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# TonB Protein and Energy Transduction between Membranes

**Kathleen Postle**[1]

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TonB protein couples cytoplasmic membrane electrochemical potential to active transport of iron–siderophore complexes and vitamin B12 through high-affinity outer membrane receptors of Gram-negative bacteria. The mechanism of energy transduction remains to be determined, but important concepts have already begun to emerge. Consistent with its function, TonB is anchored in the cytoplasmic membrane by its uncleaved amino terminus while largely occupying the periplasm. Both the connection to the cytoplasmic membrane and the amino acid sequences of the anchor are essential for activity. TonB directly associates with a number of envelope proteins, among them the outer membrane receptors and cytoplasmic membrane protein ExbB. ExbB and TonB interact through their respective transmembrane domains. ExbB is proposed to recycle TonB to an active conformation following energy transduction to the outer membrane. TonB most likely associates with the outer membrane receptors through its carboxy terminus, which is required for function. In contrast, the novel proline-rich region of TonB can be deleted without affecting function. A model that incorporates this information, as well as tempered speculation, is presented.

**KEY WORDS:** *E. coli*; TonB; iron; vitamin B12.

## INTRODUCTION

All living cells possess membranes that separate inside from outside. These membranes contain proteins involved in obtaining nutrients, concentrating them against gradients if necessary, by various energy-dependent means. The Gram-negative bacterium, *Escherichia coli,* can use the energy either from ATP hydrolysis or from a proton electrochemical potential ($\Delta\mu H^+$) to energize active transport across its cytoplasmic membrane (Nikaido and Saier, 1992). The cytoplasmic membrane is a permeability barrier that contains proteins required for generating and maintaining an electrochemical potential, as well as proteins that use the electrochemical potential for active transport of nutrients into the cell. Like other Gram-negative bacteria, *E. coli* has a second, external membrane consisting of an inner phospholipid leaflet and an outer lipopolysaccharide leaflet. Between these two membranes is an aqueous region, the periplasmic space. In addition to the murine sacculus, which confers cell shape, the periplasmic space contains numerous detoxifying enzymes, scavenging enzymes, osmoregulatory polysaccharides, and binding proteins that participate in active transport of nutrients across the cytoplasmic membrane.

The outer membrane barrier can be traversed in one of three ways: by diffusion through nonspecific protein pores (porins), by diffusion through stereospecific protein pores such as LamB, and by active transport mediated by high-affinity outer membrane receptor proteins (Nikaido and Saier, 1992). The receptor proteins are not receptors in the eukaryotic sense, but rather they are transport proteins that are also exploited as binding sites, hence *receptors*, by bacteriophages and colicins. Since molecules as large as 600 Da freely diffuse through the porins, protons can also take the same pathway, preventing the establishment and maintenance of a significant electrochemical potential. Furthermore, the outer membrane cannot access the ATP-generating reactions of the

[1] Departments of Microbiology and of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164–4233.



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cytoplasm and cytoplasmic membrane. Thus, there is no standard means by which active transport across the outer membrane can be energized. The necessity for a source of energy for the outer membrane was recognized in early experiments involving bacteriophage adsorption (Hancock and Braun, 1976). Bacteriophage $\phi$80 could bind reversibly to its outer membrane protein receptor in an energy-dependent fashion, but could not proceed to irreversible binding (and productive infection) without cytoplasmic membrane energy. Since mutations in the *tonB* gene also prevented productive infection but not reversible binding, TonB was believed to play a role in the process.

TonB mutations were isolated at the dawn of molecular biology as a class of *E. coli* resistant to bacteriophage T1 (Luria and Delbruck, 1943). It subsequently became clear that TonB is required for siderophore-mediated iron transport across the outer membrane (Frost and Rosenberg, 1975; Hantke and Braun, 1975; Williams, 1979), vitamin B12 transport across the outer membrane (Bassford *et al.*, 1976), and sensitivity to group B colicins and bacteriophage $\phi$80 (Matsushiro, 1963), even though TonB is the outer membrane receptor for none of these ligands. The role of TonB as an energy transducer was suggested by the observation that transport of vitamin B12 across the outer membrane to the periplasmic space required the electrochemical potential of the cytoplasmic membrane and a functional TonB protein (Reynolds *et al.*, 1980). A generic TonB-dependent transport system consists of: (1) an outer-membrane receptor that differs from the outer-membrane pores in its high affinity for specific transport ligands, (2) a periplasmically localized binding protein, and, (3) a complex of cytoplasmic membrane proteins for active transport of ligands across the cytoplasmic membrane (Fig. 1). Considered separately, cytoplasmic membrane transport systems are analogous to other such binding protein-dependent traffic ATPases (Ames and Lecar, 1992; Higgins, 1992). TonB does not participate in transport across the cytoplasmic membrane.

The involvement of TonB in high-affinity transport across the outer membrane reflects the relative scarcity, importance, and large size of the ligands being transported. Iron is the fourth most abundant element on the earth's surface. However, because oxygen is the most abundant element, all of the iron in an aerobic environment is present in insoluble ferric hydroxide complexes. To cope with the insolubility of iron, many microorganisms synthesize and secrete into their environment molecules called siderophores

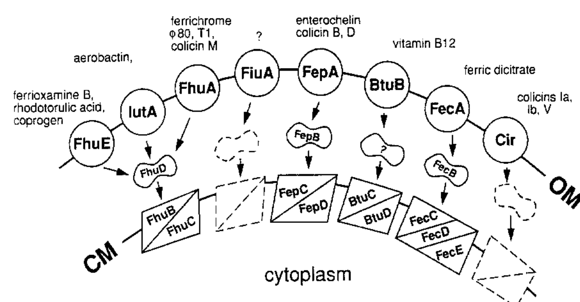


**Fig. 1.** TonB-dependent transport systems. The ligands requiring TonB-dependent transport across the outer membrane are depicted above their respective outer membrane receptors. The subsequent proteins used for siderophore and vitamin B12 uptake (but not colicin and bacteriophage infection) in each system are depicted by arrows. OM stands for outer membrane. CM stands for cytoplasmic membrane.

