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TonB-dependent energy transduction between outer and cytoplasmic membranes

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Abstract The TonB system of *Escherichia coli* (and most other Gram-negative bacteria) is distinguished by its importance to iron acquisition, its contribution to bacterial pathogenesis, and a unique and mysterious mechanism of action. This system somehow gathers the potential energy of the cytoplasmic membrane (CM) proton gradient and delivers it to active transporters in the outer membrane (OM). Our understanding of this system is confounded by the challenge of reconciling often contradictory in vivo and in vitro studies that are presented in this review.

Keywords TonB · Outer membrane · Iron transport · B-group colicins · Crystal and NMR structures

Overview

The Gram-negative envelope consists of two concentric membranes, separated by an aqueous compartment. The inner, cytoplasmic membrane (CM) is a phospholipid bilayer, rich in proteins that generate and harvest ion gradients for energy conversion, nutrient transport and other essential processes. Beyond the CM lies the periplasmic space, a viscous compartment containing a variety of proteins involved in nutrient acquisition, as well as enzymes, chaperones, and trafficking factors essential to envelope biogenesis. Also within the periplasmic space is a thin murein corset—an oligopeptide-crosslinked glycan network conferring shape and rigidity to the cell. Tethered to this matrix is a second membrane of distinct design. This outer membrane (OM) is an asymmetric lipid bilayer with an internal surface comprised of phospholipids, and an external surface of lipid-anchored oligosaccharides that presents an anionic surface to the surrounding environment. Providing a protective diffusion barrier, this surface retards the passage of lipophilic toxins soluble in typical membranes, yet still allows the diffusion of small hydrophilic nutrients via aqueous channels formed by porin proteins.

While most of the metabolic demands of Gram-negative bacteria are met by diffusible nutrients, iron demand presents a complication. In oxidizing environments, bioavailable iron is limiting, and most microbes rely upon the secretion of siderophores to capture stray ferric ions. These chelators effectively bind iron, however; the resultant iron-

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siderophore complexes exceed the diffusion limits of the OM. Consequently, efficient retrieval of iron requires high-affinity siderophore transporters. Because envelope physiology and architecture preclude energy production at the OM, energy to drive these transporters must be imported. The source of this energy is the ion electrochemical gradient of the CM—as harvested by heteromultimeric complexes of ExbB and ExbD proteins, and transduced to the OM high affinity siderophore transporters by the protein TonB (see Postle and Kadner 2003; Postle and Larsen 2004; Weiner 2005 for recent reviews). Beyond iron-siderophores, TonB-transduced energy has long been known to be essential for the high affinity transport of cobalamin, with recent studies suggesting that TonB may indeed support the transport of a much wider range of ligands in certain species (Neugebauer et al. 2005).

TonB protein

The structural and topological features of TonB are well suited for a protein that interacts with components of two distinct, and removed membranes. In *Escherichia coli*, TonB is comprised of 239 amino acids, a disproportionate percentage of which are prolyl residues (Postle and Good 1983). It can be divided into three distinct functional regions—amino-terminal (residues 2–65) and carboxy-terminal (residues 103–239) domains, separated by a central proline-rich spacer (residues 66–102). The amino-terminal domain contains a standard hydrophobic signal sequence necessary for Sec-dependent export to the CM (Skare et al. 1989), from which the majority of TonB occupies the periplasmic space (Hannavy et al. 1990; Roof et al. 1991). Uncleaved, this signal sequence ostensibly anchors TonB in the CM (Postle and Skare 1988) and clearly mediates the interaction between TonB and its energy source (Karlsson et al. 1993; Jaskula et al. 1994). Although termed a transmembrane domain, as described below, the signal anchor may not directly interact with the lipid bilayer. While the actual conformation is uncertain, if modeled as an α-helix, the signal anchor presents a surface motif (Ser16, His20, Leu27, Ser31) shared with the TonB analog TolA (Koebnik 1993). Deletion scanning analysis of the TonB transmembrane domain suggests that residues Ser16 and His20, and the register between them are important for function (Larsen and Postle 2001).

Beyond the amino-terminal domain lies a prolyl-rich spacer domain [residues 66–102; $(Glu\text{-}Pro)_4$, Ile-Pro-Glu-Pro—9 residue spacer—$(Lys\text{-}Pro)_6$]. Solution NMR studies with synthetic peptide suggests that it can achieve an extended conformation of up to 100 Å in length (Evans et al. 1986)—about half the distance across the periplasmic space and probably sufficient to extend the reach of TonB from the CM to the OM. A synthetic proline-rich spacer can specifically bind to FhuA in vitro (Brewer et al. 1990), however the relevance of this is unclear, as this spacer can be deleted in vivo with little impact on TonB activity (Larsen et al. 1993; Seliger et al. 2001).

The carboxy-terminal domain of TonB (amino acids 103–239) is essential for interaction with OM transporters. Whereas our understanding of the TonB amino-terminus has been assembled primarily from genetic and biochemical data, recent structural characterizations of the carboxy-terminus of TonB have provided new perspectives on this domain, as explored below.

