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IDENTIFICATION AND CHARACTERIZATION OF ESSENTIAL RESIDUES IN THE TONB CARBOXY TERMINUS

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by

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ABSTRACT

In gram-negative bacteria, the difficulty of iron acquisition is overcome by the secretion of siderophores and the active transport of these iron-siderophore complexes across the cell envelope. TonB is the dynamic central protein in this system, responding to energy from the proton motive force (pmf) at the cytoplasmic membrane and transducing that energy to outer membrane for transport through the TonB-gated transporters (TGTs) which bind the iron-siderophores. Determining how this protein achieves that has proven to be a difficult task with several discrepancies between the *in vivo* and *in vitro* data. TonB possesses two functional domains: an N-terminal α-helix which is required for cytoplasmic membrane association and receiving energy; and a semi-structured carboxy-terminal domain which responds to energization, interacts with the TGTs, and transduces the necessary energy for transport. This study shows that the only residue in the N-terminal helix to provide an important side-chain is the histidine at position 20. The Q160 region in the carboxy-terminus is known to interact directly with the TGTs and although the arginines at positions 158 and 166 have been proposed to form contacts essential for function, neither shows a significant loss of activity when substituted by alanine. In fact very few residues exhibit any loss of activity when substituted by cysteines and none are completely inactive. The ones that do have phenotypes are the aromatic residues, the Q160 region from positions 161-164, and the glycine at position 186. This glycine, as well as a phenylalanine at position 125 form strong disulfide linked dimers when substituted by cysteines as seen previously for the aromatic amino acids at positions 202, 213, 215, and 230. These data suggest that TonB is a protein that functions largely through the conformational changes is its backbone as

opposed to having a few catalytically important side-chains.

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CHAPTER 1: INTRODUCTION

The acquisition of essential nutrients is the most basic of functions for all living organisms. This becomes challenging however when the nutrient is scarce in the environment. Iron is one such nutrient which is essential for nearly all known organisms. It is a cofactor found predominantly in enzymes involved in electron transport, but is also catalytically important as a generator and stabilizer of free radicals as in ribonucleotide reductase (3) and members of the radical S-adenosyl methionine (SAM) superfamily of enzymes (13). Despite being one of the most abundant elements in the Earth's crust, in oxygen rich environments at neutral pH iron is found largely in insoluble ferric oxides rendering it unavailable for use by most organisms. Even parasitic organisms seeking to overcome this limitation by feeding off the iron stores of a host organism must first pry the metal away from the host's iron sequestering proteins which are often expressed in vast excess. In mammals, less than 50% of the iron sequestering protein transferrin is found associated with iron, a condition which creates an innate immunity to parasites dependent upon the metal (5).

Gram-negative bacteria are one group of organisms that often thrive in these iron-deficient environments. They do this by synthesizing and secreting iron scavenging compounds called siderophores (40). In *Escherichia coli* K12, the native siderophore is called enterochelin (enterobactin); a small molecule consisting of a three serine ring structure, with each serine also bound to a catechol group. This creates a six point coordination structure that binds to ferric iron with extremely high affinity (up to 10^{52}) (5), rendering the iron available for transport into the *E. coli* cell.

This transport, however, cannot occur by simple or facilitated diffusion, as the gram-negative cell envelope is a complex structure with greater emphasis placed on the occlusion of toxic compounds than on the ease of acquiring nutrients (Figure 1.1). The first barrier in this structure is a protein-rich lipid bilayer known as the outer membrane (OM). The OM is uncharged and the lipid composition of its outer leaflet in most species consists primarily of lipopolysaccharide. Beneath the OM is the viscous, protein-rich periplasmic space which houses the structurally essential peptidoglycan shell. The final layer is the inner or cytoplasmic membrane (CM) which separates periplasm from cytoplasm. This bilayer is charged by the proton motive force (pmf) and contains many transmembrane receptors which mediate internal cellular responses to environmental stimuli as well multiple transporters for nutrient acquisition. This cell envelope is vital for the organism's survival; however, it makes the acquisition of many essential nutrients difficult.

The OM is the first obstacle the ferric-siderophore complex encounters. Many nutrients can cross the OM by diffusing through porins, channel proteins which allow for the diffusion of molecules smaller than 600 Da (41). As the iron-loaded enterochelin is too large to fit through these channels, *E. coli* produces the outer membrane transporter (OMT) FepA, which binds the complex with high affinity. This binding is so tight, in fact, that energy is required for release into the periplasm. There is insufficient energy across the OM to energize active transport; likewise, the periplasm is devoid of nucleoside triphosphates, so it must be imported from another source. That source is the pmf, or another gradient coupled to it, which is transduced from the CM to the OM by the heteromultimeric TonB/ExbB/ExbD complex (44). Once in the periplasm, periplasmic

binding proteins deliver the ferric-siderophore complex to ATP-binding cassette (ABC) transporters in the CM where they're carried into the cytoplasm by active transport (29).

E. coli has also evolved to take advantage of the siderophores produced by other organisms by synthesizing the OMTs necessary for their transport. Most notably, ferrichrome, a hydroxamate iron siderophore produced by many species of yeast, is transported by FhuA (52). Other important *E. coli* iron transporters and their ligands are FecA for ferric dicitrate, Cir for dihydroxybenzoyl serine, ChuA for the hemoglobin heme, and IutA which transports aerobactin, a compound secreted by some pathogenic strains of *E. coli*. Iron is not the only nutrient acquired through such transporters. The most thoroughly studied of these is cobalt in the form of the cobalamin cofactor vitamin B_{12} gains entry into the periplasm through BtuB (4). Also, the SusC protein in *Bacteroides thetaiotaomicron* is responsible for the binding and transport of polysaccharides (49). And interestingly, recent evidence suggests for a TonB-dependent siderophore/transporter system for the acquisition of nickel in the ulcerating pathogen *Helicobacter pylori*. *H. pylori's* necessity for nickel when colonizing the stomach wall is created by its cytoplasmic urease enzyme used to neutralize the stomach acid (47). This enzyme contains 24 nickel ions per active complex and composes approximately 6% of total cellular protein (17).

Several crystal structures of these transporters have been solved and shown that despite differences in their ligands, they are structurally similar (reviewed in (52). Each is comprised of two domains: a C-terminal, pore-forming 22-strand β-barrel, and an N-terminal pore-occluding globular domain, which is often referred to as the plug or cork (Figure 1.2). Despite exhaustive study, the exact mechanism by which these transporters

function is still unknown. What is known is that both the barrel and plug are required for activity; and recent evidence strongly suggests that the plug is removed from the barrel during transport (12). In addition to their structural similarities, these transporters also share a requirement for the TonB protein and the pmf for transport of their respective ligands.

In addition to their ligands, these transporters are also parasitized and used as entry points for many phages such as T1 and φ80, as well as Group B colicins. Colicins are plasmid encoded proteinaceous toxins produced by one strain of *E. coli* to give it an advantage over another in ecological niches (reviewed in (9). These proteins are typically comprised of three domains: an N-terminal translocation domain which allows for transport across the OM, a central receptor domain which forms the first contact with the cell, and a C-terminal activity or killing domain which kills the infected cell. For all known Group B colicins, their specific OMT as well as energy derived from TonB are required for toxicity. There are three known mechanisms for toxicity: pore forming colicins kill their targets by forming pores in the CM of the cell, nuclease (DNase or RNase) kill targets by degrading their nucleic acids, or inhibition of peptidoglycan biosynthesis which compromises the cell wall. In addition to the aforementioned proteins, the DNase and RNase type colicins also require the leader peptidase LepB for release of their killing domain, which must then cross the CM by a mechanism not yet known. The Group B colicins used in this study are colicin B, a pore-forming colicin that enters through FepA; colicin D, an RNase colicin also using FepA as its entry point; colicin Ia, a pore former that uses Cir; and colicin M which uses FhuA for entry.

The current proposed mechanism for translocation across the OM consists of (1) the colicin contacting the extracellular side of the OMT, (2) the transporter's plug is completely pulled from the barrel into the periplasm by energized TonB concomitant with insertion of the colicin's translocation domain into the barrel, and (3) a second energized TonB pulling the colicin into the periplasm (Figure 1.3) in a similar fashion as the plug was removed (12). In this model, there is a direct interaction between TonB and the colicin in addition to the interaction between TonB and the OMT. This interaction is known to occur in OMTs and hypothesized to occur in the colicins through a five residue hydrophobic amino acid sequence at the N-terminus of these proteins called the TonB-box (39). Studies on BtuB have shown that this region will crosslink to TonB in vivo when specific residues in each protein are substituted by cysteines, and that the transporter didn't function if the TonB-box was deleted (7). It was additionally seen that flexibility was important for this region as it was tolerant to many point mutations except for when a proline was substituted for a more flexible amino acid (6).

As previously stated, the TonB/ExbB/ExbD complex is responsible for transducing the energy of the pmf (or another source coupled to it) at the CM to the transporters at the OM. Although the stoichiometry of the active complex is unknown, the ratio in the cell is 1:7:2 TonB:ExbB:ExbD (19) and TonB occurs as a dimer during at least part of the energy transduction event suggesting a possible complex of 2:14:4 respectively (16). The transmembrane proteins ExbB/D form the energy harvesting complex. ExbD is 146 amino acids long containing a short cytoplasmic domain (amino acids 1-22), a single transmembrane α-helix (amino acids 23-43), and a long periplasmic domain (amino acids 44-146), while ExbB is 244 amino acids long with a periplasmic N-

terminus (amino acids 1-15), three transmembrane α-helices (amino acids 16-39, 128-155, and 162-194), a large cytoplasmic loop (amino acids 40-127) and a short cytoplasmic C-terminal tail (amino acids 195-244) (1, 24, 25). Since both TonB and ExbD exhibit significantly reduced half-lives in its absence, ExbB is the stabilizing protein in the complex (26). Additionally, recent evidence has suggested that ExbB is also the proton translocator, as a deletion mutant of the cytoplasmic loop produces a leaky proton channel phenotype (Bulathsinghala and Postle, in press). At the present time, there is no clearly identified role for ExbD, however, it is essential for activity, and neither it nor ExbB can function independently of one another (18). There are currently two hypotheses for ExbD function: as a chaperone to regulate TonB's conformation, and as a recycling agent releasing TonB from the OMT for another catalytic cycle.

Some insight into the functions of these proteins has been provided by evaluating two other pmf-dependent inner membrane complexes: the outer-membrane integrity energy transducers TolA/TolQ/TolR, and the flagellar stator complex MotA/MotB (reviewed in (28, 37). With all components included, these complexes along with TonB/ExbB/ExbD are strikingly similar, sharing five transmembrane helices, one or two long periplasmic chains, and a single long cytoplasmic loop. This similarity is so great in fact that "crosstalk" occurs between them with both the Tol and Mot systems able to energize TonB, and ExbB/ExbD able to activate TolA. In all cases however, this crosstalk is significantly less efficient than the native proteins. While the energy harvesting proteins TolQ/R share greater homology and substitute better for ExbB/D, the Tol system is even less understood than the TonB/ExbB/ExbD system. In contrast, there has been an extensive amount of research conducted on MotA and B and a model for

activity has been proposed. In this model, a functional unit consists of four MotA peptides and two MotB peptides where two proton channels are formed in the CM, each by two transmembrane helices of a single MotA monomer and the single transmembrane helix of MotB. MotB has also been suggested to function to plug the proton channels when not active. If this model is applied to the TonB/ExbB/ExbD system, then the proton channels are likely formed between the second two transmembrane domains of ExbB and the TonB transmembrane domain to form the proton channel. Upon proton shuttling, TonB becomes energized and is then able to transducer that energy to the OM.

TonB is a 239 amino acid protein with a single transmembrane helix (residues 13-32), a proline-rich central domain (residues 70-102) and a semi-structured carboxy-terminus (residues 150-239). The N-terminal transmembrane region contains a signal sequence for sec-dependent transport into the periplasm and insertion into the CM (23). This region is also critical for interaction with ExbB/ExbD and reception of energy from the pmf. The proline rich region was indicated in vitro to be required for TonB dimerization when interacting with FhuA (46). This result, however, could not be duplicated in vivo and this region can be completely deleted from the protein without significant loss of function (36). Finally the carboxy-terminus is the region that interacts with the OMTs and the sequence surrounding Q160 is particularly important as it is known to form the interaction with the TonB-boxes of its targets for energy transduction (7).

Despite exhaustive study, the mechanism by which the energy transduction event occurs is still unclear. It has been seen that through proteolysis studies that TonB undergoes structural rearrangements to form at least three different conformations during

the energy transduction cycle. These three conformations are the uncharged state formed before acquisition of energy from the ExbB/D complex, the energized state formed after energy acquisition but before energy delivery, and the discharged state formed after energy delivery, but before being recycled back to the uncharged state. While evidence of each of these complexes has been clearly seen, the exact structure of each is still unknown. Under normal circumstances TonB is found at both the cytoplasmic and outer membranes in a ratio of approximately 70% to 30%, respectively, when fractionated on a sucrose density gradient. While this ratio is roughly maintained in strains lacking loaded OMTs (20), TonB is found completely at the OM when the cells lack ExbB, ExbD, or both; suggesting that TonB's interaction with the OM is not dependent upon readiness of OMTs, but its interaction with the CM is greatly affected by the lack of ExbB/D (32).

There are currently two models for TonB activity that reconcile most of the in vivo data. While very similar, the key difference between them is the location of the N-terminal transmembrane region throughout the energy transduction event. The first is the mechanical model in which TonB's carboxy-terminal domain (CTD) is in the uncharged state and its N-terminus is in the ExbB/ExbD complex. Energy is harvested by the ExbB/D complex and then transferred to TonB, causing the CTD to adopt the charged conformation. This charged conformation delivers its energy to the OMT by applying a mechanical pulling force from the CM where it's anchored by the transmembrane region. This allows for transfer and converts the TonB-CTD to the discharged state. TonB is then recycled to the uncharged state by the ExbB/ExbD complex for another round of catalysis. Throughout this mechanism, the transmembrane domain remains in the ExbB/D complex at the CM. For the second model, the shuttling model, the initial step

of energization of TonB is the same, except that the energized state is in the form of a chemical modification or a stored conformational change. TonB then delivers this energy to the OMT for transport, but the N-terminus must be released from the CM before transfer can occur. This creates the discharged TonB-state at the OM, which is recycled to the CM and converted to the uncharged state by the ExbB/D complex.

Structural information on the TonB carboxy-terminal domain has provided insight into its function, but hasn't always agreed with *in vivo* analysis. The first crystal structure of the CTD was resolved in 2001 (10). In this structure TonB exists as an intertwined homodimer of 25Å x 65Å with each monomer consisting of one α-helix and three β-strands (figure 1.4). Based upon this structure, the authors proposed a propeller type mechanism for energy transduction in which TonB interacts with the OMTs as a dimer and applies a rotational force upon the transporter. However, the peptide only consisted of the final 76 residues (starting at position 164), which means the protein, as seen in the structure, cannot be in its energized conformation since it lacks the N-terminal transmembrane region; or possibly in an aberrant conformation due to the lack of the first 163 amino acids.

A second crystal structure with a peptide composed of the final 92 amino acids (starting at position 148) was determined in 2005 (27). This structure was also dimeric, but showed a slightly different conformation. While the overall α-helices and β-sheets are formed much the same, there are "hinges" before and after the hairpin turn between the β1-β2 strands, which results in these strands folding on top of their own monomer instead of into a strand exchange with the other peptide (figure 1.5). The effect of these conformational changes is a dimer with significantly fewer intermolecular contacts and

reversed orientation of the individual monomers with respect to each other. Not only do the previous objections to the *in vivo* relevance of the structure apply, but the authors also observed by dynamic light scattering and analytical ultracentrifugation that the 92 residue fragment was monomeric in solution (27).