(Greek: iron bearer). The structurally diverse variety of know siderophores shares the property of an exceedingly high affinity for iron, sufficient to wrest environmental iron from its insoluble complexes or from host proteins that sequester iron. Iron–siderophore complexes are then transported back into the microorganisms. Iron is necessary for many essential biochemical processes, both as a prosthetic group and as a cofactor. Although vitamin B12 is not required for the growth of E. coli, under aerobic conditions vitamin B12 greatly enhances its growth. Furthermore, iron–siderophore complexes and vitamin B12 are too large to diffuse freely through the porins.

Bacteriophages and colicins have evolved to exploit high-affinity outer-membrane receptors to gain entry into bacterial cells. Interestingly, some outer membrane receptors, such as the vitamin B12 receptor BtuB and the ferrichrome receptor FhuA, support both TonB-dependent and TonB-independent processes (Hantke and Braun, 1975; Bradbeer *et al.*, 1976). Recently, a protein historically classified as a TonB-independent stereospecific pore for nucleosides, Tsx, has been found to be the receptor for a TonB-dependent colicin (Bradley and Howard, 1992).

## THE *E. coli tonB* DNA SEQUENCE: PREDICTIONS AND REALITY

The *E. coli tonB* gene was cloned as a 1697-bp *Hinc*II fragment by complementation of a *tonB* deletion using the Chromium$^R$ phenotype of TonB$^+$ cells (Postle and Reznikoff, 1978). The *tonB*$^+$ plasmid was shown to encode a 36-kDa protein in minicells that

was missing from minicells bearing plasmids with four different TonB⁻ IS1 insertions. Correlation of the sizes of the mutant TonB polypeptides with the positions of the IS1 elements determined by restriction enzyme mapping indicated that *tonB* is transcribed in a clockwise direction on the *E. coli* chromosomal map (Postle and Reznikoff, 1979). Following its cloning, the nucleotide sequence of *tonB* was determined (Postle and Good, 1983) and verified (Mann *et al.*, 1986). The deduced amino acid sequence predicts a protein approximately 26 kDa in size with an overall charge of +8. Translation initiates at a methionine codon in position six of the open reading frame for a total of 239 amino acids in the protein (Postle and Skare, 1988). Prolyl residues comprise 17% of the amino acids in TonB. This high percentage of proline accounts for the anomalous apparent molecular weight observed in SDS polyacrylamide gels. There is a particularly proline-rich region in the amino-terminal third of TonB (amino acids 70–102) whose sequence reads (glu-pro)$_4$-ile-pro-glu-pro-pro-lys-glu-ala-pro-val-val-ile-glu-(lys-pro)$_6$. A series of insertions in TonB (Postle and Reznikoff, 1979; Postle and Good, 1983) and a series of deletions in TonB (Traub *et al.*, 1993) suggested that this region is responsible for the aberrant migration of TonB on SDS gels. Since recently reported deletion of amino acids 66–100 restores TonB protein to a predicted molecular mass of 23 kDa on SDS gels, this proline-rich region appears to have been entirely responsible for the anomalous behavior of TonB on gels (Larsen *et al.*, 1993a).

The deduced amino acid sequence of *E. coli* TonB predicts a protein with hydrophobic amino and carboxyl termini separated by a hydrophilic central region. The amino terminus was shown to be the signal sequence for TonB using a hybrid TrpC-TonB protein that carries residues 12–239 of TonB, including the hydrophobic amino terminus (Skare *et al.*, 1989). The hybrid protein is functional and exported; however, its overexpression is lethal. Using overproduction lethality as a selection, mutant survivors were isolated where TrpC-TonB remained cytoplasmically localized as a result of a Gly26→Asp substitution in the hydrophobic amino terminus. It is likely that TonB is exported in a Sec-dependent fashion due both to the nature of the signal sequence mutation and the fact that it could be suppressed by *prlA* alleles that suppress other signal sequence mutations (Emr and Bassford, 1982; Bankaitis and Bassford, 1984). The same Gly26→Asp substitution also prevents the export of wild-type TonB (Jaskula *et al.*, 1993). The amino terminal signal sequence was shown to be uncleaved following TonB export (Postle and Skare, 1988). Subsequent analysis of various TonB-PhoA fusions demonstrated that TonB is anchored to the cytoplasmic membrane by its uncleaved amino terminus while the remainder of the protein occupies the periplasmic space (Roof *et al.*, 1991). A similar observation has been made for *Salmonella typhimurium* TonB (Hannavy *et al.*, 1990). Thus, the hydrophobic carboxyl terminus does not serve as a membrane anchor, although deletion of the TonB carboxy terminus inactivates TonB (Anton and Heller, 1991; Roof *et al.*, 1991). The topology of TonB and its localization to the cytoplasmic membrane are entirely consistent with its role in coupling cytoplasmic membrane energy to active transport across the outer membrane.

