with a carboxy-terminal periplasmic domain (more than 60% of the protein), a single transmembrane domain (TMD), and a short cytoplasmic amino terminus. ExbD is thought to direct the conformational changes of the TonB periplasmic domain [11; 18]. To date, two point mutations are the only insight into functionally important ExbD residues. ExbD D25, in the TMD, is essential for ExbD function, and a D25N substitution renders ExbD inactive [19]. Corresponding TMD asp residues are also essential in ExbD paralogues TolR and MotB [20; 21]. These residues are thought to be part of proposed proton translocation pathways of their respective systems, though this has not been directly tested. ExbD L132Q, containing a periplasmic domain substitution, is also inactive [19]. The role of L132 in ExbD function is currently unknown.

ExbD is involved in multiple protein-protein interactions *in vivo*—a homodimer and heterodimers with TonB or ExbB can be crosslinked with formaldehyde. The TonB-ExbD formaldehyde crosslinked heterodimer represents an energized interaction that occurs only when pmf is present. In contrast, ExbD-ExbB heterodimers and ExbD homodimers are observed even

in the absence of pmf, indicating the conformations of the regions where these crosslinks form do not significantly change in response to pmf [18].

Here we provide the first detailed insights into the function of ExbD protein using a 10-residue deletion scanning analysis. This "global" mutagenesis approach generated distinct phenotypes specific to particular regions of ExbD. In particular, two functional regions of the ExbD periplasmic domain were identified. These functionally important regions could have been missed by directed mutagenesis studies as no previous ExbD studies have guided focus to these regions. In addition, mutations of individual codons that give strong phenotypes are rare. The region from residues 42-61 was required for energization of TonB. The region from residues 62-141 (the carboxy terminus) was important for proper interaction with TonB and appeared to contain a 30 residue region important in supporting multiple ExbD protein-protein interactions.

Results

The ExbD periplasmic domain is important for ExbD stability and activity

Only one previous study has identified inactive variants of ExbD, by construction of TMD mutant ExbD D25N and isolation of a spontaneous periplasmic domain mutant ExbD L123Q [19]. No comprehensive studies of ExbD have been carried out to identify regions of ExbD important for function. A 10 residue deletion analysis approach previously identified several dispensible regions of the ExbD paralogue MotB [22], suggesting this method could be used to consider an entire protein and narrow in on important regions. To further define functional regions of ExbD, 14 consecutive 10 amino acid deletions were constructed along the length of the ExbD from residues 2-21 (cytoplasmic amino terminus), 22-41 (TMD is 23-43), and 42-141 (periplasmic carboxy terminus) (Fig. 4-1A). Six of the periplasmic domain deletions, from 62-

131, mapped to the region of defined tertiary structure on the ExbD NMR structure, but were not based on it (Fig. 4-1B). This structure was determined in the absence of both the ExbD TMD and pmf, both of which are essential for ExbD function [23]. It is unknown if this conformation is important for ExbD function.

Deletions were expressed from plasmids encoding *exbD* under control of the arabinose promoter. *ExbB*, which is the first gene in the operon with *exbD*, was present on the chromosome of KP1522 under control of its native promoter, and the *exbD* gene was deleted. For activity assays, attempts were made to express all ExbD deletions at levels equal to native, chromosomally-encoded ExbD. For deletions in the amino terminus or TMD, chromosomal levels could be achieved; deletions in the TMD region appeared to be highly stable (Fig. 4-2 A and B, chromosomal *exbB*, pExbD). However, 8 of the periplasmic deletions covering the region from 62-141 were proteolytically unstable, and expression levels equal to that of chromosomally-encoded ExbD could not be achieved with even the highest concentration of arabinose.

The same set of deletion variants was then re-constructed on plasmids encoding both *exbB* and *exbD* under control of the arabinose promoter. In this background, the subset of ExbD periplasmic domain ten amino acid deletions, residues 82-131, were still proteolytically unstable, but chromosomally encoded levels of expression could be supported with the concurrent expression of ExbB from the plasmid (Fig. 4-2B, pExbB, ExbD). Because all ExbD variants from these constructs could be expressed near levels of native ExbD by varying arabinose levels, further experiments used this set of plasmids. Because different deletions required different levels of arabinose as detailed in the Materials and Methods, the levels of ExbB also varied considerably.

The activities supported by chromosomally-encoded levels of the ExbD deletions were assayed by spot titers and ferrichrome-mediated iron transport. Spot titers, which are capable of detecting very low levels of TonB activity, measure sensitivity to colicins and bacteriophage that

enter and subsequently kill *E. coli* via the TonB system [24]. 13 of the 14 mutants showed complete tolerance to TonB-dependent colicins and bacteriophage (Table 4-1). Only ExbDΔ2-11 was active, supporting essentially full sensitivity to colicins (Table 4-1) and transporting ^{55}Fe-ferrichrome at a rate near wild-type, plasmid-encoded ExbD (Table 4-2). As expected, no transport was observed for the other 13 deletion variants (data not shown).

Identification of an ExbD periplasmic domain required to energize TonB

TonB undergoes a pmf-dependent change in conformation that is evidenced, in spheroplasts, by full sensitivity to 15 minute treatment with exogenous proteinase K in the presence of pmf and a proteinase K resistant fragment of TonB when pmf is collapsed, such as by addition of the protonophore CCCP [7]. Recent work suggests the proteinase K resistant conformation of TonB represents a successful pmf-independent initial interaction between TonB and ExbD periplasmic domains. This interaction occurs prior to the effect that pmf seems to have, of rearranging the nature of the initial TonB-ExbD interaction. In the absence of pmf, this pmf-independent stage is prevented from progressing further resulting in accumulation of the proteinase K resistant fragment. ExbD D25N mimics the effect of CCCP, resulting in the proteinase K resistant fragment of TonB even if cellular pmf is intact. In contrast, ExbD L132Q does not support the proteinase K resistant form of TonB, apparently because it does not properly assemble with the TonB periplasmic domain (Ollis and Postle, submitted).

The ExbD deletions were surveyed for their ability to assemble with TonB, by supporting formation of the TonB proteinase K resistant fragment. A time-course of proteinase K treatment was examined, spanning 2-15 min, since a weak or transient interaction may not stably accumulate over 15 min (Ollis and Postle, submitted). As expected, ExbD Δ2-11, which is active, fully supported formation of the TonB proteinase K resistant conformation and the pmf-

dependent conformational changes permitting accumulation only after collapse of pmf (Fig. 4-3A). ExbDΔ2-11, itself, exhibited sensitivity to proteinase K by 15 min but stable resistance after collapse of pmf, like wild-type ExbD (Fig. 4-3B). For all but three of the inactive ExbD deletions, TonB was entirely sensitive to proteinase K, both in spheroplasts and CCCP-treated spheroplasts, even after just 2 min treatment (Fig. 4-4). The same result was obtained in the same strain, RA1017, without expression of ExbB and ExbD (Fig. 4-4).

ExbDΔ12-21, ExbD Δ42-51, and ExbDΔ52-61 each supported formation of the proteinase K resistant conformation of TonB, though to different degrees. Only a faint band was detected with ExbDΔ12-21, at equal intensity in both the presence and absence of pmf (Fig. 4-4). In contrast, ExbD Δ42-51 and ExbDΔ52-61 strongly supported the proteinase K resistant conformation of TonB at 2 min, in both the presence and absence of pmf (Fig. 4-3A). In both cases the band was more susceptible to proteolysis, but not fully degraded, by 15 min in the presence of pmf and maintained more stable resistance to proteinase K after collapse of pmf. ExbDΔ42-51 and Δ52-61 themselves were resistant to proteinase K in spheroplasts under all conditions tested, similar to wild-type ExbD in the presence of CCCP (Fig. 4-3B).

Inactive ExbD ten amino acid deletions exhibit changed protein-protein interactions

ExbD has been previously shown to formaldehyde crosslink into homodimers, an ExbD-ExbB heterodimer and an ExbD-TonB heterodimer *in vivo* [18]. The ability of all the deletion mutants to replicate this crosslinking profile *in vivo* was examined. Cells expressing the deletion variants were treated with monomeric paraformaldehyde, resolved on SDS-polyacrylamide gels, and immunoblotted using polyclonal ExbD-specific antibody. As previously observed, the only active deletion, ExbDΔ2-11 formed all three known complexes at levels similar to wild-type ExbD [[18] and Fig. 4-5A].

None of the 13 inactive deletions formed a detectable crosslink to TonB. To date, this complex has only been detected with an active ExbD [18]. In cases where there appeared to be a potential TonB-ExbD heterodimer (Δ72-81, for example), the crosslinking profile in the absence of TonB remained the same (Fig. 4-5B). ExbDΔ42-51 and Δ52-61 profiles also were identical with and without TonB (data not shown), indicating that while they were clearly able to interact with TonB to support formation of the pmf-independent proteinase K resistant conformation, as indicated above, they did not form the pmf-dependent ExbD-TonB crosslink.ExbDΔ12-21 exhibited decreased levels of crosslinking to ExbB and homodimer formation. As expected, ExbDΔ22-31 and Δ32-41, each missing half of the native ExbD TMD formed no detectable complexes. Deletion of segments of the TMD may have prevented CM insertion. This was investigated below.

The five variants with deletions within the region from 42-91 could all formaldehyde crosslink to some degree with ExbB and form ExbD homodimers (Fig. 4-5A). Increased formation of the ExbB-ExbD complex was likely due in part to increased levels of ExbB in the strains where high levels of arabinose were required for expression of the ExbD variants near native ExbD levels. However, the relative level of complex formed did not correlate with the level of inducer in all cases. ExbDΔ42-51 and Δ52-61 mediated increased association with ExbB, especially compared with the active ExbDΔ2-11. These three variants were among the most stable and out of the 12 mutants with intact TMDs, required the least inducer to achieve chromosomal levels of expression (see Materials and Methods for induction levels). ExbD Δ62-71 exhibited low levels of complex formation with ExbB that were about equal to its level of homodimer. ExbDΔ72-81 and Δ82-91 formed homodimers with high efficiency.

3 deletion variants encompassing the region from residues 92-121 surprisingly were unable to significantly form any of the expected complexes, including the crosslink to ExbB, a protein with only minor soluble periplasmic domains (Fig. 4-5A). Homodimer formation was

weak for Δ92-101, and no detectable homodimers were trapped for Δ102-111 or Δ112-121. It was unlikely that this region affected ExbD export since the three deletions formed a formaldehyde crosslinked complex with an unknown protein. This unidentified complex was observed for most of the other deletions, each of which had been properly exported based on their ability to form complexes with ExbB and was not detected with either export-deficient (see below) ExbD TMD deletion variant.

The remaining two deletion variants, Δ122-131 and Δ132-141, each complexed with ExbB and into homodimers (Fig. 4-5A). Δ122-131 showed increased homodimer formation and a strong complex with an unknown protein. Intensities of the ExbDΔ132-141 homodimer and complex with ExbB were comparable to those formed with wild-type ExbD.

Deletions within the ExbD TMD prevent CM insertion

The ExbD TMD spans residues 23-43, so two ExbD deletion variants Δ22-31 and Δ32-41, each had only partial potential TMDs (Fig. 4-1A). No formaldehyde crosslinked complexes were observed for either variant, suggesting these mutants were not properly inserted in the CM. To directly test their localization, strains expressing the TMD deletion variants ExbDΔ22-31 and Δ32-41 were fractionated on sucrose density gradients. Both fractionated with soluble proteins (Fig. 4-6A), and in spheroplasts, were sensitive to exogenous proteinase K only after lysis of the CM (Fig. 4-6B), indicating they were retained in the cytoplasm, not inserted into the CM. A faint band of the stable degradation product of ExbD Δ22-31 was still resistant to 15 min proteinase K treatment in lysed spheroplasts. This may be a more proteolytically stable form of this deletion variant.

Discussion

Although ExbD is an essential protein in the TonB system, it has not been subjected to a comprehensive mutagenesis study. We initiated our study of ExbD with a 10-residue scanning mutagenesis. The conformation of the ExbD periplasmic domain was essential to support TonB activity, as all 10 periplasmic domain 10-residue deletions were inactive. They differed, however, in their ability to support pmf-independent interactions with TonB and pmf-dependent conformational changes of TonB. Two functional domains were apparent—the amino-terminal residues 42-61 and carboxy-terminal residues 62-141.

A functional unit of the ExbD amino terminal periplasmic domain and TMD

ExbD Δ42-51 and Δ52-61 were important for the conformational response of ExbD and TonB to pmf. While these deletion variants were fully capable of initial assembly with TonB, they did not support transition to energized TonB-ExbD interaction. These mutants exhibited similar phenotypes to those previously observed with ExbD D25N in all assays [[18] and submitted]. It appears that the region immediately following the ExbD TMD is directly involved in response of ExbD, and consequently TonB, to pmf. ExbD residues 44-66 are disordered in the NMR solution structure of the periplasmic domain [23], and the same region from residues 45-66 was also predicted to be disordered by PONDR™ analysis (Fig. 4-7). TonB residues 102-151 are disordered in the solution structure, with an even larger region of residues ~35-170 predicted to be disordered by PONDR™ [13; 25]. One possibility is that the disordered region of ExbD is important for the carboxy terminus of ExbD to achieve the conformation that allows it to energize TonB, with residues 45-66 serving as a flexible linker propagating changes from the TMD to the structured carboxy terminus of ExbD, involved in direct interaction with the TonB periplasmic

domain. Alternatively, the disordered regions of TonB and ExbD must find each other and collapse into a defined structure for TonB to be correctly energized.

The conformation of ExbD residues 62-141is important for assembly with TonB

While ExbD lacking 10 residues within the amino-terminal region of its periplasmic domain (42-61) was still able to fully support initial assembly with TonB, based on formation of the TonB proteinase K resistant fragment, none of the 8 carboxy-terminal ExbD deletions (62-141) supported this interaction. The 8 deletions from residues 62-141 were also highly unstable. Deletions within this span that remove a large region of the native protein almost certainly have structural ramifications. In the ExbD NMR structure, these deletions encompass the region of defined tertiary structure (Fig. 4-1B). The conformation of at least 5 deletions within this region, however, from residues 62-91 and 122-141, was not so distorted as to prevent ExbD homodimer formation, a known periplasmic domain interaction [18]. The overall conformation of this region from residues 62-141 appeared to be specifically important in mediating initial assembly with the TonB periplasmic domain.

Three deletions within the ExbD carboxy terminus, from residues 92-121, were the most unstable of the 8 C-terminal deletions and notably lacked the ability to form all three known ExbD formaldehyde-crosslinked complexes. An inability to form normally stabilizing interactions could contribute to the proteolytic instability of this region, whether due to conformational distortion preventing interaction or deletion of a region involved in direct interaction. Indeed, this region might constitute an interactive patch on ExbD. Residue, A92, was shown previously to make direct contact with TonB A150 and with another ExbD [18].

Previous work also demonstrated a propensity for ExbD residues 104-116 to be involved in homomultimeric interactions *in vitro* [23]. Consistent with that, loss of homodimer detection for

residue deletions encompassing this region could suggest a role for residues 102-121 in homodimeric interactions *in vivo*. More surprising was the requirement of residues 92-121 for formaldehyde crosslinked interaction with ExbB, since the majority of ExbB residues are localized to the cytoplasm. There is currently no evidence for direct interaction of the ExbD periplasmic domain with ExbB. While direct evidence has not been presented, periplasmic domain interaction at the CM has been suggested between homologous proteins in the Mot and Tol protein systems [26; 27; 28]. The potential for periplasmic domain interaction between ExbD and ExbB and a role for such interaction has yet to be explored.

Only the extreme amino-terminus of ExbD is expendable

TonB is surprisingly tolerant of several large internal amino acid deletions, and no single residue is essential [15; 16; 17]. MotB, a paralogue of ExbD is also tolerant of several 10 amino acid deletions [22]. In contrast, 10 residue deletions at all sites in ExbD except the extreme amino terminus resulted in complete inactivity. The fact that MotB tolerates the deletion of 5 successive 10 residue spans immediately following its TMD raises the question of how mechanistically similar the TonB and MotA/B systems are. The periplasmic domains of ExbD and MotB are entirely dissimilar, including the fact that ExbD lacks the peptidoglycan binding domain of MotB [29]. The MotB periplasmic domain is also more than twice the size of this domain of ExbD. It may be that they have functionally diverged and only require the Asp residue in the TMDs of ExbD and MotB for the same crucial aspect of each mechanism.

Reasons for the inactivity caused by deletion of the second half of the ExbD cytoplasmic domain, Δ12-21, were unclear, but this span of resides appeared to be important for stable interactions of ExbD. ExbDΔ12-21 exhibited weak protein-protein interactions, evidenced by both formaldehyde crosslinking and a low level of the TonB proteinase K resistant conformation.

No conformational response of TonB to pmf was apparent with ExbD Δ12-21 either, as the level of the proteinase K resistant TonB was unchanged after collapse of pmf. Residues 12-15 are not essential for ExbD function since ExbDΔ4-15 retains activity [19]. Residues 16 and 17 are also not essential for function since ExbD(H16A) and ExbD(D17A) are fully active (Ollis and Postle, unpublished). Therefore, residues 18-21, Ile, Asn, Val, and Thr in *E. coli*, may have an important contribution to ExbD function. All four residues have side-chains capable of hydrophobic or hydrogen-bonded interactions, properties that could stabilize protein-protein interactions of ExbD. It is unknown why this cytoplasmic region was apparently important for conformational response of TonB to pmf, but this region borders the TMD (residues 23-43), and essential Asp25 is near the cytoplasmic edge. Recently residues in the cytoplasmic C-terminus of ExbB were also shown to be important for response of TonB to pmf [30]. The importance of individual residues in this region of the ExbD cytoplasmic domain remains to be explored.

Inactivity due to deletion of the last 10 amino acids of ExbD, Δ132-141, may be an effect of removal of Leu 132, previously shown to be important for ExbD activity [19]. While the data were not shown, previous work has described almost full activity of a construct of ExbD fused at residue 134 to β-lactamase [31]. This might suggest at least the last 7 residues of ExbD are dispensable, with the important function of this region coming from Leu 132.

In summary, this work functionally divided the periplasmic domain of ExbD, with a clear separation between the amino terminus and the carboxy terminal 80 residues. A subdomain within the carboxy terminus, residues 92-121, was identified as important in mediating ExbD protein-protein interactions. This provided the first evidence for multiple functional domains of the ExbD periplasmic domain. It will be important to further determine how these specific regions of the ExbD periplasmic domain function in the energization of TonB.

Materials and Methods

Bacterial strains and plasmids

Strains and plasmids used in this study are listed in Table 4-3. KP1522 was constructed by P1*vir* transduction of Δ*exbD*::*cam* from RA1021 into RA1016 (gifts from Ray Larsen), creating W3110, Δ*exbD*::*cam*, Δ*tolQRA*.

A set of ExbD 10 amino acid deletion variants was constructed where the *exbB, exbD* operon was encoded on the plasmid. Plasmids pKP724 and pKP761 through pKP764 were constructed by in-frame deletion of ten *exbD* codons using extra-long PCR, as previously described [32]. All were derivatives of pKP660 [18]. Sequences of *exbB* and *exbD* were confirmed by DNA sequencing. A second set of ExbD deletion variants, pKP1246 through pKP1259, was constructed where the *exbB* gene was not present on the plasmid. pKP1246 through pKP1259 were derivatives of the first set of plasmids that included *exbB*. The second set was constructed by extra-long PCR to create an in-frame deletion of *exbB*. Due to a possible requirement for translational coupling between *exbB* and *exbD*, deletion of *exbB* left intact the initiating ATG plus last 25 codons of *exbB*. Sequences of the *exbB* segment and *exbD* gene were confirmed by DNA sequencing.

pKP920, which expresses only ExbB, was also a derivative of pKP660. Extra-long PCR was used to delete *exbD* from its ATG start codon through 6 bases following the *exbD* TAA stop codon. The sequence of *exbB* was confirmed by DNA sequencing.

pKP1194, *exbD* in pBAD24, was constructed by digestion of pKP999 (*exbD* in pPro24) and pBAD24 with NcoI. Fragments were separated by gel electrophoresis. The 4542 bp fragment of pBAD24 and 506 bp fragment of pKP999, containing *exbD*, were purified by gel extraction and ligated together after treatment of the vector fragment with Antarctic Phosphatase.

Proper orientation of the insert was verified by FspI digestion. The *exbD* sequence in pBAD24 was confirmed by DNA sequencing.

Induction levels for ExbD deletion variants

For assays in T broth (spot titers), the following percentages of arabinose were added at subculture to induce expression of ExbD variants near native levels of ExbD: pKP660 = no inducer, pKP761 = .0001%, pKP760 = .0003%, pKP759 = .05% glucose (to repress basal levels of overexpression), pKP758 = .0001%, pKP762 = .0003%, pKP757 = .001%, pKP756 = .0025%, pKP755 = .001%, pKP754 = .004%, pKP753 = .006%, pKP752 = .008%, pKP763 = .006%, pKP764 = .004%, pKP724 = .002%.

For assays in 1xM9, 37μM Fe (proteinase K accessibility, [^{55}Fe]-ferrichrome uptake, formaldehyde crosslinking, and sucrose density gradient fractionation), the following percentages of arabinose were added at subculture to induce expression of ExbD variants near native levels of ExbD: pKP660 = no inducer, pKP761 = .0008%, pKP760 = .0008%, pKP759 = .3% glucose (to repress basal levels of overexpression), pKP758 = .002% glucose (to repress basal levels of overexpression), pKP762 = .0006%, pKP757 = .0007%, pKP756 = .006%, pKP755 = .008%, pKP754 = .15%, pKP753 = .18%, pKP752 = .2%, pKP763 = .18%, pKP764 = .2%, pKP724 = .006%.

Activity assays

Spot titer assays were performed as described previously [24; 33]. Initial rates of [^{55}Fe]-ferrichrome uptake were determined as described previously [34].

In vivo *formaldehyde crosslinking*

Saturated overnight cultures were subcultured 1:100 into M9 minimal media (above) supplemented with L-arabinose. At mid -exponential phase, cells were treated with formaldehyde as previously described [32]. Crosslinked complexes were detected by immunoblotting with ExbD-specific polyclonal antibodies [35] or TonB-specific monoclonal antibodies [36]. To normalize levels of ExbD monomer after crosslinking, the following ODmL were loaded on the SDS-polyacrylamide gel:

W3110 = 0.2, RA1017/pKP660 = 0.25, RA1017/pKP761 = 0.2, RA1017/pKP760 = 0.4, RA1017/pKP759 = 0.15, RA1017/pKP758 = 0.2, RA1017/pKP762 = 0.2, RA1017/pKP757 = 0.2, RA1017/pKP756 = 0.5, RA1017/pKP755 = 0.35, RA1017/pKP754 = 0.4, RA1017/pKP753 = 0.5, RA1017/pKP752 = 0.45, RA1017/pKP763 = 0.45, RA1017/pKP764 = 0.4, RA1017/pKP724 = 0.42.

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Figure legends

Fig. 4-1. Consecutive 10 residue deletions span the ExbD protein. A, The *E. coli* ExbD amino acid sequence is shown, with consecutive deleted regions indicated above. The predicted secondary structure of ExbD is depicted as arrows for β-sheets and cylinders for α-helices. Secondary structure prediction used the PredictProtein Server (http://www.predictprotein.org), PROFsec output [37]. The predicted topological location of these amino acids is depicted to the right. The topology depiction is not intended to reflect predicted tertiary structure. B, Periplasmic domain deletions are mapped on the ExbD NMR structure (pdb code: 2pfu). Consecutive deletions alternate between black and grayed ribbon for distinction. The initiating reside of each deletion is labeled at the relative start of the deletion, with the exception of Δ42-51 since the structure starts at residue 43. To facilitate reading of the labels, positioning may not mark the exact residue location on the ribbon. The image was generated using Swiss-PdbViewer [38].

Fig. 4-2. ExbB protein stabilizes the ExbD 10-residue deletions. Strains expressing chromosomally encoded wild-type ExbD (W3110) or Δ*exbD*, Δ*tolQRA* (KP1522) or Δ*exbBD*, Δ*tolQRA* strains expressing plasmid-encoded ExbD deletion variants were induced uniformly with 0.2% arabinose. A, upper panel, KP1522/pKP1194 (pExbD), KP1522/pKP1246 (Δ2-11), pKP1247 (Δ12-21), or pKP1248 (Δ22-31); middle panel, RA1017/pKP660 (pExbB, ExbD), RA1017/pKP761(Δ2-11), pKP760 (Δ12-21), or pKP759 (Δ22-31). B, upper panel, KP1522/pKP1249 (Δ32-41) through pKP1259 (Δ132-141); middle panel, RA1017/pKP758 (Δ32-41), pkP762 (Δ42-51), pKP757(Δ52-61), pKP756 (Δ62-71), pKP755 (Δ72-81), pKP754 (Δ82-91), pKP753 (Δ92-101), pKP752 (Δ102-111), pKP763 (Δ112-121), pKP764 (Δ122-131), or

pKP724 (Δ132-141). The lower panel for both is a shorter exposure of the middle panel. ExbD-specific immunoblots show steady-state protein levels from samples TCA precipitated immediately after harvest and resolved on 13% SDS-polyacrylamide gels.

Fig. 4-3. ExbDΔ42-51 and Δ52-61 fully support the proteinase K resistant conformation of TonB. A, Spheroplasts were generated with a wild-type strain (W3110) or a Δ*exbBD*, Δ*tolQRA* (RA1017) strain expressing plasmid-encoded ExbB with ExbD (RA1017/pKP660), ExbDΔ2-11 (RA1017/pKP761), ExbDΔ42-51 (RA1017/pKP762), or ExbDΔ52-61 (RA1017/pKP757). Spheroplasts treated with DMSO (sph) or CCCP (sph +CCCP) were treated without (-) or with proteinase K for 2, 5, 10, or 15 min as described in Materials and Methods. The wild-type strain was included to confirm expression levels of the ExbD variants compared to chromosomally-encoded ExbD, and only the 2 and 15 min time points were included for those samples. TCA precipitated samples were resolved on 11% or 15% SDS-polyacrylamide gels and immunoblotted with (A) TonB-specific monoclonal or (B) ExbD-specific polyclonal antibodies. Data shown are representative immunoblots from at least duplicate assays. Samples shown, immunoblotted with two antibodies, came from the same assay. "*" indicates a proteinase K resistant fragment of TonB. PK = proteinase K.