Also in 2005, a solution structure of the TonB-CTD was determined by nuclear magnetic resonance (NMR) on a peptide of the final 137 amino acids (starting at position 103) (48). Although residues 103-151 were disordered, this peptide resolved to a monomeric structure that retained many of the same elements as the dimeric crystal structures (figure 1.6). This structure is comprised of four β-strands forming an antiparallel sheet and two α-helices in an oblong conformation approximately 45Å by 26Å. The long axis is slightly curved giving the protein a "bean-like" shape with concave and convex sides. The authors also compared the original structure with changes that occurred upon addition of peptides mimicking the TonB-box of OMTs and saw the largest changes in position of residues on the concave side indicating that if this structure is correct that is likely the region of interaction. However, once again, there is no transmembrane region meaning no response to the pmf and no energized state.

But perhaps the most compelling structures are those with the TonB-CTD co-crystallized with the OMTs FhuA and BtuB obtained in 2006 (42, 50). In these structures, TonB is monomeric and in a similar conformation as seen in the NMR structure. In both cases there is a strand exchange between the OMT's TonB-box and the concave portion of TonB. In this configuration, the TonB-box changes from its more disordered state and adopts a β-strand which inserts into the TonB β-sheet, displacing the β4 strand. This strand exchange is the common unifying theme in both structures and

sheds some light on the TonB-box mutations observed previously, since a proline residue at specific residues would place a kink in the strand that would inhibit its interaction with the TonB-CTD. Intriguingly however, the contacts are not oriented identically. In the FhuA co-crystal, TonB interacts with the opposite side of the plug domain and periplasmic loops of the β-barrel than it does in the BtuB structure (50). Also, the residues making what appear to be catalytically important salt bridges aren't the same, its R158 in BtuB and R166 in FhuA (42). This provides further evidence suggesting that subtle differences exist in the way TonB interacts with each of the OMTs (figures 1.7 and 1.8).

In TonB, former Postle lab member Joydeep Ghosh created cysteine substitutions in the carboxy-terminus from residues 199-216, a region proposed to be an amphipathic α-helix (15). He discovered that the aromatic amino acids in this region, F202, W213, and Y215 as well as an additional aromatic residue F230 formed three disulfide linked complexes in vivo when substituted by cysteine. These complexes, while composed of only 5-10% of the total protein, formed even in the absence of ligand loaded transporters, but didn't form when the TonB protein was rendered unresponsive to the pmf. It was also confirmed that all these complexes were TonB homodimers suggesting the differences in mobility were due to conformational changes in the protein. Additionally, while none of the substitutions were inactive, only the aromatic residue substitutions exhibited any phenotypes with regard to colicin toxicity. Intriguingly, there wasn't a universal phenotype and in fact different substitutions affected different colicin toxicities to different degrees. This effect was also seen when each aromatic residue was substituted with an alanine, but any double alanine substitution of the four previously

mentioned residues was completely inactive, as well as when combined with an aromatic that didn't form complexes, F180 (16). These results suggest that the aromatic residues form a structurally dynamic cluster whose formation is critical for TonB function, by allowing the CTD to form into the appropriate conformation for the appropriate transporter.

FIGURE 1.1

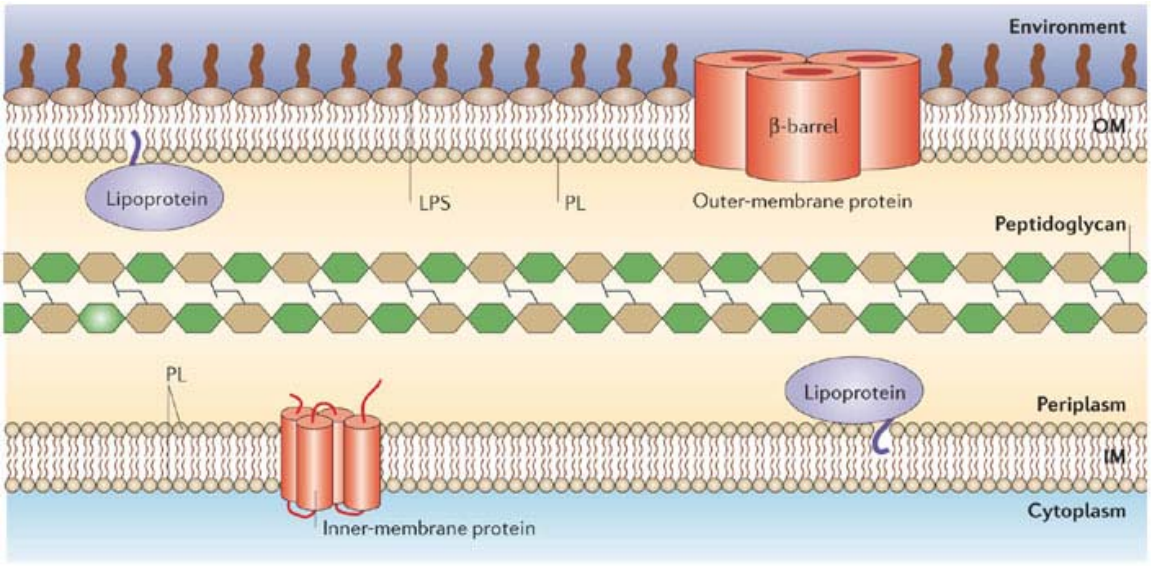

Figure 1.1 (from (45) **Gram-negative Cell Envelope**

The cytoplasm of *E. coli* is surrounded by a cell envelope, which is composed of the inner membrane (IM), the periplasm and the outer membrane (OM). The IM is a symmetric lipid bilayer composed of phospholipids (PL) and integral membrane proteins with characteristic α-helical transmembrane domains. The periplasm is an aqueous compartment located between the IM and OM, and this is where the peptidoglycan cell wall resides. The OM is an asymmetric lipid bilayer containing PLs in its inner leaflet and lipopolysaccharide (LPS) in its outer leaflet. The OM also contains integral proteins, but these are folded in β-barrel conformations. Both membranes contain lipoproteins that are anchored to their periplasmic faces.

FIGURE 1.2

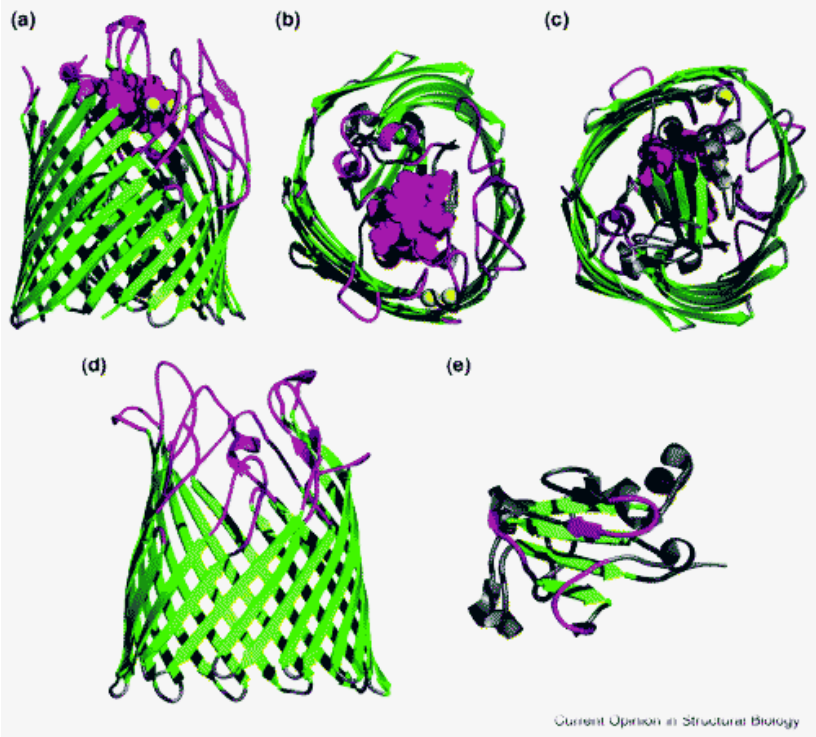

Figure 1.2 (from (52) **Structure of a TonB-gated Transporter**

Structure of a TBDT, the cobalamin transporter BtuB (11). **(a)** Structure of the substrate-bound BtuB complex, side view. The 22 β strands of the barrel and the four core β strands of the hatch (or plug or cork) domain are shown in green. The long extracellular loops of the β barrel and the apical loops of the hatch domain that contact the substrate are shown in magenta. The short periplasmic turns of the β barrel and the Ton box are shown in slate blue. Other regions (besides the core β strands and apical loops) of the hatch domain are shown in gray. The bound cyanocobalamin (vitamin B_{12}) substrate is shown in red space-filling representation; two bound calcium atoms (required for highest affinity substrate binding) are shown in yellow. **(b)** Structure of the substrate-bound BtuB complex, top (extracellular) view. **(c)** Structure of the substrate-bound BtuB complex, bottom (periplasmic) view. **(d)** Structure of the β barrel of BtuB (from the complex), side view. **(e)** Structure of the hatch domain of BtuB (from the complex), top (extracellular) view.

FIGURE 1.3

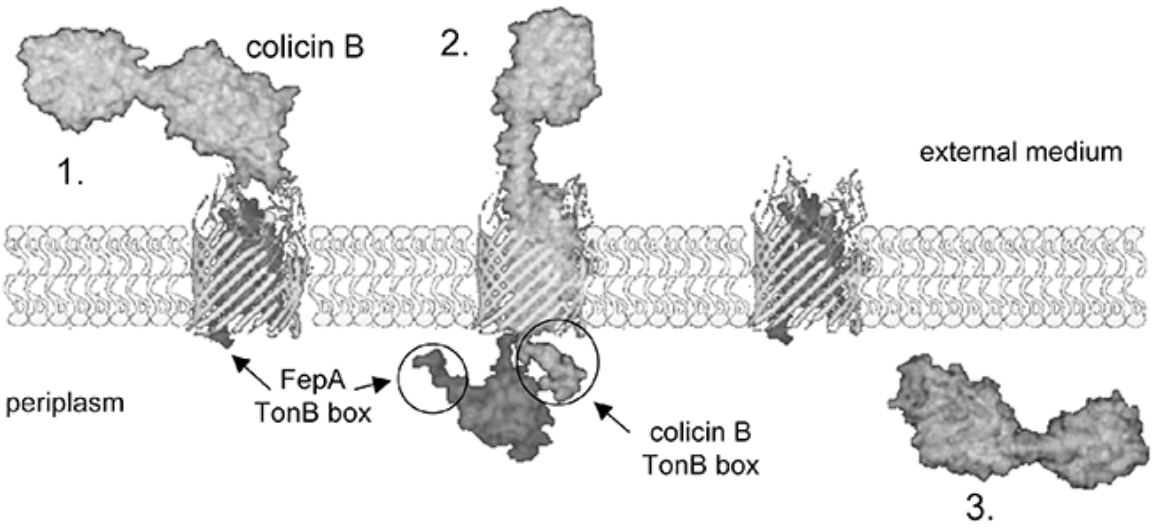

Figure 1.3 (from (12) **Model for Colicin Transport**

Model for colicin B translocation through FepA. In step 1, the amino-terminal half of the colicin B dumbbell containing a putative receptor binding domain binds to the FepA globular domain. In step 2, due to the action of TonB on the FepA TonB box, the FepA globular domain is released from the β-barrel, to be replaced by the amino-terminal dumbbell of colicin B. This also makes the TonB box of colicin B available to interact with TonB. In step 3, due to the action of TonB on the colicin B TonB box, the remainder of colicin B (the carboxy-terminal pore-forming domain) is released into the periplasm. It should be noted that there are currently no data to indicate whether or not the FepA globular domain is reinserted into the barrel following colicin B translocation. This model is adapted from (22).

FIGURE 1.4

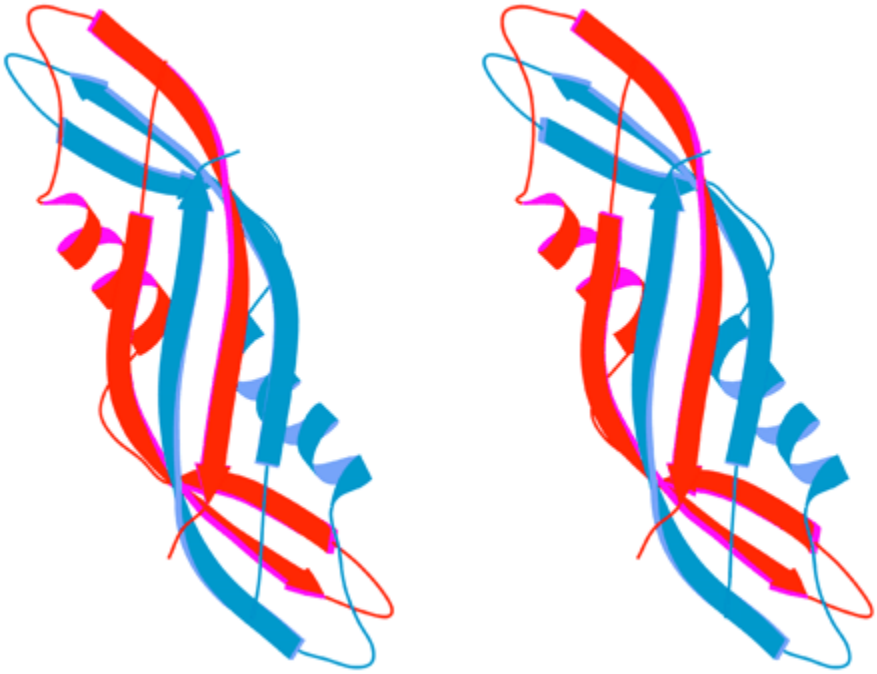

Figure 1.4 (from (10) **TonB Tight Dimer**

Stereo ribbon diagram of the C-terminal domain of TonB, showing the intertwined dimer.

FIGURE 1.5

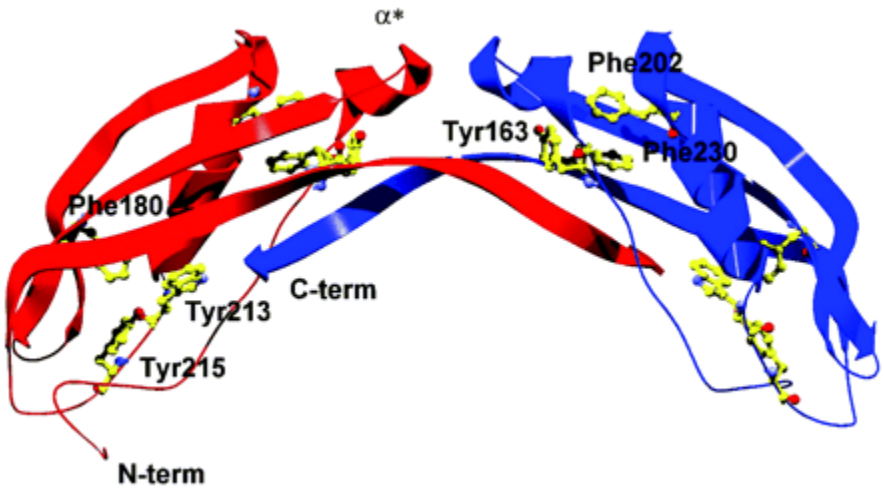

Figure 1.5 (from (27) **TonB Open Dimer**

Three-dimensional structure of the dimeric TonB-92 in ribbon representation. One molecule is shown in *red*, and the other one is *blue*. The aromatic residues forming four aromatic clusters are shown in *ball-and-stick representation*. The C-terminal β-strand forms an antiparallel β-sheet with the other TonB-92 molecule.