The 5′ *tonB* DNA sequence predicts an active promoter and mRNA start site that was confirmed by S1 nuclease mapping (Postle and Good, 1983). Subsequently, *tonB-lacZ* transcriptional fusions were used to demonstrate that, under aerobic conditions, *tonB* is regulated threefold by iron availability and Fur protein (Postle, 1990). The proposed growth-phase regulation of *tonB* (Dorman *et al.*, 1988) turned out to be due instead to increasing iron limitation, and hence derepression, as *tonB* cultures approach stationary phase in media with intermediate levels of available iron (Postle, 1990). Fur is a histidine-rich protein involved in all *E. coli* iron regulation described to date, generally as a repressor (Ernst *et al.*, 1978; Hantke, 1981; Coy and Neilands, 1991). Our recent results indicate that Fur binds *in vivo* to the Fur binding sequence (FBS) located between the −35 and −10 regions of the *tonB* promoter to decrease *tonB* transcription when iron is present in excess (Young and Postle, 1993).

Regulation of *tonB* under anaerobic conditions is more stringent. While *tonB* transcription can be induced to the same high levels when iron is limiting, when iron is present in excess, *tonB* transcription is hyper-repressed approximately 12-fold (Young and Postle, 1993; Hantke, 1981; Dorman *et al.*, 1988). Contrary to a previous proposal (Dorman *et al.*, 1988), hyper-repression does not reflect changes in supercoiling, since neither gyrase inhibitors, growth in high osmolarity, nor growth at low temperature affect transcriptional or translational expression of TonB (Young and Postle, 1993). Hyper-repression is also not due to increased binding of Fur, but rather is due to upstream sequences and an additional, uncharacterized, protein. Anaerobic hyper-repression of *tonB* is

consistent with the observation that growth on ferrous iron, which is soluble anaerobically, does not require TonB. Thus, under anaerobic circumstances, unless *E. coli* finds itself in an iron-limited environment, it does not expend energy on TonB synthesis.

## THE MECHANISM OF ENERGY TRANSDUCTION BETWEEN MEMBRANES

Some fundamental questions to be answered in order to understand the mechanism of energy transduction are the following: (1) What is the source of energy for the energy-requiring processes of the outer membrane; (2) Is TonB involved directly, or indirectly, in energy transduction; and (3) Are other proteins involved in energy transduction? In integrating the information available about TonB, it seems reasonable to assume that results from one TonB-dependent transport system will be applicable to all TonB-dependent systems. However, it may be desirable to consider high-affinity transport, colicin uptake, and bacteriophage adsorption separately. This review will focus on high-affinity transport.

*What is the source of energy for energy-requiring processes in the outer membrane?* This first question has now been clearly resolved using *btuC* mutants blocked in cytoplasmic membrane vitamin B12 transport (Reynolds *et al.*, 1980; Bradbeer, 1993). Vitamin B12 is actively transported across the outer membrane, resulting in 1000-fold concentration in the periplasmic space relative to the surrounding medium. Active transport across the outer membrane is not simply due to the presence of binding proteins in the periplasmic space. Mutations in the binding protein FhuD affect neither transport of aerobactin across the outer membrane nor its accumulation in the periplasmic space (Wooldridge *et al.*, 1992). Vitamin B12 transport across the outer membrane is prevented by either of the protonophores DNP (Reynolds *et al.*, 1980) or CCCP (J. Jaskula and K. Postle, unpublished results), suggesting the cytoplasmic membrane as the energy source. Cyanide can inhibit active transport of B12 across the outer membrane, but only in *unc* strains lacking the membrane-bound ATP synthase, clearly implicating cytoplasmic membrane PMF as energy source for TonB-dependent active transport across the outer membrane (Bradbeer, 1993).

TonB activity as an energy transducer and the necessity for the cytoplasmic membrane electrochemical potential were confirmed in experiments involving the engineering of a leader peptidase cleavage site (Glu37-Leu38-Pro39→Ala37-Leu38-Ala39) in TonB to separate its periplasmic domain from its cytoplasmic membrane domain. As expected, TonB cannot function if it has lost direct connection to the energy source, even with the amino-terminal transmembrane domain in place. Furthermore, specific sites in the transmembrane anchor are required for energy transduction since substitution with the first transmembrane domain from the cytoplasmic membrane protein TetA also inactivates TonB (Jaskula *et al.*, 1993). Similar results have been reported for *S. typhimurium* TonB (Karlsson *et al.*, 1993a). Interestingly, the cleaved *E. coli* TonB has a dominant negative effect on wild-type TonB, and remains largely associated with the cytoplasmic membrane (Jaskula *et al.*, 1993).

*Does TonB play a direct or indirect role in transport across the outer membrane?* TonB could act by generating a high-energy intermediate molecule that diffuses across the periplasmic space to activate outer membrane receptors (Reynolds *et al.*, 1980), or it could energize outer membrane receptors directly (Konisky, 1979; Holroyd and Bradbeer, 1984). While there is little evidence for or against the first hypothesis, there is much evidence in support of the latter. All *E. coli* high-affinity outer membrane receptors and B-group colicins whose deduced amino acid sequences have been determined share a region of similarity, called the TonB-box, at their amino termini (Schram *et al.*, 1987; Heller *et al.*, 1988; Schöffler and Braun, 1989; Bell *et al.*, 1990). A pentapeptide corresponding to a consensus TonB-box can inhibit TonB activity when added to the external medium (Tuckman and Osburne, 1992). Mutations in the TonB-box region of BtuB impair TonB-dependent uptake of vitamin B12, while binding to the receptor remains normal (Reynolds *et al.*, 1980). Substitution of the *btuB*451 (Leu8→Pro in the mature sequence) TonB-box mutation into the BtuB-FepA fusion protein eliminates its TonB-coupled reactions. Suppressors of *btuB*451 have been isolated in *tonB* at codon 160: D160L, D160K, and D160P (Heller *et al.*, 1988; Bell *et al.*, 1990). The suppression cannot be detected by vitamin B12 transport assays, but only by the more sensitive assay of growth on plates supplemented with vitamin B12. These may be allele-specific interactions, since TonB-box mutations in BtuB, FhuA, and Cir are differentially suppressed by the various suppressor alleles (Schöffler and Braun, 1989; Bell *et al.*, 1990; Braun *et al.*, 1991). Alternatively, it has been pro-