The energy transduction cycle

The mechanism by which TonB protein couples CM-derived energy to support events at the spatially removed OM has been the subject of much speculation. While in a cartoon sense, one can readily envision mechanical models (Chang et al. 2001), the pros and cons of which have been discussed previously (Postle and Kadner 2003), the preponderance of experimental evidence supports a dynamic cyclic model in which TonB protein disengages from the CM to physically traverse the periplasmic space—a shuttle bearing energy to the OM, with a deadhead return to the CM (Letain and Postle 1997; Larsen et al. 1999, 2003). The implications of the shuttling model are unique—unlike either mechanical (either as some variation of a lever, or as a concerted set of intramolecular conformational changes) or physical (i.e. a ‘‘proton wire’’) models, shuttling

suggests the ability to store, and subsequently release potential energy—thus in this model TonB molecules exist in distinct ''charged'' and ''uncharged'' states.

This energy transduction cycle (Fig. 1, position 1) begins with TonB at the CM in association with a heteromultimeric complex of ExbB and ExbD. Stoichiometric analysis (Higgs et al. 2002a), in vivo chemical crosslinking (Higgs et al. 1998), in vitro studies (Braun et al. 1996), and the interdependence of ExbB and ExbD (Held and Postle 2002) together suggest it is a large complex (>500 kDa) with which TonB interacts. Topological (Hannavy et al. 1990; Roof et al. 1991), genetic (Larsen et al. 1994) and biochemical (Larsen et al. 1999; Larsen and Postle 2001) studies suggest that these interactions involve the amino-terminally located ''signal anchor'' of TonB. Because shuttling requires TonB to disengage from the CM, it is likely that the association with the CM is mediated through weak interactions with residues of the ExbB/ExbD complex, rather than stronger, entropy-driven interactions with the lipid bilayer. Multiple lines of evidence suggest that, at least while associated with the ExbB/ExbD complex, TonB exists as a dimer (Sauter et al. 2003; Ghosh and Postle 2005).

In the first step of the energy transduction cycle (Fig. 1, position 2), the potential energy of the ion electrochemical gradient is harnessed to energize TonB. To be clear, we do not yet know what it specifically means for TonB to be ''energized'', nor the mechanism by which ExbB and ExbD bestow this state upon TonB. ExbB and ExbD belong to what have been respectively called the ''MotA/TolQ/ExbB'' and the ''MotB/TolR/ExbD'' families of proteins (Saier 2000; Kojima and Blair 2001; Cascales et al. 2001). The TolQ and TolR proteins are paralogues of ExbB and ExbD (Braun and Herrmann 1993) that energize the TonB analog TolA (Germon et al. 2001) and can, inefficiently, also energize TonB via crosstalk (Braun 1989; Braun and Herrmann 1993). MotA and MotB together energize flagellar rotation (Blair 2003). For each of these protein pairs there is evidence that their activities are coupled either directly or indirectly to the CM protonmotive force. Consistent with this association, these sets of proteins are most conserved in their transmembrane domains (Cascales et al. 2001; Postle and Larsen 2004). Structural predictions (based solely on topology and primary sequence) have suggested two possible proton pathways in a hypothetical ExbB/ExbD complex

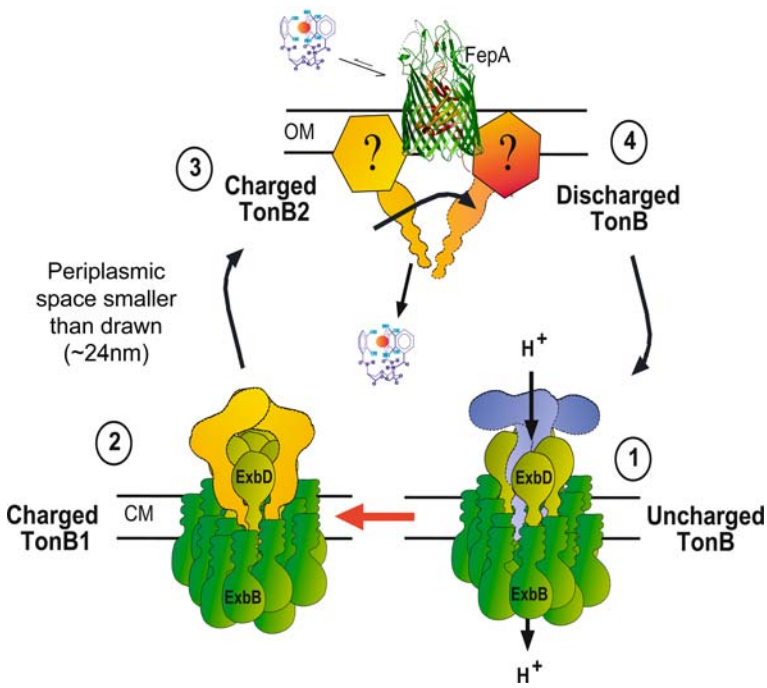

Fig. 1 Conceptual four-step model for TonB-dependent energy transduction. The potential energy of the CM proton gradient is harnessed by the ExbB/D energy-harvesting complex (*step 1*) and stored via a conformational change in the associated TonB dimer (*step 2*). TonB shuttles to the OM, where conformationally-stored potential energy is transferred to a ligand-bearing TonB-dependent transporter, energizing ligand release into the periplasmic space (*step 3*). Spent TonB is then recycled to the CM (*step 4*) to be used again

(Zhai et al. 2003), however these predictions do not take into account experimentally predicted complex stoichiometry (Higgs et al. 2002a), and are not supported by the results of a subsequent site-directed mutagenic study (Braun and Herrmann 2004). While the mechanism remains elusive, it is evident that TonB is somehow coupled to the ion electrochemical gradient by the ExbB/ExbD complex, which is required for efficient energization of TonB and evidenced by a biochemically demonstrable conformational change in TonB protein (Larsen et al. 1999).