FIGURE 1.6

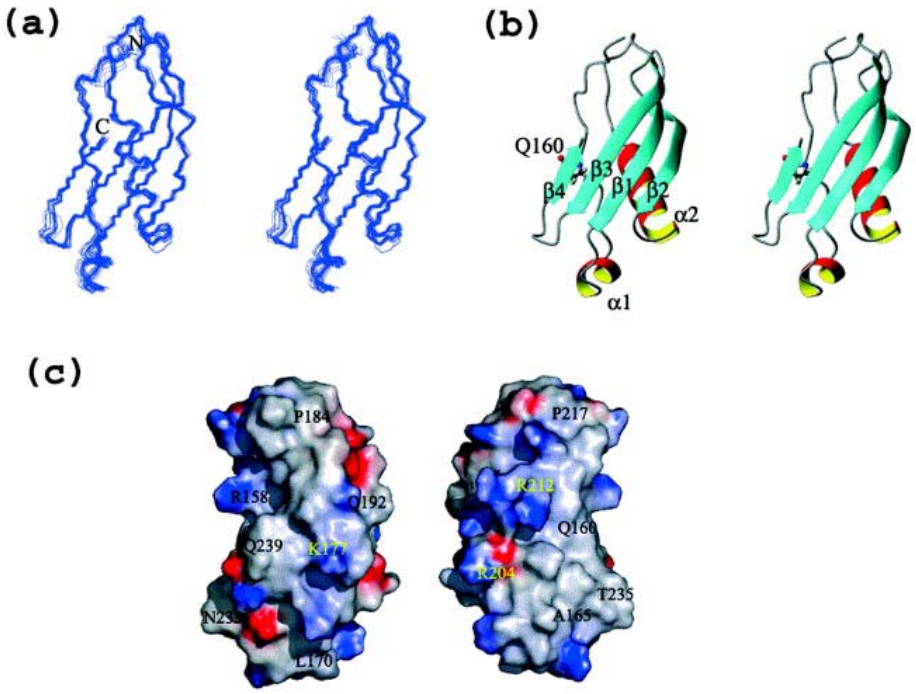

Figure 1.6 (from (48) **TonB Monomeric NMR Structure**

NMR structure of the ordered portion of TonB-CTD. (a) Overlay of the backbone of the 20 lowest-energy structures from the final iteration of structure calculations after water refinement. (b) Stereo ribbon diagram of TonB-CTD in the same orientation as in (a). (c) Surface electrostatic plots of TonB-CTD as calculated by the GRASP program.[70] The orientation on the left is as in (a). The orientation on the right is rotated 180° around the *y* axis. Red represents negative potentials, white indicates a neutral potential, and positive potentials are in blue. Several residues have been labeled for clarity.

FIGURE 1.7

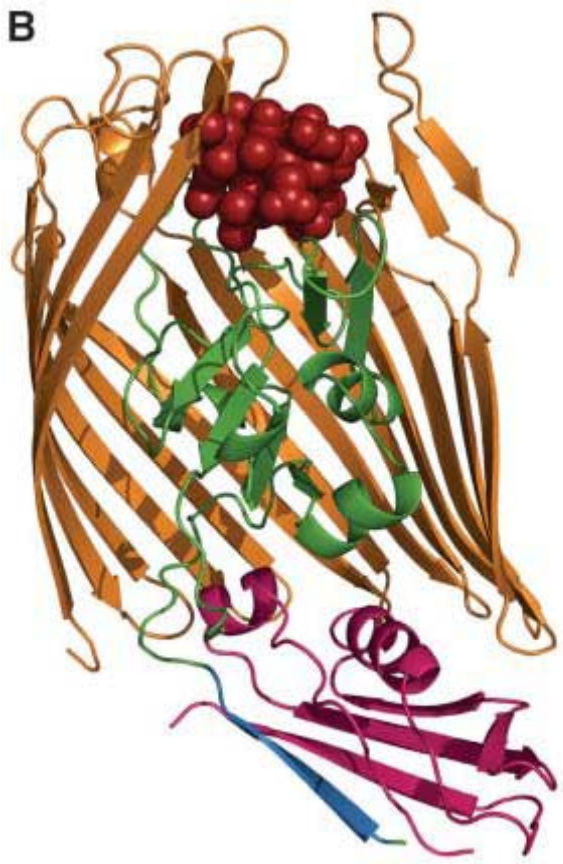

Figure 1.7 (from (50) **TonB:BtuB Co-crystal Structure**

Ribbon diagram of the BtuB:TonB complex, cutaway to show the luminal domain and Ton-box:TonB interaction. The structure is oriented such that, with respect to the bacterium, up is extracellular, down is periplasm-facing, and the β-barrel is embedded within the outer membrane.

FIGURE 1.8

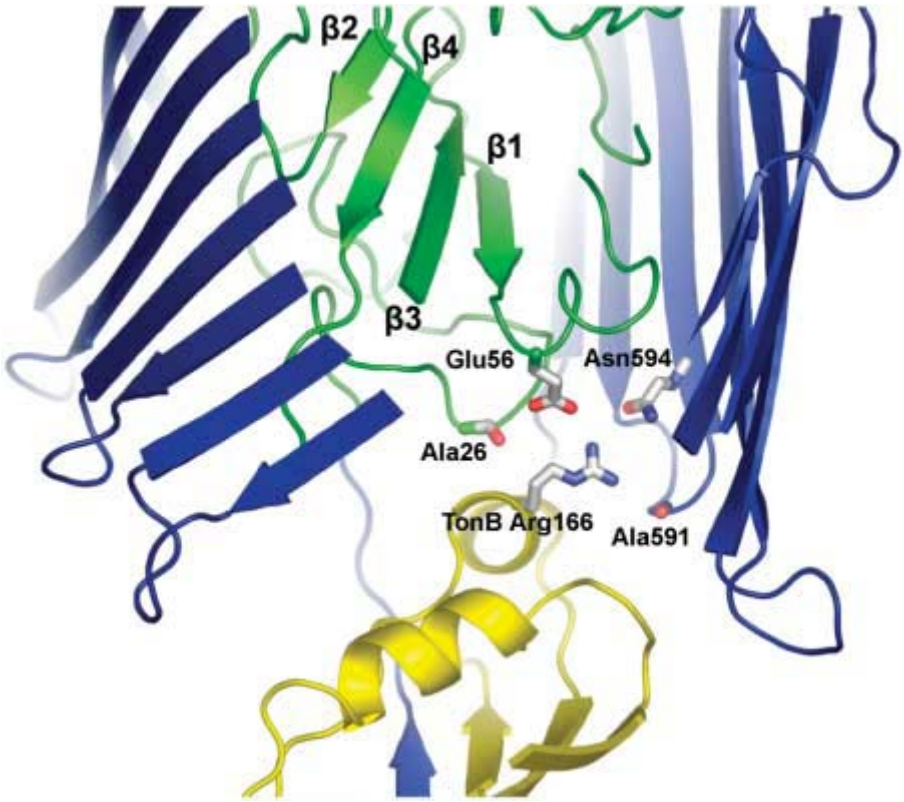

Figure 1.8 (from (42)

Residues from the FhuA cork and barrel domains interacting with TonB Arg166. Cut-away view showing FhuA and TonB protomers in cartoon representation; b strands are shown as flat arrows, helices as flat coils. The FhuA cork domain (residues 19 to 160) is colored green; the remaining FhuA residues are colored blue. TonB is colored yellow. TonB Arg166 and interacting FhuA residues (Ala26, Glu56, Ala591, and Asn594) are shown as sticks colored by atoms. Strands of the central β sheet of the FhuA cork domain (β1 to β4) are labeled.

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CHAPTER 2

INTRODUCTION

While the necessity for TonB in iron-siderophore and vitamin B_{12} acquisition has been clearly demonstrated, the mechanics behind this requirement remain ambiguous. Insight into the mechanism of TonB energy transduction has been provided by studies targeting the protein's two catalytically important domains: the amino-terminal transmembrane region (residues 12-32) which generates a conformational response to the PMF, and the carboxy-terminal domain (TonB-CTD; residues 150-239) that's known to interact with the TonB-gated transporters (TGT's) to allow active transport (7). Larsen and Postle performed deletion mutagenesis studies into the N-terminus of TonB and determined that amino acids serine-16 and histidine-20 as well as the register between them was important for activity (33). Since the amino-terminus is likely α-helical, this study it was hypothesized that S16 and H20 were catalytically important, and the spacing between them was essential to keep them on the same face of the helix. However, the authors later discovered that when serine-16 was substituted with a leucine, the resulting mutant was completely inactive and no longer formed stable interactions with the ExbB/D complex (34). This called into question the conclusions made about the deletion mutants, as each of the deletions would shift the leucine at position 15 into position 16 and create an S16L. Furthermore, work done in the *Escherichia coli* paralog TolA showed no loss of activity when serine-18 (the corresponding position of TonB S16) was changed to alanine (14), also suggested that S16 may not be essential. Here we show by S16A analysis that S16 is not essential, nor is it likely participating in the catalytic process for the N-terminus.

The Q160 region was first speculated to interact with the TonB-boxes of outer-membrane transporters when the TonB Q160K mutant was shown to suppress inactivating mutations in the TonB-box of BtuB (2). Later, direct interaction between these two regions was demonstrated by the formation of intermolecular disulfide bonds between cysteine substitutions at Q160, Q162, and Y163 of TonB and L8, V10, and A12 of BtuB (7). Recently, the co-crystal structures of the TonB-CTD with BtuB (50) and FhuA (42) have suggested that arginines within the Q160 region form salt bridges with acidic residues in the globular domain and are in proper position to deliver a perpendicular force against the transporter to remove the plug and allow transport. Here we demonstrate that in contrast to suggestions arising from the crystal structures, none of the four arginines within this region are essential for TonB activity.

MATERIALS AND METHODS

Strains and Plasmids:

The strains and plasmids used in this study are summarized in Table 2.1. All strains are derivatives of *E. coli* K-12.

Site-directed Mutagenesis:

TonB single-arginine mutants were generated from pKP325 by 30-cycle ultra-long PCR using *Pfu* Ultra DNA Polymerase from Stratagene. Forward and reverse primers were designed such that an alanine codon was substituted for the target arginine's and flanked on either side by 12-16 homologous bases. Template plasmid was removed by DpnI digestion and mutations in the TonB gene were confirmed by cycle sequencing at The Pennsylvania State University Nucleic Acid Facility. Double, triple and quadruple mutant plasmids were created by the same technique utilizing the single, double, and triple mutant-bearing plasmids, respectively, as templates. To avoid mutations occurring in un-sequenced regions of the plasmid as a result of multiple PCR amplifications, all plasmids containing multiple amino acid substitutions had a 914 bp BamHI fragment containing the TonB gene excised and ligated to pKP477 digested by the same enzyme (proper orientation confirmed by BstEII digestion) (51).

Wild-type TonB, H20A plasmids were constructed using a three primer mutagenesis technique as described previously (16, 33, 34).

^{55}Fe-ferrichrome Transport:

TonB activity was assayed for its ability to transport radiolabeled iron in complex with ferrichrome as described in (43), except that the iron and ferrichrome concentrations used to make the ligand were tripled, and samples were collected in 2 minute intervals.

Spot Titer Assays:

Spot titer assays were performed as described previously (34) with the arabinose concentrations that approximate chromosomal levels maintained in both the T-top overlay and T-plates.

RESULTS

Histidine 20 is required for a functional transmembrane helix, while, S16 and S31 are not

Previous investigations into the TonB transmembrane region indicated the conserved residues S16 and H20 as well as the respective spacing between them were critical for TonB activity(33). However, these studies were performed only on deletion mutants, which, after the discoveries that S16L was inactive (34) and there was no phenotype seen in the S16A paralog in TolA (14), raised the possibility that the deletions were simply inactive because they shifted L15 into the S16 spot, thereby creating an S16L phenotype. To investigate the role of TonB S16, an alanine substitution at codon 16 was made by a two primer extra-long PCR mutagenesis technique as described in Materials and Methods with mutation confirmed by sequencing at The Pennsylvania State University Nucleic Acid Facility. The arabinose concentration approximating protein levels of endogenously expressed TonB was determined prior to analysis by ^{55}Fe-ferrichrome transport and sensitivities to various colicins and bacteriophage Φ80, and H20A was assayed for ^{55}Fe-ferrichrome transport as well (Table 2.1). TonB H20A was completely unable to transport iron-ferrichrome, while S16A had wild-type activity in all assays.

Due to their conservation among gram-negative species and their potential positioning on a single face of the transmembrane helix, it was then speculated that perhaps S16 and S31 function to activate H20 via hydrogen bonding, in such a way that one is sufficient, but removal of both would render H20, and therefore the entirety of TonB, inactive. To test this theory TonB S16A/S31A was constructed, expression levels determined, and assayed for iron-ferrichrome transport. Although not completely active

(67% of wild-type activity retained), this double-alanine mutant was certainly capable of supporting TonB activity. While amino acid H20 is required, neither S16, nor S31 were essential for function of the TonB transmembrane region.

There are no essential arginines in the TonB-CTD Q160 region

The co-crystal structures of the TonB-CTD with the TGTs FhuA (42) and BtuB (50) suggested an important role for the arginines at positions 166 and 158, respectively. In these structures, the aforementioned arginines form a salt bridge with the globular domain of their respective transporters and are in proper orientation to deliver a perpendicular force necessary for removal of the globular domain from the barrel. To test these postulations, alanine substitutions were made at these residues and two additional arginine residues in that region, R154 and R171. The individual substitutions were also combined as all combinations of double and triple mutants and the quadruple mutant. Protein levels were again determined for each mutant and assays performed for iron-ferrichrome transport and colicin sensitivity (Table 2.2). Although R166A did exhibit the lowest activity of any single mutant with iron-ferrichrome transport, it was still 78% active and showed wild-type sensitivities to colicin M and bacteriophage Φ80, indicating that its interaction with FhuA was not disturbed. The R158A mutant retained 90% iron-ferrichrome transport but did show reduced sensitivities to colicins B and D which were perpetuated in all of its multiple mutants, suggesting that this residue is important for interaction with FepA. Interestingly, R171A had slightly higher activity with iron-ferrichrome transport and even rescued some of the activity lost to other mutants. Although minor, this indicates that there are some synergistic effects within these residues. R154A had little effect on TonB activity with any assay, and showed

little if any synergistic effects when combined with other mutations. Surprisingly, all double mutants exhibited iron-ferrichrome transport rates of 80% or higher, with the triple mutants at 70%, and the quadruple at 30%. For the triple and quadruple mutants the decrease in rates is likely due to a disruption of the natural conformation caused by loss of so many positive charges in the protein.

DISCUSSION:

It has long been known that the TonB N-terminal transmembrane region formed by amino-acids 13-32 is essential for activity as well as cytoplasmic membrane localization and interaction with the ExbB/D complex (23, 35), however the specific contacts and catalytically essential amino-acids are still unclear. Initial deletion mutagenesis studies indicated that serine 16 and histidine 20 as well as the spacing between them were essential for activity making the most likely requirement being presence of the two residues on the same face of the transmembrane α-helix (33). The data presented here confirms that neither S16 nor S31 are essential for activity and suggests that the loss of activity in the deletion mutants is likely due to steric hindrance created by moving L15 into the 16 position (34). Further studies into the TonB transmembrane region using multiple alaninyl substitutions at every position except for S16 and H20 (positions 22 and 25 are native alanines) showed that none of the other residues in the transmembrane helix are essential for function (30). This would suggest that H20 is the only residue that supplies an essential side chain, however, when S16A was placed in this background leaving only H20 as the sole non-alaninyl residue in the transmembrane region, the protein was inactive. This would seem to indicate that, although not essential per se, S16 does provide some role, most likely in forming a stable interaction between TonB and the ExbB/D complex.