posed, based on mutational analysis of the TonB-box in BtuB, that the local structure rather than specific amino acid sequence is most important for its function (Gudmundsdottir *et al.*, 1989), thus implying that the suppressors are not allele-specific. If the TonB-box is required for TonB binding, the apparently contradictory finding that the TonB-box may be embedded in the outer membrane will have to be resolved (Murphy *et al.*, 1990).

It is interesting to speculate why colicins have TonB-box regions. If TonB can interact directly with colicins, it would also explain the absence of a TonB box in Tsx protein, even though Tsx is required for TonB-dependent uptake of colicin 5 (Bradley and Howard, 1992). Alternatively, the TonB-box regions of the outer membrane receptors may not be involved in TonB binding. Several other regions of homology exist among the TonB-dependent outer-membrane receptors (Lundrigan and Kadner, 1986; Nau and Konisky, 1989), and it has recently been shown that while the *Pseudomonas putida* TonB-dependent receptor PupA contains a TonB-box (Bitter *et al.*, 1991), TonB-dependent PupB outer membrane receptor lacks the TonB-box (Koster *et al.*, 1993). Since PupB retains three other regions of homology with TonB-dependent outer membrane receptors, the most important interaction between TonB and the outer membrane receptors may not be through the TonB-box (Koster *et al.*, 1993).

The biochemical evidence wherein a synthetic peptide corresponding to the proline-rich region of TonB was shown by NMR analysis to interact specifically with FhuA receptor *in vitro* must have been due to nonfunctional interactions (Brewer *et al.*, 1990). Recent studies of a mutant TonB deleted for amino acids 66–100 (the proline-rich region) indicate that, surprisingly, the deleted region plays no role in TonB activity. The deletion strain can irreversibly absorb $\phi$80, which uses FhuA as its receptor, equally as well as its nonmutant parent strain (Larsen *et al.*, 1993b).

Direct *in vivo* biochemical evidence for the interaction of TonB with the outer membrane receptor FepA comes from crosslinking studies using 1% formaldehyde. TonB can be crosslinked into a 195-kDa complex *in vivo*. This complex is absent in either *tonB* or *fepA* mutants (but appears when the latter is complemented with a $fepA^+$ plasmid) and can be detected on immunoblots by either anti-TonB or anti-FepA monoclonal antibody. The apparent size of the complex is consistent with a composition of one TonB and two FepA polypeptides; however, it could alternatively consist of one TonB, one FepA, and additional, uncharacterized protein(s). The complex forms in the absence of the ligand enterochelin, indicating that bound ligand is not required for a detectable TonB-FepA interaction (Skare *et al.*, 1993). A review of the high-affinity outer membrane receptors will also appear within this series (Klebba, 1993).

*Are other proteins involved in energy transduction?* The first evidence for the interaction of TonB with other proteins, in hindsight, came from observations that plasmid encoded-TonB is proteolytically unstable (Postle and Skare, 1988). At the time, this was believed to reflect the functional instability of TonB that had been detected earlier (Kadner and McElhaney, 1978). It then became apparent that plasmid-encoded TonB could be stabilized by plasmid-encoded ExbB (Fischer *et al.*, 1989). Mutations in *exbB* have a leaky TonB phenotype and reduced, but not entirely absent, levels of vitamin B12, and siderophore-mediated iron transport (Hantke and Zimmerman, 1981). Accordingly, *exbB* mutants hypersecrete enterochelin and are insensitive to B-group colicins, but both to a lesser extent than *tonB* mutants (Gutermann and Dann, 1973). Overexpression of TonB can compensate for an *exbB* mutation, but overexpression of ExbB cannot compensate for a *tonB* mutation (Fischer *et al.*, 1989).

ExbB is a 26-kDa cytoplasmic membrane protein (Eick-Helmerich and Braun, 1989) with an unusual membrane topology. Its amino terminus protrudes into the periplasm, while the majority of the protein remains in the cytoplasm. The cytoplasmic domain is punctuated by two transmembrane domains, for a total of three. The small portion of ExbB that protrudes into the periplasmic space is inaccessible to trypsin (Kampfenkel and Braun, 1993). Although plasmid-encoded TonB is unstable, chromosomally encoded TonB has been shown to be a stable protein that becomes unstable in the absence of ExbB protein (Skare and Postle, 1991), suggesting that the two may form a complex, and explaining why plasmid-encoded TonB is unstable: Overexpression of TonB relative to ExbB results in a significant fraction of unprotected, thus degraded, TonB. TonB can be crosslinked *in vivo* to ExbB protein, confirming the physical association of ExbB and TonB (Skare *et al.*, 1993). Given the respective membrane topologies of TonB and ExbB, they would have to interact through transmembrane domains. Indeed, a Val17 deletion in the transmembrane region of TonB significantly decreases its

activity and prevents crosslinking with ExbB. Suppressors of that mutation map in *exbB* and restore both the activity of TonB and its ability to crosslink with ExbB (Larsen, *et al.*, 1993b). Mutations in OmpT protease, which is located in the outer membrane, stabilize TonB in the absence of ExbB, suggesting that the proteolytic protection afforded by ExbB is not so much due to physical exclusion of potential proteolysis sites as it is to conversion of TonB to a protease-resistant configuration (Skare *et al.*, 1993).