Fig. 4-4. The majority of inactive ExbD deletion variants do not support formation of the TonB proteinase K resistant conformation. Spheroplasts were generated with a wild-type strain (W3110), a Δ*exbBD*, Δ*tolQRA* (RA1017) strain expressing plasmid-encoded ExbB with ExbDΔ12-21 (RA1017/pKP760), ExbDΔ22-31 (RA1017/pKP759), ExbDΔ32-41 (RA1017/pKP758), pKP756 (Δ62-71), pKP755 (Δ72-81), pKP754 (Δ82-91), pKP753 (Δ92-101), pKP752 (Δ102-111), pKP763 (Δ112-121), pKP764 (Δ122-131), or pKP724 (Δ132-141), and a Δ*exbBD*, Δ*tolQRA* (RA1017) strain. Spheroplasts treated with DMSO (sph) or CCCP (sph

+CCCP) were treated without (-) or with proteinase K for 2 or 15 min as described in Materials and Methods. TCA precipitated samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal. "*" indicates a proteinase K resistant fragment of TonB. PK = proteinase K.

Fig. 4-5. 10-residue deletions alter known ExbD interactions. A, Strains expressing chromosomally- or plasmid-encoded ExbD (W3110 and RA1017/pKP660, respectfully) and ExbD deletion variants [from left to right: RA1017/pKP761 (Δ2-11), pKP760 (Δ12-21), pKP759 (Δ22-31), pKP758 (Δ32-41), pKP762 (Δ42-51), pKP757 (Δ52-61), pKP756 (Δ62-71), pKP755 (Δ72-81), pKP754 (Δ82-91), pKP753 (Δ92-101), pKP752 (Δ102-111), pKP763 (Δ112-121), pKP764 (Δ122-131), or pKP724 (Δ132-141)] were crosslinked with formaldehyde as described in Materials and Methods. Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted. ExbD was visualized with ExbD-specific polyclonal antibodies. Topological location of each deletion is indicated above each lane. Positions of molecular mass standards are indicated on the left. Identities of ExbD-specific crosslinked complexes and the ExbD monomer are indicated on the right. B, Strains expressing chromosomally- or plasmid-encoded ExbB, ExbD (W3110 and RA1017/pKP660, respectfully) and ExbB, ExbD Δ72-81 (RA1017/pKP755) and a Δ*tonB* strain expressing plasmid-encoded ExbB, ExbD (KP1503/pKP660) or ExbB, ExbD Δ72-81 (KP1503/pKP755) were crosslinked with formaldehyde and processed as above.

Fig. 4-6. Deletions removing halves of the ExbD TMD prevent CM insertion. A, Strains expressing chromosomally-encoded (RA1016) or plasmid-encoded (RA1017/pKP660) ExbD and ExbD deletion variants (RA1017/pKP759 and RA1017/pKP758) were fractionated by sucrose density gradient fractionation, as described in Materials and Methods. TCA precipitated samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with ExbD-specific

polyclonal antibodies. Positions of soluble, cytoplasmic membrane (CM), and outer membrane (OM) fractions are indicated above. B, Spheroplasts were generated with a wild-type strain (W3110) or a Δ*exbBD*, Δ*tolQRA* (RA1017) strain expressing plasmid-encoded ExbB and ExbD (RA1017/pKP660), ExbDΔ22-31 (RA1017/pKP759), or ExbDΔ32-41 (RA1017/pKP758). Whole cell (WC), intact spheroplasts (sph), lysed spheroplasts (lysed sph), and collapsed pmf spheroplasts (sph +CCCP) were treated with or without proteinase K as described in Materials and Methods. TCA precipitated samples were resolved on 15% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies. Data shown are representative immunoblots from at least duplicate assays. "+" or "-" above each lane indicates presence or absence, respectively, of added proteinase K. PK = proteinase K

Fig. 4-7. ExbD residues 45-66 are predicted to have significant disorder. The *E. coli* ExbD amino acid sequence (SwissProt accession no. P0ABV2) was analyzed using PONDR®. The line traces values for ExbD residues from the amino to carboxy terminus. Points with PONDR scores exceeding a threshold of 0.5, indicated by regions above the horizontal line, are predicted to be disordered. ExbD residues 45-66 are indicated by the arrow spanning the length of the trace.

Table 4-1: Spot titer assay results

Strain	Phenotype	Susceptibility[a]			
		ϕ80	Colicin B	Colicin M	Colicin Ia
W3110	wild type	9,9,9	8,8,8	6,6,6	7,8,8
RA1017	ΔExbBD, ΔTolQRA	T,T,T[b]	T,T,T	T,T,T	T,T,T
RA1017/pKP660	ExbB, ExbD	7,7,7	5,5,5	4,4,4	6,6,6
RA1017/pKP761	ExbB, ExbDΔ2-11	7,7,7	4,4,4	4,4,4	6,6,6
RA1017/pKP760	ExbB, ExbDΔ12-21	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP759	ExbB, ExbDΔ22-31	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP758	ExbB, ExbDΔ32-41	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP762	ExbB, ExbDΔ42-51	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP757	ExbB, ExbDΔ52-61	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP756	ExbB, ExbDΔ62-71	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP755	ExbB, ExbDΔ72-81	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP754	ExbB, ExbDΔ82-91	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP753	ExbB, ExbDΔ92-101	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP752	ExbB, ExbDΔ102-111	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP763	ExbB, ExbDΔ112-121	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP764	ExbB, ExbDΔ122-131	T,T,T	T,T,T	T,T,T	T,T,T
RA1017/pKP724	ExbB, ExbDΔ132-141	T,T,T	T,T,T	T,T,T	T,T,T

[a]Scored as the highest ten-fold dilution of bacteriophage ϕ80 or five-fold dilution of a standard colicin preparation that provided an evident zone of clearing on a cell lawn.

[b]"T" indicates tolerance to undiluted colicin or phage (no clearing of the lawn).

The values of three platings are presented for each strain/plasmid and colicin or phage pairing. Expression of ExbD variants to near levels of chromosomally-encoded ExbD was verified by immunoblots with ExbD-specific antibodies (not shown).

Table 4-2: Transport of ^{55}Fe-loaded ferrichrome

Strain	Phenotype	Initial Rate of Transport[a]	% Wild-type Activity[b]
KP1522	ΔExbBD, ΔTolQRA	-4.167 ± 2.309	0
KP1522/pKP1194	ExbD	672.2 ± 15.97	100
KP1522/pKP1246	ExbDΔ2-11	654.7 ± 11.55	97

[a]Strains/plasmids indicated were assayed for ability to transport ^{55}Fe-loaded ferrichrome as described in Materials and Methods. Plasmid-encoded ExbD variants were induced with the following percentages of arabinose: pKP1194 = .005%, pKP1246 = .08%

[b]Percent wild-type activity was recorded as the initial rate of transport of the variant strain divided by the initial rate of transport of the wild-type strain (multiplied by 100). Rate of transport by the strain expressing ExbDΔ2-11 was also compared to the rate supported by plasmid-encoded wild-type ExbD (in parentheses). Expression levels for plasmid-encoded ExbD and ExbDΔ2-11 equal to chromosomally-encoded ExbD (W3110) levels were confirmed by Western blot with ExbD-specific polyclonal antibodies (data not shown).

Table 4-3. Strains and plasmids used in this study.

Strain or Plasmid	Genotype or Phenotype	Reference
Strains		
W3110	F⁻ IN*(rrnD-rrnE)1*	[39]
RA1016	W3110, Δ*tolQRA*	Ray Larsen
RA1017	W3110 Δ*exbBD*::*kan*, Δ*tolQRA*	[13]
RA1021	W3110, Δ*exbD*::*cam*	Ray Larsen
KP1503	Gm1, *exbB*::*Tn10*, $tolQ_{am}$, Δ*tonB*::*kan*	[18]
KP1522	W3110 Δ*exbD*::*cam*, Δ*tolQRA*	Present study
Plasmids		
pBAD24	L-arabinose-inducible, pBR322 *ori*	[40]
pPro24	Sodium propionate (2-methyl citrate)-inducible, pBR322 *ori*	[41]
pKP920	*exbB* in pBAD24	Present study
pKP999	*exbD* in pPro24	[18]
pKP660	*exbB, exbD* in pBAD24	[18]
pKP761	ExbB, ExbDΔ2-11	[18]
pKP760	ExbB, ExbDΔ12-21	Present study
pKP759	ExbB, ExbDΔ22-31	Present study
pKP758	ExbB, ExbDΔ32-41	Present study
pKP762	ExbB, ExbDΔ42-51	Present study
pKP757	ExbB, ExbDΔ52-61	Present study
pKP756	ExbB, ExbDΔ62-71	Present study
pKP755	ExbB, ExbDΔ72-81	Present study
pKP754	ExbB, ExbDΔ82-91	Present study
pKP753	ExbB, ExbDΔ92-101	Present study
pKP752	ExbB, ExbDΔ102-111	Present study
pKP763	ExbB, ExbDΔ112-121	Present study
pKP764	ExbB, ExbDΔ122-131	Present study
pKP724	ExbB, ExbDΔ132-141	Present study
pKP1194	*exbD* in pBAD24	Present study
pKP1246	ExbDΔ2-11	Present study
pKP1247	ExbDΔ12-21	Present study
pKP1248	ExbDΔ22-31	Present study
pKP1249	ExbDΔ32-41	Present study
pKP1250	ExbDΔ42-51	Present study
pKP1251	ExbDΔ52-61	Present study

pKP1252	ExbDΔ62-71	Present study
pKP1253	ExbDΔ72-81	Present study
pKP1254	ExbDΔ82-91	Present study
pKP1255	ExbDΔ92-101	Present study
pKP1256	ExbDΔ102-111	Present study
pKP1267	ExbDΔ112-121	Present study
pKP1258	ExbDΔ122-131	Present study
pKP1259	ExbDΔ132-141	Present study

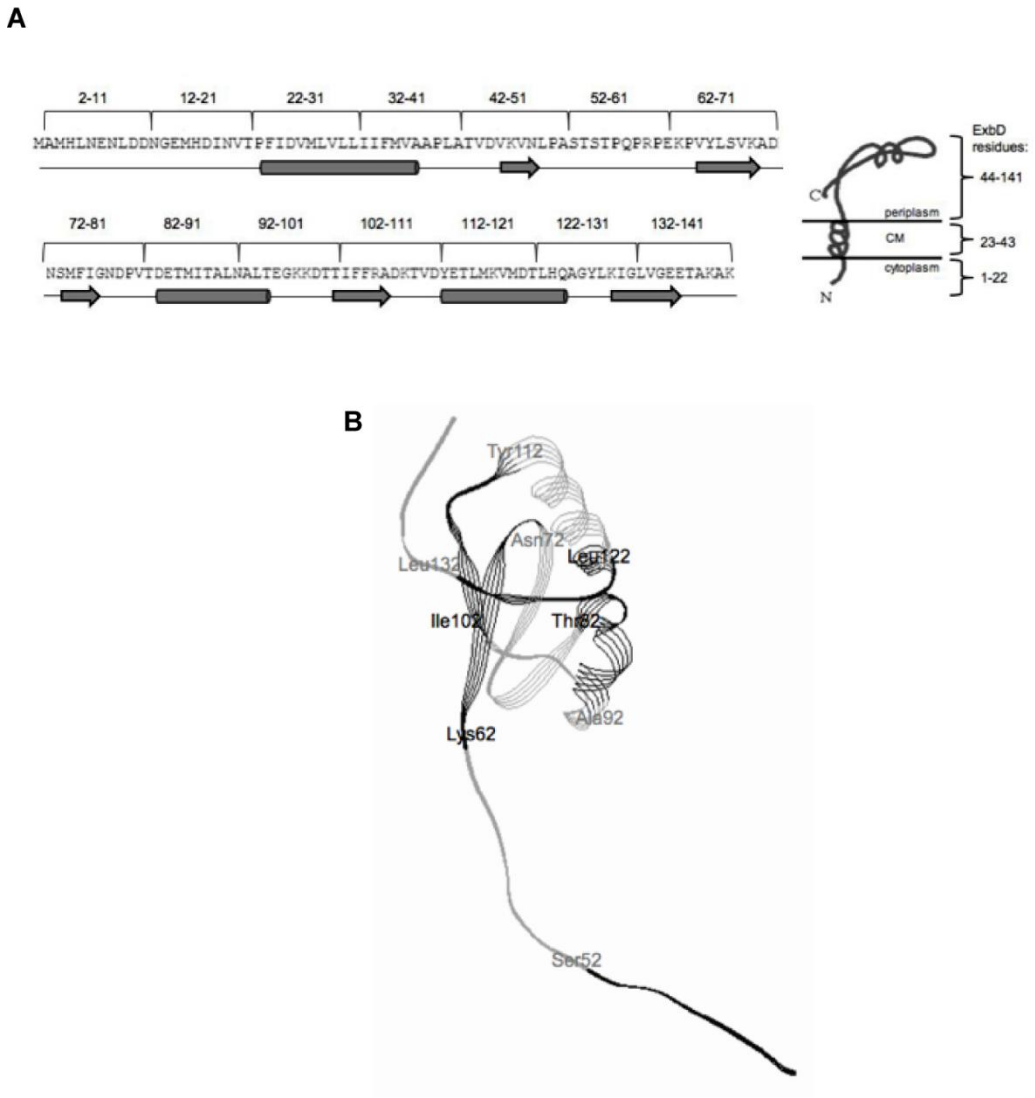

Figure 4-1, Ollis *et al*.

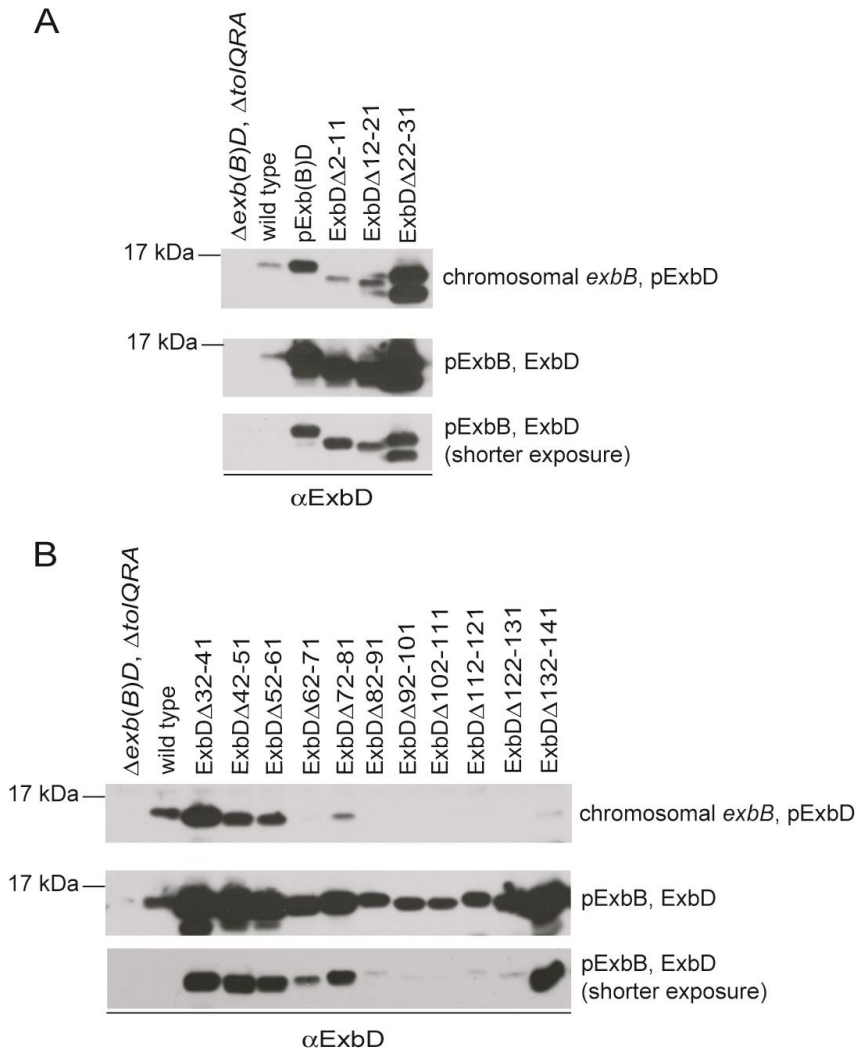

Figure 4-2, Ollis *et al*.

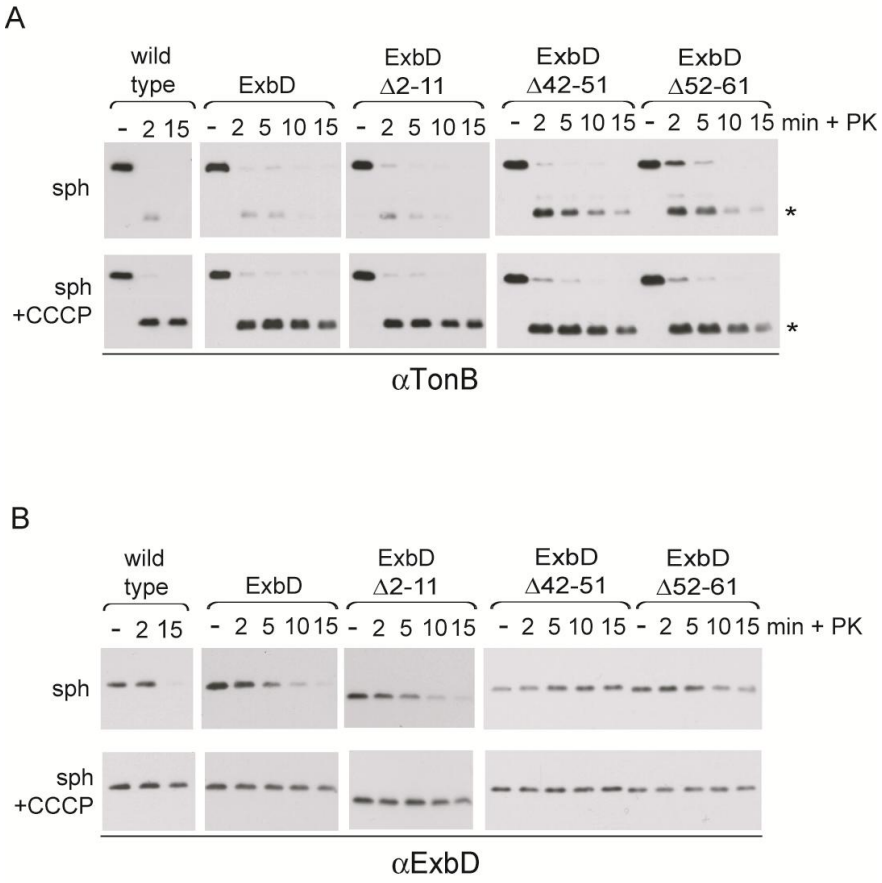

Figure 4-3, Ollis *et al*.

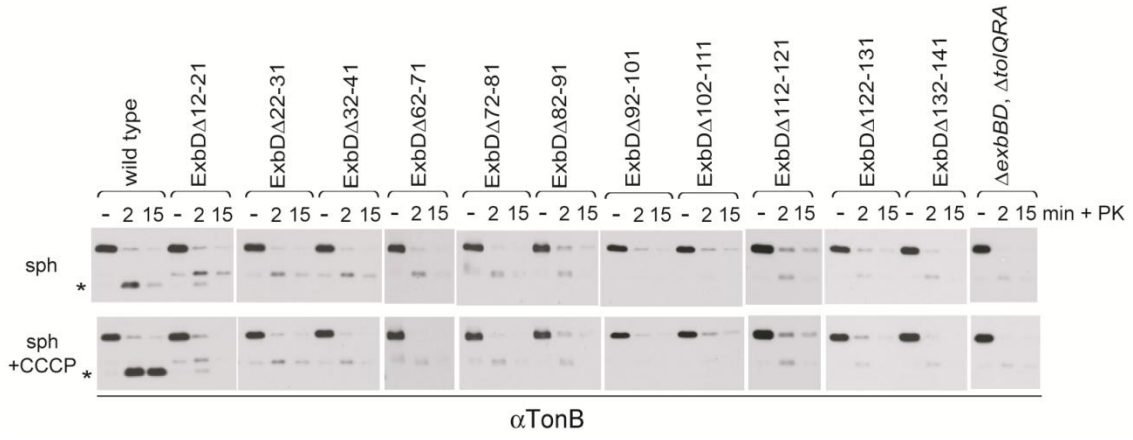

Figure 4-4, Ollis *et al*.

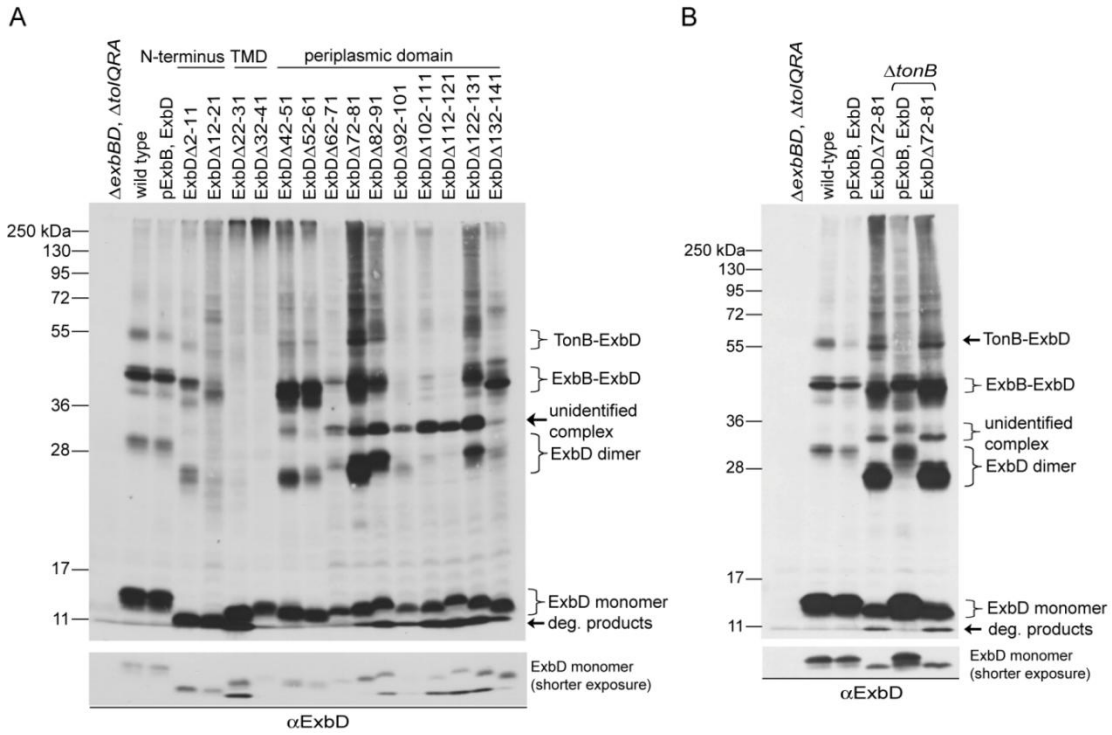

Figure 4-5, Ollis *et al*.

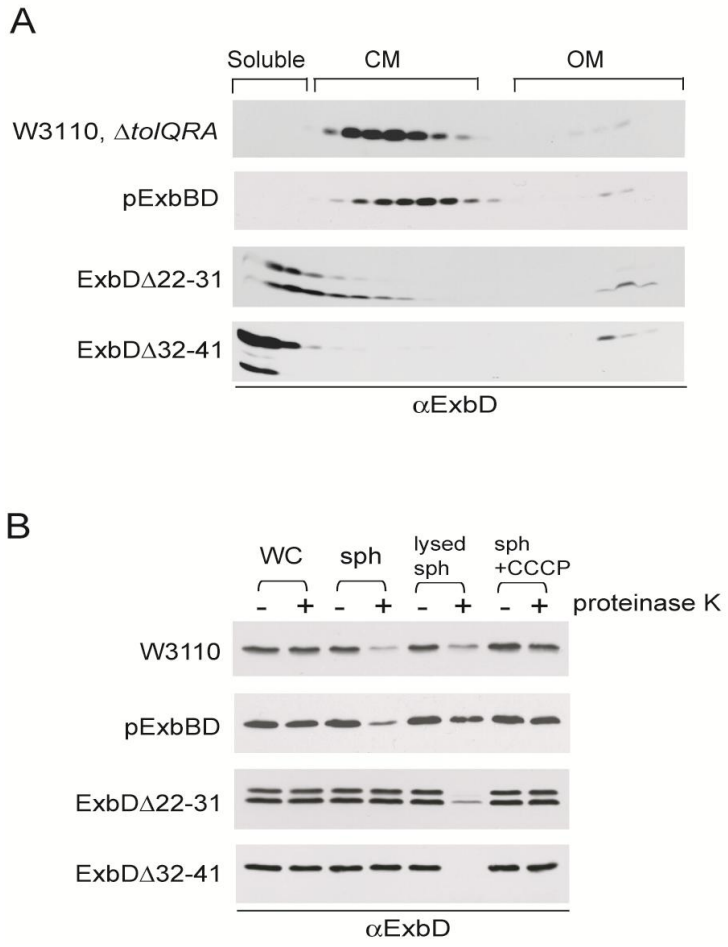

Figure 4-6, Ollis *et al*.