In the second step of the energy transduction cycle (Fig. 1, position 3), TonB has shuttled to the OM. The lack of an identifiable periplasmic intermediate (Letain and Postle 1997) and the inability of shortened TonB derivatives to energize OM transport under conditions that expand the distance between the CM and the OM (Larsen et al. 1993; Seliger et al. 2001) suggests that TonB does not freely diffuse between membranes, but rather must contact the OM before releasing from the CM. The carboxy-terminal region of TonB appears to mediate interaction with the OM, with derivatives lacking the final 64 residues found only in the CM (Letain and Postle 1997). As discussed below, it is unclear whether TonB exists as monomer or dimer at the OM. Interestingly, the ability of TonB to shuttle between the CM and the OM does not seem to depend upon its energization state; amino-terminal mutants lacking the ability to store and release energy partition between the CM and OM much like wild-type TonB (Larsen et al. 1999), suggesting that the energy source for shuttling itself is distinct from the energy that is transduced.

In the third step of the energy transduction cycle (Fig. 1, position 4), TonB recognizes a ligand-loaded TonB-dependent transporter, with a subsequent transfer of energy that drives the release of the ligand into the periplasmic space. While it remains entirely unclear how this energy transfer is achieved and how ligand transport occurs, at least some of the required interactions and features of the process have been identified. Mutations in TonB-dependent transporters that uncouple ligand binding from ligand transport map to a short, conserved amino-terminally located motif called the ''TonB-box'' (Table 1, Heller and Kadner 1985; Pressler et al. 1988). This motif is also shared by Group B colicins, and is essential for the TonB-dependent transport of these protein toxins (Schramm et al. 1987). The idea that TonB recognizes this motif was strengthened by the recovery of second-site mutations in TonB (at and around TonB residue 160) that weakly suppressed certain TonB-box mutations (Heller et al. 1988; Schöffler and Braun 1989; Bell et al. 1990). Subsequent disulphide-trapping experiments demonstrated the ability of cysteinyl substitutions in the TonB box of the cobalamin transporter BtuB to interact with cysteinyl residues substituted into the region surrounding TonB residue 160 (Cadieux and Kadner 1999). However, in vivo chemical cross-linking studies had indicated that this region alone was not sufficient for interaction, with a region within the carboxy-terminal 48 residues of TonB also essential for interaction with the TonB-dependent siderophore transporter FepA

Table 1 TonB-box alignment for TonB-dependent outer membrane (OM) transporters and Group B (TonB-dependent) colicins of *E. coli* (adapted from Braun et al. 2002; Shultis et al. 2006 [online supplement])

Transporters	TonB box sequence
BtuB	**DTLVVTA**
ChuA	**ETMTVTA**
Cir	**ETMVVTA**
FecA	**DALTVVG**
FepA	**DTIVVTA**
FhuA	**DTITVTA**
FhuE	**ETVIVEG**
Fiu	**DTLVVEA**
FyuA	**STLVVTA**
IutA	**NEIIVSA**
Consensus	**dtlvvta**
Colicins	
ColB	**DTMVVWP**
ColD	**HSMMVWP**
Colla	**EIMAVDI**
ColM	**ETLTVHA**
Col 5	**DTITATL**
Pesticin	**DTMVVNG**
Consensus	**dtm-v--**

(Larsen et al. 1997). This supposition was confirmed in studies using chimeric TonB molecules, wherein a carboxy-terminal region of *E. coli* TonB exclusive of the region surrounding residue 160 (specifically, residues 175–239) rendered *Vibrio cholerae* TonB1 able to support siderophore transport by both FepA and FhuA proteins (Mey and Payne 2003). Further, this study identified a proline residue in the extreme carboxy-terminus of *V. cholerae* TonB1 to be involved in interactions with the TonB box of *V. cholerae* TonB-dependent OM transporters. Consistent with a role in recognition by TonB, the solved crystal structures of FhuA and FecA suggest that ligand binding produces a dramatic change in the positioning of the TonB box (Ferguson et al. 1998, 2002; Locher et al. 1998), presumably signaling receptor occupancy and rendering the TonB box accessible to TonB.

In the fourth step of the energy transduction cycle (Fig. 1, transit from position 4 to 1), TonB has transferred its stored energy to the OM transporter, and must now deadhead back to the ExbB/ExbD energy-harvesting complex in the CM. At present, relatively little is known regarding this recycling step; however, the degradation of certain TonB signal anchor mutants at the OM following energy transfer suggests a role for the amino-terminal domain in the process (Larsen et al. 1999).

This working model is consistent with the available data, and would seem to satisfy the apparent demands of inter-membrane energy transduction. Nonetheless, it is undoubtedly too simple, with a number of unanswered questions, the resolution of which will either refine, or overturn it. The most fundamental question: ''what constitutes an energized state for TonB?'' remains unknown. The approaches to this question have been tangential, relying upon biochemical and genetic strategies to define the machinery and glean the specifics of the interactions that facilitate energy transduction. More recently, solved structures for the carboxy-terminal domain of TonB have become available, initially by itself (Chang et al. 2001; Ködding et al. 2004, 2005; Peacock et al. 2005) and most recently, in association with TonB-dependent receptors (Shultis et al. 2006; Pawelek et al. 2006). While it is clear that, by their very nature, none of these structures can represent the energy-bearing form of TonB (having neither opportunity to interact with an ExbB/D energy-harvesting complex, nor the signal anchor region to facilitate such an interaction), they have provided a more refined view of the potential interactions between TonB and the receptors it services. As described below, certain of these in vitro structural findings are consistent with, and provide explanations for in vivo biochemical and genetic observations, while others have not withstood the rigors of experimental analysis. Importantly, these structures provide a variety of testable hypotheses.