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Escherichia  MTLD---LPR RFPWPTLLSV CIHGAVVAGL LYTSVHQVIE LPAPAQ-PIS  46
Salmonella   MTLD---LPR RFPWPTLLSV GIHGAVVAGL LYTSVHQVIE LPAPAQ-PIT  46
Klebsiella   MTLD---LPR RFPWPTLLSV AIHGAVVAGL LYTSVHQVIE QPSPTQ-PIE  46
Enterobacter MTLD---LPR RFPWPTLLSV AIHGAVVAGL LYTSVHQVIE KPSPSQ-PIE  46
Serratia     MPLKKMFLNR RISVPFVLSV GLHSALVAGL LYASVKEVVE LPKPEDAPIS  50
Yersinia     MQLNKFFLGR WLTWPLAFSV GIHGSVIAAL LYVSVEQMRI QPEIEDAPIA  50
Pseudomonas  MTKTRHNLA- RYSGSLALVL GVHAVAVLLT LNWSVPQAIE LPPAA-----  44

               !!  ▽    ▽▽▽         ▽  ▽!▽▽ ! ▽▽▽    ! !
Escherichia  VTMVTPADL- EPPQAVQPPP EPV-VEPEPE PEP--IPEPP KE-----APV  87
Salmonella   VTMVSPADL- EPPQAVQPPP EPV-VEPEPE PEPEPIPEPP KE-----APV  89
Klebsiella   ITMVAPADL- EPP-PAQPVV EPV-VEPEPE PEPEVVPEPP KE-----A-V  87
Enterobacter ITMVAPADL- EPPQAAQPVV EPV-VEPEPE PEPEVVPEPP KE-----VPV  87
Serratia     VMMVNTAAMA EPPPPAPAEP EPPQVEPEPE PEPEPIVEPP PK--------  92
Yersinia     VTMVNIDTF- AAPQPAAAEP QAE-PEPEPE PEPEPIDEAP PEPEVLPEPV  98
Pseudomonas  -MMVELAPLP EPAPPPPPKA APQPPAPVEE LPLPKLVEAP KP-----KIA  88

                ▽!   !! !▽!!         !     ▽▽     ▽          !▽ ▽
Escherichia  VIEKP---KP KPKPKPK--P VKKVQEQPKR DV-KPVE-SR PASPFEN--T 128
Salmonella   VIEKP---KP KPKPKPKPKP VKKVEEQPKR EV-KPAA-PR PASPFEN--S 132
Klebsiella   VIHKPEP-KP KPKPKPKPKP EKKV-EQPKR EV-KPAAEPR PASPFENNNT 134
Enterobacter VIHKPEP-KP KPKPKPKPKP EKKV-E-PKR EV-KPA-EPR PVSPFENNNT 132
Serratia     AIVKPEPVKP KPKPKPKPKV EK----QVKP EPKK-VE-PR EPSPFNNDSP 136
Yersinia     PVPIPEPVKP KPKP--VKKE VKK-PEVKKP DVKKTVA-PP DDKPFKSDEP 144
Pseudomonas  IA-KPP--KP KAKPQP-PKP EKK--PEPPK EA-PPTE-EV VDAPPSN--T 128

             ▽ ▽                                    !        ▽ !! ▽! ▽▽
Escherichia  A-PARLTSS- --TATAATSK PVTSVAS--- GP-RALSRNQ PQYPARAQAL 170
Salmonella   A-PVRPTSS- --TASA-TSK PAVSVPT--- GP-RALSRNQ PQYPARAQAL 173
Klebsiella   A-PAR-TAP- --STSTAAAK PTVTAPS--- GP-RAISRVQ PSYPARAQAL 174
Enterobacter A-PAR-TAP- --STTAATAK PMTTAPS--- VP-KALKRGD PSYPQRAQAL 173
Serratia     AKPID-KAPV KQAPAAPVQG NSREV----- GP-RPISRAN PLYPPRAQAL 178
Yersinia     ALVST-NAPV KSAPKASVPG VSTS--T--- GP-KALSKAK PTYPARALAL 186
Pseudomonas  P-PQKSAAP- --APSIASNS NAL--PTWQS DPVRHLAKYK R-YPEDARRR 170

               ▽! ▽ ▽ ▽ ▽    !▽▽ ▽   ▽▽ ▽ ▽ ▽  ▽▽! ▽▽  ▽ ! ▽▽
Escherichia  RIEGQVKVKF DVTPDGRVDN VQILSAKPAN MFEREVKNAM RRWRYEPGKP 220
Salmonella   RIEGRVKVKF DVTSAGRVEN VQILSAQPAN MFEREVKNAM RKWRYEAGKP 223
Klebsiella   RIEGTVRVKF DVSPDGRIDN LQILSAQPAN MFEREVKSAM RRWRYQQGRP 224
Enterobacter RIEGDVRVKF DVTADGRVEN IQILSAKPAN MFERDVKTAM RKWRYEAGRP 223
Serratia     QIEGNVRVQF DIDSAGRVSN VRILSAEPRN MFEREVKQAM RKWRYEA-KE 227
Yersinia     GVEGQVKVQY DIDENGRVTN VRILEATPRN TFEREVKQVM RKWRFEA-VA 235
Pseudomonas  GLQGINRLRF VVDAEGKVVS YAMAGGSGSA ALDRATLEMI RR-AGTVPKP 219