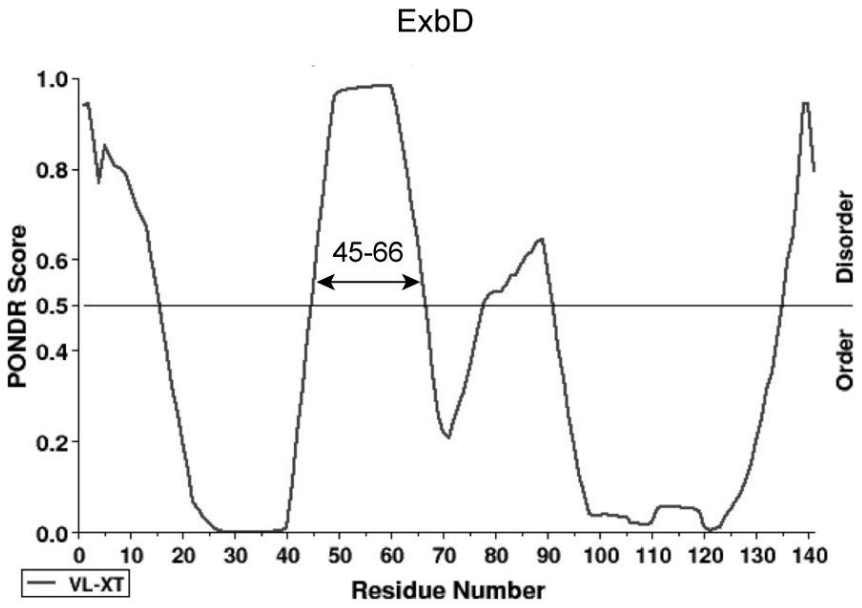

Figure 4-7, Ollis *et al*.

CHAPTER 5

THE SAME PERIPLASMIC EXBD RESIDUES MEDIATE *IN VIVO* INTERACTIONS BETWEEN EXBD HOMODIMERS AND EXBD-TONB HETERODIMERS

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Chapter 5

The Same Periplasmic ExbD Residues Mediate *In Vivo* Interactions Between ExbD Homodimers and ExbD-TonB Heterodimers

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Abstract

The TonB system couples cytoplasmic membrane protonmotive force to TonB-gated outer membrane transporters for active transport of nutrients into the periplasm. In *Escherichia coli*, cytoplasmic membrane proteins ExbB and ExbD promote conformational changes in TonB, which transmits this energy to the transporters. The only known energy-dependent interaction occurs between the periplasmic domains of TonB and ExbD. This study identified sites of *in vivo* homodimeric interactions within ExbD periplasmic domain residues 92-121. ExbD was active as a homodimer ($ExbD_2$) but not through all cys substitution sites, suggesting the existence of conformationally dynamic regions in the ExbD periplasmic domain. A subset of homodimeric interactions could not be modeled on the NMR structure without significant distortion. Most importantly, the majority of ExbD cys substitutions that mediated homodimer formation also mediated ExbD-TonB heterodimer formation with TonB A150C. Consistent with the implied competition, ExbD homodimer formation increased in the absence of TonB. Although ExbD D25 was not required for their formation, ExbD dimers interacted *in vivo* with ExbB. ExbD-TonB interactions required ExbD transmembrane domain residue D25. These results suggested a model where $ExbD_2$ assembled with ExbB undergoes a transmembrane domain-dependent transition and exchanges partners in localized homodimeric interfaces to form an $ExbD_2$-TonB heterotrimer. Findings here were also consistent with our previous hypothesis that ExbD guides the conformation of the TonB periplasmic domain, which itself is conformationally dynamic.

Introduction

The TonB system of Gram negative bacteria couples the protonmotive force (pmf) of the cytoplasmic membrane (CM) to the active transport of a diverse range of large, scarce, or important nutrients across the unenergized outer membrane (1, 24, 25). Active transport across the outer membrane requires TonB-gated transporters, which are 22 stranded β-barrels with lumens occluded by an amino-terminal globular domain called the cork (37).

In addition to the TonB-gated transporters in the outer membrane, the TonB system consists of 3 integral CM proteins, TonB, ExbB, and ExbD. TonB and ExbD have identical topologies, each with a single transmembrane domain (TMD) and more than two-thirds of each protein in the periplasm (15, 20, 43). ExbB has 3 TMDs and significant cytoplasmic domains (21, 22). ExbB and ExbD couple the energy of the CM pmf to conformational changes in the TonB carboxy terminus (33). The TonB carboxy terminus directly contacts the TonB-gated transporters and somehow transmits energy for active transport of substrates. Evidence suggests all three proteins form a complex in the CM, but the stoichiometry of this complex is unknown (2, 38). The cellular ratio of TonB:ExbD:ExbB is 1:2:7 (16).

The only step in TonB energization currently known to require the pmf is characterized by a formaldehyde crosslinkable interaction between the TonB and ExbD periplasmic domains. While TonB is conformationally responsive to changes in the pmf, the absence of protonatable residues in the TonB TMD, among other data, suggests that TonB only responds to pmf by virtue of TonB-ExbD interactions through their periplasmic domains (5, 11, 29, 38, 45).

ExbD can also be trapped in formaldehyde crosslinked homodimers *in vivo*. Unlike the formaldehyde-mediated TonB-ExbD complex, homodimer formation does not require pmf, TonB H20, or ExbD D25 (38). It is unknown if ExbD homodimers are functional. *In vitro* studies pinpointed a specific region of the ExbD periplasmic domain, residues 104-116, as a

homomultimeric interface. Nuclear magnetic resonance (NMR) studies of the ExbD periplasmic domain (residues 43-141) demonstrated extensive aggregation of this isolated domain, with 4-7 copies of the domain forming a homomultimer with 1mM protein at pH 7.0 (10). It is not known if this region mediates homo-dimerization *in vivo*.

In this study, cysteine scanning of a 30-residue region of the ExbD periplasmic domain identified regions involved in homodimer formation *in vivo*, some of which mapped to the region identified in the NMR studies and some of which mapped to the opposite end of the solution structure, suggesting that the ExbD TMD contributes substantially to the conformation of a dynamic ExbD carboxy terminus. Most importantly, the same set of ExbD cys substitutions that mediated spontaneous disulfide-linked homodimer formation also mediated spontaneous heterodimer formation with TonB A150C *in vivo*.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 5-1. Single amino acid substitutions were generated for all plasmids using 30-cycle extra-long PCR. All ExbD cys substitutions were constructed using this method with pKP999 (*exbD*) as the template unless otherwise noted. pKP885, pKP899, pKP905, and pKP911 were derivatives of pKP660 (*exbB, exbD*). All ExbD D25N cys substitutions were derivatives of pKP1064 (*exbD* D25N) except for the following: pKP1050 was a derivative of pKP1005 (*exbD* K97C). pKP1051 was a derivative of pKP1011 (*exbD* F103C). pKP1052 was a derivative of pKP1017 (*exbD* T109C). pKP1053 was a derivative of pKP1023 (*exbD* L115C). pKP1082 was a derivative of pKP1029 (*exbD* T121C). pKP1429 was a derivative of pKP1024 (*exbD* M116C). 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. Sequences of the *exbB* segment and *exbD* gene were confirmed by DNA sequencing at the Penn State Genomics Core Facility – University Park, PA.

To construct pKP1005, pKP1011, pKP1017, and pKP1023, forward and reverse primers were designed to amplify the last 22 codons of *exbB* through the stop codon of *exbD* from a pKP885 (*exbB*, *exbD* K97C), pKP899 (*exbB*, *exbD* F103C), pKP905 (*exbB*, *exbD* T109C), or pKP911 (*exbB*, *exbD* L115C) template respectively, introducing flanking NcoI sites. The PCR-amplified, NcoI-digested fragment was cloned into the unique NcoI site in pPro24 (34). Proper orientation was determined by FspI digestion. Sequences of the *exbB* segment and *exbD* gene were confirmed by DNA sequencing at the Penn State Genomics Core Facility – University Park, PA.

Media and culture conditions

Luria-Bertani (LB), tryptone (T), and M9 minimal salts were prepared as previously described (35, 44). Liquid cultures and agar plates were supplemented with 100 μg ml^{-1} ampicillin and plasmid-specific levels of sodium propionate, pH 8, as needed for expression of ExbD (Table 5-S1). When coexpression of plasmid-encoded TonB(C18G, A150C) was examined, cultures and agar plates were also supplemented with 34 μg ml^{-1} chloramphenicol and plasmid-specific levels of L-arabinose as needed for TonB expression. 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 37 μM $FeCl_3 \cdot 6H_2O$. Cultures were grown with aeration at 37°C. All assays were performed using mid-exponential phase cells ($A_{550} \approx 0.43$-0.5, as measured on a Spectronic 20 with a path length of 1.5 cm).

Activity assays

Initial rates of [^{55}Fe]-ferrichrome uptake were determined as described previously (32, 40). For assays where cultures were treated with copper-(1,10-phenanthroline)$_3$ (CuoP), harvested cells were pelleted, resuspended in 1xM9 supplemented as described above (no sodium propionate added), and treated with an equal volume 0.06mM CuoP ([final] 0.03mM) or 0.5mM sodium phosphate buffer, pH 7.4 (buffer only) for 5 min, at 37ºC with aeration. Cells were pelleted, and the standard iron transport protocol was followed, starting with resuspension in the assay medium.

In initial assays testing CuoP treatment, it was found that resuspension of harvested cells in unsupplemented 1xM9 and subsequent treatment with 0.03mM CuoP inhibited iron transport of a wild-type strain to about 25% (data not shown). However, treatment with the same concentration, 0.03mM CuoP, in supplemented 1xM9 did not inhibit activity of a wild-type strain. In all assays the CuoP solution was removed and assays carried out in identical media, but the medium present during the 5 min CuoP treatment was important and differentially affected activity of a wild-type strain.

In vivo *disulfide crosslinking*

Saturated LB overnight cultures were subcultured 1:100 in T broth. Equivalent ODmL of mid-exponential phase cultures were harvested and precipitated by addition of an equal volume of 20% trichloroacetic acid (TCA). Cell pellets were solubilized in non-reducing Laemmli sample buffer (26) containing 50mM iodoacetamide, as previously described (11). Samples were resolved on non-reducing 15%, 13%, or 11% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies (16) or TonB-specific monoclonal antibodies (31). Disulfide-linked complexes still formed when samples were not TCA precipitated (Fig. 5-S1). For reasons that were unclear, anti-ExbD immunoblots from non-reducing gels consistently

showed lower detection compared to reducing gels. Typically a 10-minute exposure from a non-reducing gel immunoblot was comparable in intensity of a 1-minute exposure from a reducing gel immunoblot. To determine total protein expression levels, cell pellets of TCA precipitated samples harvested at the same time as the above samples were solubilized in reducing Laemmli sample buffer containing β-mercaptoethanol (βME), resolved on reducing SDS-polyacrylamide gels, and immunoblotted as described above.

For assays catalyzing oxidative crosslinking with copper-(1,10-phenanthroline)$_3$, saturated LB overnight cultures were subcultured 1:100 in M9 minimal media. Harvested cells were resuspended in unsupplemented 1xM9 and treated with an equal volume 0.06mM CuoP ([final] 0.03mM) or 0.5mM sodium phosphate buffer, pH 7.4 (buffer only) for 5 min, at 37ºC with aeration. Following treatment, samples were precipitated by addition of an equal volume of 20% TCA. All cell pellets were resuspended in non-reducing Laemmli sample buffer containing 50mM iodoacetamide. At the time of initial harvest, samples from each culture were also TCA precipitated and pellets resuspended in reducing Laemmli sample buffer containing βME. These served as the reduced sample controls for total protein expression levels. Equal A_{550}-mL were loaded for all samples. Immunoblotting was performed as described above.

In vivo *formaldehyde crosslinking following oxidative crosslinking*

Saturated LB overnight cultures were subcultured 1:100 into M9 minimal media supplemented with sodium propionate (see Table 5-S1 for induction levels). At mid-exponential phase, cells were harvested and treated with an equal volume 0.6mM CuoP (0.3mM [final]) for 5 min, at 37ºC with aeration. Cell pellets were washed once with 1xM9, then resuspended in sodium phosphate buffer pH 6.8, and treated with formaldehyde as previously described (17). Half of the samples were resuspended in non-reducing Laemmli sample buffer containing 50mM iodoacetamide and half in reducing Laemmli sample buffer containing βME. Samples were

resolved on 13% non-reducing or reducingSDS-polyacrylamide gels, respectively, and immunoblotted with ExbD-specific polyclonal antibodies.

Results

Cysteine substitutions between ExbD residues 92-121 form spontaneous disulfide-linked dimers

In vivo, ExbD A92C, a periplasmic domain subsititution, forms disulfide-linked homodimers (38). *In vitro*, the ExbD periplasmic domain formed higher order homomultimers through residues 104-116 (10). Our unpublished *in vivo* studies suggested a 30-residue region of the ExbD periplasmic domain, from residues 92-121, was important in mediating protein-protein interactions of ExbD (Ollis *et al*., in preparation). To investigate the role of this region of ExbD in homodimer formation *in vivo*, cysteine substitutions were individually constructed at each of the remaining 29 residues. ExbD has no native cysteine residues so wild-type ExbD encoded on plasmid pKP999 was used as the template.

Each plasmid-encoded substitution was expressed in strain RA1045 (Δ*exbD*, Δ*tolQR*), where deletion of *tolQR* prevented the activity attributable to crosstalk of TonB with this homologous system (3). Attempts were made to express each ExbD cys substitution at levels equal to native ExbD when analyzed under reducing conditions. Immunoblots with ExbD-specific polyclonal antibody of steady-state levels of TCA precipitated proteins resolved on SDS-polyacrylamide gels showed all but one of the substitutions could be stably expressed (Fig. 5-1, reduced). ExbD I102C was proteolytically unstable and only faintly detected on longer exposures, even when induced with the highest concentration of sodium propionate. The instability was not specific to substitution with cysteine, since ExbD I102A was also proteolytically unstable (data not shown). Cys substitutions at T94, K98, and D107 also

decreased ExbD stability, but these substitutions could be expressed to native ExbD levels with higher concentrations of sodium propionate (Table 5-S1). ExbD L93C showed aberrant migration, migrating slower than wild-type ExbD. ExbD L93A also exhibited slower migration (data not shown). Single amino acid substitutions can alter protein mobility on SDS-polyacrylamide gels (36).

To see if any of the ExbD periplasmic domain cys substitutions were capable of forming spontaneous disulfide-linked homodimers, samples were prepared under non-reducing conditions. Bacterial cell pellets were solubilized in sample buffer containing iodoacetamide to alkylate free cysteines and prevent dimer formation after cell lysis. ExbD homodimers would theoretically migrate at about 31 kDa. Accordingly, ExbD-specific immunoblots of non-reducing SDS-polyacrylamide gels showed a number of ExbD cys substitutions formed disulfide-linked homodimers, with the highest level of complex formation observed for G96C, D99C, I102C, D107C, K108C, Y112C, and E113C (Fig. 5-1, non-reduced). Notably, dimer formation appeared to stabilize ExbD I102C. Reducing conditions completely hydrolyzed the homodimer, yet only a small amount of monomeric ExbD I102C was detected on long exposures of immunoblots (Fig. 5-S2). Lower levels of dimer formation were observed for A92C, E95C, K98C, T109C, V110C, D111C, M116C, and D120C. For a few substitutions, a higher complex was formed, migrating around 40 kDa and most significantly detected with G96C, K98C, and D99C. This complex was still detected in a strain lacking ExbB, ruling out the possibility that it was a disulfide-linked complex between ExbD and ExbB, which has a single native cysteine (Fig. 5-S3 and data not shown). The identity of this complex is unknown.

It is important to note that, for certain cys substitutions, the nature of the native side chain would not favor interaction with the same residue of another ExbD. Asp99, for example, formed prominent homodimeric interactions when substituted with cys, but two side chains with the same charge are unlikely to interact. Disulfide-linked interactions served as evidence that the regions

of ExbD where these substitutions are located came into close contact *in vivo*. Disulfide bonds may trap normally transient conformations in addition to any native interfaces.

ExbD residues 92 through 121 are tolerant to substitution with cysteine

The ability of each cys-substituted ExbD to support ferrichrome-mediated iron transport under non-reducing conditions was determined, with each substitution expressed to native ExbD levels (Fig. 5-2, lower immunoblots). The corresponding degree of dimer formation for each strain assayed was also determined. All 30 substitutions were active, with Y112C supporting the lowest initial rate of transport at about 80% the rate of wild-type plasmid-encoded ExbD (Fig. 5-2C).

The 80% activity of ExbD I102C, for which a monomer was not detected, suggested that ExbD was active as a homodimer (Fig. 5-2A). ExbD G96C, K108C, and Y112C also had significant levels of disulfide-linked dimers present (Fig. 5-2A and B). Dimers observed here for F103C were only detected when this substitution was overexpressed. ExbD G96C, with ~ 2/3 present as a homodimer and 1/3 present as a monomer, supported 85% activity, also suggesting that the dimeric form was active. ExbD K108C and Y112C, which were initially expressed higher levels than native ExbD and exhibited dimer levels equal to or greater than monomer levels, supported 110% and 90% activity, respectively (Fig 5-2 B). When inducer was decreased to achieve the desired native ExbD levels of expression for these substitutions, significantly less dimer was present for K108C and activity was the same, near 110%. Y112C now showed equal dimer and monomer levels and activity decreased to 80% (Fig. 5-2C). The activities of these substitutions could be attributed to the significant level of monomer present.

ExbD homodimers differentially affect ExbD activity

To increase the ratio of dimer to monomer so that the activity of the dimer could be estimated, strains expressing cys-substituted ExbDs were treated with the oxidizing agent copper-(1,10-phenanthroline)$_3$ (CuoP) as described in Materials and Methods. After treatment, only 3 substitutions, K97C, T114C, and K117C, still showed no homodimer formation (Fig. 5-3). For E95C, G96C, D99C, and I102C, homodimer levels remained unchanged after CuoP treatment. Significant increases in homodimers were observed for T109C through E113C, L115C and M116C.

The degree of dimer formation could only be gauged by the decrease in monomer levels. This was due to the fact that, although treated and untreated samples came from the same culture, the detectability of ExbD in the dimer band had greatly increased-- exemplified by D111C, Y112C and E113C-- suggesting a change in conformation that allowed greater access to antibody binding. Only a slight increase in total ExbD levels was observed when CuoP-treated samples of these cys substitutions were reduced after treatment (data not shown). Inducer was absent during the 5 min CuoP treatment, ruling out an actual increase in protein synthesized, which would otherwise have been evident in all treated samples.

Since CuoP treatment catalyzed almost 100% dimer formation for some of the cys substitutions, attempts were made to determine the activities of the dimeric forms. It was necessary to adapt standard CuoP treatments to the demands of the iron transport assays because the standard treatment inhibited iron transport in wild-type cells [(27); data not shown]. To retain full iron transport activity of a wild-type strain after CuoP treatment, the concentration of CuoP was reduced 10-fold to 0.03 mM, and cells had to be suspended in fully supplemented M9 medium as described in Materials and Methods (Fig. 5-2D, pExbD).

Unfortunately this treatment no longer catalyzed high levels of dimer formation for the majority of ExbD cys substitutions (data not shown). Only Y112C and M116C exhibited nearly 100% homodimers. K108C also showed a significant increase in homodimers, though monomer was still present (Fig 5-2D, non-reduced). This limited our ability to examine activity or inactivity of observed ExbD homodimers to these few substitutions.

For both Y112C and M116C, the dimeric form inhibited iron transport activity. CuoP-treated ExbD Y112C and M116C supported only 5% and 20% activity, respectively, compared to about 90% for samples treated with buffer only (Fig. 5-2D). In contrast, ExbD K108C exhibited monomer levels similar to those of ExbD M116C after CuoP treatment but was still at least 70% active. This was similar to the result, described above, where ExbD I102C was highly active as a spontaneously formed homodimer.

ExbD dimers are assembled with ExbB

It was not known if the inactive ExbD disulfide-linked dimers were inhibited in their ability to assemble as part of a normal complex with ExbB, such as if non-native conformations were trapped after translocation of the periplasmic domain across the CM, or if dimer formation blocked later conformational changes after assembly. Both active and inactive ExbD (ExbD D25N or wild-type ExbD after collapse of pmf) can be crosslinked with formaldehyde to ExbB (38). Since CuoP catalyzed almost complete dimerization for certain ExbD cys substitutions, attempts were made to formaldehyde crosslink these disulfide-linked dimers to ExbB to determine if the trapped ExbD homodimeric conformations could still assemble with ExbB.

Cultures expressing wild-type ExbD or the 4 ExbD cys substitutions that showed the highest dimer levels (Fig. 5-3), K108C, Y112C, E113C, and M116C, were treated with 0.3mM CuoP 5 min and then crosslinked with formaldehyde under non-reducing conditions. Crosslinked

samples were divided in half. One half was reduced with β-mercaptoethanol to hydrolyze disulfide crosslinks, and the other half kept non-reduced. All were solubilized at 60º C to retain formaldehyde crosslinks. Samples were resolved on reducing or non-reducing SDS-polyacrylamide gels, respectively, and immunoblotted with ExbD-specific polyclonal antibodies (Fig. 5-4). An ExbD disulfide-linked homodimer (~31 kDa) formaldehyde crosslinked to ExbB (~26 kDa) would theoretically migrate at approximately 57 kDa. Due to the similar migration to a heterodimeric complex of ExbD and TonB (52 kDa), each substitution was also crosslinked in a Δ*tonB* background (KP1509) to rule out those complexes due to TonB-ExbD crosslinks.

As seen previously under reducing conditions (38), here, wild-type monomeric ExbD formaldehyde crosslinked into homodimers and heterodimeric complexes with ExbB and TonB, that were detected under both non-reducing and reducing conditions (Fig. 5-4A). The formaldehyde crosslinked homodimer is typically the complex of lowest abundance (38) and was apparent here on long exposures (Fig. 5-4B). Under non-reducing conditions, crosslinking profiles for ExbD cys substitutions had much lower levels of the ExbD-ExbB complex and appeared to show a TonB-ExbD crosslink (Fig. 5-4A, left immunoblot). However, the ~57 kDa bands were still present in a Δ*tonB* strain, ruling out a crosslink to TonB. The size of the complex suggested it could represent a novel complex of an ExbD disulfide-linked homodimer that was formaldehyde crosslinked to ExbB ($ExbD_2$-ExbB). Due to the CuoP treatment, much higher levels of homodimers and no monomers were detected. It was not clear why ExbD homodimer bands appeared as a doublet; however, both bands decreased in the presence of reducing agent, suggesting disulfide-linked dimers were in both bands. Doublet bands were also apparent for the ExbD monomer, including cysteine-less wild-type ExbD. These may represent two conformations of ExbD, an unknown modification, or a degradation product.

The same samples were also immunoblotted under reducing conditions that hydrolyzed the disulfide bond of the ExbD homodimer and retained ExbD monomers formaldehyde

crosslinked to other proteins (Fig. 5-4A, right immunoblot). Under these conditions, levels of the suspected $ExbD_2$-ExbB heterotrimer were greatly lowered with concomitant restoration of the typical ExbD-ExbB heterodimer and ExbD monomer bands (Fig. 5-4A and C). This confirmed the identity of the 57 kDa band as an $ExbD_2$-ExbB heterotrimer, demonstrating that the ExbD disulfide-linked dimers assembled with ExbB *in vivo*. This also confirmed the supposition, based on data from the Mot and Tol systems, that dimeric ExbD interacts with ExbB (4, 6, 23). The absence of TonB had no effect on the crosslinking profiles in the reduced samples, indicating that while ExbD was trapped as a disulfide-linked homodimer, it could not formaldehyde crosslink to TonB.

ExbD homodimer formation does not require ExbB or ExbD D25

While it was observed that select ExbD disulfide-linked homodimers could still complex with ExbB, it was not known if they formed before or after interaction with ExbB. Since ExbD homodimers formaldehyde crosslink in the absence of ExbB, it was possible disulfide-linked dimer formation occurred before interaction with ExbB (38). The ability of each cys substitution to form spontaneous disulfide-linked dimers was compared in a Δ*exbBD*, Δ*tolQRA* strain (RA1017). All dimers still formed, and no new dimers were detected in the absence of ExbB (Fig. 5-S3 and data not shown). Apparent decreases in the levels of dimers mostly reflected variations in ExbD expression, since ExbB stabilizes ExbD (9). For ExbD D111C, Y112C, and E113C, the decrease in dimer suggested that they interacted with ExbB.

D25 is an essential residue in the ExbD transmembrane domain, and a D25N mutation prevents the energized interaction between TonB and ExbD periplasmic domains (38). A functional ExbD TMD, however, was not important for homodimer formation. Inactive ExbD D25N can be crosslinked into homodimers with formaldehyde (38), and accordingly, all ExbD

D25N cys substitutions still formed disulfide-linked dimers (Fig. 5-S3). No new dimers were detected in the presence of the D25N mutation (data not shown).

ExbD homodimers increase in the absence of TonB

The results discussed above, where formaldehyde crosslinking of disulfide-linked ExbD dimers was analyzed, suggested that the ExbD periplasmic domain could form disulfide-linked homodimers or formaldehyde crosslinked TonB-ExbD heterodimers, but not both at the same time. For the four cys substitutions examined in that assay, dimer formation was efficiently catalyzed even in the absence of TonB. To determine if TonB was important for spontaneous ExbD homodimer formation, disulfide-linked dimer formation was examined in a Δ*exbD*, Δ*tolQR*, Δ*tonB* strain (KP1509). All14 dimer-forming cys substitutions showed increased disulfide-linked dimers in the absence of TonB, suggesting possible competition existed between homodimers and heterodimers and a TonB-dependent change in the dynamics of the ExbD periplasmic domain (Fig. 5-5).

Most ExbD cys substitutions that can dimerize also form heterodimers with cys-substituted TonB

It was previously observed that ExbD A92C forms a homodimer and a disulfide-linked heterodimer with TonB(C18G, A150C) (38). A150 is a non-essential residue of the TonB carboxy terminus (residues 150-239), a functionally essential region of the TonB periplasmic domain (30, 41). To determine if any of the other ExbD cys-substituted residues could be trapped in specific interaction with TonB, each was co-expressed with TonB(C18G, A150C), and disulfide-linked complex formation was examined for both ExbD and TonB. A number of ExbD cys substitutions were trapped in disulfide-linked heterodimers with TonB (Fig. 5-6). There was

a strong correspondence between those cys substitutions that participated in ExbD-TonB heterodimer formation and those that formed homodimers: A92C, E95C through D99C, K108C, T109C, Y112C, and E113C. Both interactions, however, were not observed through all sites. ExbD I102C and V110C formed homodimers but not ExbD-TonB heterodimers. K97C and K98C formed heterodimers but not homodimers.