While histidine-20 is the only residue providing a functional side-chain, it is unknown exactly what its capacity is in TonB activity. One potential role is as a site for modification, most likely a phosphorylation event. This likelihood is somewhat diminished by the ability to suppress the H20A mutation by mutations in ExbB V25D

and A39E (35) as an alanine residue is incapable of being phosphorylated. Even if the acidic residues in the ExbB suppressors were becoming phosphorylated, the subsequent allosteric effect would then have to be transferred to TonB. Another possible role is formation of a salt bridge with ExbB, however experiments reversing the charges between the mutant and suppressor still resulted in no detectable TonB activity (Manning and Postle, unpublished results). Its most likely role then becomes a site for protonation in transfer across the membrane. This could explain why partial suppression is achieved by replacement of hydrophobic residues in ExbB with acidic ones, as the carboxylate groups could be protonated, but not to the degree of a histidine.

While it has been shown that the residues in the Q160 region of TonB interact with the TonB-boxes of the TonB-gated Transporters (TGTs), the specific contacts are unclear. When deletion mutants were employed to identify the essential specific residues, it was seen that when only Q160 was removed, or when R158-Q162 were removed, TonB activity was near wt, but when N159-P161 were removed there was almost no TonB activity (Table 3). While this would suggest that at least R158 wasn't essential, it was possible that the deletions shifted one of the arginines at the nearby positions 154, 166, or 171 into the appropriate spot (51). Additionally, two co-crystal structures of the TonB carboxy terminus with the transporters BtuB and FhuA suggested electrostatic interactions between arginines at positions 158 and 166 and acidic residues on the plug domain of the TGT in such a way that they are likely to be involved in a catalytic pulling interaction (42, 50). However, neither of these residues significantly affected TonB individually or together and only exhibited moderate loss of activity when combined with alanine substitutions of the arginines at positions 154 and 171 (51).

Taken together, these data indicate that these arginines do not play an active role in TonB activity.

TABLE 2.1 STRAINS AND PLASMIDS USED IN THIS STUDY

Strains used in this study	Genotype	Reference
W3110	wt	(21)
KP1344	W3110, ΔtonB::blaM	(34)

Plasmids used in this study	Mutation	Reference
pKP477	pBAD empty vector	(16)
pKP325	pBAD regulated TonB	(34)
pKP813	TonB R154A	This study
pKP873	TonB R158A	This study
pKP814	TonB R166A	This study
pKP815	TonB R171A	This study
pKP887	TonB R154/158A	This study
pKP874	TonB R154/166A	This study
pKP875	TonB R154/171A	This study
pKP888	TonB R158/166A	This study
pKP889	TonB R158/171A	This study
pKP876	TonB R166/171A	This study
pKP890	TonB R154/158/166A	This study
pKP891	TonB R154/158/171A	This study
pKP892	TonB R154/166/171A	This study
pKP893	TonB R158/166/171A	This study
pKP935	TonB R154/158/166/171A	This study
pKP381	TonB H20A	(30)
pKP894	TonB S16A	This study
pKP942	TonB S16/31A	This study

TABLE 2.2 ARGININE TO ALANINE SUBSTITUTION ACTIVITY

Strain	Fe-transport %	Col B	Col D	Col Ia	Col M	φ80
ΔtonB	0 ± 0	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T
Chromosomal	110 ± 8	8,8,8	6,6,6	7,7,7	5,5,6	8,9,8
Wild-type	100 ± 6	8,8,7	6,6,5	7,7,7	5,5,5	9,8,9
R154A	87 ± 8	8,9,8	6,6,6	7,7,7	5,5,6	8,8,8
R158A	89 ± 9	3,3,3	T,T,T	6,6,6	5,4,5	8,7,8
R166A	78 ± 6	7,7,7	6,6,5	7,7,7	5,6,6	8,7,8
R171A	110 ± 10	7,7,7	5,4,5	7,7,7	5,5,5	8,9,8
R154/158A	96 ± 6	5,4,4	T,T,T	7,7,7	3,4,3	8,9,8
R154/166A	79 ± 6	6,6,7	3,3,4	7,7,7	3,3,4	8,7,8
R154/171A	123 ± 10	7,7,8	5,5,5	7,7,7	5,5,4	7,8,7
R158/166A	97 ± 7	3,4,4	T,T,T	7,7,7	3,3,4	8,8,9
R158/171A	112 ± 10	7,7,7	1,1,2	8,7,8	5,5,5	9,8,9
R166/171A	81 ± 4	6,6,6	3,3,3	8,7,8	4,5,4	8,8,8
R154/158/166A	69 ± 7	T,T,T	2,2,2	7,7,7	4,4,4	8,7,7
R154/158/171A	75 ± 7	T,T,T	T,T,T	7,8,8	4,4,4	9,8,9
R154/166/171A	76 ± 6	5,5,5	2,2,2	8,8,8	5,5,5	9,9,9
R158/166/171A	72 ± 9	T,T,T	2,2,2	7,7,7	T,T,T	8,8,8
R154/158/166/171A	32 ± 6	1,1,1	T,T,T	8,8,8	3,3,3	9,8,8
S16/31A	67 ± 11	ND	ND	ND	ND	ND

T = tolerant
ND = not determined

Table 2.2

All alanyl substitutions and plasmid encoded wild-type TonB were expressed to a level approximating endogenously expressed and verified by Western analysis (data not shown). Colicins B and D gain entry through FepA, Colicin Ia through Cir, and colicin M, bacteriophage Φ80, and Fe-ferrichrome through FhuA.

FIGURE 2.1

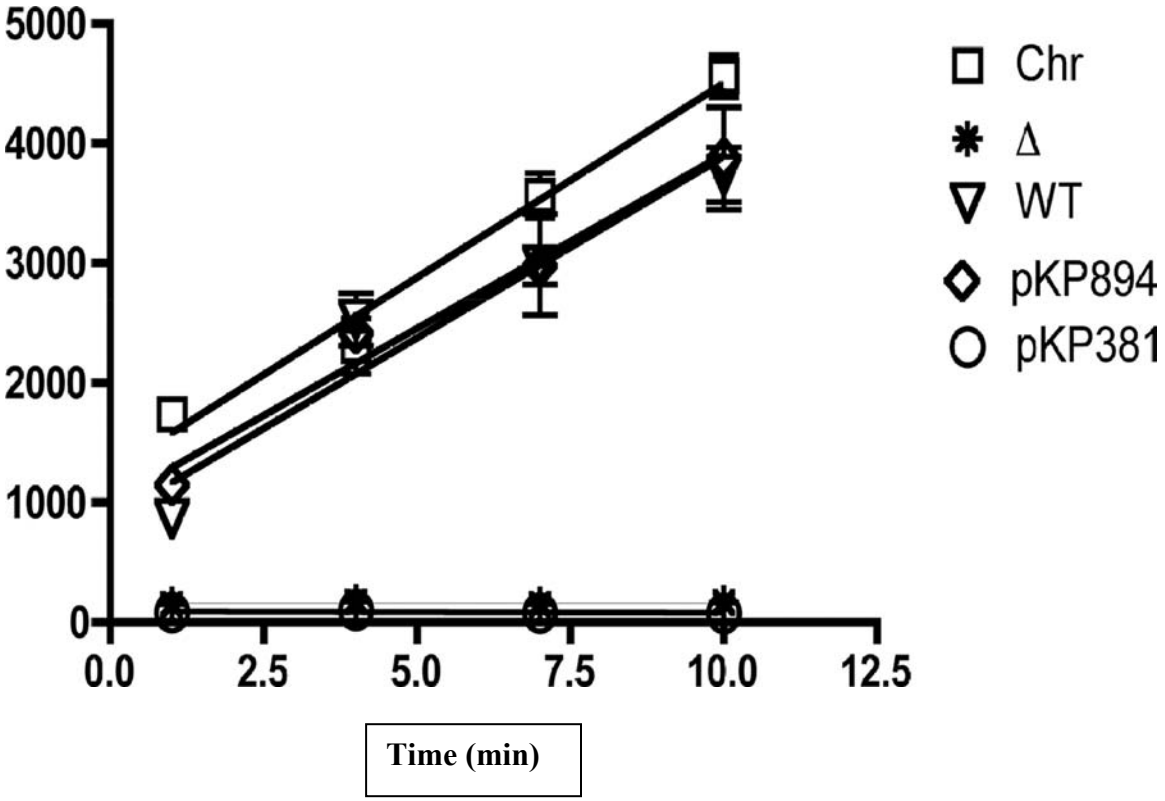

Figure 2.1 Ferrichrome Transport of N-terminal Alanine Substitutions. TonB $Ser_{16}Ala$ supports the transport of Fe(III) siderophores, but TonB $His_{20}Ala$ does not. KP1344 strains expressing TonB-$Ser_{16}Ala$ and TonB-$His_{20}Ala$ at chromosomal levels (data not shown) were grown and assayed for the uptake of [^{55}Fe]ferrichrome as described in Materials and Methods. All time points were sampled in triplicate, with transport rates calculated by linear regression of the entire data set collected for each derivative for the time frame displayed. Relative transport rates for each strain/plasmid pair, expressed in counts per minute per 0.35 A_{550} ml of cells per minute, are as follows: for W3110, 324 ± 27; for KP1344/pKP477, 0.5 ± 1.3; for KP1344/pKP325, 303 ± 45; for KP1344/pKP381, 291 ± 35; and for KP1344/pKP894, –1.6 ± 1.7.

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CHAPTER 3

INTRODUCTION

The carboxy terminus of TonB is essential for contacting the outer membrane and transducing energy to the TonB-gated transporters. The means by which this domain accomplishes this, however, is still unclear. As mentioned in the previous chapter, the region around Q160 has been shown to interact directly with the TonB-box of the transporters (7) and removal of this region renders the protein inactive (51). Several other sources, most notably the co-crystal structures have indicated other locations of interaction between the TonB carboxy-terminal domain (TonB-CTD) and the outer membrane transporters (8, 12, 42, 50).

After the release of the first TonB crystal structure (10), Joydeep Ghosh, a previous member of the Postle Lab, investigated an amphipathic helix region, amino-acids 199-216, in the TonB-CTD by cysteine scanning. He found that the aromatic residues within this region, F202, W213, and Y215 formed disulfide linked complexes when substituted by a cysteine and electrophoresed under non-reducing conditions(figure 3.1 and (15). These complexes were shown to be homodimers (figure 3.2), localizing to the cytoplasmic membrane (figure 3.3) and only formed when the TonB molecules possessed a functional N-terminus and could therefore become energized (figure 3.4). They also formed to an equal degree in an *aroB* strain, which, being unable to synthesize its own siderophores, will have no ligand-loaded transporters in the outer membrane (figure 3.5). Taken together, these data indicate that the structures represented by the complexes were formed after energization, but before energy delivery at the outer membrane. Additionally, each substitution also exhibited a unique phenotype when

assayed for its abilities to support colicin toxicity. This trend continued when the other two aromatic residues represented in the crystal structure, F180 and F230, were substituted with cysteines, although F180 failed to make disulfide-linked complexes (figure 3.1). Similar phenotypes were observed when each of the aforementioned amino acids was substituted by an alanine, and the effects on activity were synergistic, as the loss of any combination of two aromatic side-chains completely abolished TonB activity (16).

In this chapter I will show that very few residues in the TonB-CTD from amino-acids 150-239 have any effect on TonB activity and none have shown to be essential. The ones that do affect activity are localized around the Q160 region and G186. I also investigated F125, the only aromatic residue in the carboxy terminus within the unstructured region from amino-acids 103-149, and showed that while its substitution to a cysteine does not affect activity, it does form disulfide-linked complexes. In fact, aside from the previously mentioned aromatics, the only residues in the TonB-CTD to form these complexes are F125 and G186, however several residues form complexes with less efficiency. As seen previously (15), the monomeric forms of these proteins localize to both membranes, but the crosslinked complexes are only seen in the cytoplasmic membrane fractions except for F125C whose crosslinks fractionate with both membranes.

MATERIALS AND METHODS

Strains and Plasmids:

The strains and plasmids used in this study are summarized in table 3.1. All strains are derivatives of *E. coli* K-12.

Site-directed Mutagenesis:

TonB single mutants were generated from pKP568 by 30-cycle ultra-long PCR using *Pfu* Ultra DNA Polymerase from Stratagene. Forward and reverse primers were designed such that an alanine codon was substituted for the target residue's and flanked on either side by 12-16 homologous bases. Template plasmid was removed by DpnI digestion and mutations in the TonB gene were confirmed by cycle sequencing at The Pennsylvania State University Nucleic Acid Facility (51). Mutant plasmids deleted for residues 70 to 102 were constructed in the same fashion except the plasmid containing the cysteine substitution was the template and the forward primer annealed starting at residue 103 and the reverse, in the opposite polarity, annealed starting at residue 69, and PCR products were ligated before sequencing.

Wild-type plasmids were constructed using a three primer mutagenesis technique as described previously (16, 33, 34).

^{55}Fe-ferrichrome Transport:

TonB activity was assayed for its ability to transport radiolabeled iron in complex with ferrichrome as described in (43), except that the iron and ferrichrome concentrations used to make the ligand were tripled, and samples were collected in 2 minute intervals.

Spot Titer Assays:

Spot titer assays were performed as described previously (34) with the arabinose concentrations given in Table 3.1 maintained in both the T-top overlay and T-plates.

In vivo *Disulfide Crosslinking:*

Samples were prepared for SDS-PAGE as described in (34) except that the LSB contained 50mM iodoacetamide and no β-mercaptoethanol, then analyzed as described in (15).

Sucrose Density Gradient Fractionation

Sucrose density gradient fractionation was performed as described in (38) except that 50mM iodoacetamide was present in all buffer and sucrose solutions.

RESULTS:

No single amino acid in the region from 150-239 of the TonB carboxy terminus is essential for activity

Residues from the structured region of the carboxy-terminus, positions 150-239, were substituted with cysteine residues (mutant plasmids for A165C – Q239C were constructed by Joydeep Ghosh), expressed at levels approximating endogenously expressed TonB (data not shown) and assayed for their ability to transport radiolabeled iron-ferrichrome as well as their sensitivities to colicins B, Ia, and M. Those worthy of interest were also tested for sensitivity to colicin D and bacteriophage Φ80. Surprisingly, no single substitution was completely inactive and only very few showed any loss of activity at all (table 3.1 and figure 3.12). Within the Q160 region, P161C, Y163C, and P164C all showed some loss of activity with colicins D and M as well as ferrichrome transport rates that were 50-70% of wild-type rates. Also, Q162C showed wild type activity with iron-ferrichrome transport when assayed in the *tonB* strain KP1344, but was completely inactive to all colicins, bacteriophage Φ80, and even iron-ferrichrome transport when assayed in the *aroB tonB* strain KP1406. Western analysis showed that the protein was present in wt concentrations, so this would indicate that the presence of enterochelin is important for Q162C, even when interacting with transporters other than FepA. Intriguingly, R166C exhibited only 33% of wt activity, despite the fact that R166A was 80% active. The loss of activity in these proteins is likely due to a reduced affinity for the TonB-boxes of either the transporters or the colicins themselves. The only non-aromatic residue to exhibit a dramatic change in activity is the glycine at position 186. Cysteine replacement of this residue resulted in a 90% loss of the iron-

ferrichrome transport rate and severely diminished sensitivities to all bacteriocidal agents tested except for colicins B and D. Since colicins B and D both gain entry through FepA, this would indicate that while important for interaction/energy transduction with FhuA and Cir, G186 does not play a major role when transducing energy to FepA.