                           ▽   ▽
Escherichia  GSGIV----V NILFKINGTT EIQ 239
Salmonella   GSGLV----V NIIFRLNGTA QIE 242
Klebsiella   GTGVT----M TIKFRLNGV- EIN 242
Enterobacter GTGLT----M NIKFRLNGV- KMD 241
Serratia     AKDRT----V TIRFKLNGTT ELN 246
Yersinia     AKDYV----T TVVFKIGGTT EMD 254
Pseudomonas  PPELLNNGTI EVVAPFVYSL DRR 242
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**Fig. 2.** Comparison on TonB amino acid sequences. Deduced amino acid sequences from *Escherichia coli* (Postle and Good, 1983), *Salmonella typhimurium* (Hannavey *et al.*, 1990), *Klebsiella pneumoniae* (Bruske *et al.*, 1993), *Enterobacter aerogenes* (Bruske and Heller, 1993), *Serratia marsescens* (Gaisser and Braun, 1991), *Yersinia enterocolitica* (Koebnik *et al.*, 1993), and *Pseudomonas putida* (Bitter *et al.*, 1993) are compared. Inverted triangles represent six indentical residues; exclamation marks represent residues conserved in all seven species known to date. The large boxed region encompasses the hydrophobic amino terminal transmembrane anchor of *E. coli* (Roof *et al.*, 1991) and *Salmonella* TonB (Hannavy *et al.*, 1990), and the corresponding regions of other species. The smaller boxed region delineates a segment of *E. coli* TonB whose deletion has no effect on function (Larsen *et al.*, 1993a). Within the region spanned by amino acids 66–120 of the *E. coli* sequence, one possible alignment among several is presented.

Since the absence of ExbB results in proteolytic degradation of TonB, it has been difficult to directly address the role of ExbB in energy transduction. When TonB is stabilized in an *ompT,exbB* strain such that steady-state levels of TonB are identical to $exbB^{+}$, the absence of ExbB diminishes TonB-dependent vitamin B12 transport to approximately 10% of the wild-type activity (Skare *et al.*, 1993). The residual TonB activity is due to evolutionary crosstalk with the Tol system (Braun, 1989; Braun and Herrmann, 1993). Thus ExbB is required to activate TonB, or, more likely, to recycle it to an active conformation, an idea that is explored later in this review. Recently an additional function for ExbB as a chaperonelike molecule has been proposed. Under circumstances where TonB has been confined to the cytoplasm by deletion of its signal sequence, the absence of ExbB increases the proteolysis of cytoplasmically localized TonB (Karlsson *et al.*, 1993b). It is not clear how this observation pertains to wild-type TonB since (1) the PhoA activity of a full-length TonB-PhoA fusion is not significantly affected by the loss of ExbB, suggesting that the fusion protein, which has functional TonB activity as well (Roof *et al.*, 1991), is exported efficiently in either case (S. K. Roof and K. Postle, unpublished observations), and (2) mutations in the outer membrane protease OmpT prevent much of the TonB proteolysis that occurs in the absence of ExbB (Skare *et al.*, 1993). Crosslinking experiments have identified two other TonB complexes *in vivo*, with apparent molecular weights of 43.5 and 77 kDa (Skare *et al.*, 1993), suggesting that there are undiscovered loci involved in energy transduction.

*Information from sequence comparisons.* Deduced TonB amino acid sequences have been obtained from a number of Gram-negative bacteria (Fig. 2). TonB sequences from the enteric bacteria are all rather similar, with significant stretches of identical residues and even larger regions of conserved residues (Fig. 2). All heterologous *tonB* genes from enteric bacteria can function to some extent in *E. coli* (Hannavy *et al.*, 1990; Gaisser and Braun, 1991; Bruske *et al.*, 1993; Bruske and Heller, 1993; Koebnik *et al.*, 1993); however, *Pseudomonas* and *E. coli* TonB are not functionally interchangeable (Bitter *et al.*, 1993).

Comparison of residues conserved only among enteric TonBs with residues additionally conserved in *P. putida* TonB provides confirmation of some experimental data and suggests additional possibilities. At the amino terminus, most of the membrane-spanning domain amino acids are conserved among enterics. It has been recently noted that a Ser His Leu Ser motif is present in the transmembrane region that is highly conserved in *E. coli*. *Yersinia enterocolitica*, *Serratia marcescens*, and *S. typhimurium* (Koebnik *et al.*, 1993). This motif, which also occurs in *Klebsiella pneumoniae* and *Enterobacter aerogenes* (Fig. 2), would be entirely localized on one face of an α-helix and is clearly important since a His20→Arg substitution results in an inactive TonB protein (Traub *et al.*, 1993). The Val17 deletion in *E. coli tonB* would result in the disruption of the motif and displacement of the Ser relative to His Leu Ser. Since the Val17 deletion cannot be crosslinked to ExbB, it is likely to be through this face of the transmembrane domain that TonB can contact and interact with ExbB (Larsen *et al.*, 1993b). Further support for this idea comes from the extent of divergence of *P. putida* TonB transmembrane domain amino acid sequences from the enteric consensus for that region. Although there is significant divergence in amino acid sequence (they are all still hydrophobic amino acids, however), the His Leu Ser portion of the ExbB interaction motif has been retained. Consistent with that observation, the *P. putida* ExbB and ExbD proteins are functionally similar to, and can complement, mutations in their *E. coli* analogues (Bitter *et al.*, 1993). Furthermore, the *exb* genes are significantly more homologous (57 and 65%, respectively) than the two *tonB* genes (28%) (Bitter *et al.*, 1993). The homology among Exb proteins suggests that they may have been conserved for interactions with additional, equally conserved, proteins.