The TonB carboxy-terminus—in vitro

Rigorous in vitro characterization of TonB protein is problematic. From a practical standpoint, the susceptibility of TonB molecules to proteolysis in the absence of sufficient ExbB complicates over-expression strategies; recovering adequate amounts of protein to work with has not been trivial. This instability highlights a more important consideration—over-expressed TonB is unlikely to be in a biologically relevant conformation. Many or most full-length TonB molecules will not have had opportunity to interact with an ExbB/D energy harvesting complex. Furthermore, simple lysis of the CM, necessary to recover any full-length TonB, is known to alter TonB conformation, most likely to an un-energized form (Larsen et al. 1999). In fact the in vitro studies all use TonB derivatives that lack the TonB signal anchor and are over-expressed in the cytoplasm, precluding the possibility of TonB achieving an ''energized'' conformation. Despite these inherent limitations, observations from several in vitro approaches hint at interesting features of the interactions between TonB and TonB-dependent OM transporters. Analytical ultracentrifugation of TonB derivatives in mixture with purified FhuA have suggested that ligand-bearing receptors enhance, but are not required for, dimerization of TonB (Khursigara et al. 2004). In this same study, surface plasmon resonance experiments identified two distinct sets TonB interactions with receptors—a weak, ligand-independent binding involving components of the

TonB carboxy-terminal domain (residues 155–239 in this study), and a stronger, ligand-dependent binding that appeared to require an additional region not contained within the carboxy-terminal domain (Khursigara et al. 2004). More recently, panning of phage display libraries against the purified TonB carboxy-terminus identified several motifs in TonB-dependent transporters with which TonB may specifically interact. Surprisingly, the motifs identified were not limited to periplasmically-accessible surfaces of the transporters, suggesting the possibility that unfolding events in ligand transport expose normally sequestered regions of the transporter that engage in relevant interactions with TonB (Carter et al. 2006). It is not yet clear whether these interactions occur in vivo. Potential alterations in folding may not be limited to the transporter—indeed NMR characterizations of mixtures of purified amino terminal globular domain of FepA and the carboxy-terminus of TonB suggest that specific interactions promote transition of TonB to a disordered conformation (Peacock et al. 2006). Together, these studies support a model of ligand transport that requires dynamic and structurally plastic mediators.

The possibility of a flexible, structurally-mobile TonB is not entirely consistent with first solved TonB structures. Initial structures derived from 85- and 77-residue carboxy-terminal domain fragments (Chang et al. 2001; Ködding et al. 2004) depicted a highly ordered, strand-exchanged dimer, composed of three antiparallel β-strands and a single α-helical region (Fig. 2, panel A). While both of these fragments also formed dimers in solution (Chang et al. 2001; Ködding et al. 2004), a longer, 92-residue fragment occurred as a monomer in solution (Ködding et al. 2005), and upon crystallization rendered a significantly less rigid dimeric structure, with strand exchange occurring only with the third β-strand of each molecule, and an additional short α-helical region just prior to the first β-strand (Fig. 2, panel B). A third structure has been solved by NMR (Peacock et al. 2005), using a 137-residue TonB fragment, in which the amino-terminal 49 residues were disordered, but the remaining residues (amino acids 152–239) yielded a well-structured, monomeric carboxy-terminal region (Fig. 2, panel C). This structure contains the same basic secondary features apparent in the solved crystal structures, with the division of the third β-strand into two shorter, interacting β-strands. Interestingly, the folding pattern of this structure is similar to that solved for the carboxy-terminal region of the TonB analog TolA of *E. coli* (Lubkowski et al. 1999) and *Pseudomonas aeruginosa* (Witty et al. 2002). When examined in the presence of peptides corresponding to the TonB boxes of the TonB-dependent transporters BtuB, FepA, and FhuA, conformational shifts consistent with specific binding to these peptides occurred. Together with the relative position of TonB residue Gln160 (the location of second site suppressors of TonB box mutants) and similarity to the structure of TolA in association with a transportable ligand (the N1 domain of the gp3 protein of F1 phage; Lubkowski et al. 1999), these data suggested that the third β-strand of TonB mediates specific interactions with the TonB box motif.

The recently solved structures for TonB co-crystallized with the ligand-loaded TonB-dependent transporters BtuB (at 2.1 Å) and FhuA (at 3.3 Å; Fig. 2, panels D, E) are consistent with this prediction (Shultis et al. 2006; Pawelek et al. 2006). In the BtuB-TonB structure, the TonB box of the transporter participates in an anti-parallel interaction with the third β-strand of TonB (residues 226–232), with the fourth TonB β-strand (residues 234–239) displaced and disordered. In the FhuA structure, there are two crystallographically independent structures: one in which the last TonB β-strand is swapped with the TonB box and the other where the TonB box was not located at all, although other contacts with FhuA are similar. The presence of two different complexes in the same structure may indicate that the free energy cost for the TonB box swap with TonB β-strand 4 is not large (Dick van der Helm, personal communication). Coincident with this arrangement, the region of TonB surrounding Gln160 is clearly visible to be in tight association with the TonB box of BtuB (modeled with FhuA). In the case of FhuA, additional hydrophilic interactions are evident between TonB and the transporter, involving both the cork domain (via TonB Arg166) and residues of the barrel (via TonB Arg166, Asn200, & Arg204). It has been