ExbD D25 is specifically important for disulfide-linked TonB-ExbD heterodimers

We had previously observed that ExbD D25N still formed disulfide-linked homodimers, but did not form the ExbD A92C-TonB A150C heterodimers (38). To determine if a functional ExbD TMD was also important for the other observed disulfide-linked heterodimers, ExbD cys substitutions with a wild-type TMD or containing a D25N TMD substitution were co-expressed with TonB(C18G, A150C) and examined under non-reducing conditions. ExbD cys substitutions formed homodimers with or without residue D25, but significant interaction with TonB A150C required D25 (Fig. 5-7). In the presence of wild-type TonB, ExbD D25N cys homodimers were increased compared to substitutions with a wild-type TMD, as expected if disulfide-linked homodimer formation prevented subsequent interactions with TonB (Fig. 5-S3). In studies of these same substitutions with the co-expression of TonB A150C, the differences between ExbD cys and ExbD D25N cys homodimers were not as obvious (Fig. 5-7). Disulfide-linked heterodimer formation of the wild-type ExbD cys with TonB A150C may also have resulted in increased ExbD homodimers, similar to the effect of the D25N substitution, possibly by blocking further cycles of ExbD-TonB associations.

Discussion

ExbD can formaldehyde crosslink *in vivo* into a homodimer or a heterodimer with TonB through its periplasmic domain. ExbD also formaldehyde crosslinks efficiently to ExbB through unknown regions. ExbB and pmf are required to form the ExbD-TonB heterodimer but not the ExbD homodimer. The functional significance of the ExbD homodimer is unknown. ExbD A92C was previously demonstrated to be a site of *in vivo* ExbD homodimeric and ExbD A92C-TonB A150C heterodimeric interaction. ExbD A92C formed homodimers even if the inactivating D25N TMD mutation was present, but the heterodimeric complex with TonB A150C required functional TMDs of both proteins (38).

The same periplasmic ExbD residues mediate in vivo *interactions between ExbD dimers and ExbD-TonB heterodimers*

We recently identified a 30-residue region of ExbD that was important in supporting ExbD formaldehyde crosslinked protein-protein interactions, including homodimers and ExbD-TonB heterodimers (Ollis *et al*., in preparation). To determine if this region was directly involved in these interactions, ExbD residues 92-121 were scanned with cys substitutions for analysis of *in vivo* disulfide crosslinking with another ExbD and with TonB A150C.

Here we showed that most of the fourteen ExbD cys substitutions that mediated disulfide crosslinking of ExbD homodimers also mediated formation of heterodimers with TonB. This suggested that ExbD periplasmic domain homodimeric interactions were not formed through static interfaces, since a permanent interaction would have prevented interaction with TonB in that region. Consistent with this idea, the absence of competing TonB led to an increase in ExbD homodimerization.

Because ExbD homodimers could associate with ExbB and because ExbD homodimers appeared to be the default arrangement for ExbD in the absence of other members of the complex, it appeared that the $ExbD_2$-ExbB complex might be an initial assembly intermediate. TonB was also not required for the formation of the $ExbD_2$-ExbB complex. It should be noted that Pramanik *et al*. recently identified an $ExbB_6$-$ExbD_1$ complex *in vitro* and could find no evidence for ExbD dimers (42). In that study, however, ExbD carried an 8-residue StrepII affinity tag at its carboxy terminus, and ExbB carried a His6 tag. Epitope tags have resulted in artifactual protein interactions or prevented native interactions, described in multiple protein systems where histidine tags were used (8, 19, 47). Within the TonB system, ExbD oligomerization states are affected by epitope tagging. A T7 epitope tag at the amino terminus of ExbD prevents *in vivo* formaldehyde crosslinking between ExbD and TonB or ExbB and results in artifactual formation of T7-ExbD homotrimers (38). It could be that the presence of the StrepII or His6 tag prevented detectable association of ExbD homodimers with ExbB *in vitro*.

The functional distinction between homodimeric ExbD and TonB-ExbD heterodimeric interactions became apparent when the ExbD D25N substitution was examined. Because the disulfide linked complexes in this study occurred spontaneously, it was not possible to directly assess the role of pmf. Newly synthesized proteins require pmf for CM insertion, and disulfide-linked complexes would be preexisting in any cultures treated with a protonophore to collapse the pmf. ExbD TMD residue D25 is a candidate residue for promoting direct response of ExbD to pmf and is essential for energized ExbD-TonB periplasmic domain interaction (38). In this study ExbD D25N largely prevented the formation of ExbD-TonB heterodimers, which validated the biological relevance of these specific sites of disulfide-linked interaction.

A model is therefore proposed where ExbD homodimers constitute the initial interaction of the ExbD periplasmic domain. ExbD homodimers assembled with ExbB are able to interact with TonB. Homodimerization of ExbD appears to involve multiple interfaces, and specific

regions of the ExbD homodimer transition to heterodimeric interactions with TonB. The ExbD TMD, specifically residue D25, is important for the conformational transitions of the ExbD periplasmic domain to these specific heterodimeric associations with the TonB carboxy terminus. The specific signal that initiates the transition is unknown.

The theme of homodimeric to heterodimeric transitions involving shared interfaces has been seen in other systems. In *Agrobacterium tumifaciens*, TraM inhibits the transcriptional activator TraR through formation of a heterodimeric antiactivation complex. Both TraM and TraR form homodimers, with the homodimeric interface of TraM also serving as its interactive site with TraR. Initial non-specific binding of TraR to the TraM homodimer has been proposed to facilitate homodimer dissociation and formation of the inactive TraR-TraM heterodimer (7). If present, initial pmf-independent interaction between ExbD and TonB periplasmic domains could serve a similar purpose. In the case of the TonB system, however, it is likely that ExbD-TonB periplasmic domain interaction activates TonB, since TonB lacks TMD residues that could promote a direct, independent, response of TonB to pmf (45). The TonB-ExbD periplasmic domain interaction, detected by formaldehyde crosslinking, requires pmf (38). Thus an equilibrium between ExbD and TonB-ExbD interactions might be more like the yeast copper chaperone yCCS and copper, zinc superoxide dismutase SOD1. Both form homodimers, as do both TonB and ExbD (11, 38), with activation of SOD1 by yCCS occurring through heterodimeric interaction between conserved homodimer interface residues from both proteins (28). Copper binding to yCCS promotes and stabilizes heterdimeric interaction with SOD1 (46).

Similarities and differences between the solved structure of the ExbD periplasmic domain and in vivo *results*

Previous *in vitro* studies using NMR diffusion or dynamic light scattering assays with the isolated ExbD periplasmic domain (1mM at pH 3.0 or 7.0) showed multimerization of 4-7 copies of the ExbD periplasmic domain. Based on amide chemical shifts, when protein concentration was increased from 0.2mM (monomeric) to 1mM (multimeric), the region of residues 104-116 was proposed as the homo-multimeric interface. The opposite end of the structure, including residues 92-103, was not involved in multimerization based on no change with increased protein concentration (10). The proposed region of homo-multimerization was not investigated *in vivo*.

When all sites of spontaneous *in vivo* disulfide-linked dimer formation in this study were mapped onto the ExbD periplasmic domain NMR structure, homo-dimerization sites clustered at both ends of the structure (Fig. 5-8). These two distinct interactive domains suggested the possibility of at least two homodimeric interfaces *in vivo*. If these residues form a single interface, the interactive conformation is not represented by the solution structure, where residue side chains of even just the most prominent interaction sites face in multiple directions. One of the *in vivo* interactive regions, residues 106-116, overlapped most of the identified *in vitro* interactive region, residues 104-116. *In vivo* interaction through these substitution sites did not require function of the ExbD TMD, supporting the fact that this region supports homo-multimeric associations of an inactive ExbD periplasmic domain, even lacking the entire TMD. In the monomeric NMR structure, this region has predominantly hydrophobic, surface-exposed residues, F104, A106, V110, L115, and M116, which could promote non-specific aggregation of the 4-7 subunits. While *in vivo* results support interactions through this region, it is unknown if higher order homo-multimers, in addition to a homodimer, occur *in vivo*.

A conformationally dynamic ExbD periplasmic domain

This study provided evidence that ExbD periplasmic domain conformation is dynamic *in vivo*. Interaction sites observed *in vivo* extend beyond those observed *in vitro*. ExbD I102, which was unstable when substituted with cys unless trapped as a dimer, is a buried residue on the β4 strand of the NMR structure (Fig. 5-8). ExbD I102C was fully active as a dimer, suggesting that this region of the β4 strand is surface exposed in the monomers prior to forming the homodimer. The conformation of a monomeric unit of the active ExbD I102C dimer would require changes from the solved structure. Additional sites of interaction *in vivo* not observed *in vitro* clustered at the opposite end of the NMR structure from the identified sites and included A92, E95, G96, K98, and D99. Perhaps membrane insertion of ExbD is important for interaction through this region.

Specific regions of the ExbD periplasmic domain required conformational flexibility. Y112C, the site of highest dimer formation, and M116C, both located on the external face of the same helix in the NMR structure, were only active as monomers. Complete homodimer formation through these sites inhibited activity, indicating that these residues must be free to move *in vivo*. This could suggest that interaction at the interface of this helix is normally resolved within a cycle of conformational changes or that non-native conformations trapped by disulfide bonds reflect a transient close association of the helices. ExbD K108C, like Y112C and M116C, formed both homodimers and heterodimers, yet high levels of K108C homodimers did not significantly inhibit TonB activity. K108C may be in a region that comes into close association during conformational changes but not itself an essential residue for direct interactions. Sites such as Y112 or M116 could still be accessible to TonB interaction in a disulfide-linked K108C homodimer. That the trapped dimer of ExbD I102C was fully active

indicated that homodimeric ExbD had a functional role and suggested some regions of ExbD remained associated as homodimers even during heterodimeric interaction with TonB.

In the presence of an oxidizing agent, only 3 of the 30 ExbD cys substitutions were not trapped in homodimeric interaction—K97, T114, and K117. This suggested that ExbD dynamically sampled many environments prior to establishing its dimeric interfaces. Findings in this study were also consistent with earlier suggestions that ExbD guides the conformation of the TonB periplasmic domain, which itself is conformationally dynamic (12, 29, 33, 41).

Comparison to the TolR periplasmic domain

The solution structure of residues 59-130 of the paralogous TolR periplasmic domain (residues 39-139) from *Haemophilus influenzae* is a homodimer, and the monomers have secondary and tertiary structures similar to the *Escherichia coli* ExbD monomer solution structure (39). ExbD I102C, which was active as a dimer and unstable as a monomer, is located on the β4-strand in the ExbD structure. In the TolR structure, the corresponding β4strands of each monomer do form part of the homodimeric interface (39). However, the strands are oriented anti-parallel, so Leu104, which corresponds to ExbD Ile102, is not in close interaction between monomers, with a C^{β}-C^{β} distance of about 16 Å (data not shown). It is unknown how the presence of the TolR C-terminal tail (residues 131-139) would alter the structure—in the ExbD monomeric structure, the corresponding tail region lies partially along the β4 strand. The TolR dimeric structure, however, supports the idea of homodimeric interaction involving the β4strand of ExbD *in vivo*.

In vivo disulfide crosslinking of *E. coli* TolR cys substitutions, which overlapped ExbD cys substitutions from 111-121 in this study, show similarities to ExbD homodimeric interactions, although TolR Y117C, corresponding to ExbD Y112C, formed the only spontaneous dimer. Like

ExbD Y112C, TolR Y117C is non-functional as a homodimer and is hypothesized to block conformational changes of the TolR periplasmic domain (13). As reported, however, in the *H. influenzae* TolR dimer structure, the corresponding residue Y114, although located on a helix that forms part of the homodimer interface, is too distant for interaction between monomers (39). Overall, the *in vitro* and *in vivo* homodimeric interactions suggest the β4 strand and α2 helix form homodimeric interfaces for both the ExbD and TolR periplasmic domains. The structural similarities of these proteins suggest they may form very similar homodimers. The functional differences of these similar proteins may be defined by the way their homodimers change in response to TMD interactions with their respective CM complexes and/or pmf.

In summary, this work demonstrated the conformational plasticity of the ExbD periplasmic domain as it interacted with either itself or with a TonB periplasmic domain *in vivo*. The results led to a model where some homodimeric interactions of ExbD must be released for subsequent heterodimeric interaction with TonB. Asp25 in the ExbD TMD was important to promote this transition. Sequestering specific ExbD periplasmic domain residues in homodimeric interactions could provide a level of regulation for TonB-ExbD interactions, restricting unproductive interactions of the ExbD periplasmic domain and controlling the transition with signals from the ExbD TMD.

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Figure Legends

Fig. 5-1. Cysteine substitutions in the ExbD periplasmic domain form spontaneous disulfide-linked dimers *in vivo*. TCA precipitated proteins from strains expressing chromosomally-encoded wild-type ExbD (W3110) or a Δ*exbD*, Δ*tolQR* strain (RA1045) expressing plasmid-encoded ExbD variants near native ExbD levels (see Table 5-S1 for induction levels) were resolved on non-reducing or reducing 15% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies. Reduced and non-reduced samples came from the same culture. On the right, "d" indicates the position of the homodimer, and "m" indicates the position of the monomer. Positions of non-reducing molecular mass standards are indicated on the left.

Fig. 5-2. ExbD residues 92-121 are tolerant to substitution with cysteine, but disulfide-linked dimer formation can inhibit activity. Strains expressing chromosomally-encoded wild-type ExbD (W3110) or a Δ*exbD*, Δ*tolQR* strain (RA1045) expressing plasmid-encoded wild-type ExbD (pExbD) or ExbD cys substitutions near native ExbD levels (see Table 5-S1, 1xM9, for induction levels) were assayed for the ability to support transport of iron-loaded ferrichrome (Fe-Fc) as described in Materials and Methods. Results presented are representative data where activity was within 5% across at least two sets of triplicate assays. Y-axis indicates the initial rate of transport. ExbD variants assayed are indicated along the x-axis. TCA precipitated samples harvested just prior to each assay were resolved on 15% non-reducing or reducing SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibody. "Δ" indicates the Δ*exbD*, Δ*tolQR* strain (RA1045), "w" indicates wild-type strain W3110, and "pE" indicated plasmid-encoded wild-type ExbD. Residue numbers indicate the position of the ExbD cys substitution. On the right, "d" indicates the position of the homodimer, and "m" indicates the

position of the monomer. A, B, and C, Sets of activity assays performed on the same days. D, Activity assays for select ExbD homodimers, catalyzed by CuoP treatment. "-" or "+" indicates buffer only or 0.03mM CuoP treatment, respectively, prior to assaying. Gray bars highlight CuoP-treated strains.

Fig. 5-3. Addition of an oxidizing agent increases the number of ExbD cys substitutions trapped in disulfide-linked homodimers. Non-reduced, A Δ*exbD*, Δ*tolQR* strain (RA1045) expressing plasmid-encoded ExbD variants near native ExbD levels (see Table 5-S1, 1xM9 for induction levels) were treated with buffer only (-) or 0.03mM CuoP (+) for 5 min at 37ºC, with aeration. TCA precipitated samples were resolved on non-reducing 15% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies. Positions of non-reducing molecular mass standards are indicated on the left. On the right, "d" indicates the position of the homodimer, and "m" indicates the position of the monomer. Reduced, TCA precipitated samples taken from the same cultures prior to treatment were resolved on reducing 15% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies.

Fig. 5-4. ExbD disulfide-linked homodimers assemble with ExbB. Strains expressing chromosomally-encoded wild-type ExbD (W3110), in the absence of TonB (KP1344), or a Δ*exbD*, Δ*tolQR* strain (RA1045) or a Δ*exbD*, Δ*tolQR*, Δ*tonB* (KP1509) strain expressing plasmid-encoded wild-type ExbD (pExbD) or ExbD cys substitutions near native ExbD levels (see Table 5-S1 for induction levels) were treated with CuoP, washed, then crosslinked with formaldehyde as described in Materials and Methods. A, Samples were resolved on 13% non-reducing or reducing SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies. Positions of monomeric ExbD and ExbD-specific crosslinked complexes are indicated on the left. Positions of molecular mass standards are indicated between the

immunoblots. B, Long exposures of the immunoblots in A, showing only the strains expressing wild-type ExbD, are shown. "NR" indicates non-reduced. "Red" indicates reduced. C, Short exposures of the immunoblots in A, cropped to the region of the ExbB-ExbD heterodimer are shown for better comparison of relative levels of the complex under non-reducing or reducing conditions.

Fig. 5-5. ExbD disulfide-linked dimers increase in the absence of TonB. TCA-precipitated samples of wild-type chromosomally-encoded ExbD (W3110) and a Δ*exbD*, Δ*tolQR* strain (RA1045) or a Δ*exbD*, Δ*tolQR*, Δ*tonB* (KP1509) expressing plasmid-encoded ExbD cys substitutions near native ExbD levels (see Table 5-S1 for induction levels) were resolved on non-reducing or reducing 15% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies. Reduced and non-reduced samples came from the same culture. "+" indicated the presence (RA1045) and "-" indicated the absence (KP1509) of TonB. ExbD cys substitutions are indicated across the top. Positions of non-reducing molecular mass standards are indicated on the left. On the right, "d" indicates the position of the homodimer, and "m" indicates the position of the monomer. The middle panel of immunoblots is a shorter exposure of the top panel, showing the monomer band only.

Fig. 5-6. ExbD cysteine substitutions share common interfaces between homodimeric and heterodimeric interactions. TCA precipitated samples of strains expressing chromosomally-encoded wild-type ExbD and TonB (W3110) or a Δ*exbD*, Δ*tolQR*, Δ*tonB* strain (KP1509) coexpressing plasmid-encoded ExbD cys substitutions and TonB(C18G, A150C) near native levels were resolved on non-reducing or reducing 13% and 11% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies or TonB-specific monoclonal antibodies. Reduced and non-reduced samples came from the same culture. Positions of the

monomeric proteins or disulfide crosslinked complexes are indicated on the left. L93C, T94C, D107C, D111C, and D120C ExbD-TonB heterodimers were detected on longer exposures (data not shown). Positions of non-reducing molecular mass standards are indicated between immunoblots. Strain designations across the top apply to both immunoblots below. All samples were processed the same day.

Fig. 5-7. ExbD D25 is important for ExbD-TonB disulfide-linked heterodimer formation. TCA precipitated samples of strains expressing chromosomally-encoded wild-type ExbD and TonB (W3110) or a Δ*exbD*, Δ*tolQR*, Δ*tonB* strain (KP1509) coexpressing plasmid-encoded TonB(C18G, A150C) and ExbD cys substitutions without (-) or with (+) a D25N TMD substitution near native levels were resolved on non-reducing or reducing 13% and 11% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies or TonB-specific monoclonal antibodies. Reduced and non-reduced samples came from the same culture. Positions of the monomeric proteins or disulfide crosslinked complexes are indicated on the left. Positions of non-reducing molecular mass standards are indicated between immunoblots. Strain designations across the top apply to both immunoblots below. All samples were processed the same day.

Fig. 5-8. Sites of disulfide-forming cys substitutions map to opposite ends of the ExbD periplasmic domain solution structure. Side chains of residues where cys substitutions were trapped in disulfide-linked homodimers *in vivo* are mapped on the ExbD periplasmic domain NMR structure, pdb code 2pfu. The image was generated using Swiss-PdbViewer (14). Grayed ribbon indicates the cys scanned region examined in this study. Black side chains indicate significant spontaneous ExbD homodimer formation. Gray side chains indicate weak spontaneous homodimer formation. All side chains pictured except I102, V110, and M116,

showed significant ExbD cys heterodimer formation with TonB A150C. “C” and “N” indicate the carboxy and amino terminus of the domain, respectively.

Table 5-1. Strains and plasmids used in this study

Strain or Plasmid	Genotype or Phenotype	Reference
Strains		
W3110	F⁻ IN*(rrnD-rrnE)1*	(18)
RA1017	W3110, Δ*exbBD*::*kan*, Δ*tolQRA*	(29)
RA1045	W3110, Δ*exbD*, Δ*tolQR*	(5)
KP1344	W3110, *tonB, P14*::*BlaM*	(33)
KP1509	W3110, Δ*exbD*, Δ*tolQR*, Δ*tonB*::kan	(38)
[a]Plasmids		
pKP381	TonB(H20A)	(29)
pKP945	TonB(C18G, A150C)	(38)
pKP660	*exbB, exbD* in pBAD24	(38)
pKP885	ExbB, ExbD(K97C)	Present study
pKP899	ExbB, ExbD(F103C)	Present study
pKP905	ExbB, ExbD(T109C)	Present study
pKP911	ExbB, ExbD(L115C)	Present study
pKP1000	ExbD(A92C)	(38)
pKP1049	ExbD(D25N, A92C)	(38)
pKP999	*exbD* in pPro24	(38)

pKP1001 (L93C)	pKP1011 (F103C)	pKP1021 (E113C)
pKP1002 (T94C)	pKP1012 (F104C)	pKP1022 (T114C)
pKP1003 (E95C)	pKP1013 (R105C)	pKP1023 (L115C)
pKP1004 (G96C)	pKP1014 (A106C)	pKP1024 (M116C)
pKP1005 (K97C)	pKP1015 (D107C)	pKP1025 (K117C)
pKP1006 (K98C)	pKP1016 (K108C)	pKP1026 (V118C)
pKP1007 (D99C)	pKP1017 (T109C)	pKP1027 (M119C)
pKP1008 (T100C)	pKP1018 (V110C)	pKP1028 (D120C)
pKP1009 (T101C)	pKP1019 (D111C)	pKP1029 (T121C)
pKP1010 (I102C)	pKP1020 (Y112C)	

pKP1064	ExbD(D25N)	(38)
pKP1217 (D25N, L93C)	pKP1051 (D25N, F103C)	pKP1234 (D25N, E113C)
pKP1216 (D25N, T94C)	pKP1226 (D25N, F104C)	pkP1274 (D25N, T114C)
pKP1218 (D25N, E95C)	pKP1227 (D25N, R105C)	pKP1053 (D25N, L115C)
pKP1219 (D25N, G96C)	pKP1228 (D25N, A106C)	pKP1429 (D25N, M116C)
pKP1050 (D25N, K97C)	pKP1261 (D25N, D107C)	pKP1290 (D25N, K117C)
pKP1233 (D25N, K98C)	pKP1270 (D25N, K108C)	pKP1291 (D25N, V118C)
pKP1203 (D25N, D99C)	pKP1052 (D25N, T109C)	pKP1294 (D25N, M119C)

pKP1204 (D25N, T100C)	pKP1237 (D25N, V110C)	pKP1297 (D25N, D120C)
pKP1260 (D25N, T101C)	pKP1262 (D25N, D111C)	pKP1082 (D25N, T121C)
pKP1189 (D25N, I102C)	pKP1269 (D25N, Y112C)	

[a]Plasmids listed below the lines are derivatives of pKP999 and pKP1064, respectively, unless otherwise noted in Materials and Methods. The ExbD substitutions expressed from each plasmid are listed in parentheses.