Although several residues in the TonB-CTD crosslink, only F125C and G186C form structures to the same intensity as the previously observed aromatic residues

The structures previously seen by Joydeep Ghosh were triplet dimers (figure 3.2), meaning three dimeric complexes of TonB in different conformations. To see if any other residues in the carboxy terminus from A150 – Q239 (minus the twenty assayed previously) would form similar complexes, each amino acid substituted with a cysteine was electrophoresed on an 11% polyacrylamide gel in buffers lacking β-mercaptoethanol and containing 50mM Iodoacetamide. Additionally, F125 was also analyzed in the same fashion, as it was the only remaining aromatic amino acid terminal to the proline-rich domain. Of the remaining seventy-one carboxy-terminal residues, when expressed at levels approximating endogenously encoded TonB, only cysteine substitutions at F125 and G186 produced similar triplet dimeric complexes (figures 3.6 and 3.7) which also formed to equal intensity in aroB strains (data not shown). It was also discovered that the complexes formed by F230C and G186C exhibit apparent molecular masses slightly heavier than those seen for F202C, W213C, and Y215C; and the complexes formed by F125C appear even heavier still (figures 3.7). Further distinguishing F230C and G186C is the detection of a lighter degradation product that is also observed under reducing conditions, but not in wild type or other cysteine substitutions. While no other cysteine substitution analyzed here forms triplet complexes, several do form a single complex

corresponding to the highest apparent molecular weight complex (residues from A150 – L170 shown in figure 3.6). These structures also vary slightly in apparent molecular weights between variants, but do not form consistently. All these data suggest that while TonB exhibits a general trend of conformational rearrangement, there are subtle differences in the structures formed.

The Disulfide-linked Complexes Formed Appear to be Homodimers

Joydeep Ghosh determined that the complexes formed by the aromatic amino acid to cysteine substitutions were dimers as shown in figure. Several crosslinking residues were selected and variants lacking the proline-rich domain (Δ70-102) were constructed as described in Material and Methods. These proteins were expressed and assayed identically to their full length counterparts. Although exact molecular weights weren't determined as in Ghosh and Postle 2005, the new crosslinks were compared to the complexes formed in F202C and Δ70-102 F202C; all formed structures similar to those seen in the previously characterized peptides (Figure 3.8). Furthermore, none formed structures that would indicate an apparent molecular mass shift of 8.5 kDa lighter which would indicate formation of a heterodimer.

The Complexes Formed by F230C and G186C are Localized to the Cytoplasmic Membrane, While Those Formed by F125C Localize to Both the Cytoplasmic and Outer Membranes

As seen in Ghosh and Postle 2005, upon sucrose density fractionation, the monomeric forms of the TonB proteins W213C and F202A/Y215C reside in both the cytoplasmic and outer membranes with an approximate ratio of 70% to 30%, respectively, as is the case with wild-type TonB; whereas the crosslinked complexes are only found in the

cytoplasmic membrane fractions. The cysteine substitutions at F230C, G186C, and F125C were subjected to a similar fractionation protocol as described in Materials and Methods. In the cases of F230C and G186C, the monomers and crosslinked complexes exhibited the same trend as seen with the previous cysteine substitutions (figures 3.9 and 3.10). This trend, however, does not continue in the case of F125C. This variant has a higher affinity for the outer membrane and exhibits a distribution of approximately 20% inner membrane association to 80% outer membrane association after accounting for the substantial amount of degraded protein seen in the outer membrane fractions (figure 3.11). This abundance of TonB degradation at the outer membrane is commonly seen in fractionations and is not unexpected. Intriguingly, however, is the observation of dimeric complexes at the outer membrane, which appear to fractionate equally with the monomeric form, i.e. 20% at the cytoplasmic membrane and 80% at the outer membrane. This effect is not altered by the absence of ligand-loaded transporters, as the same approximate distribution was seen when the variant is assayed in an *aroB* strain.

DISCUSSION:

The lack of a single essential amino acid in the carboxy-terminal residues from 150-239 was an extremely surprising result. This would lead to speculation that any globular domain may substitute for the TonB-CTD. This, however, cannot be the case as TolA, with an amino-terminus so similar to TonB's that it can be energized by ExbB/D, but dissimilar carboxy-terminus; cannot substitute for TonB. This tolerance to mutation becomes even more intriguing considering that the unstructured region between the transmembrane and the proline-rich domains [residues 33-65, (unpublished results)], and the proline-rich domain itself [residues 66-100, (36)] can each be deleted individually with minimal loss of TonB function. While an essential residue in the unstructured region from residues 103-149 cannot be ruled out, the TonB protein with an amber mutation at position 168 is energizable, but inactive (31), indicating a necessity for the extreme carboxy-terminal residues. All together, these data suggests that TonB is a protein that's truly greater than the sum of its parts.

Since enterochelin cannot enter the cell through any transporter other than FepA, the unusual results for Q162C would suggest that this protein needs to interact with either FepA specifically or any other ligand-loaded transporter before it can transport. This could be answered by testing for activity in a *fepA* strain and in an *aroB* strain in medium supplemented with iron-ferrichrome. The R166C result is also difficult to explain as to how a cysteine could be less active than an alanine when replacing an arginine. The protein exists largely in its monomeric form, which suggests that trapping of its crosslinked form is not causing the loss of activity. This could be tested by assaying the protein in the presence of a reducing agent to prevent crosslink formation.

Ghosh and Postle, 2005 identified four aromatic amino acids that formed disulfide-linked dimers when a cysteine was substituted in its position and one that didn't. This work has added two more substitutions to this list; another aromatic in the case of F125C and a non-aromatic in G186C. Much like F202C, W213C, and Y215C, G186C forms triplet-dimeric structures despite the distance between residues within respective monomers in either dimeric crystal structure. G186C also continues the trend of triplet crosslinkers showing significant losses of activity which are specific to certain transporters or colicins. This adds to the mounting evidence that TonB functions with subtle differences between its transporters. This property may be the reason there's so little sequence conservation among the TonB C-termini of known gram-negative organisms. As a species no longer needed a certain class of transporter, or the transporter's sequence was altered by mutation, the TonB residues important for interaction/energy transduction with that class would no longer need to be conserved.

F125C, unlike all other known triplet dimer formers, does not have an effect on TonB function. Formation of the dimers would indicate participation in the aromatic cluster identified in Ghosh and Postle, 2004, however no loss of function likely means that F125C doesn't alter the ability of the TonB C-terminus to rearrange itself. This is perhaps due to its location within the unstructured region of the protein; far enough away from the structured carboxy-terminus to allow normal interaction with the outer-membrane transporters.

Table 3.1 ACTIVITY OF TONB-CTD CYSTEINE SUBSTITUTIONS

Mutant	Plasmid	Arabinose %	Col B	Col D	Col Ia	Col M	Φ80	Fc-transport %	Disulfide Crosslinking
Chromosomal	pKP477	0.0	9,9,9	6,7,7	8,8,8	6,6,6	8,8,8	103 ± 5	None
ΔtonB	pKP477	0.0	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T	0 ± 0	None
C18G	pKP568	0.0005	9.9.9	7,6,6	7,8,8	7,6,6	8,8,9	100 ± 4	None
F125C	pKP1070	0.001	8,8,9	6,6,7	8,9,8	5,5,6	7,6,7	76 ± 4	Triplet Dimers
A150C	pKP945	0.0005	8,9,8	6,6,6	7,8,8	6,6,6	8,8,8	98 ± 2	Strong
S151C	pKP946	0.0005	8,8,8	6,6,6	7,8,8	6,6,6	8,8,8	85 ± 3	Strong
G152C	pKP947	0.0005	8,8,8	6,7,6	7,7,7	6,6,6	8,8,9	124 ± 5	Strong
P153C	pKP948	0.0005	8,8,8	6,6,6	8,8,8	6,6,6	8,8,8	98 ± 4	Weak
R154C	pKP949	0.0005	8,8,8	6,6,6	8,8,8	6,6,6	9,8,8	100 ± 5	Strong
A155C	pKP950	0.0005	8,8,8	6,7,6	7,7,7	6,6,6	8,8,8	104 ± 6	None
L156C	pKP951	0.0002	9,9,9	7,7,7	8,8,8	7,7,7	8,8,9	94 ± 3	None
S157C	pKP952	0.0005	8,9,8	6,6,7	7,8,8	5,5,5	8,8,8	107 ± 4	Strong
R158C	pKP953	0.0005	8,8,8	4,4,4	8,8,8	6,5,6	9,9,8	94 ± 5	Weak
N159C	pKP934	0.0005	9,8,9	6,7,7	8,8,8	5,6,6	8,9,8	78 ± 2	Strong
Q160C	pKP588	0.0005	9,9,9	7,7,7	8,7,7	6,5,5	8,9,9	85 ± 4	Weak
P161C	pKP592	0.0005	7,7,8	2,2,2	8,8,8	5,5,5	9,9,8	74 ± 3	Weak
Q162C	pKP587	0.0005	T,T,T	T,T,T	T,T,T	T,T,T	T,T,T	116 ± 7	Weak
Y163C	pKP586	0.0005	6,6,6	T,T,T	8,8,8	T,T,T	9,8,9	58 ± 2	Weak
P164C	pKP585	0.0005	8,7,7	5,5,5	8,8,8	4,4,4	8,8,8	67 ± 5	Strong
A165C	pKP584	0.0005	9,9,9	7,7,7	8,8,8	5,5,5	7,7,8	70 ± 3	Strong
R166C	pKP589	0.0005	7,8,8	6,6,6	8,8,8	5,5,5	6,6,6	33 ± 0	Strong
A167C	pKP590	0.0005	8,8,8	6,6,6	8,8,8	6,6,6	7,7,8	79 ± 1	None
Q168C	pKP593	0.0005	9,8,8	6,6,6	8,8,8	6,6,6	7,7,7	73 ± 3	Strong
A169C	pKP594	0.0005	8,8,8	6,6,6	8,8,8	6,6,6	7,7,7	66 ± 2	Strong
L170C	pKP591	0.0005	9,9,9	7,7,7	8,8,8	6,6,6	7,8,8	72 ± 2	Weak
R171C	pKP595	0.0005	9		8	5		97 ± 4	Weak
I172C	pKP600	0.0005	9		8	4		95 ± 3	None

Mutant	Plasmid	Arabinose %	Col B	Col D	Col Ia	Col M	Φ80	Fc-transport %	Disulfide Crosslinking
E173C	pKP596	0.0005	9		8	4		91 ± 5	None
G174C	pKP597	0.001	9		8	4		91 ± 1	None
Q175C	pKP620	0.0005	9		8	4		97 ± 2	None
V176C	pKP598	0.0005	9		8	4		92 ± 4	None
K177C	pKP599	0.0005	9		8	4		104 ± 3	None
V178C	pKP601	0.0005	9		8	4		98 ± 3	None
K179C	pKP602	0.0005	9		8	4		95 ± 3	None
F180C	pKP569	0.001	7	4,5,5	5	4	6,6,6	48 ± 2	None
D181C	pKP603	0.0005	10		7	4		67 ± 2	None
V182C	pKP604	0.001	9		6	4		72 ± 4	Weak
T183C	pKP605	0.0005	10		7	4		95 ± 4	None
P184C	pKP610	0.0005	9,9,9	6,6,6	9,9,9	5,5,5	7,8,8	78 ± 2	Triplet Dimers
D185C	pKP611	0.0005	9,9,9	6,6,6	8,9,8	3,3,3	7,8,8	76 ± 4	Strong
G186C	pKP612	0.002	7,7,7	4,4,4	5,5,5*	T,T,T	6,6,6†	9 ± 0	Triplet Dimers
R187C	pKP613	0.0005	10		7	4		89 ± 4	None
V188C	pKP614	0.0005	10		7	4		76 ± 2	None
D189C	pKP615	0.0005	10		7	4		93 ± 4	None
N190C	pKP638	0.0005	9		7	4		88 ± 2	None
K191C	pKP642	0.0005	9		7	4		80 ± 3	None
Q192C	pKP639	0.0005	9		7	4		85 ± 2	None
I193C	pKP640	0.0005	9		7	4		66 ± 2	Weak
L194C	pKP641	0.0005	9		7	4		76 ± 3	None
S195C	pKP606	0.0005	9		7	4		66 ± 4	None
A196C	pKP607	0.0005	9		7	4		95 ± 2	None
K197C	pKP608	0.0005	9		7	4		98 ± 3	Strong
P198C	pKP609	0.0005	8,8,8	5,5,5	9,8,8	4,4,4	7,7,7	58 ± 2	Triplet Dimers
A199C	pKP468	0.0005	9		8	4		80 ± 3	Strong
M200C	pKP469	0.0005	9		8	3		76 ± 2	Strong
M201C	pKP470	0.0005	9		8	4		97 ± 4	Strong

Mutant	Plasmid	Arabinose %	Col B	Col D	Col Ia	Col M	Φ80	Fc-transport %	Disulfide Crosslinking
F202C	pKP415	0.001	4	T,T,T	8	T	7,6,6	38 ± 1	Triplet Dimers
E203C	pKP417	0.0005	9		8	4		68 ± 5	None
R204C	pKP418	0.0005	9		8	4		92 ± 3	Strong
E205C	pKP462	0.0005	9		8	4		85 ± 2	Weak
V206C	pKP463	0.0005	9		8	4			None
K207C	pKP464	0.0005	9		8	5		120 ± 6	None
N208C	pKP416	0.0005	9		8	4		89 ± 2	None
A209C	pKP465	0.0005	9		8	4		90 ± 4	None
M210C	pKP466	0.0005	9		8	4		108 ± 4	None
R211C	pKP467	0.0005	9		8	4		108 ± 4	None
R212C	pKP471	0.0002	9		9	4		106 ± 4	Weak
W213C	pKP472	0.002	6	T,T,T	5	3	6,6,6	20 ± 1	Triplet Dimers
R214C	pKP473	0.0005	9		8	5		101 ± 3	None
Y215C	pKP474	0.001	8	3,3,3	3	3	7,6,7	35 ± 2	Triplet Dimers
E216C	pKP475	0.0005	9		8	4		98 ± 4	None
P217C	pKP621	0.0005	9		7	4		77 ± 3	None
G218C	pKP643	0.0005	9		7	4		88 ± 3	None
K219C	pKP616	0.0005	9		7	4		104 ± 5	None
P220C	pKP617	0.0005	9		7	4		74 ± 3	None
G221C	pKP618	0.0005	9		7	4		93 ± 4	None
S222C	pKP619	0.0005	9		7	4		79 ± 2	None
G223C	pKP622	0.0005	9		7	4		96 ± 2	None
I224C	pKP623	0.0005	9		7	4		95 ± 2	None
V225C	pKP624	0.0005	9		7	5		101 ± 3	None
V226C	pKP625	0.0005	9		7	5		91 ± 5	None
N227C	pKP626	0.0005	9		7	5		98 ± 4	None
I228C	pKP627	0.0005	8		7	5		80 ± 2	None
L229C	pKP632	0.0005	8		7	5		89 ± 5	None
F230C	pKP570	0.001	7	T,T,T	8	2	6,7,7	38 ± 1	Triplet Dimers

Mutant	Plasmid	Arabinose %	Col B	Col D	Col Ia	Col M	Φ80	Fc-transport %	Disulfide Crosslinking
K231C	pKP628	0.0005	8		7	4		85 ± 5	None
I232C	pKP629	0.0005	8		7	4		96 ± 2	None
N233C	pKP630	0.0005	8		7	4		58 ± 2	None
G234C	pKP631	0.0005	9		7	4		91 ± 3	None
T235C	pKP633	0.0005	8		7	5		80 ± 3	None
T236C	pKP634	0.0005	8		7	4		86 ± 4	None
E237C	pKP635	0.0005	9		7	4		65 ± 2	None
I238C	pKP636	0.0005	8		7	4		77 ± 4	None
Q239C	pKP637	0.0005	9		7	5		86 ± 2	None

* Very faint zones of clearing

[†]Faint, fuzzy plaques

Mutant plasmids made by Joydeep Ghosh and screened by Gail Deckert except for F125C and A150C-A165C.

Plasmid pKP1070 made by Cheryl Swayne

Colicin B, Ia, and M data for F180C, A199C-E216C, and F230C obtained by Joydeep Ghosh

Col B and Ia data for R171-K179C, D181C-T183C, R187C-K197C, P217C-N227C, and Q239C obtained by Lisa van Gamert.