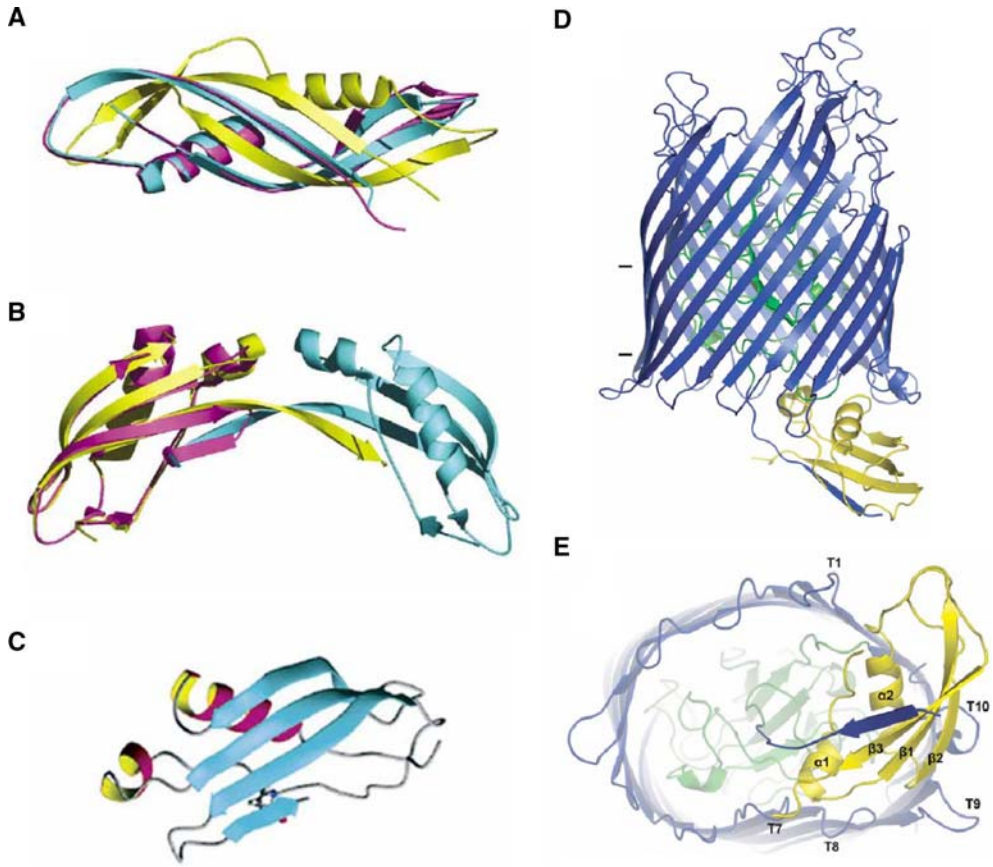

Fig. 2 Solved structures of TonB. Panels **A** and **B** depict dimeric crystal structures solved for carboxy-terminal TonB domains (adapted from Weiner 2005). In Panel **A**, the original structure of two tightly interwoven 75-residue peptides (in yellow and blue) is depicted (Chang et al. 2001), with one peptide from a later-solved 76-residue structure (Ködding et al. 2004) superimposed in magenta. In Panel **B** the domain-swapped dimer structure solved for 92-residue peptides (Ködding et al. 2005) is presented. Individual peptides are depicted in yellow and blue, with the 87-residue ordered region of the solution NMR structure for monomeric TonB (Peacock et al. 2005) superimposed on the left-hand monomer in magenta. Panel **C** presents the solution NMR structure for 87-residue ordered portion of the 136 carboxy-terminal residues of a monomeric TonB peptide (Peacock et al. 2005). Panels **D** and **E** depict two views of the solved crystal structure for FhuA (residues 1–725, in blue and green) in complex with a monomeric TonB (residues 33–239) polypeptide, in which TonB residues 158–235 (in yellow) are observed (Pawelek et al. 2006)

suggested that one such interaction, the electrostatic bond evident between TonB Arg166 and the FhuA domain residue Glu56, located in a region of the cork domain shared between various TonB-dependent transporters (Chimento et al. 2005), may play a specific role in triggering ligand transport (Pawelek et al. 2006).

Interestingly the two crystal structures are different in the means by which they contact TonB. In the BtuB structure, TonB interacts with periplasmic loops 3, 4, 5, 6, 7, and 8. In the FhuA structure, TonB also interacts with periplasmic loops 7 and 8, but in contrast to BtuB, is shifted to interact with periplasmic loops 9 and 10, and the FhuA carboxy terminus. As these differences suggest, when the TonB structures are overlaid, it is impossible to line up the transporter structures because TonB approaches BtuB at an angle that is 20° different than the angle at which it approaches FhuA. Taken together the differences

amount to a rotation about 90° around the axis of the barrel for TonB interaction (Dick van der Helm, personal communication). It will be important to establish whether similar crystal structures can be obtained in the absence of ligand, since TonB does not appear to transduce energy to unliganded transporters in vivo (Larsen et al. 1999).