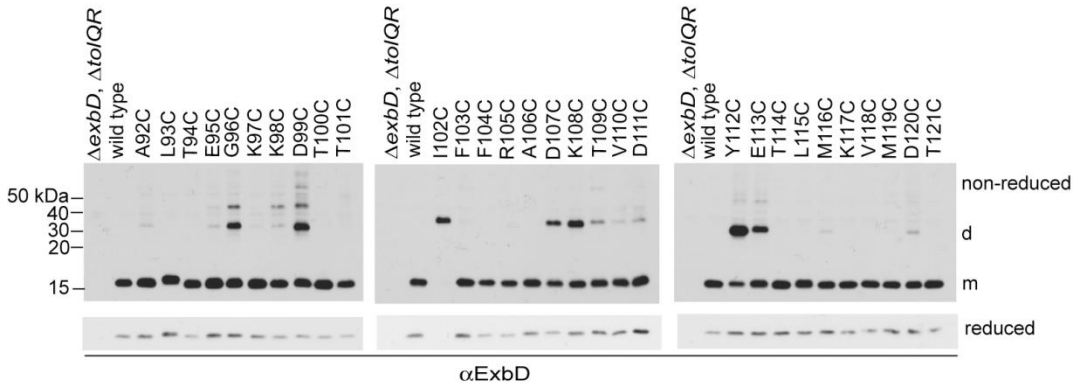


Figure 5-1, Ollis and Postle

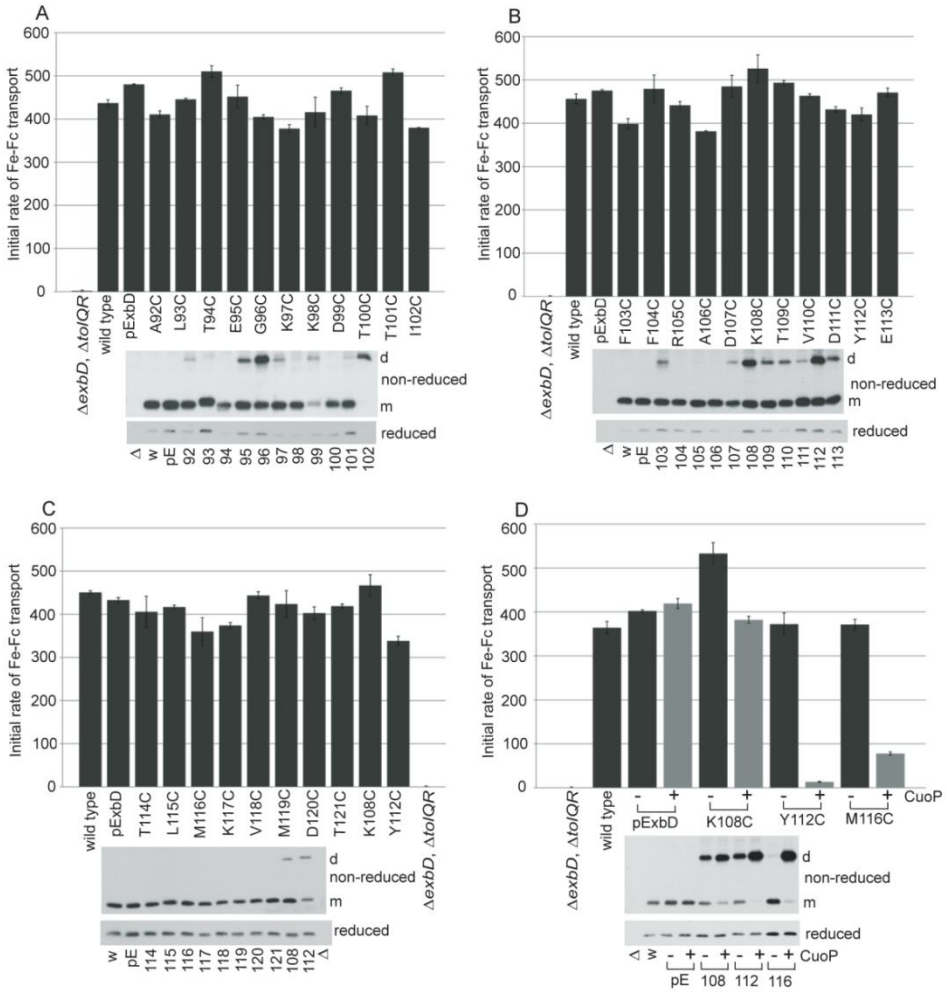
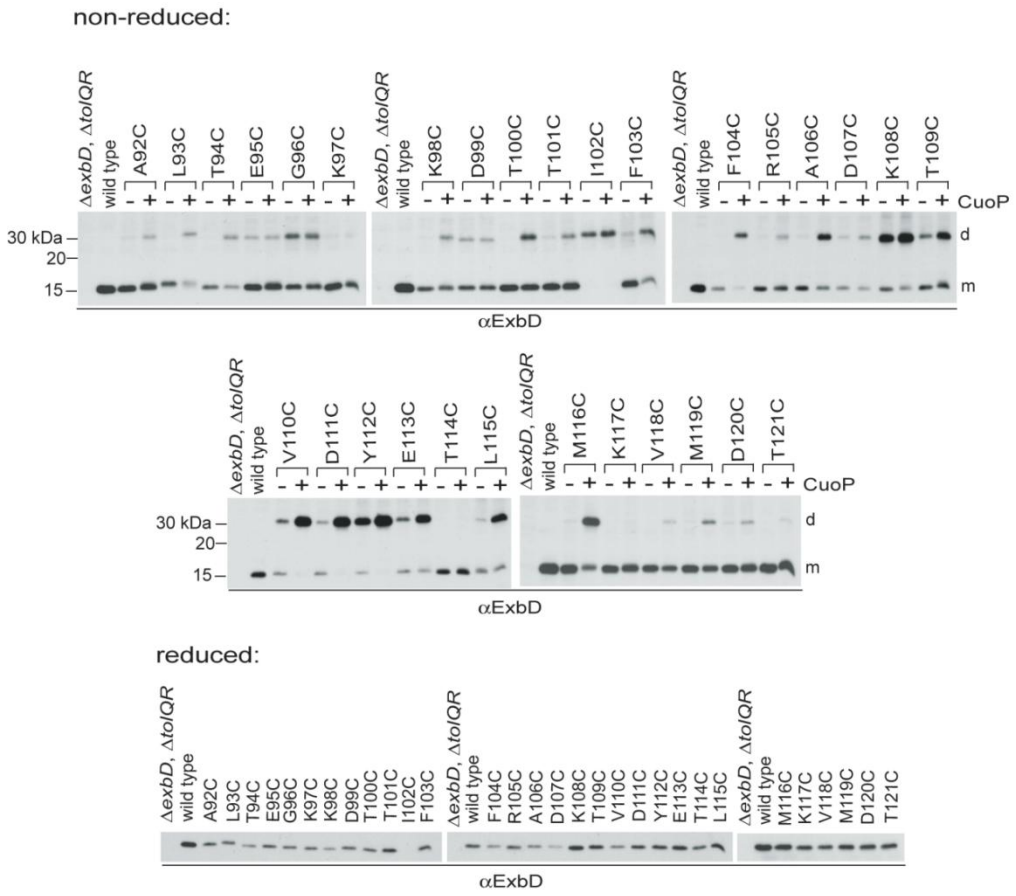


Figure 5-2, Ollis and Postle



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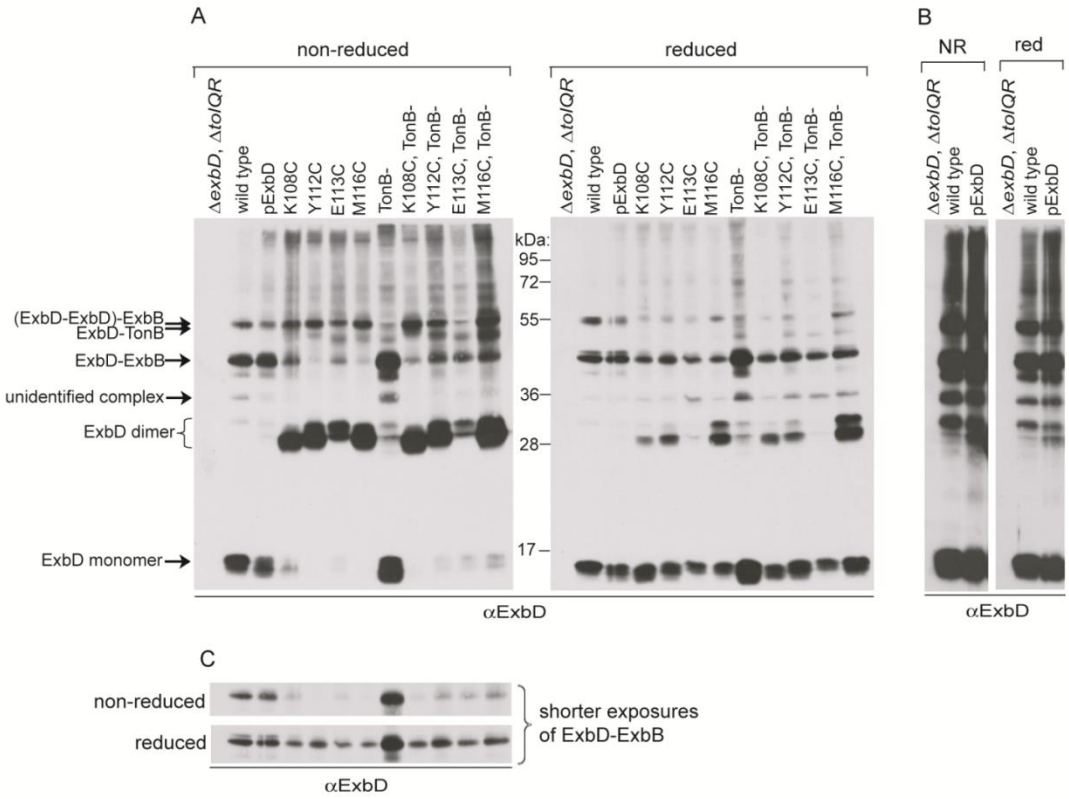

Figure 5-4, Ollis and Postle

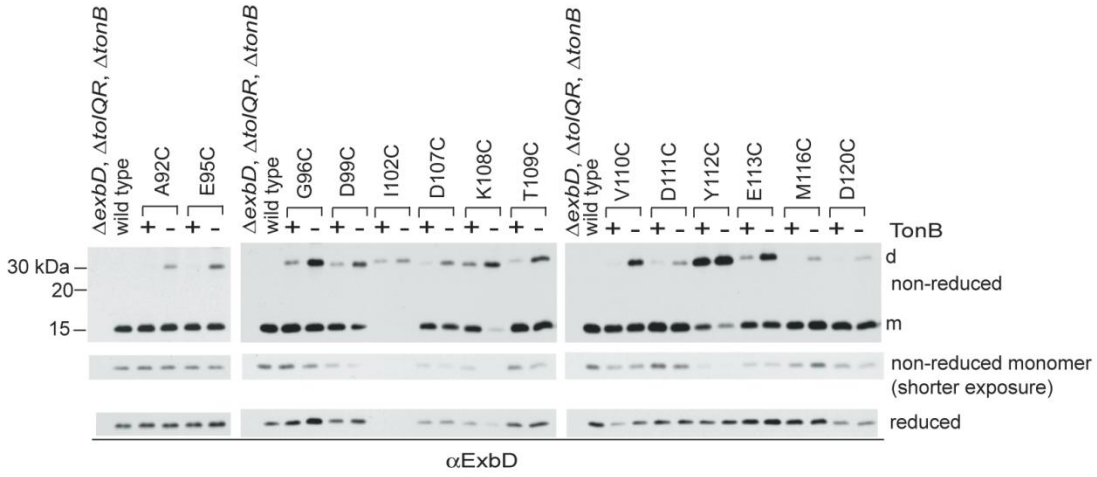

Figure 5-5, Ollis and Postle

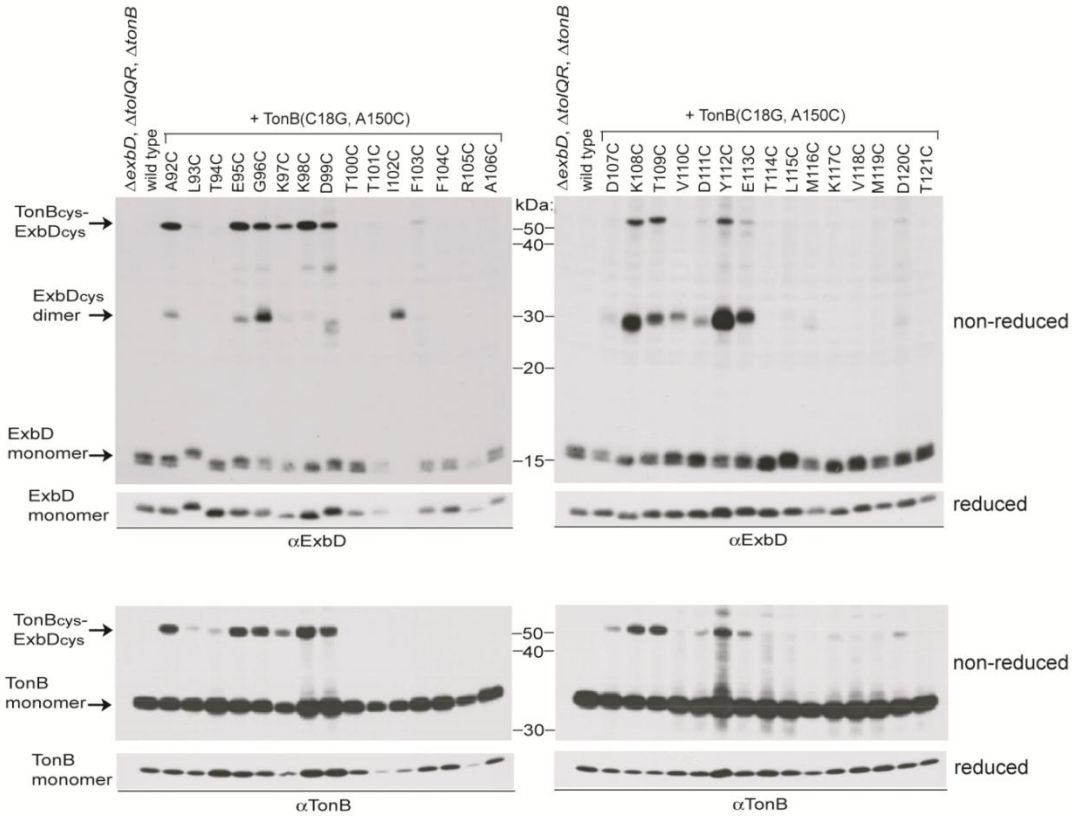

Figure 5-6, Ollis and Postle

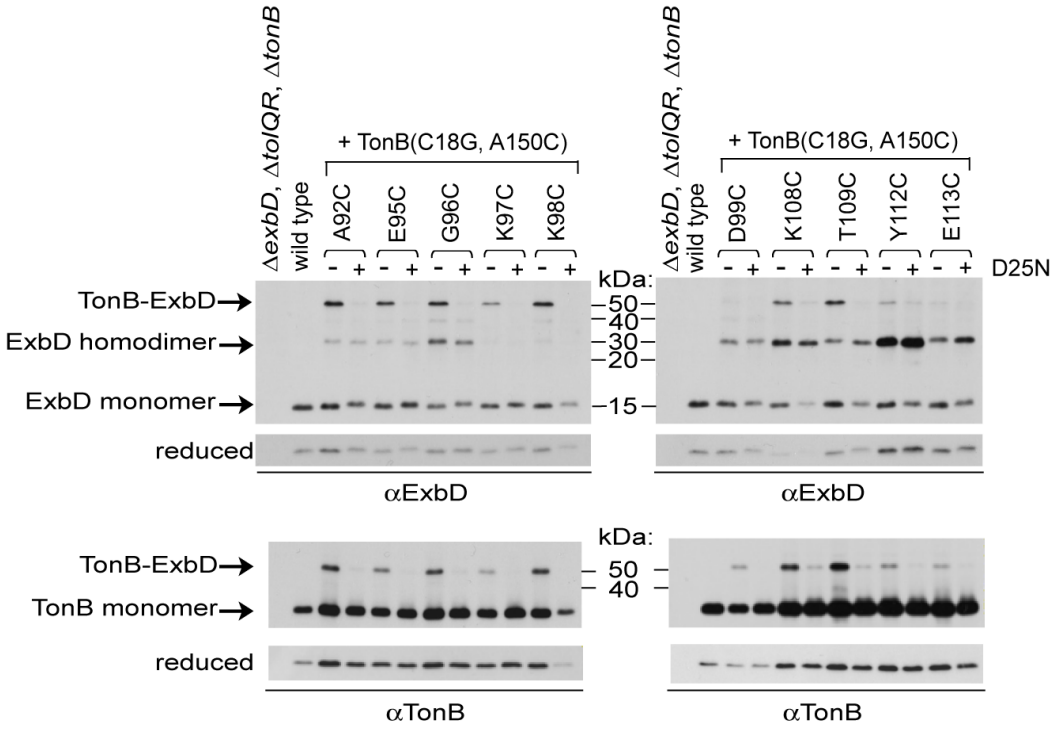

Figure 5-7, Ollis and Postle

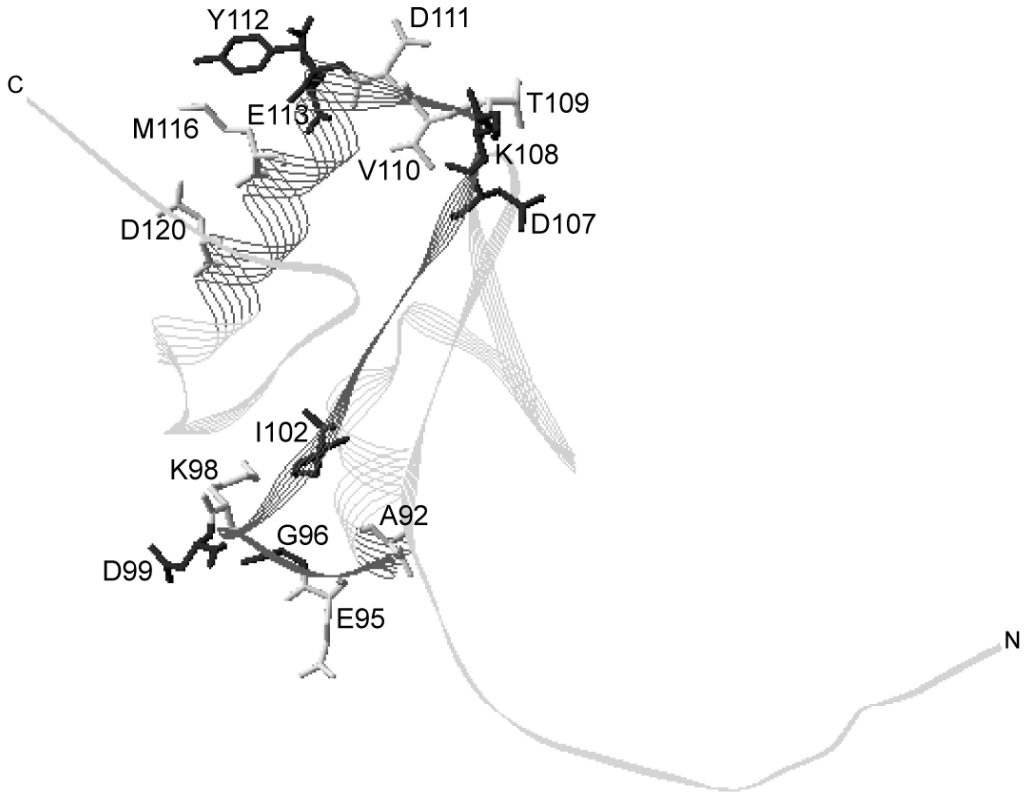

Figure 5-8, Ollis and Postle

Table 5-S1. Induction levels for plasmids used in this study.

		Strain (Phenotype)		
		RA1045 (ExbD-, TolQR-)	KP1509 (ExbD-, TolQR-, TonB-)	RA1045 (ExbD-, TolQR-)
Plasmid	Substitution	mM sodium propionate in T broth[a]		in 1xM9
pKP999	wt ExbD	0.5		3
pKP1000	A92C	0.4	0.5	3
pKP1001	L93C	2		50
pKP1002	T94C	15		35
pKP1003	E95C	0.4	0.4	5
pKP1004	G96C	2	1.8	10
pKP1005	K97C	.0004% glucose		0.5
pKP1006	K98C	35		60
pKP1007	D99C	8	7	15
pKP1008	T100C	1		10
pKP1009	T101C	.0001% glucose		4
pKP1010	I102C	25	25	45
pKP1011	F103C	0.1		0.8
pKP1012	F104C	0.8		10
pKP1013	R105C	.00005% glucose		8
pKP1014	A106C	0.3		3
pKP1015	D107C	35	30	45
pKP1016	K108C	0.2	0.8	10
pKP1017	T109C	0.2	0.2	8
pKP1018	V110C	3	2	16
pKP1019	D111C	0.8	0.7	20
pKP1020	Y112C	0.2	0	3
pKP1021	E113C	1	0.8	12
pKP1022	T114C	0.3		3
pKP1023	L115C	1		7
pKP1024	M116C	0.2	0.1	3
pKP1025	K117C	1.5		8
pKP1026	V118C	1.5		8
pKP1027	M119C	0.5		3
pKP1028	D120C	0.75	0.4	4
pKP1029	T121C	0.5		3

[a]concentrations are not listed (blank spaces) for data not shown

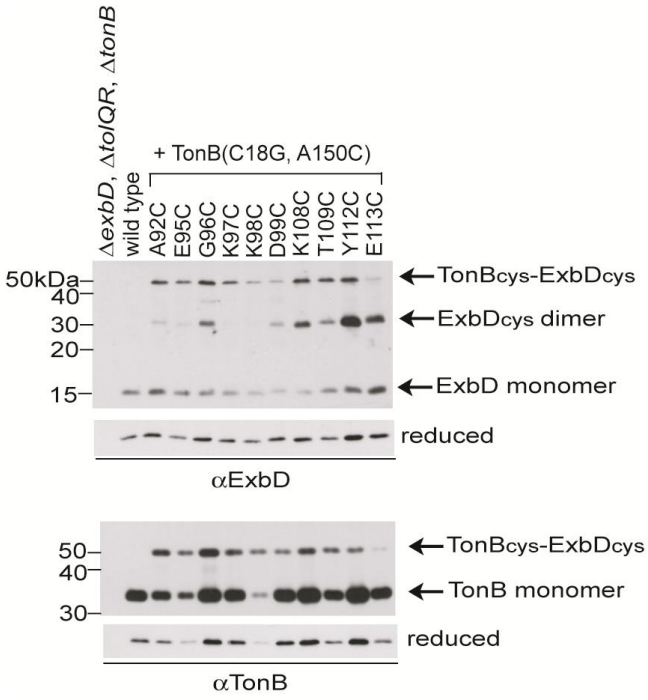

Fig. 5-S1. ExbD disulfide-linked complexes are not induced by TCA precipitation. Cell pellets of a wild-type strain expressing chromosomally-encoded ExbD (W3110) and a Δ*exbD*, Δ*tolQR*, Δ*tonB* strain (KP1509) co-expressing plasmid-encoded ExbD cys substitutions and TonB C18G, A150C were solubilized in non-reducing sample buffer containing 50mM iodoacetamide and immediately resolved on non-reducing 15% and 11% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies or TonB-specific monoclonal antibodies. TCA precipitated samples from the same cultures were solubilized in reducing sample buffer containing β-mercaptoethanol and resolved on gels and immunoblotted as above. ExbD cys substitutions are indicated across the top. Lanes for the lower immunoblot are the same as labeled across the top immunoblot. Positions of ExbD and TonB monomers and disulfide-linked complexes are indicated on the right. Positions of non-reducing molecular mass standards are indicated on the left. The following concentrations of sodium propionate (for ExbD cys substitution) and percentages of arabinose (for TonB C18G, A150C) were used for induction, listed as ExbD cys substitution (mM sodium propionate, % arabinose): A92C (0.4, .00025), E95C (0.2, .00025), G96C (0.6, .0003), K97C (.0006% glucose, .00035), K98C (10, .0002), D99C (5, .00025), K108C (0.4, .00028), T109C (0.3, .00028), Y112C (1.5, .0002), E113C (2, .00028).

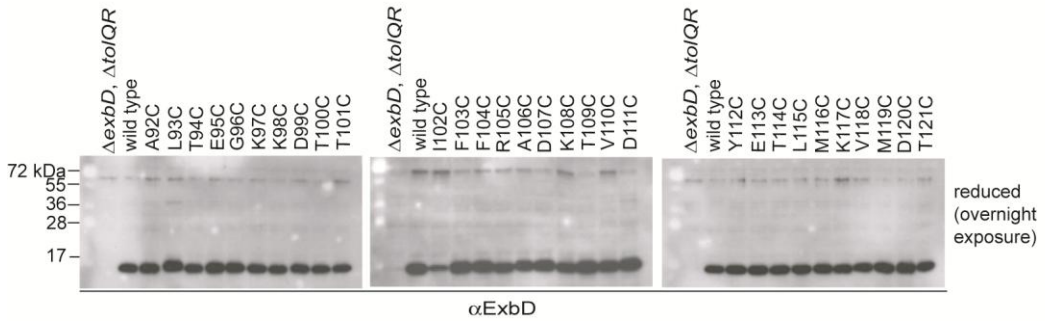

Fig. 5-S2. No ExbD disulfide-linked homodimers are resistant to reduction. TCA precipitated proteins from strains expressing chromosomally-encoded wild-type ExbD (W3110) or a Δ*exbD*, Δ*tolQR* strain (RA1045) expressing plasmid-encoded ExbD variants near native ExbD levels (see Table 5-S1 for induction levels) were resolved on reducing 15% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies. Immunblots shown are overnight exposures of the reduced, cropped immunoblots in Fig. 5-1.

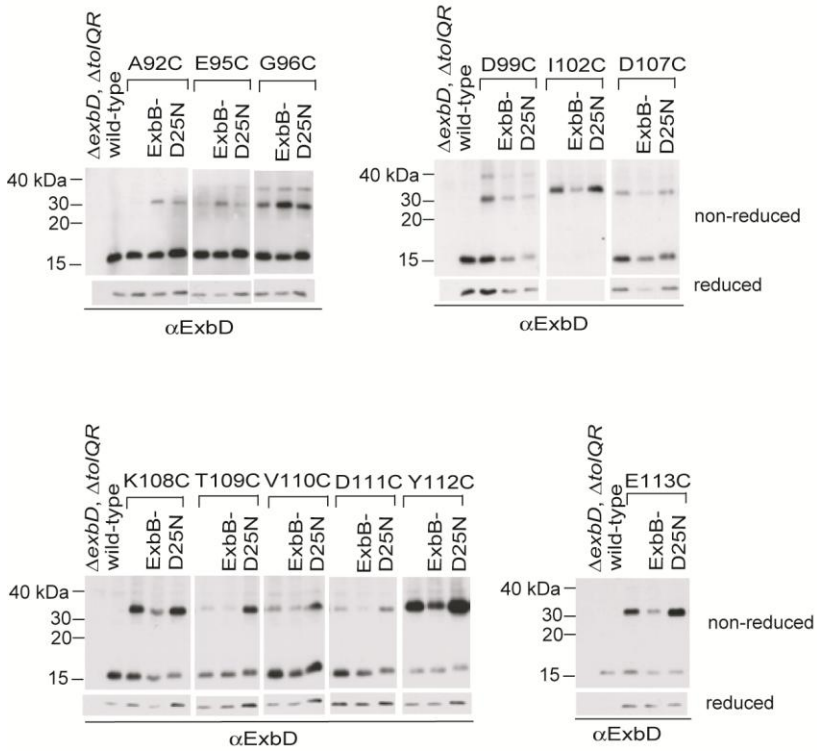

Fig. 5-S3. ExbD disulfide-linked dimers do not require ExbB or TMD residue D25. TCA-precipitated samples of wild-type chromosomally-encoded ExbD (W3110), a Δ*exbD*, Δ*tolQR* strain (RA1045, blank space), a Δ*exbBD*, Δ*tolQRA* (RA1017, ExbB-) expressing plasmid-encoded ExbD cys substitutions, and RA1045 expressing ExbD D25N cys substitutions (D25N) near native ExbD levels were resolved on non-reducing or reducing 15% SDS-polyacrylamide gels and immunoblotted with ExbD-specific polyclonal antibodies. Reduced and non-reduced samples came from the same culture. ExbD cys substitutions are indicated across the top. Positions of non-reducing molecular mass standards are indicated on the left. Combined immunoblots were exposed on the same film, and samples were processed the same day. See Table 5-S1 for induction levels in RA1045. The following concentrations of sodium propionate were used for induction, listed as substitution (ExbB-, D25N samples): A92C (10mM, 0.5mM), E95C (5mM, 0.5mM), G96C (15mM, 3mM), D99C (10mM, 0mM), I102C (40mM, 20mM), D107C (60mM, 9mM), K108C (60mM, 5mM), T109C (30mM, 0.8mM), V110C (60mM, 10mM), D111C (30mM, 1mM), Y112C (4mM, 0.15mM), E113C (25mM, 0.15mM).