Several residues that are identical in all TonBs examined to date are candidates to encode either important structural information, or protein interaction domains (Fig. 2). Of particular note are Gly174 and Gly186, the only glycine residues conserved in all TonBs. Since glycine bears only a hydrogen atom as a side chain, and can accommodate a wide range of different conformations, it can potentially play a major structural role in proteins by allowing unusual backbone conformations. These two conserved glycines are therefore likely to be structurally significant in TonB. The glycines occur near the carboxy terminal end of TonB, along with a number of other residues conserved in all seven species (Y163, P164, A167, R211). This observation, together with the essential nature of the extreme carboxy terminus (Anton and Heller, 1991; Roof *et al.*, 1991) and the lack of energy-transducing

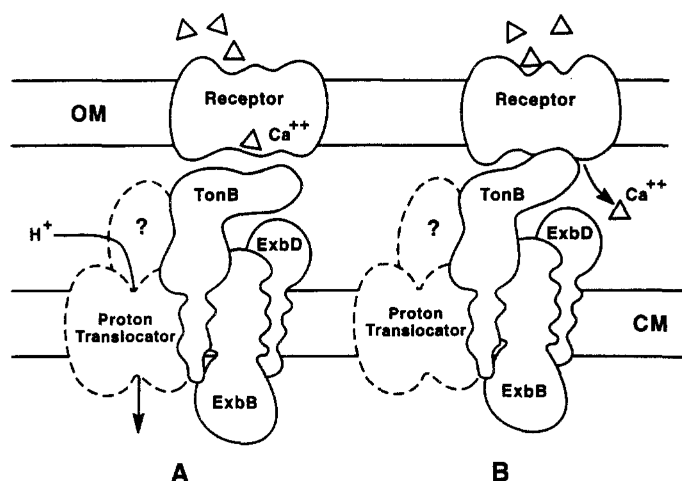


**Fig. 3.** A model for TonB-dependent energy transduction and active transport across the outer membrane. A. The movement of a proton down the concentration gradient results in a conformation change transmitted to TonB. B. The conformational change causes TonB to interact with the outer membrane receptor such that $Ca^{++}$ and the bound ligand are released into the periplasmic space (to date, no evidence exists regarding the temporal order of these events). TonB and ExbB are depicted by solid lines to indicate that their existence and physical interaction are proven. ExbD is also drawn in a solid line since mutations in the corresponding gene are known to affect energy transduction. TonB, ExbB, and ExbD are all drawn to reflect their known membrane topologies. Since the existence of the proton channel is tentative, it is delineated by a broken line. The protein labelled with a question mark (broken line) represents unidentified proteins discovered in *in vivo* crosslinking experiments that interact with TonB. Triangles represent transport ligands. The text contains a more detailed discussion of the model and presents the evidence for various aspects of it.

function of the proline-rich region (Larsen *et al.*, 1993b), suggest that the region of TonB that interacts with the outer membrane receptors is at the carboxy terminus. Consistent with that hypothesis, none of the carboxy terminal deletions recently characterized in TonB exhibits dominance (Anton and Heller, 1993), while the TonB cleavage mutant that loses its amino terminus but retains its carboxy terminus shows a dominant negative phenotype (Jaskula *et al.*, 1993). The possibility for formation of an amphipathic helix exists at the carboxy terminus of TonB (amino acid, 202–220) in a region highly conserved among the six enterics. The amino acid sequence of *P. putida* is significantly divergent in this region, however, and while any helical conformations postulated within that region have a hydrophobic face, they also have other hydrophobic residues variably arranged around the rest of the putative helix. Nonetheless, this region may be important for interaction with either the outer membrane or the outer membrane receptors and could explain why *P. putida* TonB cannot function in *E. coli*.

Although the *P. putida* sequence is much different from the enteric TonBs, it still contains 18% prolyl residues and a number of arginyl residues (13, compared to 14 in *E. coli*). With the abundance of prolyl residues in all TonB sequences, the problem of cis–trans isomerization of the prolyl residues must be addressed, either intramolecularly or by a periplasmic peptidyl-prolyl *cis–trans*-isomerase (Liu and Walsh, 1990). One appealing possibility is that, analogous to dihydrofolate reductase (Texter *et al.*, 1992), the isomerization occurs intramolecularly, catalyzed by arginyl residues, two of which—Arg204 and Arg211—are conserved in all TonB sequences (Fig. 2). If the isomerization is carried out by proline isomerase or some other enzyme, one might expect mutants in the corresponding *rot* gene to have arisen from *tonB* mutant hunts, which they have not. However, if the *rot* gene is an essential one, precluding its discovery in mutant hunts, it might encode one of the unidentified proteins to which TonB crosslinks *in vivo*.