Col M data for R171C-K179C, D181C-T183C, R187C-K197C, P217C-L229C, and K231C-Q239C obtained by Amber Dilhoff.

Table 3.2 STRAINS AND PLASMIDS USED IN THIS STUDY

Plasmids used in this study	Mutation	Reference
pKP486	C18G Δ70-102	(15)
pKP482	C18G Δ70-102 F202C	(15)
pKP1107	C18G Δ70-102 A150C	This study
pKP1102	C18G Δ70-102 P164C	This study
pKP1096	C18G Δ70-102 Q168C	This study
pKP1103	C18G Δ70-102 A169C	This study
pKP1108	C18G Δ70-102 L170C	This study
pKP1105	C18G Δ70-102 P198C	This study
pKP1104	C18G Δ70-102 N233C	This study

Strains used in this study	Genotype	Reference
W3110	F^- λ^- IN(*rrnD-rrnE*)1 *rph-1*	(21)
KP1344	W3110 (*tonB::blaM*)	(34)
KP1270	W3110 (*aroB*)	(34)
KP1406	W3110 (*aroB tonB::blaM*)	(34)

FIGURE 3.1

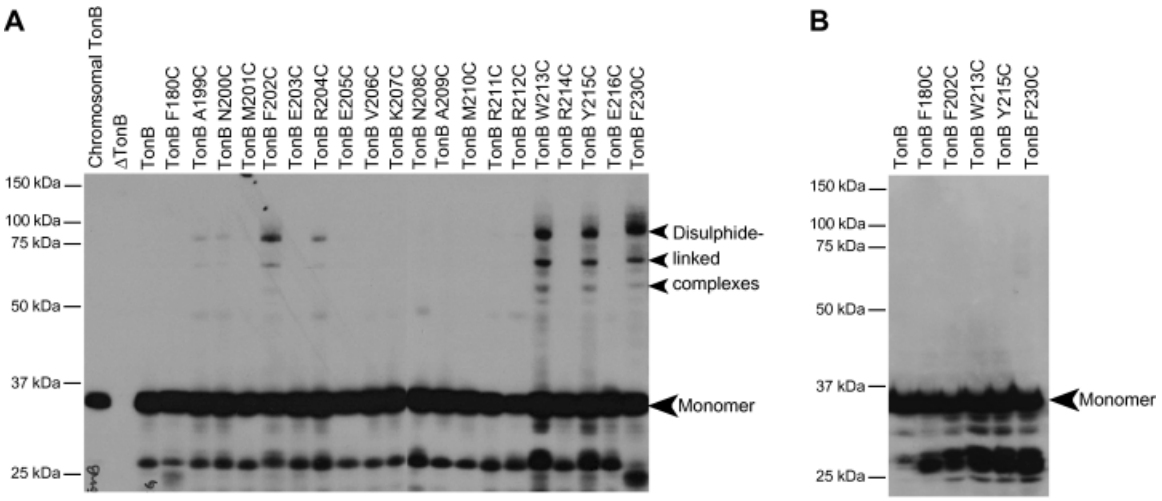


Figure 3.1 (from (15) **Disulfide Crosslinking in C-terminal Amphipathic Helix and Aromatic Residues**

Cysteine substitutions at four out of five aromatic residues in the TonB carboxy-terminus spontaneously form significant levels of disulphide-linked complexes.

A. An immunoblot of all plasmid-encoded cys substitutions in strain KP1344, resolved on an 11% non-reducing SDS-polyacrylamide gel, is shown. Positions of uncross-linked TonB and disulphide-linked complexes are indicated. Strains W3110 and KP1344 both carrying the vector pKP477 were used as the wild-type and Δ*tonB* controls respectively.

B. An overnight exposure of an immunoblot of cys substitutions at aromatic amino acids, resolved on an 11% reducing SDS-polyacrylamide gel. All TonB proteins except chromosomally encoded TonB are also C18G. Bands with masses smaller than TonB are TonB degradation products.

FIGURE 3.2

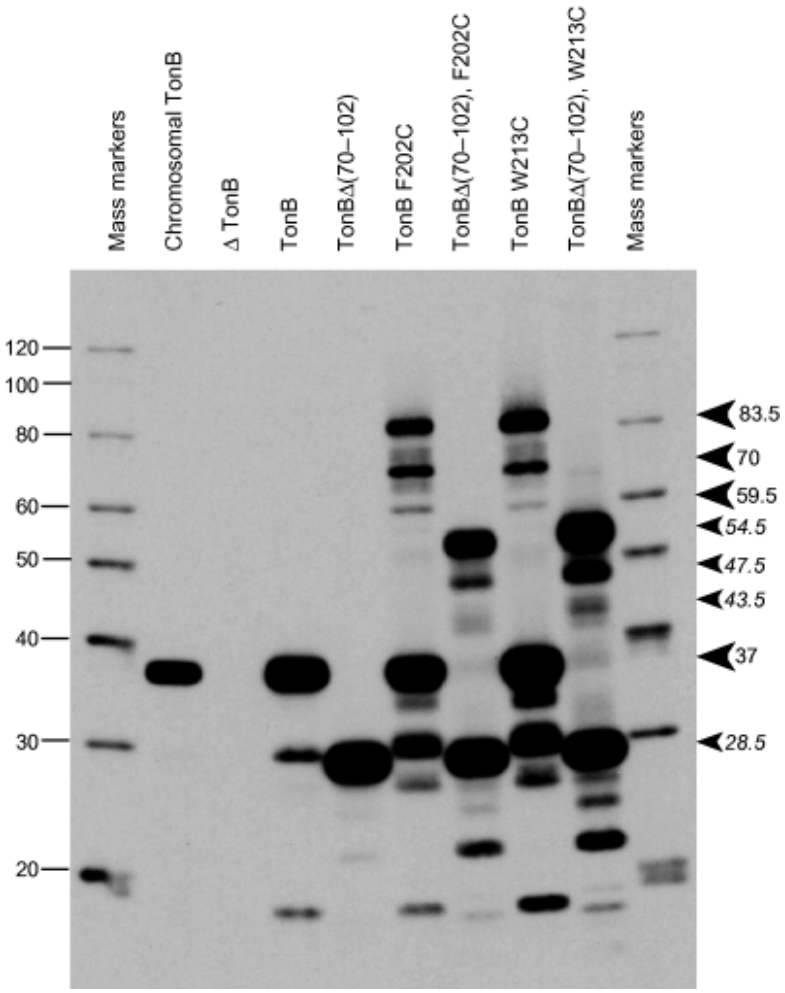


Figure 3.2 (from (15) **Crosslinks Formed are Homodimers**

Each of the three disulphide-linked complexes appears to be a TonB homodimer. An immunoblot of complexes formed by two full-length TonB mutants and their corresponding size variants TonBΔ(70–102) and resolved on an 8–16% non-reducing SDS-polyacrylamide gradient gel is shown. Apparent molecular masses of monomers and complexes are indicated on the right. Mass standards are indicated on the left. Positions of monomer and disulphide-linked complexes are shown. All TonBs except chromosomally encoded TonB are also C18G. Bands with masses smaller than TonB or TonBΔ(70–102) are TonB degradation products.

FIGURE 3.3

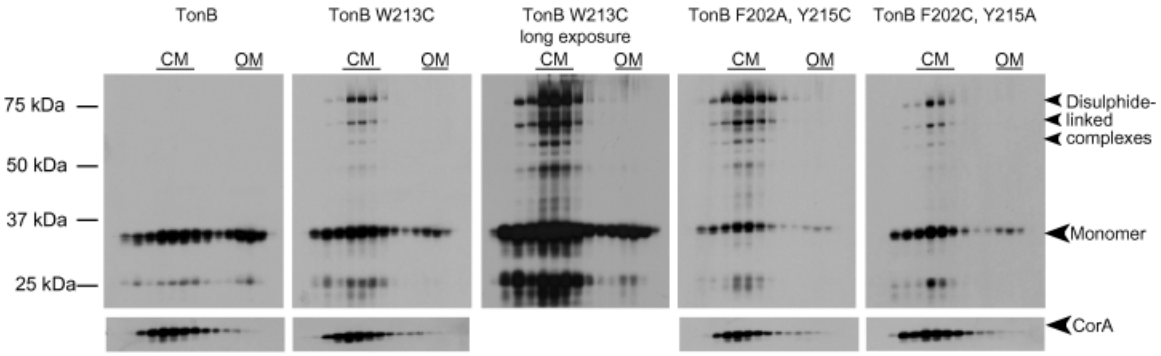


Figure 3.3 (from (15) **Dimers Localize Exclusively with Cytoplasmic Membrane Fractions**

Disulphide-linked TonB dimers are located only in cytoplasmic membrane fractions. Anti-TonB immunoblots of sucrose density gradient fractions displayed on 11% non-reducing SDS-polyacrylamide gels are shown (top). Immunoblots of cytoplasmic membrane protein CorA (Smith *et al.*, 1993) from the same fractions are shown (bottom). Positions of uncross-linked TonB and disulphide-linked dimers are indicated. All TonB proteins are also C18G. Bands with masses smaller than TonB are TonB degradation products. CM, cytoplasmic membrane; OM, outer membrane.

FIGURE 3.4

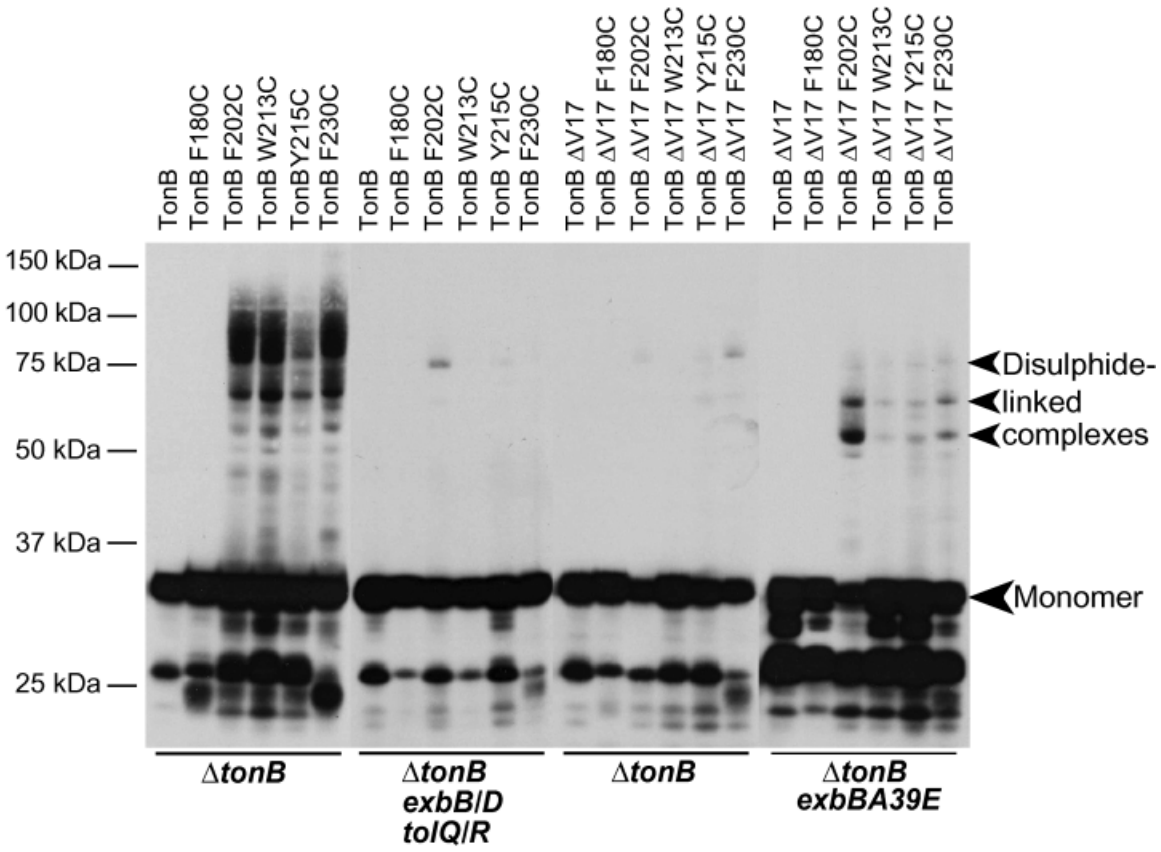


Figure 3.4 (from (15) **Crosslinks form After TonB Energization**

Formation of disulphide-linked complexes requires pmf mediators and the TonB energy coupling transmembrane domain. An immunoblot of cultures resolved on a non-reducing 11% SDS-polyacrylamide gel is shown. The four strains in which each plasmid was examined are indicated at the bottom of the four panels. Δ*tonB* is KP1344; Δ*tonB exbB/D, tolQ/R* is KP1440; Δ*tonB exbBA39E* is KP1348. For the two right-hand panels, the inactive TonBΔV17 mutation has been engineered into each mutant plasmid. Positions of uncross-linked TonB and disulphide-linked complexes are indicated. All TonB proteins are also C18G. Bands with masses smaller than TonB are TonB degradation products.

FIGURE 3.5

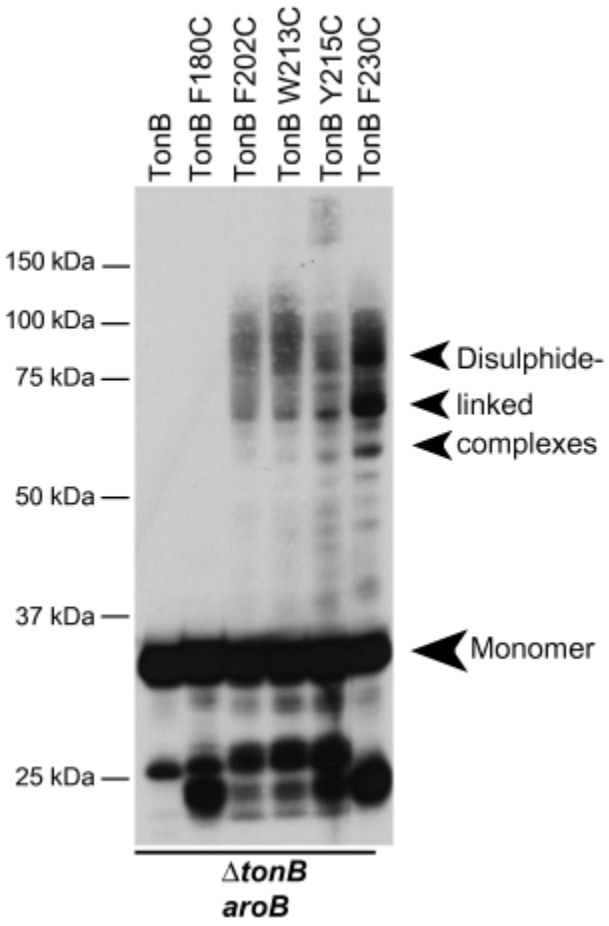


Figure 3.5 (from (15) **Crosslinks Form Before Energy Transduction**

Disulphide-linked TonB dimers can form before the release of energy at ligand-loaded outer membrane transporters. The Δ*tonB*, *aroB* strain (KP1406) is unable to synthesize enterochelin thus eliminating the presence of any TonB-dependent ligand. Anti-TonB immunoblots resolved on non-reducing 11% SDS-polyacrylamide gels are shown. Positions of uncross-linked TonB and disulphide-linked dimers are indicated. All TonB proteins are also C18G. Bands with masses smaller than TonB are TonB degradation products.