CHAPTER 6

MAPPING FUNCTIONALLY IMPORTANT TONB-EXBD PERIPLASMIC DOMAIN INTERACTIONS *IN VIVO*

This chapter is in preparation for submission. The format of the following chapter is slightly different than the preceding chapters to accommodate publication guidelines.

Chapter 6

Mapping functionally important TonB-ExbD periplasmic domain interactions *in vivo*

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ABSTRACT

In Gram negative bacteria, the cytoplasmic membrane protonmotive force energizes active transport of TonB-dependent ligands through outer membrane TonB-gated transporters. Cytoplasmic membrane proteins ExbB and ExbD couple the protonmotive force to conformational changes in TonB, which are hypothesized to form the basis of energy transduction through direct contact with the transporters. While the role of ExbB is not well understood, contact between periplasmic domains of TonB and ExbD is required, with conformational response of TonB to the presence or absence of protonmotive force being modulated through ExbD. A region (residues 92-121) within the ExbD periplasmic domain was previously identified as important for TonB interaction. Here the specific sites of periplasmic domain interactions between that region and the TonB carboxy terminus were identified by examining 270 combinations of 45 TonB and 6 ExbD individual cysteine substitutions for disulfide-linked heterodimer formation. ExbD residues A92C, K97C, and T109C interacted with multiple TonB substitutions in 4 regions of the TonB carboxy terminus known to be conformationally important for TonB function. Because the effect of protonmotive force collapse on spontaneous disulfide-linked complex formation cannot be assayed, the same 270 combinations were examined in the presence a D25N TMD mutation in ExbD, which mimics loss of protonmotive force. ExbD D25 was important to support efficient TonB-ExbD heterodimerization at these specific regions.

Introduction

The TonB system of Gram negative bacteria couples the protonmotive force (pmf) of the cytoplasmic membrane (CM) to energize active transport across the outer membrane (OM). OM TonB-gated transporters bind large, scarce, and essential nutrients that are otherwise excluded

from passive diffusion into the periplasm through OM porin proteins [recently reviewed in (9, 25, 26, 34)]. Multiple specific, high-affinity transporters bind TonB-dependent ligands, including iron-chelating siderophores, vitamin B_{12}, heme, maltodextrin, sucrose, and nickel for subsequent active transport across the OM (2, 6, 32, 44, 45). In *Escherichia coli* K12, at least 7 OM transporters are TonB-dependent (34).

The TonB system includes three integral CM proteins, TonB, ExbB, and ExbD. While the stoichiometry of the potentially hetero-oligomeric complex of these proteins is unknown, TonB, ExbB, and ExbD are present in a cellular ratio of 1:7:2 (17). TonB (239 residues) has a transmembrane domain signal anchor from residues 12-32 and carboxy-terminal periplasmic domain from residues 33-239 (16, 20, 24, 41). ExbD (141 residues) has an identical topology, with TMD residues 23-43, followed by a 98-residue periplasmic domain (21). ExbB (244 residues) contains three TMDs and adopts an N-out, C-in topology. In contrast to TonB and ExbD, the significant soluble domains of ExbB are exposed to the cytoplasm (22, 23).

TonB is the physical connection from the CM pmf energy source to the unenergized OM, spanning the periplasm to make direct contact with TonB-gated transporters (4, 35, 49). ExbB and ExbD appear to couple the protonmotive force to TonB energization, which can be detected *in vivo* as an ExbB-dependent formaldehyde crosslink between the periplasmic domains of TonB and ExbD (36). ExbD TMD D25 is an essential residue and the D25N substitution renders ExbD inactive (3). Cognate aspartate residues in the TolR and MotB transmembrane domains are proposed to participate in proton translocation (5, 53). ExbD D25N mimics the effect of protonmotive force collapse on TonB conformations and *in vivo* formaldehyde crosslinking, and prevents the conformational response of ExbD to protonmotive force (36, 37).

The TonB TMD anchors TonB in the CM throughout its energy transduction cycle (14). The TonB TMD lacks residues for direct response to the pmf, but is important for interaction with ExbB (20, 28, 50). The periplasmic carboxy terminus is the region through which TonB contacts

both TonB-gated transporters and the ExbD periplasmic domain (4, 36). The TonB periplasmic domain is unusual in its high tolerance to internal deletions or residue substitutions. Internal deletions within the region of residues 33-149 retain detectable activity, even with as many as 47 residues (103-149) deleted, suggesting the conformation of the domain immediately following the transmembrane domain is not essential for TonB function (31, 40, 47). Between residues 150-239, only seven residues are functionally important. Substitutions at these residues exhibit idiosyncratic phenotypes depending on the residue being substituted and the transporter-specific assay being used. While no single residue is essential for TonB function, simultaneous substitution of any two of the seven residues with alanine inactivates TonB, a synergistic phenotype suggesting that they all interact (12, 13, 40). Because the majority of TonB is predicted to have a disordered structure (28), it is logical to propose that the overall conformation of the TonB periplasmic domain is determined by its association with another protein. Based on its topology, ExbD is a logical candidate.

The TonB periplasmic domain assumes multiple conformations *in vivo*. Six different TonB cysteine substitutions in the carboxy terminus each appear to exhibit three conformations of disulfide-linked homodimers on non-reducing SDS-polyacrylamide gels. These TonB "triplet homodimers" form primarily through substitutions at aromatic residues. The TonB conformations represented by the triplet homodimers are dependent on the presence of ExbB and ExbD, and a wild-type TonB transmembrane domain (12). These *in vivo* TonB homodimers represent unique conformations compared to the homodimeric TonB observed in the crystal structure of a $TonB_{165-239}$ carboxy terminal fragment that lacks input from the TonB transmembrane domain, ExbB, and perhaps most importantly, ExbD (7, 40).

ExbD also forms homodimers *in vivo* through its periplasmic domain, with specific interactions identified between residues 92-121 (38). The ExbD periplasmic domain appears to function as a homodimer because ExbD I102C, which dimerizes completely, is still fully active.

While the nuclear magnetic resonance (NMR) structure of the monomeric ExbD periplasmic domain shows ExbD residues 92-121 as part of a region of defined tertiary structure, conformational changes in that structure are required to account for all the sites of homodimerization observed *in vivo* (11, 38). The conformation of ExbD is responsive to changes in protonmotive force, dependent on TMD residue D25 (37). D25 is also important for an apparent transition of localized regions of the ExbD periplasmic domain from specific sites of homodimerization to heterodimeric interaction with the TonB periplasmic domain *in vivo* (38).

Consistent with those data, the TonB carboxy terminus appears to conformationally respond to protonmotive force only indirectly, through modulation of its conformation by ExbD. Three stages in the energization of TonB through its relationship with ExbD have been recently identified. Analysis of the effects of ExbD mutants on ExbD and TonB conformation, demonstrated that the ExbD transmembrane domain responds to changes in the protonmotive force, mediating changes in the ExbD periplasmic domain that are then transmitted to the TonB carboxy terminus (37).

Here we mapped specific interactions between a subdomain of the ExbD periplasmic domain and the extreme carboxy terminus of TonB *in vivo*. Multiple significant interactions with TonB residue substitutions were observed for 3 of the 6 ExbD residue substitutions examined, with 3 remaining non-reactive. Interactions clustered in 4 important regions of the TonB carboxy terminus. In the presence of an ExbD D25N TMD mutation, all interactions were significantly reduced.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 6-1.

Media and culture conditions

Luria-Bertani (LB) and tryptone (T) broth were prepared as previously described (33). 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. Cultures were grown with aeration at 37°C.

In vivo *disulfide crosslinking*

Saturated LB overnight cultures were subcultured 1:100 in T broth. Equivalent ODmL of mid-exponential phase cultures were harvested and precipitated by addition of an equal volume of 20% trichloroacetic acid (TCA). Cell pellets were solubilized in non-reducing Laemmli sample buffer (27) containing 50mM iodoacetamide, as previously described (12). Samples were resolved on non-reducing 11% and 13% SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal antibodies (29) or ExbD-specific polyclonal antibodies (17). Disulfide-linked complexes still formed when samples were not TCA precipitated (data not shown).

Results

3 of 6 ExbD cys substitutions interact significantly with the TonB carboxy terminus in vivo.

Recently, 71 cys substitutions in the extreme TonB carboxy terminus were characterized, completing a set of 90 single substitutions from TonB A150C through TonB Q239C. From those studies, only 7 TonB residues, F125, G186, F202, W213, Y215, and F230, were found to be

partially intolerant to substitution with cys, exhibiting idiosyncratic phenotypes across multiple activity assays (12, 40). 83 cys substitutions were fully active, providing a large pool for studies of TonB interactions through *in vivo* disulfide crosslinking.

A92 initiates a 30-residue region of ExbD, from 92-121, where the conformation is especially important in supporting ExbD protein-protein interactions (Ollis, Kumar, & Postle, in preparation). Individual cys substitutions were previously constructed spanning these ExbD residues, and all 30 ExbD cys substitutions fully supported TonB activity (38). Previous work showed that ExbD A92C is trapped in a disulfide-linked heterodimer when co-expressed with TonB A150C (36). In addition to ExbD A92C, E95Cthrough D99C, K108C, T109C, Y112C and E113C also formed significant heterodimers with TonB A150C (38). Here we extended our study of specific periplasmic ExbD-TonB interactions to a more comprehensive scan of the extreme carboxy terminus of TonB (every other residue from 150-239).

To examine specific TonB-ExbD interactions *in vivo,* all plasmids expressing TonB cys substitutions (pACYC *ori*, $cam^{R)}$ in this study were compatible with plasmids expressing the ExbD cys substitutions (pBR322 *ori*, amp^{R}). The introduced cys in each protein was the only site of potential disulfide crosslinking, as ExbD has no native cysteine residues, and all TonB cys substitutions were constructed on a cys-less TonB (C18G) (12, 40). *TonB* and *exbD* expression were under control of the P_{BAD} and P_{prpB} promoters, respectively, allowing control of expression of both TonB and ExbD substitutions at native levels of each respective protein, as assayed under reducing conditions. Iodoacetamide was also present in the sample buffer to alkylate free sulfhydryl groups and prevent disulfide-linkage from occurring after cell lysis.

Initially ExbD A92C and 45 individual TonB cys substitutions (every even-numbered residue from A150C to I238C) were co-expressed in a Δ*exbD,* Δ*tonB,* Δ*tolQR* strain and analyzed for spontaneous disulfide-linked heterodimer formation on non-reducing SDS-polyacrylamide gels. ExbD- and TonB-specific immunoblots showed the previously observed complex at

approximately 52 kDa for ExbD A92C with TonB A150C and significant complexes, designated here as strong interactions, of the same apparent molecular mass for TonB cys substitutions, at 156, 164, 168, 170, 200, 202, 204, 208, and 212. Those interactions are summarized in Fig. 6-1A, by solid lines. A number of weaker interactions were also observed (Fig. 6-1A, dashed lines). Any complexes that formed less inefficiently than the "weak" classification were not mapped in this study. Immunoblots of ExbD A92C in combination with TonB N200C through R214C are shown as examples of strong (R212C), weak (R214C), and below detection interactions (V206C) (Fig. 6-2). Subsequent examples show only the anti-TonB immunoblot.

Results with ExbD A92C suggested that specific interactions between TonB and ExbD span the extreme carboxy terminus of TonB. To extend these studies, TonB interactions with ExbD K97C, F103C, T109C, L115C, and T121C were examined to include sites spanning the entire ExbD subdomain. This study, therefore, included 3 previously identified ExbD interactive sites and 3 ExbD sites through which interaction with TonB was not previously detected. Like ExbD A92C, ExbD K97C and T109C exhibited strong and weak interactions with a number of TonB cys substitutions that clustered in similar regions (Fig. 6-1B and 6-1C, solid lines). TonB substitution sites of strong interaction with ExbD K97C were at 150, 170, 184, and residues from 202-212 with the exception of 206 and 210. Strong TonB interactions with ExbD T109C occurred with TonB residue substitutions at 150, 152, 164, 166, 200, 204, and 208 . Three ExbD cys substitutions at F103C, L115C, and T121C were designated as non-reactors because no interactions were detected between them and any TonB cys substitutions. Degree of interactions among ExbD A92C, K97C, F103C, T109C, L115C, or T121C with TonB R166C, Q168C, or L170C are shown as examples (Fig. 6-3).

A D25N mutation in the ExbD TMD significantly reduces TonB-ExbD disulfide-linked heterodimer formation

To assess the biological relevance of the disulfide crosslinking between ExbD and TonB periplasmic cys substitutions, the same 270 TonBcys-ExbDcys combinations were examined but with a D25N mutation in the TMD of all ExbD cys substitutions. Disulfide-linked dimer formation was significantly reduced in the presence of the D25N TMD mutation (examples are shown in Fig. 6-4). All heterodimers detected with ExbD D25N cys substitutions were below our arbitrary limit of detection, with the recognition that upon much longer exposures, some complexes were evident to various degrees depending on how strong the initial interaction with the ExbD D25 variants were. Complexes of ExbD A92C with TonB Q162C or P164C were approximately 4 times or 10 times less when D25N was present, for example, based on dilutions (Fig. 6-4). No new heterodimers were detected. The low level detection of heterodimers with ExbD D25N cys substitutions suggested D25 was important for either the formation of the complexes, such that they formed inefficiently in the presence of D25N, or for maintaining ExbD-TonB periplasmic domains in the crosslinkable conformations. The significant but incomplete loss of interactions supports the previous finding that ExbD D25N and TonB can assemble. Strong interactions in the presence of D25 may reflect the conformational changes due to response to pmf (37). Similar to results here with ExbD D25N-TonB interactions, treatment of flagellated cells with the protonophore CCCP prior to BMOE crosslinking through introduced cys substitutions significantly reduces but does not eliminate MotB-FlgI heterodimers compared to when pmf is present (19).

Discussion

Recent results from our lab have demonstrated that ExbD appears to be in equilibrium between homodimers and TonB-ExbD heterodimers *in vivo,* with nearly the same ExbD residues involved in both interactions (38). Three stages of TonB energization have been identified based on effects of ExbD mutations D25N in the transmembrane domain and L132Q in the periplasmic domain (37). ExbD L132Q is required for an initial correct pmf-independent assembly between TonB and ExbD that subsequently leads to formation of a pmf-dependent conformation detectable through formaldehyde crosslinking through the TonB and ExbD periplasmic domains (36). ExbD D25 is required for the pmf-dependent interaction. Residues important in mediating specific interaction between the TonB and ExbD periplasmic domain, however, were largely unknown.

Here we examined 270 combinations of TonB and ExbD periplasmic domain cys substitutions in the presence of either an active or inactive (D25N) ExbD TMD, totaling 540 combinations. The targeted regions in this study were the TonB extreme carboxy terminus (residues 150-239), where the only functionally important TonB periplasmic domain residues are located, and a 30-residue region of the ExbD periplasmic domain important in supporting protein-protein interactions of ExbD (Ollis, Kumar, & Postle, in preparation). A surprising number of TonB cys substitutions were trapped in spontaneous disulfide-linked interaction through 3 of the 6 ExbD cys substitutions examined, with the other 3 showing essentially no reactivity. These were designated non-reactors. When all sites of heterodimer formation were compared, 4 regions of ExbD-TonB interactions were apparent, based on TonB sites where at least one ExbD cys substitution exhibited strong interaction or at least 2 exhibited weak interactions, TonB 150-152, 164-170, 184-186, and 198-212.

The first and second regions included TonB A150C and G152C, and TonB P164C, R166C, Q168C, and L170C, respectively. Both of these regions are located towards the carboxy terminal end of a large region of TonB periplasmic domain (residues 33-169) that is predicted to be disordered (28). The predicted unstructured nature of this region may facilitate interaction of ExbD at multiple sites. Close interaction of ExbD with this region could support a previously proposed role for ExbD in the potential disorder-to-order transitions of this region of TonB (51).

The first and second regions are also found on either side of a region of TonB, residues 160-163, that makes direct *in vivo* contact with the conserved amino terminal TonB boxes of the OM TonB-gated transporters BtuB, specific for vitamin B_{12}, and FecA, specific for ferric citrate (4, 35). For the majority of interactions, the strongest ExbD-TonB interaction sites did not overlap the strongest TonB box-TonB interaction sites, suggesting ExbD could theoretically interact simultaneously with TonB that is bound to the TonB box or direct bound TonB to the TonB box. Two possibilities seem likely: ExbD-TonB interaction through these regions may occur within a cycle of energy-independent TonB contact and release with OM transporters. Alternatively, energy may serve to release initial TonB contact at the OM or energized interaction between TonB and ExbD may result in a TonB conformation that only permits productive interaction with ligand-bound transporters. It is unknown if the specific TonB box interactions with TonB represent contacts for functional interaction. To date, less specific crosslinking methods, such as formaldehyde crosslinking, have trapped only energy-independent interactions between TonB and TonB-gated transporters *in vivo* {Ogierman 2003;Skare 1993;Ghosh 2005).

The third region of ExbD-TonB interaction included TonB P184C and G186C, which exhibited primarily weak interactions with 2 of the 3 reactive ExbD cys substitutions, A92C and K97C. G186 is one of the only 7 residues in the TonB periplasmic domain that are functionally important and one of the 6 sites (the only non-aromatic site) where cys substitution resulted in formation of TonB triplet homodimers *in vivo*. These results along with the high conservation of

G186 led to the proposal that it may have a fundamental mechanistic role as opposed to being a site of direct transporter recognition by TonB, as the other aromatic residues appear to be {Postle 2010}. Two additional functionally important TonB residues, capable of forming triplet dimers as cys substitutions, were also examined as part of this study—F202 and F230. Heterodimeric interactions observed for TonB G186C with ExbD A92C or K97C and F230C with ExbD A92C were weak. F202C, however, exhibited strong interaction with A92C and K97C. F230C forms the highest levels of TonB triplet homodimers, and this interaction requires ExbB/D (12). Homodimeric rather than heterodimeric interactions may dominate the F230C sites of assembled TonB, as interactions that were observed with ExbD K97C and T109C were below our threshold weak interaction level. Alternatively F230 is not a primary site of interaction but low levels of interaction were trapped during conformational changes. TonB homodimers that spontaneously arise through disulfide crosslinking of F202C and F230C form only at the CM, and preclude interaction of TonB with the OM, indicating homodimeric interfaces must be reorganized before the TonB periplasmic domain can interact with OM proteins (12). The formation of TonB-ExbD heterodimers through these sites further supports that G186, F202, and F230 residues do not constitute permanent TonB homodimeric interfaces. Because TonB-ExbD interactions required ExbD TMD residue Asp 25, these heterodimeric interactions may occur after response to pmf. Taken together, these data extend ExbD-directed remodeling of the TonB periplasmic domain (36) to involve a network of specific D25-promoted ExbD-TonB interactions and further suggest that one role of these interactions might be to resolve TonB homodimeric interactions to free these sites for interaction with OM TonB-gated transporters.

The fourth region included strong interaction of ExbD cys substitutions with TonB N200C, F202C, R204C, N208C, and R212C. This region is found within a predicted amphipathic helix (residues 199-216) of TonB, one of the most highly conserved TonB features across Gram negative bacteria (8). Of the even-numbered TonB residues exhibiting strong ExbD

interactions, N200, R204, N208, and R212, mapped to one face of this helix, as seen in the crystal structures of TonB periplasmic domain fragments (Fig. 6-5, for example). The exception was F202C, a functionally important aromatic residue, discussed above, which maps to an opposite face. In the monomeric TonB NMR structure or crystal structures of TonB in complex with BtuB or FhuA, N200 and F202 are not part of the helix but in an adjacent loop (Fig. 6-5B, for example). It is unknown if this helix is important for TonB functions. The alignment of strong interactive sites almost exclusively to one face of the predicted helix suggests primarily partial solvent exposure of this secondary structural element within monomeric TonB. While these 4 regions of TonB represent the sites of strongest interaction with ExbD cys substitutions, the arbitrary threshold chosen for this study excludes mapping a number of weaker interactions observed upon long exposures. Overall, these sub-weak interactions still group to 4 regions, but expand the residues at the limit of each region (for example, sub-weak interactions not mapped from TonB E216 through S222 extending region 4). It may be that these represent transient associations of ExbD or TonB searching for the conformational state that supports their functional interaction or undergoing conformational changes triggered by their initial assembly to reach the interactive state.

The available structures for fragments of the TonB carboxy terminus have similar overall structures, with two main conformational variations observed, whether the TonB fragment is monomeric or in complex (39, 48, 52). The specific sites of TonB-ExbD interactions observed *in vivo* map primarily along one side of these structures, and here, two representative structures are shown (Fig. 6-5). In the $TonB_{165-239}$ homodimer, most sites exhibiting strong interactions with ExbD are exposed (Fig. 6-5A). This differs from $TonB_{153-239}$ crystallized in complex with the OM transporter BtuB, where the general region involved in interaction with ExbD interfaces with the periplasmic face of the transporter (Fig. 6-5B). TonB residues forming interactions with BtuB *in vitro* span the regions from 158 to 172, 199 to 213, and 225 to 233 [analyzed using the

program MONSTER (42), data not shown]. TonB R166, L170, N200 and R204, which all exhibited strong interactions with ExbD *in vivo*, are involved in specific interactions with the β-barrel of BtuB *in vitro*. R166, Q168, and L170 also exhibit interactions with the plug domain of BtuB. It is unknown if these specific TonB-BtuB interactions occur *in vivo*, but this could potentially suggest some common TonB interactive sites with both ExbD and TonB-gated transporters. It is known that the TonB box is not the only site through which TonB interacts with the tranporters *in vivo* (10). Interestingly, TonB sites of interaction with BtuB that were not significantly detected to interact with ExbD in this study, primarily form interactions with the TonB box of BtuB (residues 6-12). It is unknown if ExbD is required for TonB interaction with the TonB box of transporters, but these results could suggest TonB bound by ExbD can freely interact with the TonB box region of transporters. Perhaps ExbD "primes" TonB for proper TonB box interaction by stabilizing a specific conformation of the periplasmic domain, similar to the priming of pilus subunits, for subsequent donor strand exchange, by a periplasmic chaperone in the chaperone-usher pathway (43). In both TonB structures, interactive sites such as F202, are buried and inaccessible for ExbD interaction, even if just the monomeric unit of TonB is isolated, suggesting ExbD interaction with TonB involves multiple TonB conformations.

Since the disulfide-linked heterodimers in this study formed spontaneously, the effect of protonophores on complex formation could not be meaningfully examined. When the cys substitutions are expressed, disulfide-linked complexes are pre-existing in cells. If expression of the cys substitutions is induced, protonmotive force cannot be collapsed without preventing insertion of newly synthesized proteins into the CM. ExbD D25 is important for the energized interaction between ExbD and TonB periplasmic domains and is involved in the conformational response of ExbD to the pmf. Both functions are prevented by a D25N mutation (36). The effect of a D25N mutation on ExbD cys substitutions was significant, but not complete, reduction in specific interactions with the TonB extreme carboxy terminus, suggesting pmf-dependent

conformational changes promote these interactions. While the observed interactions between the TonB carboxy terminus and ExbD D25N were below our chosen threshold for significant interactions, the detection of low levels of ExbD D25N-TonB heterodimers provided the first direct evidence to confirm our previous study suggesting that ExbD D25N assembled with TonB (37). The current study shows, in addition, that ExbD D25N interactions did not strongly involve the TonB carboxy terminus (residues 150-239). Accordingly, the TonB carboxy terminus is specifically sensitive to proteinase K at this stage of ExbD-TonB interaction, with the pmf-independent interactions represented by proteinase K resistance of the amino-terminal 1 to ~156 residues of TonB (30). Response of ExbD to pmf may transmit a signal from the ExbD TMD that alters the conformation of the ExbD periplasmic domain, promoting interaction with the carboxy terminus of TonB. Specific interactions of the ExbD paralogue MotB are influenced by the pmf. In *E. coli*, the periplasmic domain of MotB can bedisulfide crosslinked to FlgI, a periplasmic component of the rotor of the flagellar motor, through introduced cysteines in both proteins in the presence of an oxidizing agent or a bismaleimide crosslinker such as BMOE, and interactions are significantly reduced after collapse of pmf (19).

When the three sites of significant ExbD interaction with TonB are mapped on the ExbD periplasmic domain NMR structure, A92 and K97 are located at one end of the structure, with T109 at the opposite end, suggesting the possibility of multiple interfaces of interaction of the ExbD periplasmic domain with TonB (Fig. 6-6). The non-reactors F103, L115, and T121 are each positioned closer to the middle of the structure. If this conformation of ExbD were to interact with TonB, interactive sites would be expected to be surface exposed. A92 and T109, both highly interactive *in vivo* as cys substitutions are highly solvent exposed in the NMR structure, at 76% and 72% respectively [determined using ASA-View (1), data not shown]. Non-interactive cys substitutions *in vivo* were at sites of F103 and T121, with about only 36% and 32% solvent exposure in the NMR structure, respectively. K97 and F103, however, had similar

relative solvent exposure in the solution structure, 48% and 50% respectively, but K97C was highly interactive *in vivo* and F103C was not.