The total region of contact between TonB and the transporter was limited to about one half of the periplasmic face of the transporter molecule, leaving room for a second TonB to bind (as part of a putative TonB dimer—as discussed below). As there is only one TonB box per transporter, the interactions through which a second TonB might engage the transporter would be necessarily different; and the absence of a second TonB in these co-crystalized structures suggests that if TonB is actually a dimer at this point in the energy transduction cycle, the additional interactions mediated by the second TonB molecule would be weaker and/or less ordered than those involving the TonB molecule that does interact with the TonB-box. Alternatively, if the shuttle model is correct, there is room for the entire TonB molecule to bind the periplasmic face. Unfortunately, there are currently no definitive data on the oligomeric state of energized TonB at the OM to guide our thinking, and indeed some of the in vitro data are internally contradictory. For example, the binding data posit dimeric interactions whereas the crystal structures suggest TonB is a monomer when it interacts with the OM transporters. In addition, phage panning experiments identify different TonB contacts with FhuA than the FhuA–TonB crystal structure identifies (Fig. 3).

The TonB carboxy-terminus—in vivo

In vivo studies have shed little light on the oligomerization state of TonB in association with transporters in the OM, however; two lines of evidence indicate that TonB can occur as a dimer while associated with the CM. First, using a ToxR transcriptional activation system to assay for dimer formation, the Braun lab (Sauter et al. 2003) convincingly demonstrated an ExbB/D-dependent dimerization of TonB-ToxR chimeras via the TonB signal anchor. As one might expect from the in vitro studies, chimeras partnering only the carboxy-terminal 76 residues of TonB with ToxR also dimerized—but importantly, without regard to the presence or absence of ExbB/D. Second, cysteinyl substitutions for certain aromatic residues which cluster in the TonB carboxy-terminal domain result in the spontaneous formation of disulphide-linked TonB dimers that no longer shuttle to the OM (Ghosh and Postle 2005). As seen with the ToxR chimeras, these dimers also require the TonB signal anchor and the presence of ExbB/D to form. These observations are consistent with obligatory dimerization of TonB at some point in the energy transduction cycle prior to the association with the OM.

The idea that specific aromatic residues (Phe180, Phe202, Trp213, Tyr215, Phe230 depicted in Fig 4, panel B) were clustered in functional TonB in vivo stemmed from the observation that individual alanyl substitutions at these sites differentially altered TonB activity, and that multiple substitutions were synergistic, suggesting that they interacted with each other

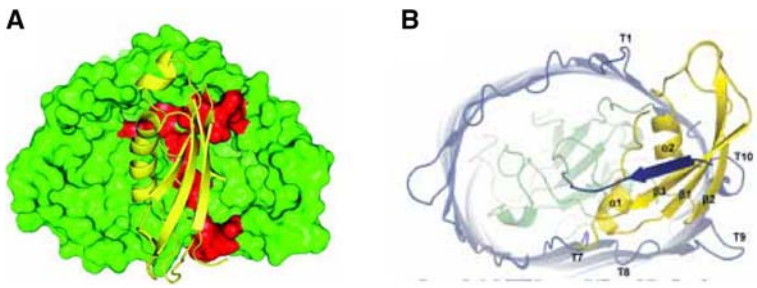

Fig. 3 Comparison of TonB contacts identified by phage panning and by crystallography. Panel **A**. TonB–FhuA contacts identified by phage panning (Carter et al. 2006). Panel **B**. TonB–FhuA contacts identified by crystallography (Pawelek et al. 2006)