FIGURE 3.6

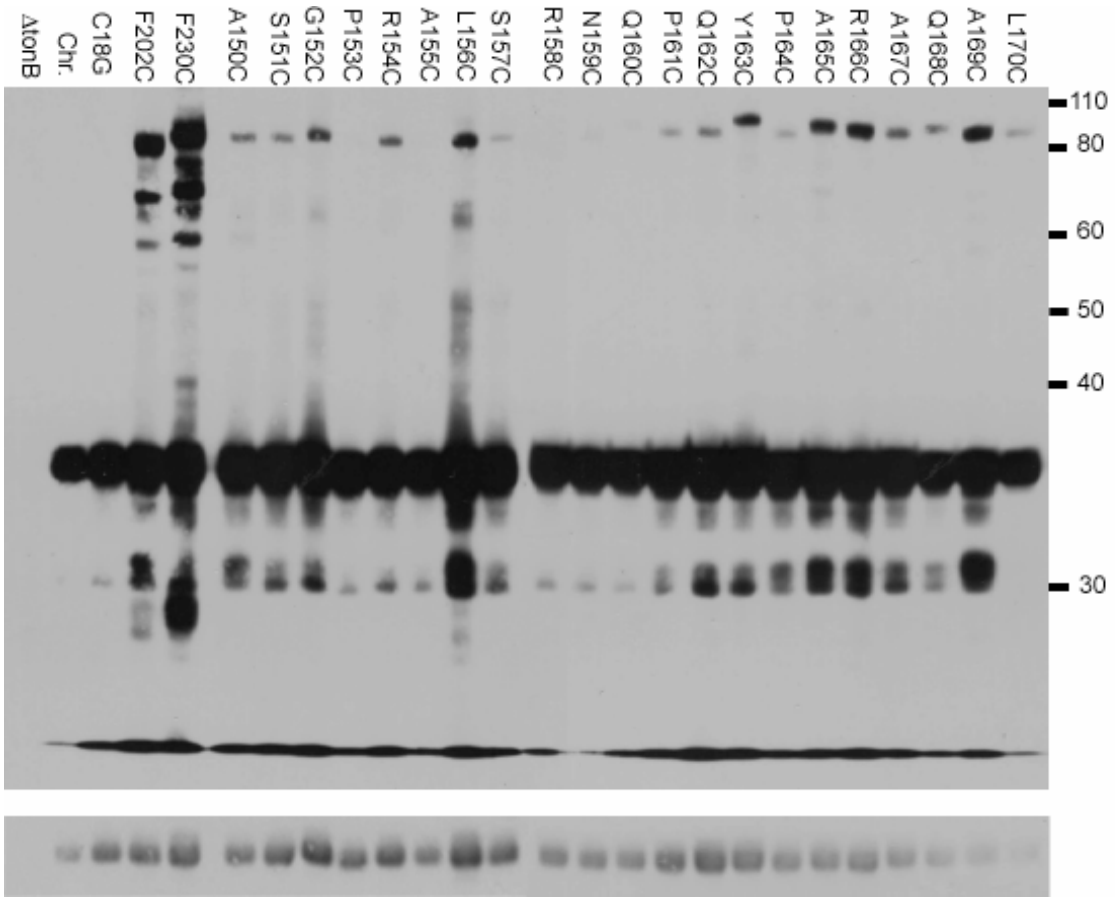


Figure 3.6 Disulfide-linked Dimer Formation in the Q160 Region

Disulfide-linked dimer formation in the Q160 region is sparse and inconsistent. Anti-TonB immunoblot of plasmid encoded cysteine substitutions expressed in KP1344 displayed on an 11% non-reducing polyacrylamide gel (top) with a lighter exposure (bottom) to indicate monomer protein levels. Molecular weight markers are shown on the right in kDa. Strains KP1344 and W3110 transformed with pKP477 were used as *ΔtonB* and chromosomally encoded TonB (Chr.), respectively. All TonB substitutions except Chr. are also C18G.

FIGURE 3.7

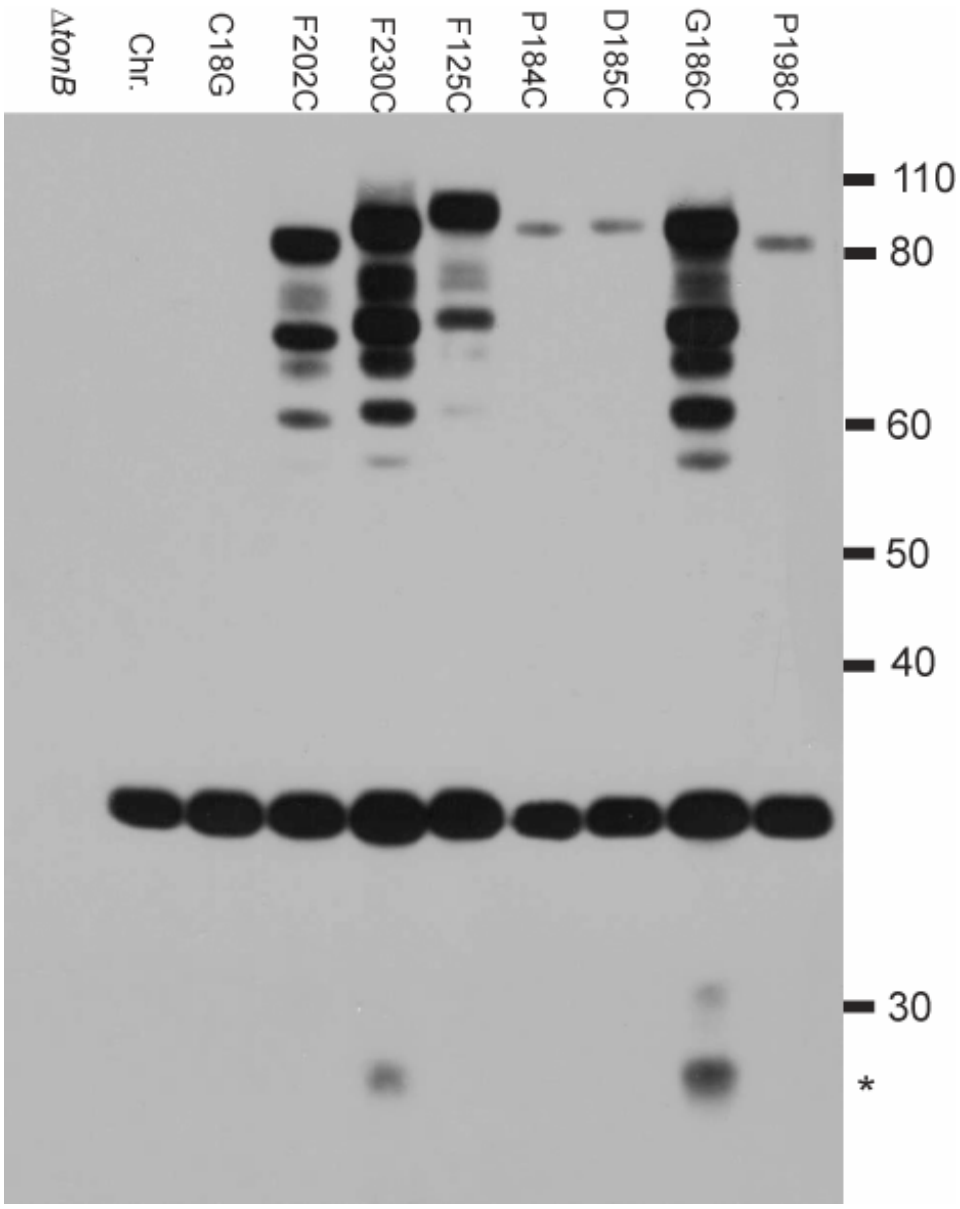


Figure 3.7 G186C and F125C Also Form Triplet Dimers

Disulfide crosslinks in F125C and G186C form with intensity equivalent to F202C and F230C. Anti-TonB immunoblot shown as in Figure 3.6 with no lighter exposure displayed. The position of the unique degradation product is marked by an asterisk. All TonB proteins except Chr. are also C18G

FIGURE 3.8

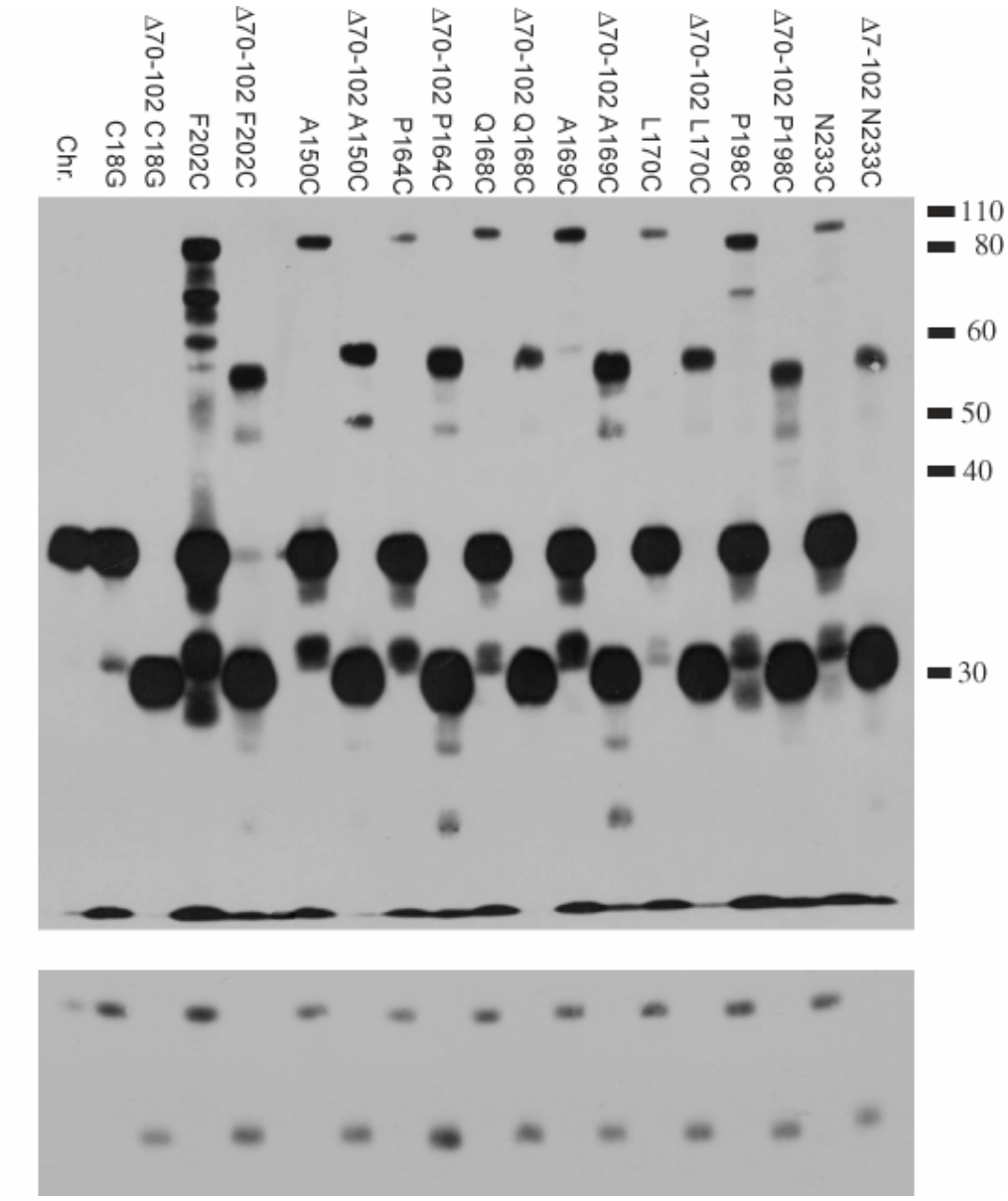


Figure 3.8 TonB Cysteine Disulfides Appear to be Homodimers

Anti-TonB immunoblot shown as in Figure 3.6.

FIGURE 3.9

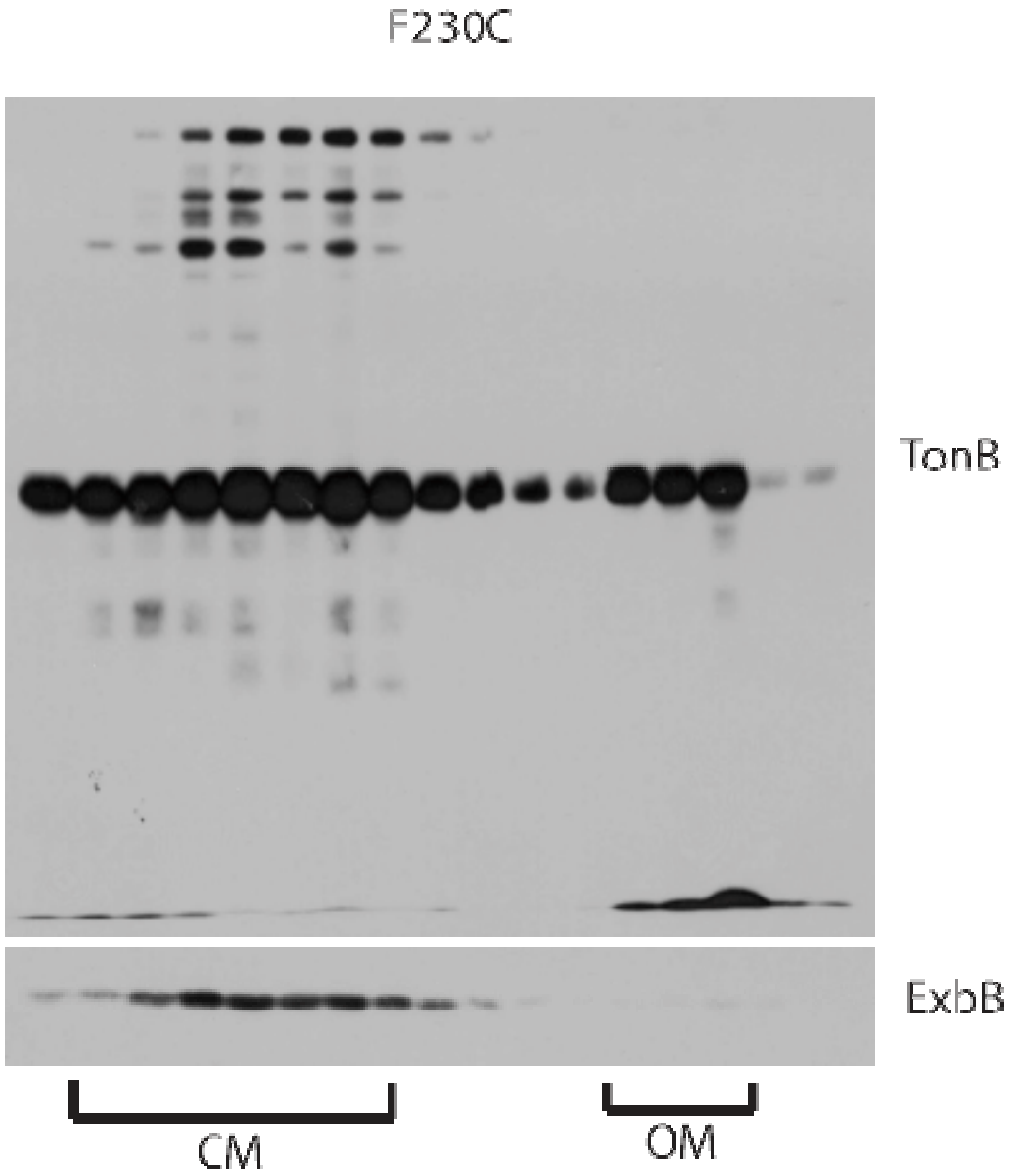


Figure 3.9 Sucrose Density Gradient Fractionation of F230C

Disulfide complex formation in C18G F230C is localized to the cytoplasmic membrane. Anti-TonB immunoblot (top) and of sucrose density fractionation in KP1344 displayed on a non-reducing 11% polyacrylamide gel. An anti-ExbB immunoblot (bottom) of the same fractions is shown for visualization of the cytoplasmic membrane. CM, cytoplasmic membrane; OM, outer membrane.