It is important to note in the context of the NMR structures of both the ExbD and TonB periplasmic domains that, *in vitro*, no significant interactions were observed between them at either pH 3, which allowed solution of the monomeric ExbD structure or pH 7, approximately the pH of the periplasm (11). The TonB carboxy terminal fragment used in those studies (residues 103-239) assumes a nearly identical conformation to the monomeric unit of $TonB_{153-239}$ crystalized in complex with BtuB [(46, 48) and Fig. 6-5B]. This suggests these observed conformations do not represent the interactive states of the ExbD and TonB periplasmic domains *in vivo*. Elements of the structures, however, may be important in energy transduction. Localized regions could represent interactive surfaces, but perhaps the stable tertiary structures assumed *in vitro* lack the proper orientation of these elements for these interactions to occur.

This work provided the first extensive view of specific ExbD D25-dependent interactions with the extreme TonB carboxy terminus. ExbD interaction with functionally important regions of TonB, thought to be involved in defining TonB conformations important for recognition of OM transporters, supports a role for ExbD in regulating TonB conformation. ExbD TMD residue D25 appears to define the extent of ExbD-TonB periplasmic domain interactions through its role in regulating ExbD periplasmic domain conformation, potentially in response to the pmf.

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Figure Legends

Fig. 6-1. Mapping specific sites of TonB-ExbD periplasmic domain interactions supported by a functional ExbD TMD. ExbD and TonB cysteine substitutions examined and their abilities to form disulfide crosslinked heterodimers are depicted. TonB cys substitutions are listed on the left and right, and the ExbD cys substitution each was co-expressed with is listed in the center. Solid lines indicate a relatively strong interaction. TonB substitutions supporting strong interactions are highlighted in bold. Dashed lines indicate a relatively weak or inefficient interaction. A-C, Interactions supported by ExbD cys substitutions with a wild-type ExbD TMD.

Fig. 6-2. Strong and weak interactions are supported by ExbD A92C and TonB substitutions between N200C and R214C. A strain expressing wild-type ExbD and TonB (W3110) or a Δ*exbD*, Δ*tonB*, Δ*tolQR* strain (KP1509) co-expressing ExbD A92C (pKP1000) with TonB C18G, N200C (pKP469), F202C (pKP415), R204C (pKP418), V206C (pKP463), N208C (pKP416), M210C (pKP466), R212C (pKP471), or R214C (pKP473) were processed in non-reducing sample buffer containing iodoacetamide as described in Materials and Methods. Samples were resolved on a 13% non-reducing SDS-polyacrylamide gel and immunoblotted with ExbD-specific polyclonal antibodies (left) or TonB-specific monoclonal antibodies (right). Samples from the same cultures were processed in reducing sample buffer containing βME and resolved on 11% or 13% SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal or ExbD-specific polyclonal antibodies (lower immunoblots). The combinations of substitutions specific to each lane are indicated across the top. Positions of molecular mass standards are indicated in the center. Positions of the ExbD or TonB monomers and disulfide-linked complexes are indicated on the left and right, respectively.

Fig. 6-3. ExbD A92C, K97C, and T109C but not F103C, L115C, and T121C form significant complexes with TonB cys substitutions. A strain expressing wild-type ExbD and TonB (W3110) or a Δ*exbD*, Δ*tonB*, Δ*tolQR* strain (KP1509) co-expressing ExbD A92C (pKP1000), K97C (pkP1005), F103C (pKP1011), T109C (pKP1017), L115C (pKP1023), or T121C (pKP1029) with TonB C18G, R166C (pKP589), Q168C (pKP593), or L170C (pKP591) were processed in non-reducing sample buffer containing iodoacetamide as described in Materials and Methods. Samples were resolved on an 11% non-reducing SDS-polyacrylamide gel and immunoblotted with TonB-specific monoclonal antibodies. Samples from the same cultures were processed in reducing sample buffer containing βME and resolved on 11% or 13% SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal or ExbD-specific polyclonal antibodies (lower immunoblots). Division of the immunoblot is to indicate ExbD F103C combinations came from a separate gel and immunoblot. All other combinations came from the same gel and immunoblot. The combinations of substitutions specific to each lane are indicated across the top. Positions of molecular mass standards are indicated on the left. Positions of the ExbD or TonB monomers and disulfide-linked complexes are indicated on the right.

Fig. 6-4. A D25N TMD mutation in ExbD significantly reduces TonB-ExbD disulfide-linked interactions. A strain expressing wild-type ExbD and TonB (W3110) or a Δ*exbD*, Δ*tonB*, Δ*tolQR* strain (KP1509) co-expressing ExbD A92C (pKP1000); D25N, A92C (pKP1049); K97C (pkP1005); D25N, K97C (pKP1050); T109C (pKP1017); or D25N, T109C (pKP1052) with TonB C18G, Q160C (pKP588), Q162C (pKP587), or P164C (pKP585) were processed in non-reducing sample buffer containing iodoacetamide as described in Materials and Methods. Samples were resolved on an 11% non-reducing SDS-polyacrylamide gel and immunoblotted with TonB-specific monoclonal antibodies. Samples from the same cultures were processed in reducing sample buffer containing βME and resolved on 11% or 13% SDS-polyacrylamide gels

and immunoblotted with TonB-specific monoclonal or ExbD-specific polyclonal antibodies (lower immunoblots). The combinations of substitutions specific to each lane are indicated across the top. "-" or "+" indicates the absence or presence, respectively, of the D25N mutation in the TMD of the ExbD cys substitution. Positions of molecular mass standards are indicated on the left. Positions of the ExbD or TonB monomers and disulfide-linked complexes are indicated on the right.

Fig. 6-5. Sites of *in vivo* disulfide-linked heterodimer formation mapped on two TonB periplasmic domain fragment crystal structures. Side chains depicted in black show sites where TonB cys substitutions formed strong disulfide-linked heterodimers with at least 1 ExbD cys substitution. Two representative TonB structures, homodimeric $TonB_{165-239}$ (A, 1ihr) and BtuB-$TonB_{153-239}$ (B, 2gsk), are shown. Interactive sites A150, G152, and L156 (for 1ihr only) are not part of the structures and could not be mapped. The image was generated using Swiss-PdbViewer [http://www.expasy.org/spdbv/, (15)].

Fig. 6-6. ExbD cys substitutions showing strong interactions with TonB cys substitutions *in vivo* map to opposite ends of the ExbD periplasmic domain NMR structure. The locations of the six ExbD cys substitutions examined in this study, with side chains of the native residue shown, are mapped on the ExbD periplasmic domain NMR structure, pdb code 2pfu. The image was generated using Swiss-PdbViewer [http://www.expasy.org/spdbv/, (15)]. Black side chains indicate sites of significant spontaneous ExbD-TonB heterodimer formation. Gray side chains indicate sites of no significant spontaneous heterodimer formation. "C" and "N" indicate the carboxy and amino terminus of the domain, respectively.

Table 6-1. Strains and plasmids used in this study.

Strain or Plasmid	Genotype or Phenotype	Reference
Strains		
W3110	F⁻ IN*(rrnD-rrnE)1*	(18)
KP1509	W3110, Δ*exbD*, Δ*tonB*::*kan*, Δ*tolQR*	(36)
[a]Plasmids		
pKP945	TonB C18G, A150C	(36)
pKP1000	ExbD A92C	(36)
pKP1005	ExbD K97C	(38)
pKP1011	ExbD F103C	(38)
pKP1017	ExbD T109C	(38)
pKP1023	ExbD L115C	(38)
pKP1029	ExbD T121C	(38)
pKP1049	ExbD D25N, A92C	(36)
pKP1050	ExbD D25N, K97C	(38)
pKP1051	ExbD D25N, F103C	(38)
pKP1052	ExbD D25N, T109C	(38)
pKP1053	ExbD D25N, L115C	(38)
pKP1082	ExbD D25N, T121C	(38)

pKP947 (G152C)	pKP597 (G174C)	pKP609 (P198C)	pKP508 (F180C)
pKP949 (R154C)	pKP598 (V176C)	pKP643 (G218C)	pKP469 (N200C)
pKP951 (L156C)	pKP601 (V178C)	pKP617 (P220C)	pKP415 (F202C)
pKP953 (R158C)	pKP604 (V182C)	pKP619 (S222C)	pKP418 (R204C)
pKP588 (Q160C)	pKP610 (P184C)	pKP623 (I224C)	pKP463 (V206C)
pKP587 (Q162C)	pKP612 (G186C)	pKP625 (V226C)	pKP416 (N208C)
pKP585 (P164C)	pKP614 (V188C)	pKP627 (I228C)	pKP466 (M210C)
pKP589 (R166C)	pKP638 (N190C)	pKP629 (I232C)	pKP471 (R212C)
pKP593 (Q168C)	pKP639 (Q192C)	pKP631 (G234C)	pKP473 (R214C)
pKP591 (L170C)	pKP641 (L194C)	pKP634 (T236C)	pKP475 (E216C)
pKP600 (I172C)	pKP607 (A196C)	pKP636 (I238C)	pKP510 (F230C)

[a]Plasmids listed below the line express TonB C18G with the cys substitution listed in parentheses. Plasmids in the first 3 columns are from (40). Plasmids in the last column are from (12).

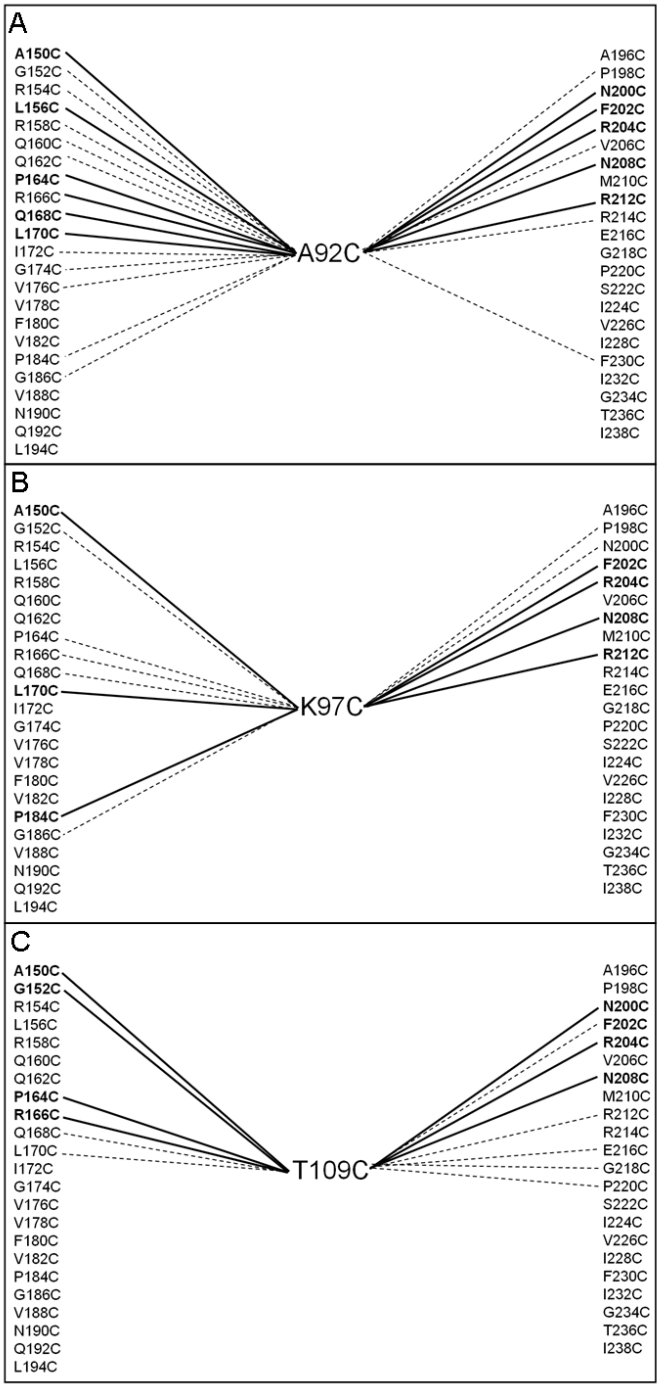

Figure 6-1, Ollis and Postle

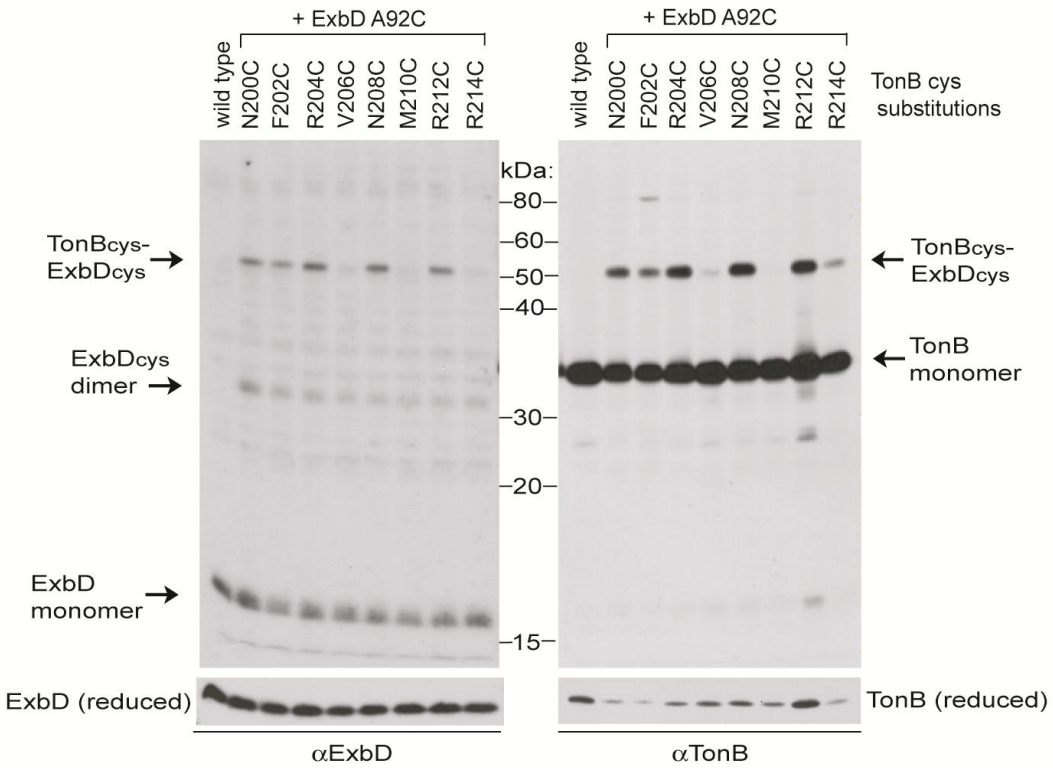

Figure 6-2, Ollis and Postle

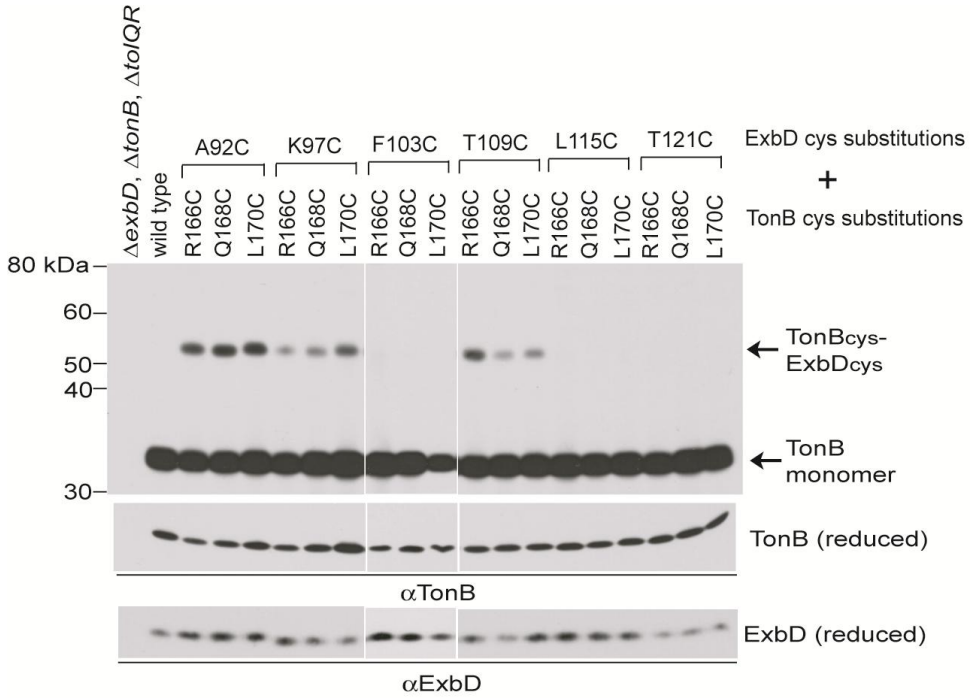

Figure 6-3, Ollis and Postle

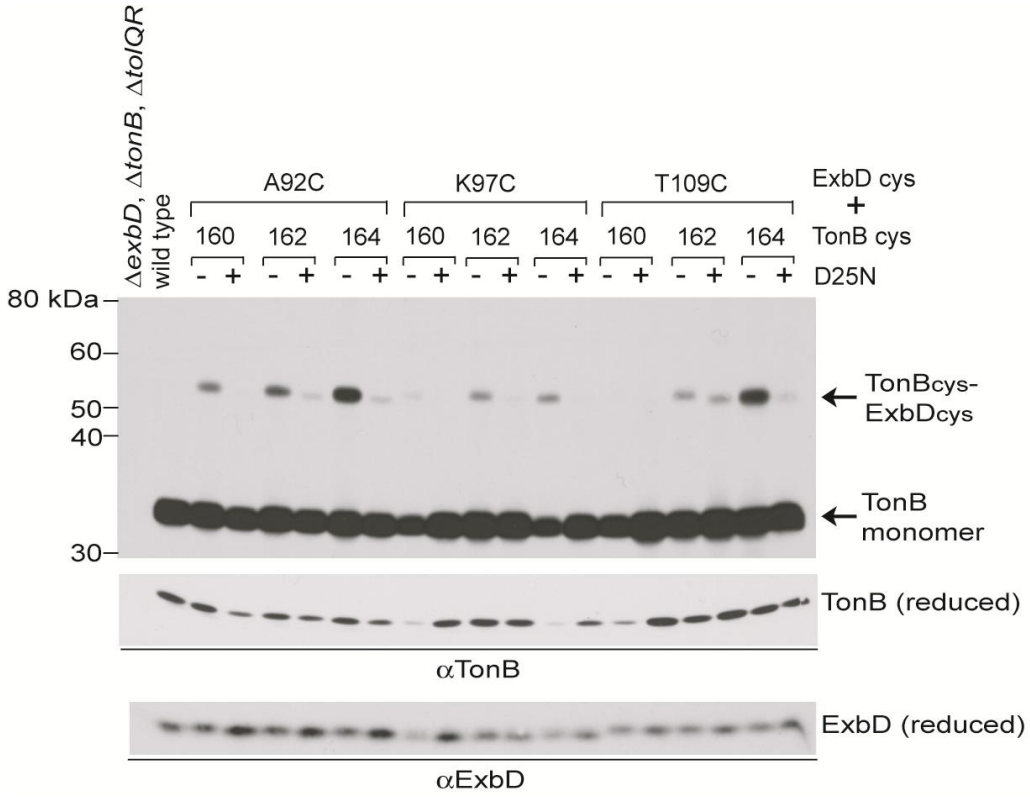

Figure 6-4, Ollis and Postle

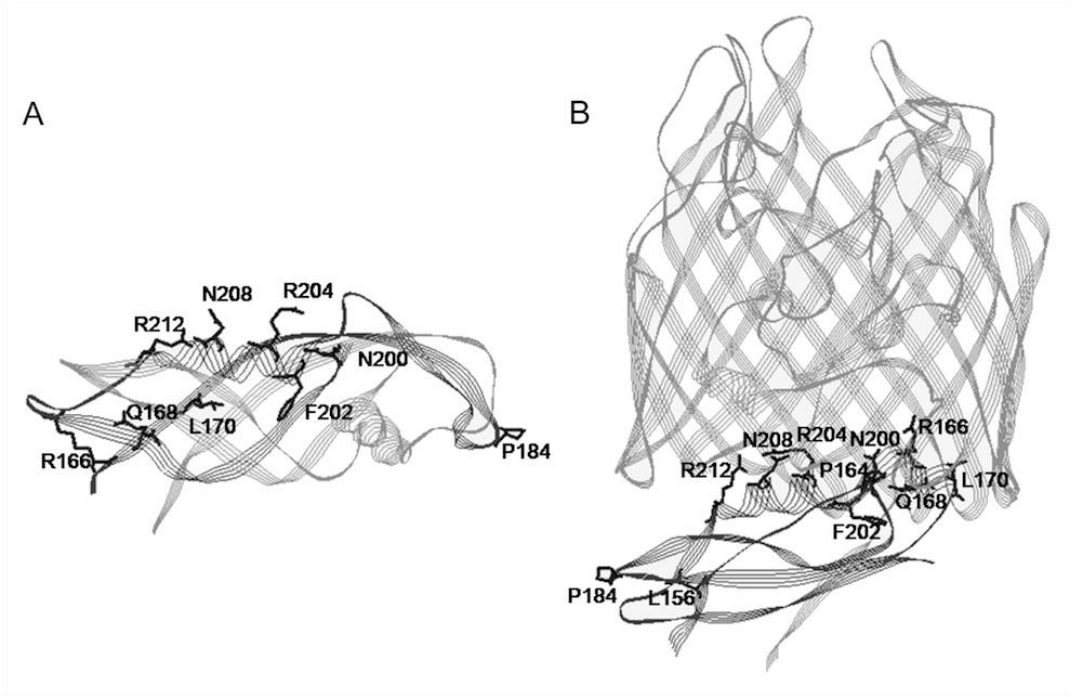

Figure 6-5, Ollis and Postle

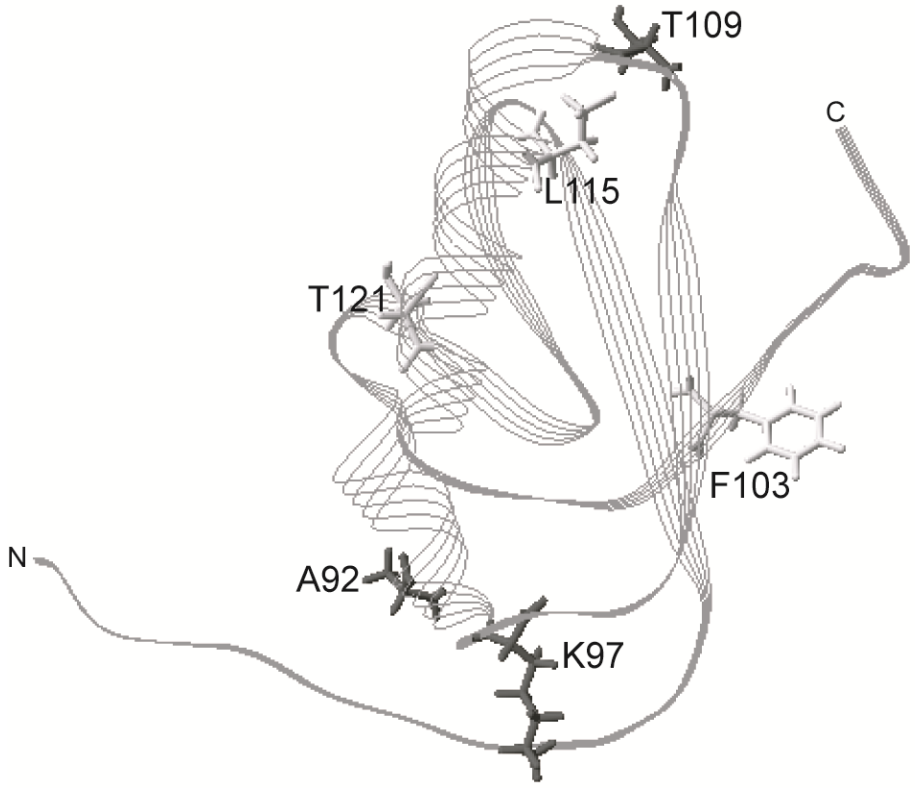

Figure 6-6, Ollis and Postle

CHAPTER 7

DISCUSSION

ExbD is an essential protein in TonB-dependent energy transduction but its role was relatively unexplored. While the findings that ExbD and ExbB both function to support TonB activity, stability, and conformational changes solidified the importance of their apparent functional unit, studies had not defined the individual functional contributions of these two proteins (Ahmer *et al*., 1995, Held & Postle, 2002). The hypothesized role of ExbD as a mediator of TonB conformational changes was developed from studies of TonB, with mainly the topology of ExbD contributing an ExbD-specific perspective (Ghosh & Postle, 2005, Vakharia-Rao *et al*., 2007). Two known point mutations, D25N in the ExbD TMD and L132Q in the periplasmic domain, indicated the importance of these regions in ExbD function, but the functional significance of these sites was unknown (Braun *et al*. 1996). This study provided the first comprehensive analysis of ExbD, shedding light on the role of the periplasmic domain and providing evidence of signaling from the TMD. This work has also revised and extended the interpretations of established assays used for analysis of TonB function.

The studies presented here support the proposed function of ExbD, providing specific evidence for ExbD-directed conformational changes of TonB and provide novel evidence that the assembly of TonB is important for ExbD conformational changes. In basic consideration, the proposed function of ExbD minimally requires both interaction between the ExbD and TonB periplasmic domains and a conformationally dynamic ExbD. Evidence for both was presented in this study. The simple view of this function ends with those observations. ExbD function appears to involve an interplay of dynamic interactions through its periplasmic domain, with both

specific homodimeric and heterodimeric interactions occurring through common regions of the periplasmic domain (Fig. 7-1). Specific ExbD-TonB interactions required Asp25 but ExbD homodimers did not. A transition from localized homodimeric to heterodimeric interactions was proposed, dependent on ExbD TMD residue Asp25. Asp25 was also important for the conformational response of ExbD to pmf. Pmf may induce the homodimer to heterodimer transitions of the ExbD periplasmic domain. While TonB lacks a direct means to respond to the pmf (Swayne & Postle, 2011), TonB was required for conformational response of ExbD to the pmf. Initial pmf-independent interaction of TonB and ExbD could trigger changes that potentially open a proton channel, allowing harness of the protonmotive force by ExbB/D and subsequent energized TonB-ExbD interaction. ExbD activity is inhibited if regions of the periplasmic domain cannot release from homodimeric interaction. Specific regions of ExbD, such as I102, however, did not require disruption of homodimeric interaction for ExbD function—ExbD functioned as a homodimer. This study examined a 30-residue region of the 99 residue ExbD periplasmic domain so it will be important to further define residues involved in an apparent stable homodimeric interface and regions requiring conformational flexibility. A stable homodimeric interface could form a hinge-like region, allowing movement of other regions but maintaining the homodimer. The significance of the homodimer to ExbD function remains to be determined.