## A MODEL

Figure 3 is a scheme depicting TonB in an active conformation. In response to a proton moving through a postulated proton translocator, active TonB interacts with outer membrane receptors, causing a conformational change that releases calcium into the periplasm, lowers the very high ligand affinity for the receptor (Kenley *et al.*, 1978; Bradbeer *et al.*, 1986; Bradbeer and Gudmundsdottir, 1990), and results in ligand release into the periplasm. The current known cast of characters in TonB-dependent energy transduction is shown in the cytoplasmic membrane.

Newly synthesized TonB may already be in an active conformation, ready to interact with the outer membrane receptors and transduce energy. Evidence for this idea comes from experiments where both *exbB* and its analogue in the *tol* system, *tolQ*, are mutated (Braun, 1989). While *exbB* mutants exhibit low level TonB activity due to crosstalk with TolQ protein, the double mutants have no detectable TonB activity. If, however, TonB is overexpressed from a plasmid, some level of activity can be restored. This new level of activity most likely reflects the fact that TonB is synthesized in an active state or that some small portion can independently achieve an active state.

TonB can be crosslinked to the FepA outer membrane receptor whether ligands are present or not (Skare *et al.*, 1993). It is not clear whether the active conformation of TonB could also be dissipated by interaction with unliganded receptor. It may be that there is a kinetic sampling of receptors with the TonB occupancy time increased whenever ligand is present. TonB is proposed to interact with the receptor through the TonB carboxy terminus, for reasons described earlier.

The actual role of the protonmotive force—which is clearly the energy source (Bradbeer, 1993)—is unknown at this time. In keeping with the idea that TonB is synthesized in an active conformation, it seems likely that the PMF is necessary to drive the conformational change in TonB that, when transmitted to an outer membrane receptor, could pump ligand across the outer membrane, whether ligand is present or not. It has recently been proposed that the high-affinity outer membrane receptor proteins function as gated pores and that TonB acts to open the gate to allow ligands to move through the pores (Rutz *et al.*, 1992). It is not realistic, however, to ignore the very real pumping nature of the outer membrane receptors. The vitamin B12 receptor is capable of concentrating vitamin B12 1000-fold in the periplasm relative to the external environment (Reynolds *et al.*, 1980). It is therefore in this active pumping that the role for protonmotive force is most likely to be manifested.

It may then be that TonB spontaneously releases the outer membrane receptor for which, due to the conformational change, it no longer has any affinity. If this is the case, what is the role for ExbB and ExbD? It is in consideration of their obvious importance that a role for them is postulated in the release of TonB from the outer membrane receptors and recycling of TonB back to an active conformation. TonB is not used once and then discarded. Were that the case, in order to account for rates of siderophore and vitamin B12 transport, it would necessarily be an abundant and easily detectable species in the envelope—which it is not. Thus, without ExbB and ExbD, TonB is postulated to remain attached to the outer membrane receptors. In order to avoid the deleterious situation where the cytoplasmic and outer membranes are attached permanently at an abnormally high number of sites, a fail-safe mechanism in the form of envelope proteases exists that degrades TonB in that conformation. Thus, one way or another, the membranes can be released from their temporary sites of apposition.

The topologies of ExbB and ExbD (Kampfenkel and Braun, 1992, 1993; Karlsson *et al.*, 1993b) suggest the interesting possibility that they function together as a transmembrane signaling molecule. Although both are anchored in the cytoplasmic membrane, the topology of ExbB places it largely in the cytoplasm, while the topology of ExbD, like TonB, places it largely in the periplasm (Fig. 3). The fact that *exbB* and *exbD* form an operon suggests that they need to be concomitantly expressed. Why the necessity of a cytoplasmic domain? It seems reasonable to propose that communication with the cytoplasm must also play a role in energy transduction. It is known that arsenate significantly increases active transport of vitamin B12 across the outer membrane (Reynolds *et al.*, 1980). I propose that ExbB may be chemically modified, most likely by phosphorylation, in order to transmit a signal to ExbD allowing the recycling of TonB to an active conformation. There is no obvious nucleotide-binding site in ExbB that exactly conforms to a P-loop motif; however, since not all nucleotide-binding proteins employ the P-loop motif (Saraste *et al.*, 1990), the lack of sequence homology does

not invalidate the hypothesis. Alternatively, cytoplasmic communication could occur via other chemical modifications, such as methylation. Recycling of ExbB, then, may be the source of the short functional half-life of TonB in the absence of protein synthesis (Kadner and McElhaney, 1978). Perhaps the protein that modifies ExbB has a short chemical half-life. ExbD is stabilized by ExbB (Fischer *et al.*, 1989). To date, TonB–ExbD interactions have not been detected by formaldehyde crosslinking (Skare *et al.*, 1993), perhaps because either the interaction is too transitory or crosslinkable sites between the two molecules are more distantly located than can be readily spanned by the methyl bridge provided by formaldehyde. Unlike the implications of Fig. 3, it is not yet clear whether TonB will be found in a large, energy-transduction complex, or whether it will remain in the general vicinity of the required proteins but establish the close contact required for energy transduction in a sequential fashion.

At this juncture—the 50th anniversary of TonB's discovery—many tantalizing observations have been made concerning TonB and the phenomenon of energy transduction. It is only recently, however, that the complexity of this unique process has become apparent. I think the key to the next few years in understanding energy transduction will be to (1) discover the source of the short functional half-life of TonB, (2) identify all the players in the game, either genetically or biochemically, and (3) expand the available assays for TonB activity so that its different functional aspects can be addressed. The rapid pace at which TonB-related research has proceeded in the past few years bodes well for unravelling the mechanism of energy transduction between membranes.

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