FIGURE 3.10

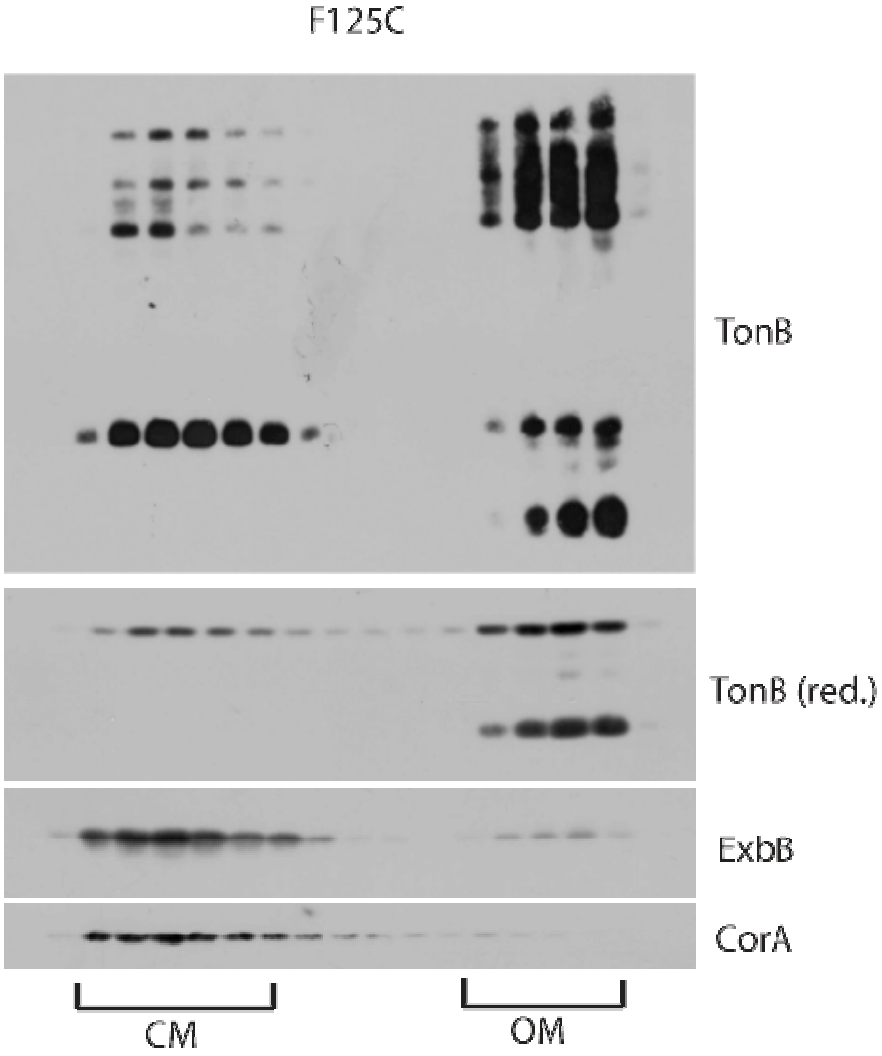


Figure 3.10 Sucrose Density Gradient Fractionation of F125C

Disulfide linked complexes formed by C18G F125C localize to both the cytoplasmic and outer membrane fractions. Anti-TonB immunoblot of sucrose density gradient fractionation in KP1344 under non-reducing and reducing conditions (top two panels) displayed on an 11% polyacrylamide gel. Anti-ExbB and anti-CorA immunoblots are displayed for visualization of the cytoplasmic membrane. red., reduced; CM, cytoplasmic membrane; OM, outer membrane.

FIGURE 3.11

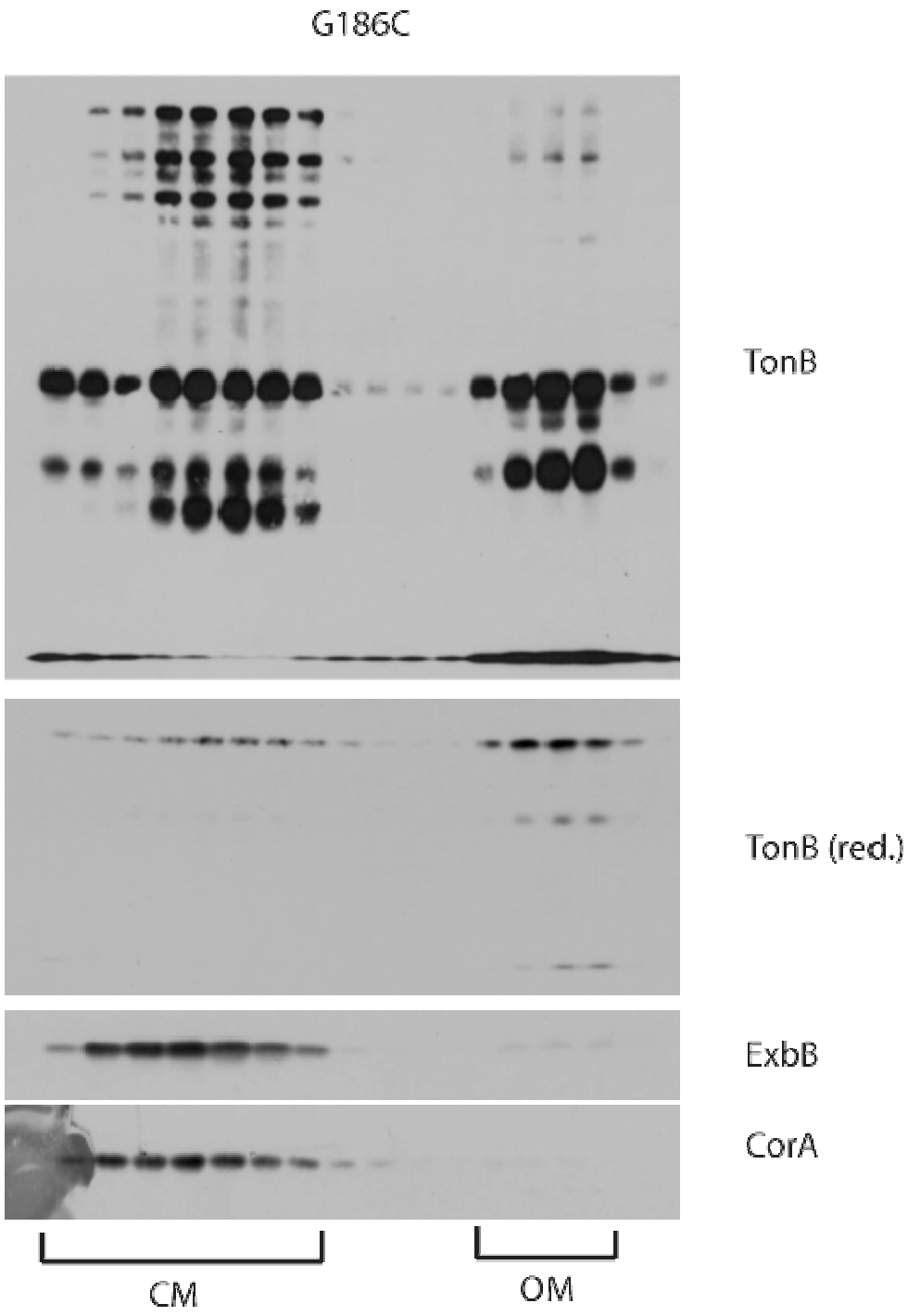
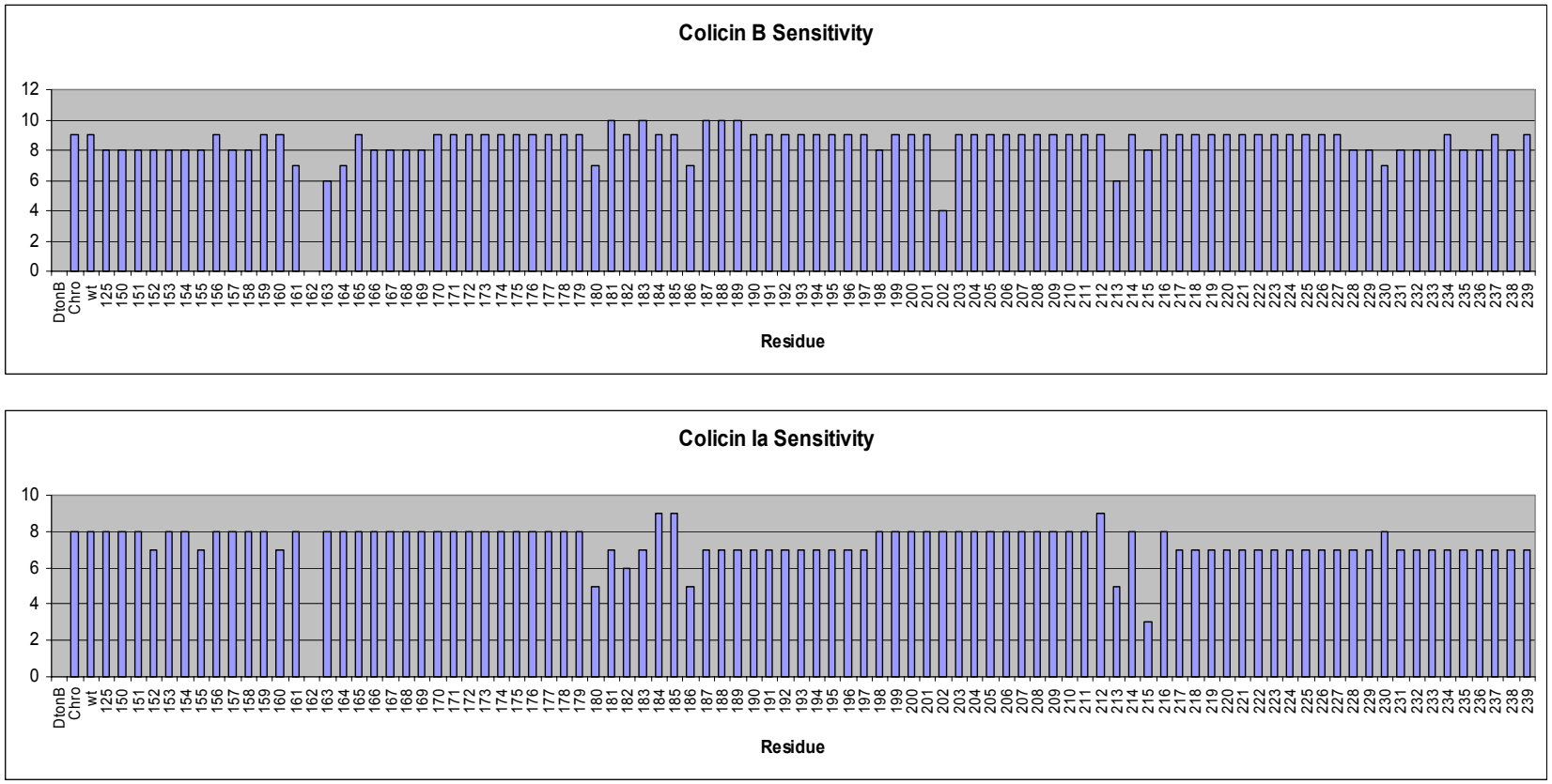
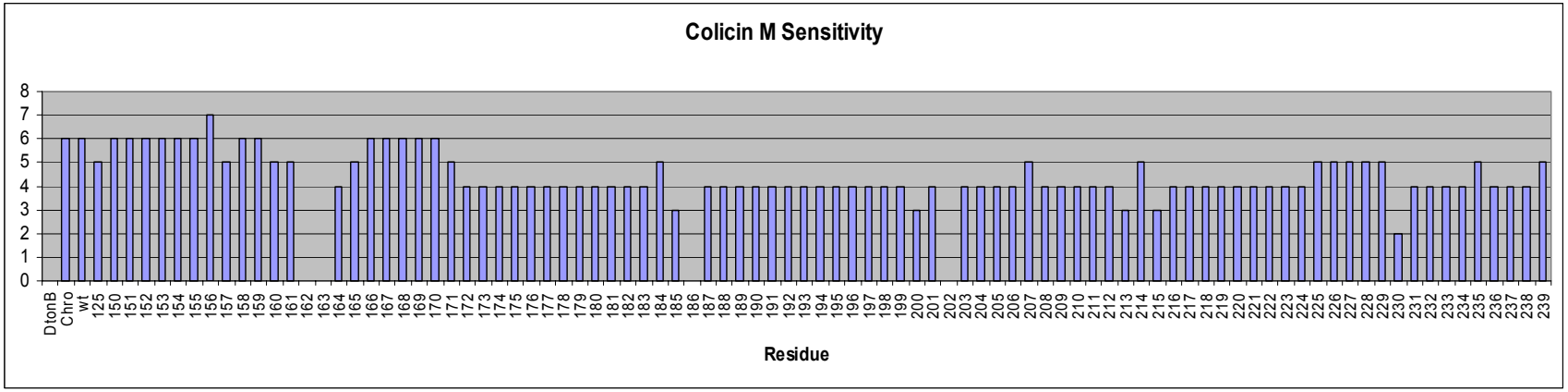


Figure 3.11 Sucrose Density Gradient Fractionation of G186C

G186C disulfide crosslinks form entirely at the cytoplasmic membrane. Immunoblots shown as in figure 3.10.

Figure 3.12 SUMMARY OF ACTIVITY DATA





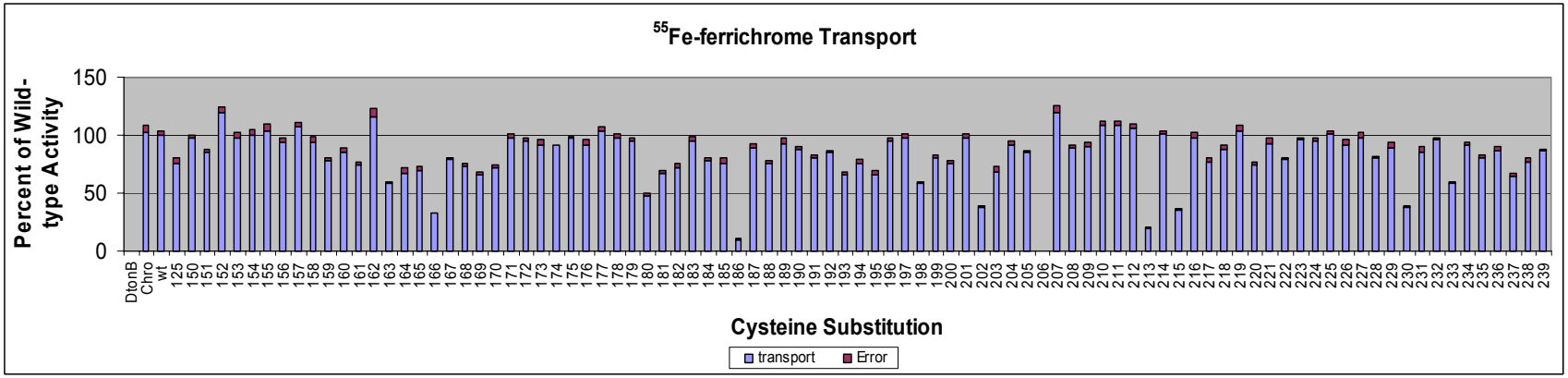


Figure 3.12

The colicin sensitivities are given as the reciprocal of the highest five-fold serial dilution that showed clearing on a lawn of cells (an average of three trials). ^{55}Fe-ferrichrome transport data is shown as the percentage of wild-type activity. The proteins were expressed at levels approximating wild-type activity for all assays (data not shown)

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