In the homologous MotA/B system, it is thought that initial contact of MotA/B with the flagellar rotor triggers conformational changes of the MotB periplasmic domain that open the putative MotA/B proton channel, which is "plugged" by a predicted amphipathic helix located almost adjacent to the MotB TMD (Hosking *et al.*, 2006, Kojima *et al.*, 2009). ExbD does not contain the specific sequence of this plug region (Goemaere et al., 2007). It is unknown if a potential ExbB/D proton channel is "plugged" by any domains of ExbB or ExbD. Speculation could assign the homodimeric ExbD carboxy terminus a similar role, but there is no current

evidence the potential ExbB/D channel needs a physical plug. The conformation of ExbD residues 92-121 was important for formaldehyde crosslinked ExbD-ExbB interaction, however, and a potential amphipathic helix spanning residues 112-125 could promote interaction of this domain near the CM. Perhaps the arrangement of the TMDs is such that assembly of ExbB/D is not the sole determinant of channel formation or more generally, initiation of response by ExbD to the pmf. In the homologous Tol system, disulfide crosslinking of a cys-substituted TolR TMD suggests the TMD undergoes functionally important rotation (Zhang *et al.*, 2009). Changes in the association of the ExbD periplasmic domain near or with ExbB could induce conformational changes resulting in an open channel. Such a role would require further investigation, in particular, determining if this region of ExbD directly interacts with ExbB, which has limited regions exposed to the periplasm, or if the ExbD periplasmic domain is in contact near the CM. Previous studies successfully generated a leader peptidase recognition site in TonB that resulted in cleavage of the TonB periplasmic domain from its TMD and allowed study of both the protein associations of this fragment by formaldehyde crosslinking and sucrose density gradient fractionation (Jaskula *et al.*, 1994). A similar approach could be used to study protein interactions or membrane association of the ExbD periplasmic domain, where cleavage would result in about an 11 kDa ExbD periplasmic domain fragment. This method seems advantageous to alternatively exporting an ExbD periplasmic domain fragment since cleavage is not instantaneous, and temporary attachment to the TMD could allow pmf-dependent associations to be observed. Another approach could use disulfide crosslinking to ask if ExbD residues 92-121 are involved in direct interaction with residues of ExbB's soluble periplasmic domains. Cys substitutions could be constructed in the amino terminus or periplasmic loop of ExbB and individually co-expressed with the ExbD periplasmic domain cys substitutions constructed in chapter 5and examined for the ability to form disulfide-linked heterodimers.

This work provided the first evidence of distinct interactions between the ExbD and TonB periplasmic domains occurring before and after the action of the pmf. The carboxy terminus of TonB, referring here to TonB residues 150-239, appears to be primarily involved in interactions requiring ExbD Asp25, potentially pmf-dependent ExbD-TonB interactions. Less defined are the pmf-independent interactions, which could involve a more amino-terminal region of the TonB periplasmic domain if ExbD physically protects the TonB proteinase K resistant fragment, 152-156 amino-terminal residues of TonB (Larsen *et al*., 1999). In chapter 3 it was demonstrated that collapse of the pmf reversibly stalls TonB in the proteinase K resistant conformation, and TonB can be toggled between the two ExbD-dependent conformational stages by changes in the pmf. The nature of this reversibility could be further defined. When pmf is collapsed must the population of TonB in the energized conformation continue through the energy transduction cycle until it once again achieves the proteinase K resistant conformation, where it is then stalled at that stage, or can energized TonB revert to the proteinase K resistant, "de-energized" conformation when pmf is collapsed with no energy transduction event required? Two methods could be used to address this, both using *aroB*- strains which lack transportable ligand and therefore, prevent energized TonB from transmitting energy to the TGT (Larsen *et al*., 1999). First, pmf could be collapsed with CCCP in an *aroB*- strain followed by formaldehyde crosslinking to determine if the energized TonB-ExbD formaldehyde crosslinked complex is still present after pmf collapse when TonB cannot continue its normal cycle to de-energize by transmitting energy to ligand-loaded TGTs. If the TonB-ExbD formaldehyde crosslinked complex is still pmf-dependent in an *aroB*- strain, it would suggest TonB can "de-energize" without a full energy transduction cycle. Additionally it could be examined if in an *aroB*- background, 100% of TonB coverts to the proteinase K resistant conformation following collapse of the pmf, as observed in wild-type strains. If the ability to achieve the proteinase K resistant conformation requires de-energization of TonB by transmittance of energy to ligand-loaded

TGTs, then only a fraction of the TonB population would be able to assume the proteinase K resistant conformation, and any energized TonB would be proteinase K sensitive and degraded. If 100% of TonB is observed to form the proteinase K resistant conformation in an *aroB*- background, this would suggest TonB can revert to the de-energized conformation without requiring energy transfer to TGTs.

While this work overall has proposed a sequence of interactions involving the ExbD periplasmic domain, interaction with ExbD is not necessarily the first in a cycle of TonB carboxy terminus interactions. The carboxy terminus of TonB has multiple known interaction partners *in vivo* (Fig. 7-1), including OM proteins and homodimeric interactions with another TonB (Skare *et al*., 1993, Higgs *et al*., 2002, Ghosh &Postle, 2005). Interactions of unenergized TonB do not appear to have requirements for a sequence of interactions. In the absence of ExbD and/or ExbB, the TonB carboxy terminus associates primarily with OM sucrose density gradient fractions (Held & Postle, 2002). TonB association with OM fractions is disrupted by treatment with high salt, so this association is thought primarily to be mediated by protein-protein interactions (Higgs *et al*., 2002). Formaldehyde crosslinkable interaction with the TGT FepA also increases in the absence of ExbB/D (Brinkman & Larsen, 2008). Pmf-independent interactions of ExbD and TonB, defined in chapter 3, occur even with truncated $TonB_{am175}$, which does not associate with OM proteins (Larsen *et al*., 1999). Chapters 2 and 3 identified changes in ExbD-TonB interactions dependent on the pmf, where energized interaction involved conformations distinct from pmf-independent interactions. It is unknown if the pmf-independent TonB-TGT interactions also change when TonB becomes energized for function or if this interaction is a maintained contact through which TonB exerts its function. To date, the only identified sites of direct interaction of TonB with TGTs *in vivo* involves the TonB Q160 region, with studies demonstrating significant disulfide-linked interaction between individual TonB cys substitutions at residues 160-163 and the cys substitutions in the TonB box region of TGTs. (Cadieux & Kadner, 1999, Cadieux *et al.*,

2000, Ogierman & Braun, 2003). As pointed out in chapter 6, sites of specific ExbD interaction flank this region and extend to more carboxy-terminal regions of TonB, theoretically permitting simultaneous ExbD-TonB and TonB-TGT interaction. The question remains of whether ExbD can make contact with this or other regions of TonB while it is in interaction with the TGT or if the TonB-TGT interaction must dissociate prior to interaction with ExbD. Disulfide crosslinking could be used to see if simultaneous ExbD-TonB-TGT interactions could be detected. TonB double cys substitutions, TonB(Q160C, N200C) for example, could be constructed, choosing substitution sites known to prominently interact with only one partner, Q160C with TGTs and N200C with ExbD in this example. This would require co-expression of the TonB double cys substitution, an ExbD cys substitution, and a TGT TonB box cys substitution.

It is unknown if signals from the OM, in the form of changes in TonB-TGT interactions initiate changes in ExbD-TonB interactions. Interactions of specific sites in the TonB Q160 region with the TonB box of the TGT BtuB significantly increase in the presence of ligand, suggesting changes involving this region perhaps occur to signal ligand occupancy or during transport of ligand (Cadieux & Kadner, 1999). Specific mutations in the TonB box of the TGT BtuB result in a "TonB-uncoupled phenotype" where ligand still binds the transporter, but active transport into the periplasm does not occur (Bell *et al*., 1990). TonB can still bind the TonB box region of these uncoupled mutants, but the specific interaction sites are changed, suggesting the nature of TonB-TGT TonB box interactions is important for successful transmission of energy for active transport (Cadieux & Kadner, 1999). Mutants known to alter TonB-TonB TGT interactions could potentially be used to ask if proper TonB-TonB box interaction is important for specific TonB-ExbD interactions. In chapter 6, ExbD-TonB disulfide-linked interactions were observed at 4 important regions of the TonB carboxy terminus. It could be examined if TonB uncoupled TGT mutations prevent or increase interaction of ExbD at any of these 4 regions of TonB. This could potentially provide insight into the dynamics of ExbD-TonB-TGT interactions

and if/how TonB interactions at the OM alter TonB-ExbD interactions or the timing of those interactions.

It is also unknown if ExbD interaction at these four regions of the TonB carboxy terminus are sequential interactions. Since the same ExbD cys substitutions showed significant interaction at most of the same substitution sites on TonB, native interactions at one region of TonB are likely not static interactions. A TonB cys substitution from one of the four regions known to interact with ExbD cys substitutions could be combined individually with inactivating or disruptive mutations in one of the remaining three regions of known interaction. It could then be asked if inactivation or disruption of each interactive region prevents interaction of ExbD at another of the known interactive sites. This could provide insight into a potential sequence of interactions.

The role of ExbD in regulating TonB interactions at the OM is also an area with unknown details. While the fact that almost all TonB is found strongly associated with OM proteins in the absence of ExbB/D and TolQ/R could suggest a default interaction between TonB and OM proteins (Letain & Postle, 1997, Held & Postle 2002), some information argues against "docking" of the TonB periplasmic domain at the OM until association of TonB with ExbB/D. The proteolytic instability of TonB in the absence of ExbB and/or ExbD could suggest this predominant OM association of TonB is not a stable state and therefore unlikely to serve a mechanistic role under wild-type conditions (Fischer *et al.*, 1989, Skare & Postle, 1991, Ahmer *et al*., 1995). It is unknown if TonB exists in the CM unassociated with ExbB and/or ExbD, but TonB can complex with ExbB in the absence of ExbD, and TonB is still proteolytically unstable under these conditions, again suggesting a potentially unfavorable state [(Ollis *et al.*, 2009) and data not shown]. Later studies, have suggested proteolytic instability of TonB may actually occur after energy transduction by TonB, where stability is proposed to be conferred by action of ExbD in recycling TonB (Brinkman & Larsen, 2008). However, this does not explain the fact

that TonB is still proteolytically unstable in a strain lacking both ExbB/D and TolQ/R and found predominantly associated with the OM (Held & Postle, 2002, Brinkman & Larsen, 2008). There may be multiple conformations of TonB sensitive to proteolysis *in vivo*, similar to the observations in this study of two proteinase K sensitive conformations of TonB in spheroplasts. In studies of activity-dependent instability, inefficient assembly of TonB with TolQ/R, mimicking an ExbB/D-, TolQR- strain, was not ruled out as a cause of susceptibility to proteolysis in the ExbB/D-, TolQ/R+ background. Since TonB does not transmit energy to transporters in absence of ligand (Larsen *et al*., 1999), the same studies could be carried out in a strain lacking transportable ligand, such as an *aroB*- strain which cannot synthesize enterochelin. The authors' results would be supported if TonB exhibits increased resistance to degradation in an ExbB/D-, TolQ/R+, *aroB*- background. Evidence for direct interaction of ExbD with TonB following energy transmission is lacking. Combinations of ExbD and TonB cys substitutions observed to form disulfide-linked heterodimers in this study could be co-expressed in an *aroB*- strain to look for interactions specific to recycling TonB.. ExbD-TonB interactions absent in an *aroB*- background may occur after TonB-dependent transport. This could be confirmed by asking if addition of exogenous enterochelin subsequently induces the interaction.

In addition to proteolytic instability of TonB under conditions where its periplasmic domain is primarily associated with the OM, the cellular levels of TonB also do not favor the idea of TonB docking at the OM. Of the components in the TonB system, TonB is the limiting protein (Higgs 2002). For a protein that interacts with multiple transporters specific for different ligands, TonB likely needs to be able to freely scan for ligand-loaded TGTs. Docking to a single receptor, waiting for ligand, would not facilitate efficient servicing of multiple transporters. This could be reconciled if TonB docked specifically to ligand-loaded transporters, where it would then be positioned for functional interaction with ExbD. TonB does show increased interaction with transporters in the presence of ligand, based on formaldehyde or disulfide crosslinked

interactions, but the presence of ligand is not required for these interactions (Moeck *et al.*, 1997, Cadieux & Kadner, 1999, Ogierman & Braun, 2003, Higgs *et al*., 2002).

Further studies of TonB-TGT interactions are needed to develop these speculations. An apparent question to be addressed is if active ExbD is required to remove TonB from OM protein association. Sucrose density gradient fraction studies of TonB may provide some insight, since a phenotype of TonB predominantly associated with the OM is readily distinguishable. Studies could start with Δ*exbD,* Δ*tolQR* strains, and induction of wild-type ExbD or ExbD D25N later in growth would introduce these proteins into a strain where TonB should already be predominantly associated with the outer membrane. Synthesis of TonB could be inhibited prior to induction of ExbD expression and strains could be fractionated to determine if less TonB is present in OM fractions dependent on ExbD. This could also be examined in the presence or absence of ligand.

Pmf-dependent TonB-ExbD periplasmic domain interaction occurs even in the absence of transportable ligand, indicating this association occurs prior to TonB transmitting energy for active transport , considered the "front half" of the energy transduction cycle (Larsen *et al.*, 1999, Ollis et al., 2009). Perhaps transport is initiated immediately after this energized TonB-ExbD interaction. One possible way to test this would be to ask if the TonB-ExbD disulfide-crosslinked periplasmic domain interaction represents energized TonB. If this is the case, reduction of this disulfide-linked complex could initiate a transport event. Strains co-expressing heterodimer-forming TonB and ExbD cys substitutions could be treated with CCCP to collapse pmf, known to prevent transport by monomeric TonB, which can be monitored by ^{55}Fe-ferrichrome transport. A reducing agent could subsequently be added to samples in the presence of CCCP to determine if the previously crosslinked TonB was being held in an energy transmission competent conformation. The levels of TonB-ExbD disulfide-linked heterodimers currently observed may not be high enough for the sensitivity level of this assay. Overexpression of the proteins does increase the level of the disulfide crosslinked complexes. Since excess monomeric forms of the

proteins would be inactivated by CCCP, this may be a viable option to increase complex formation to test this. A similar approach could potentially be used to ask if the TonB disulfide-linked homodimers represent an energized form of TonB. This would not, however, distinguish which conformation of homodimer could be energized.

It is interesting to consider that residues of TonB heterodimeric interaction with ExbD, G186C, F202C, and F230C, were also involved in TonB homodimeric interaction. Three conformations of TonB homodimers are known, and detection of these "triplet dimers" requires the presence of ExbB/D (Ghosh & Postle, 2005). Triplet homodimer formation is observed with TonB F125C, G186C, F202C, W213C, Y215C, and F230C (Ghosh & Postle, 2005, Postle *et al*., 2010), but not all of these sites were examined for specific interaction with ExbD. This could be investigated to examine if, like ExbD, TonB may transition between homodimeric and heterodimeric interactions through common sites. It is currently unknown what the triplet homodimer conformations represent in terms of the TonB energy transduction cycle. Assembly with ExbD might normally promote resolution of TonB homodimeric interactions. Perhaps conformational changes meant to accomplish this could result in the observed unique multiple TonB homodimer conformations. It is unknown if the triplet homodimers represent conformational transitions from an initial trapped interaction or independently trapped conformations. The dynamics of the TonB homodimer populations and the influence of ExbD on TonB homodimer formation could be examined if TonB cys substitutions are identified where absence of ExbD alone (ExbB present) results in loss of triplet homodimers but at least one homodimer band present. Cultures could be grown expressing the TonB cys substitution but initially not inducing plasmid-encoded wild-type ExbD. An alkylating agent could potentially be added during cell growth to isolate the existing population of TonB homodimers and prevent new disulfide bond formation. ExbD could subsequently be induced and non-reducing samples harvested over time. If the multiple TonB homodimer populations form by conformational

transitions after initial homodimeric interaction, expression of ExbD could result in formation of the TonB triplet homodimer bands. No changes could suggest the populations form from separate associations of the interactive site, and formation of the other homodimers is prevented. Results would be compared to a culture where ExbD remains uninduced.

How might TonB homodimeric interactions fit into the picture of dynamic associations? TonB triplet dimers formed through sites in the TonB carboxy terminus (residues 150-239) are observed only in CM sucrose density gradient fractions, indicating they prevent the high affinity OM interactions observed to result in TonB fractionating primarily with outer membrane sucrose density gradient fractions. This is thought to mean homodimeric interactions through these sites must be resolved before TonB can interact with TGTs (Ghosh & Postle, 2005). OM association is also not required for formation of these homodimeric interactions since TonB carboxy terminal triplet homodimers form even when TonB also contains a deletion of residues in the Q160 region that prevents TonB fractionation with the outer membrane (Vakharia-Rao *et al*., 2007). Perhaps TonB energization by ExbD normally resolves TonB carboxy-terminal homodimeric interactions, freeing previously sequestered sites, for productive interaction with TGTs. In chapter 3, three stages in TonB energization were described based on relative association of the ExbD and TonB periplasmic domains. It is interesting to speculate that the three conformations of TonB homodimers could fall into these stages or in transitions normally leading to these stages. When pmf is collapsed, ~100% of TonB is observed stalled in the proteinase K resistant conformation, proposed to represent an initial assembly of ExbD and TonB. Cultures expression TonB cys substitutions capable of triplet homodimer formation could be treated with CCCP to collapse pmf and non-reducing samples harvested over time. Under these conditions, monomeric TonB would be expected to accumulate in a conformationally homogenous population. If this conformation or a transition to this conformation is represented by one or more of the triplet homodimer populations, homodimer formation could increase after collapse of pmf. It remains to be

determined how functionally important residues of the TonB carboxy terminus fit into the picture of complex interactions.

Models of the TonB system provide a basis for comparison or hypotheses of the homologous Tol and Mot protein systems. The greatest similarity between ExbD and TolR or MotB is in the TMDs of these proteins. ExbD and TolR have similarities in their periplasmic domains; however, the periplasmic domains of ExbD and MotB are quite dissimilar. As comparison, the periplasmic domain of TonB aligns better than ExbD with the periplasmic domain of MotB, though no significance has been assigned with this (data not shown). TolQ/R can partially substitute for ExbB/D and vice versa (Braun & Herrmann, 1993, Brinkman & Larsen, 2008). This work has identified diverging functions in heterodimeric interactions of the ExbD periplasmic domain compared to TolR. Both pmf-independent and pmf-dependent interactions differed. Both ExbD and TolR interact with the corresponding protein in each system, TonB or TolA, but the manner of interaction or initiation of interaction was distinct. Such differences may contribute to the inability of TolR to fully functionally substitute ExbD.

Based on sequence, ExbD and TolR have almost identical TMD's, suggesting functional association with TonB or TolA is more likely defined by the periplasmic domains of ExbD and TolR. The periplasmic domain of ExbD and TolR have similar sequences, predicted secondary structural elements, and isoelectric points (pI = 5.3 for ExbD, 4.8 for TolR), so perhaps differences in localized regions define their functional interactions. In chapter 6, ExbD residues 92-121 were shown to contain sites of specific interaction with sites in the TonB carboxy terminus (residues 150-239). This 30-residue region of ExbD has a calculated pI of 5.12. In contrast, the corresponding 30-residue region in TolR, residues 97-126, has a pI of 9.44. The TonB carboxy terminus has a pI of 10.42, perhaps making interaction with this region of TolR unfavorable. It would be interesting to exchange ExbD residues 92-121 and TolR residues 97-126 and assay each for ability to support TonB and TolA function. If ExbD residues 92-121

define specific interaction with TonB, perhaps the presence of this 30-residue region in the TolR periplasmic domain would increase its ability to functionally substitute for ExbD.

In contrast, homodimeric interactions observed for ExbD were similar to those observed previously for TolR, and activity of both proteins was inhibited by disulfide-linked homodimer formation through the same corresponding site (Goemaere *et al.*, 2007). It seems common to these systems that some homodimeric interactions must be resolved for function. It is unknown if sites of TolR homodimer formation are involved in heterodimer formation with TolA, corresponding to ExbD interaction with TonB. Specific sites of TolR-TolA interaction are unknown. It seems likely, however, that this stage of transition differs since unlike ExbD-TonB, TolR-TolA heterodimers crosslinked with formaldehyde do not require pmf to form. TolA-Pal interaction (Fig. 1-4) is pmf-dependent (Cascales *et al.*, 2000). Like ExbD, the TolR periplasmic domain is conformationally responsive to changes in pmf, and these changes require the highly conserved TMD asp residue (Goemaere et al., 2007). It is unclear, however, how these changes relate to energization of TolA.

While the MotB and ExbD periplasmic domains lack sequence similarity, similar conformational changes have been proposed for each. For MotB, it is thought that the predicted initial compact, structured homodimeric carboxy terminus is too small (~50Å) to span the periplasm to interact with the peptidoglycan layer (~100Å from the CM) (Kojima et al., 2009). It is proposed that when a MotA/B complex assembles with the flagellar rotor, interaction between the cytoplasmic domain of MotA with rotor component FliG (Zhou *et al.*, 1998) induces conformational changes in MotB that open the MotA/B proton channel and allow MotB to span the periplasm for putative interaction with the peptidoglycan layer (Hosking et al., 2006; Van Way *et al.*, 2000). It may be that ExbD, TolR, and MotB undergo similar conformational transitions, initiated by signals from their conserved TMDs, in particular Asp25, Asp23, or

Asp32, respectively. The diverging functional outputs of these proteins appear to be defined by residues or conformations that define their specific interactions.

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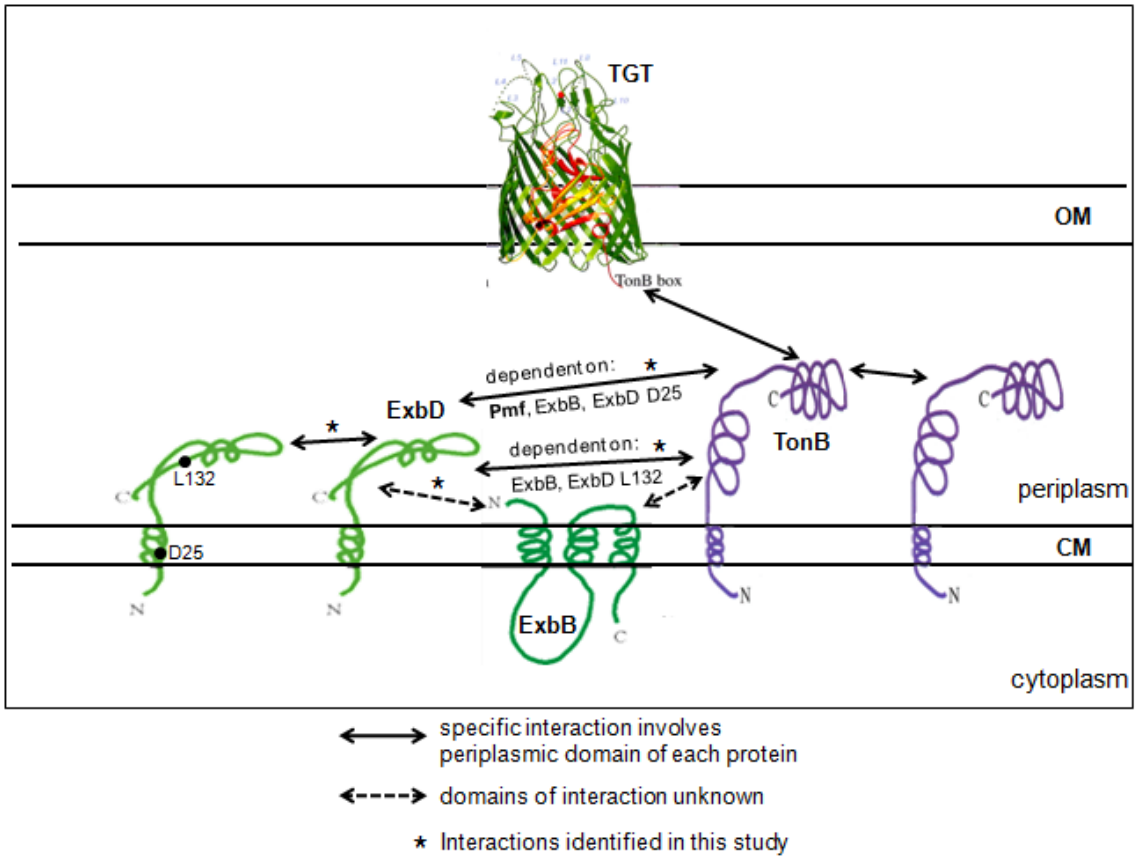

Figure 7-1. I*n vivo* interactions in the TonB system of *Escherichia coli*. A cartoon illustrates the multiple protein-protein interactions of the ExbD and TonB periplasmic domains and other known interactive partners. Solid lines indicate knowledge of specific sites of interaction. Dashed lines indicate interactions are known but specific regions involved in interaction are unknown. The two distinct ExbD-TonB interactions are distinguished by the determinants of the interactions listed below each line. The TonB-gated transporter (TGT) shown is FepA (Buchanan *et al*., 1999). OM indicates outer membrane. CM indicates cytoplasmic membrane. Relative positions of ExbD residues D25 and L132 are indicated on the left-most depiction of ExbD.

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