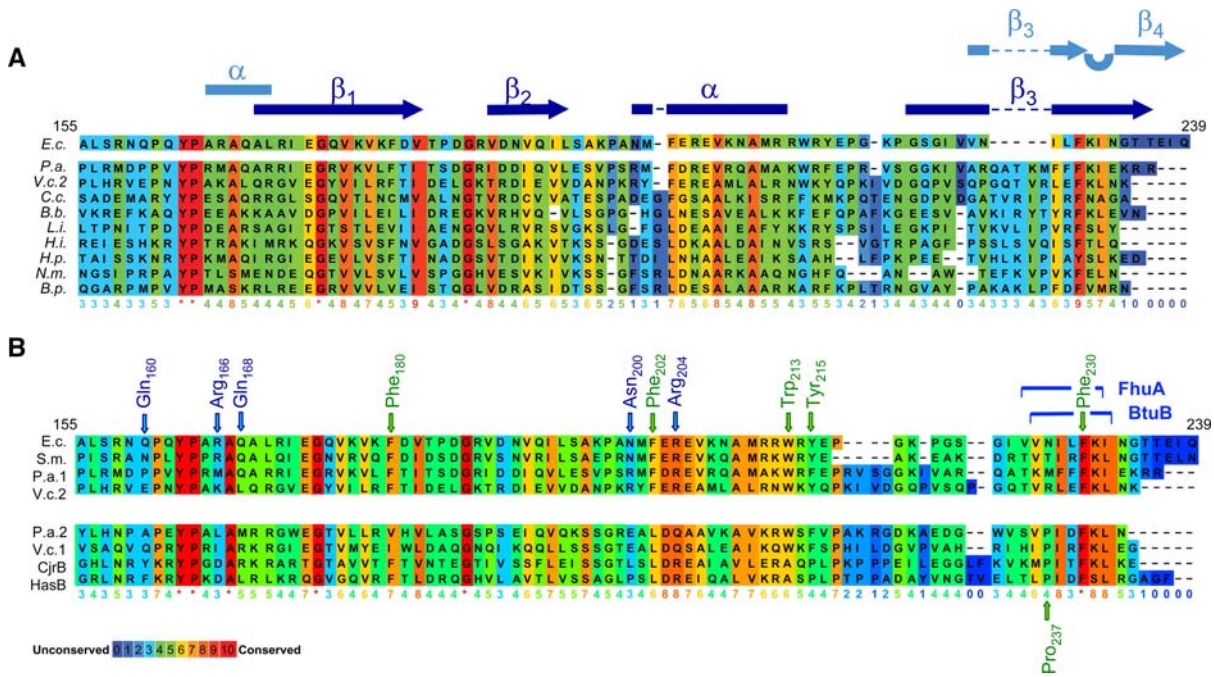

Fig. 4 Praline alignments (Heringa 1999, Simossis et al. 2005) of TonB carboxy-termini, with corresponding structural features. Panel **A**. The carboxy-terminal region of *E. coli* TonB (residues 155–239) is presented at the top of the figure (E.c.; GenBank accession # NP_415768), with the major secondary structural features depicted as lines (α-helix) and arrows (*β*-strand). The three *β*-strands and one α-helix described in the initial crystallographic studies of a dimeric molecule (Chang et al. 2001) are depicted in dark blue, with addition/alternative secondary features identified by NMR of a fluid phase monomer (Peacock et al. 2005) presented in light blue. The conservation of individual residues in the carboxy-terminal region is depicted relative to three other *γ*-Proteobacteria species (*Pseudomonas aeruginosa* [P.a.; # AAB18654], *Haemophilus influenzae* [H.i.; # AAA60460], and *Vibrio cholerae* [V.c.2: # AAF94698]), two *β*-Proteobacteria species (*Neisseria meningiditis* [N.m.; # AAC44834] and *Bordetella pertussis* [B.p.; # CAB53383]), one α-Proteobacterium (*Caulobacter crescentus* [C.c.; # NP_421130]), one *δ*-Proteobacterium (*Bdellovibrio bacteriovorus* [B.b.; # CAE78385]), one *ε*-Proteobacterium (*Helicobacter pylori* [H.p.; # AAD08383), and one Spirochaete (*Leptospira interrogans* [L.i.; # YP_000866]). Degrees of conservation are color-coded as indicated by the scale at the figure bottom—with the numeric value assigned to each position scored as the final row in the sequence set. Panel **B**. The TonB carboxy-termini from *E. coli* and three additional *γ*-Proteobacteria species (*Serratia marcescens* [S.m.; # P26185], *P. aeruginosa* [P.a.1; # AAB18654], and *V. cholerae* [V.c.2: # AAF94698]) at the panel top are compared with the carboxy-termini from additional TonBs and TonB-like molecules from these same species that recognize different OM targets than their typical TonB counterpart (*E. coli* [CjrB; # AAK67306], *S. marcescens* [HasB; # CAE46937], *P. aeruginosa* [P.a.2; # AAF04082], *V. cholerae* [V.c.1; # AAF96807]) at the panel bottom. The regions of the *E. coli* TonB identified to closely associate with the TonB boxes of the OM receptors FhuA and BtuB (Pawelek et al. 2006; Shultis et al. 2006) are indicated by the blue brackets at the panel top. Other specific residues believed to interact with OM receptors (as discussed in the text) are identified by blue arrows at the panel top; while the functionally-important aromatic residues (Ghosh and Postle 2004) are identified by green arrows. An additional residue identified as important in dictating OM receptor specificity in *V. cholera* TonB1 (Mey and Payne 2003) is identified by a green arrow at the panel bottom. As per Panel **A**, the relative conservation at each position is color-coded as indicated by the scale at the figure bottom with assigned numeric values presented as the final row in the sequence set. Note that the alignment presented in Panel **B** was performed independent of the alignment presented in Panel **A**

(Ghosh and Postle 2004). These studies also indicated that the specifics of the TonB interaction varied with the particular receptor, resulting in unique substitution-dependent profiles for bacteriophage *ϕ*80 and ferrichrome transport, and sensitivity individual group B colicins (Fig. 5, panel A).

Despite their apparent importance in *E. coli* TonB, with one exception, the aromatic residues of the carboxy-terminal domain are not widely

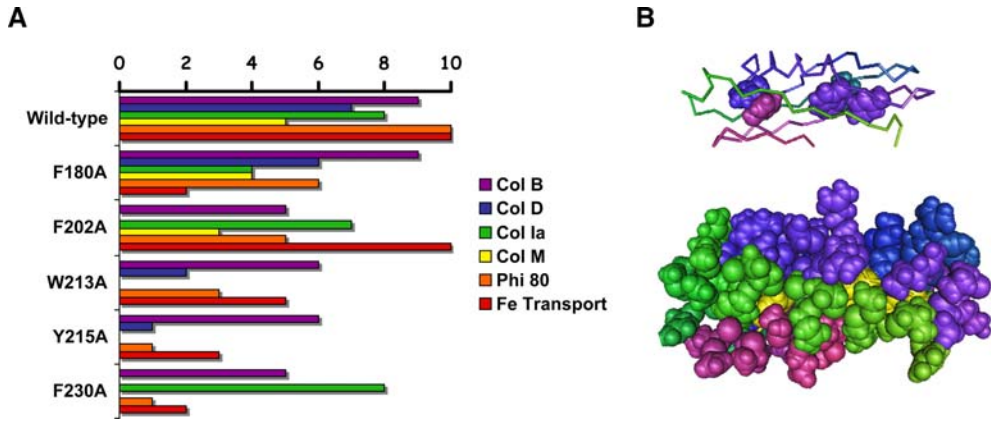

Fig. 5 Comparison of TonB in vivo and in vitro. Panel **A**. TonB in vivo. Normalized colicin (B, D, Ia and M) and phage (ϕ80) sensitivity and iron (Fe) transport activity results from Ghosh and Postle (2004) are shown. Higher numbers on upper horizontal scale indicate a greater degree of TonB function. Profiles for alanyl substitutions at TonB aromatic residues (labels *left*) are shown, compared to the sensitivity/activity profile of wild-type TonB. Panel **B**. TonB in vitro. *Upper.* Amino acid backbone representation of the TonB carboxy terminal domain NMR structure with buried aromatic amino acid side chains shown in space-filling mode. *Lower.* space-filling model of TonB carboxy terminal domain NMR structure, with aromatic amino acids shown in gold. Both representations were generated by Cn3D from NCBI using TonB NMR data from Peacock et al. (2005)

conserved (Fig. 4, panel A). In fact, sequence comparisons suggest that the TonB carboxy-terminus can tolerate a great deal of variation, with relatively few highly conserved residues. Of these, two seemingly invariant glycyl residues (positions 174 & 186) can nonetheless tolerate a variety of substitutions (Traub et al. 1993), as can the more highly conserved aromatic residue Phe230 (Ghosh and Postle 2004). The potential malleability of the conserved tyrosyl-prolyl pair (positions 163 & 164) has not been tested to our knowledge.

In retrospect this should not be surprising. It seems reasonable to presume that TonB and the transporters it services continually coevolve, with selection fine-tuning these systems for specific environments. This becomes particularly evident when one considers species in which multiple TonB and TonB-like molecules function (Fig. 4 panel B). In the examples presented here, the ''primary'' TonB molecules from four γ-Proteobacteria species are strikingly similar, with marked conservation of five aromatic amino acids known to play a role in the activity of *E. coli* TonB (discussed further below) as well as some (but not all) residues identified as contact points between *E. coli* TonB and FhuA (Pawelek et al. 2006). The corresponding ''secondary'' TonB molecules from these species, which in each case recognize OM receptors distinct from those served by the ''primary'' TonB (reviewed in Postle and Larsen 2004), show a marked departure in residue composition at many of these sites. Interestingly, the third β-strand region of *E. coli* TonB region identified in the co-crystal structures as specifically interacting with the TonB box remains fairly conserved between ''primary'' and ''secondary'' TonB molecules, except for the inclusion of a prolyl residue in the ''secondary'' TonBs, demonstrated to be important in receptor discrimination in *V. cholerae* (Mey and Payne 2003).

What do the crystal/NMR structures represent?

TonB is a dynamic molecule, with energy transduction involving a number of conformational changes (Larsen et al. 1999). The present working model (Fig. 1) includes three distinct states, based on in vivo evidence: Are any of these states captured by the solved structures? Certainly, some of the structural information meshes well with experimental data. Consistent with in vivo disulphide crosslinking studies (Cadieux and Kadner

1999), the co-crystallization studies reveal strand exchange between the third β-strand of TonB and the TonB box and help to explain why the TonB box tolerates such a diversity of substitution. Interaction of TonB Gln160 and surrounding residues with the TonB box of BtuB in the co-crystal satisfyingly suggests why some TonB box mutants are inactive (Gudmundsdottir et al. 1989). Conversely, some of the structural information is inconsistent with experimental data. For example, structural data arrange the carboxy-terminal aromatics in two buried clusters unlikely to engage in transporter/colicin recognition and sufficiently removed from one another to preclude synergy between clusters (Fig. 5, panel B). Thus, whatever stage they might represent, it is unlikely that any of the crystal structures mimic the energized conformation of TonB (Weiner 2005).

What about other conformations? When expressed in vivo, carboxy-terminal TonB fragments can compete with wild-type TonB (Howard et al. 2001), suggesting some type of biological activity of the carboxy-terminus (Carter et al. 2006). However, this conclusion must be tempered by remembering that addition of a synthetic TonB box penta-peptide is also inhibitory in vivo (Tuckman and Osburne 1992) and that inactive degradation productions of TonB are also likely to participate in competitive inhibition, as evident in the dominant negative gene dosage effect associated with the over-expression of TonB (Mann et al. 1986). Furthermore, it is clear that un-energized TonB can crosslink in a ligand-specific manner to the OM transporter FepA in vivo (Ghosh and Postle 2005).

Physiological relevance of the TonB crystal and NMR structures has yet to be demonstrated. This would, perhaps, not even arise as an issue for many proteins, but because the structures represent only part of the TonB protein and omit a domain required for TonB activity, the question is a fair one. It can be raised also in the context of contradictory results for TonB examined in vivo and in vitro. For example, despite significant in vitro ligand-independent interaction between TonB and FhuA (Khursigara et al. 2004), in vivo TonB cannot transduce energy to OM transporters in the absence of ligand (Larsen et al. 1999). Furthermore, while the proline rich domain is not essential for efficient TonB function in vivo (Larsen et al. 1993; Seliger et al. 2001) in vitro data suggest it is required for the dimerization of TonB into a 2:1 complex with FhuA (Khursigara et al. 2005b), implying the existence of an OM-associated TonB dimer—for which in vivo evidence is presently lacking.

The current tension between TonB in vivo and TonB in vitro must be resolved for the field to progress. Certainly both sets of results are telling us something, but the question is: What?

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