ROSET Model of TonB Action in Gram-Negative Bacterial Iron Acquisition

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The **rotational surveillance and energy transfer (ROSET)** model of TonB action suggests a mechanism by which the electrochemical proton gradient across the Gram-negative bacterial inner membrane (IM) promotes the transport of iron through ligand-gated porins (LGP) in the outer membrane (OM). TonB associates with the IM by an N-terminal hydrophobic helix that forms a complex with ExbBD. It also contains a central extended length of rigid polypeptide that spans the periplasm and a dimeric C-terminal- $\beta\alpha\beta$ -domain (CTD) with LysM motifs that binds the peptidoglycan (PG) layer beneath the OM bilayer. The TonB CTD forms a dimer with affinity for both PG- and TonB-independent OM proteins (e.g., OmpA), localizing it near the periplasmic interface of the OM bilayer. Porins and other OM proteins associate with PG, and this general affinity allows the TonB CTD dimer to survey the periplasmic surface of the OM bilayer. Energized rotational motion of the TonB N terminus in the fluid IM bilayer promotes the lateral movement of the TonB-ExbBD complex in the IM and of the TonB CTD dimer across the inner surface of the OM. When it encounters an accessible TonB box of a (ligand-bound) LGP, the monomeric form of the CTD binds and recruits it into a 4-stranded β -sheet. Because the CTD is rotating, this binding reaction transfers kinetic energy, created by the electrochemical proton gradient across the IM, through the periplasm to the OM protein. The equilibration of the TonB C terminus between the dimeric and monomeric forms that engage in different binding reactions allows the identification of iron-loaded LGP and then the internalization of iron through their trans-outer membrane β -barrels. Hence, the ROSET model postulates a mechanism for the transfer of energy from the IM to the OM, triggering iron uptake.

The 239-amino-acid TonB protein underlies several aspects of Gram-negative bacterial cell envelope physiology, including obligatory involvement in metal (iron) transport through the outer membrane (OM), susceptibility to numerous bacteriocins/microcins, and infection by certain bacteriophages. Regarding the role in iron transport, bacteria generally acquire iron by the elaboration of siderophores (1) that complex it in the environment. The subsequent active transport of ferric siderophores through OM receptor proteins (2, 3) requires TonB (4, 5).

FepA is one such OM protein that selectively recognizes and internalizes the native siderophore of *Escherichia coli*, ferric enterobactin (FeEnt) (6). The N-terminal 150-residue globular portion of FepA (N-domain) resides within a C-terminal 22-stranded β -barrel (Fig. 1). The C-terminal β -barrel places FepA and its relatives in the porin superfamily (7, 8). Their selectivity for ligands (9–11) and the fact that high-affinity ligand binding (12) triggers uptake through their transmembrane channels led to the designation ligand-gated porins (LGP) (13). They require energy and TonB for functionality, so they are also energy- and TonB-dependent receptors or TonB-dependent transporters (TBDT) (14). None of these designations is fully appropriate, in that OM proteins in this class are not diffusive porins (15) and neither are they true transporters, a term usually reserved for ATP- or proton motive force (PMF)-driven inner membrane (IM) permeases (16). With the additional stipulation that their activities are TonB and energy dependent, in this paper, I will refer to FepA and its orthologs/paralogs as LGP.

The need for TonB in the energy-dependent uptake of metals (ferric siderophores [3], heme [17], and cobalt as vitamin B₁₂ [2]) is a key aspect of cellular nutrition and an enigmatic feature of cell envelope physiology. FeEnt transport through the OM requires the electrochemical proton gradient (PMF) across the IM (3). TonB-dependent phages and colicins also require metabolic en-

ergy as they penetrate the OM (4, 18), and these facts connect TonB action to transport bioenergetics. Together, these findings portray TonB as an energy transducer that bridges the Gram-negative cell envelope to allow metal transport through LGP into the periplasm (19). Nuclear magnetic resonance (NMR) descriptions of the central rigid portion of TonB (20), bioinformatic predictions about its hydrophobic and helical N terminus (21), and the structures of the dimeric (22) and monomeric (23) forms of its C-terminal domain (CTD), especially those in complex with ligand-bound OM receptors (24, 25), revealed it as an IM-anchored protein spanning the periplasm to interact with the underside of the OM bilayer. When LGP bind metal complexes, large surface loop motions occur (26–28) that disseminate through the protein interior and relocate the TonB box to the center of the β -barrel (29). Subsequent protein-protein interactions between the TonB box of LGP and the TonB CTD were postulated (30–33) and demonstrated (24, 25). Thus, ligand binding by LGP initiates trans-OM signal transduction that exposes their TonB box for recognition by the TonB CTD in the periplasm (Fig. 1).

The actions of TonB in the IM were first genetically (4) and later biochemically linked to ExbBD (34). Various findings led to the “TonB shuttle hypothesis” (35), which proposed that the elec-

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FIG 1 Structures of LGP and TonB CTD. The TonB CTD may exist in dimeric (bottom left; PDB identification [ID] [1IHR](#)) or monomeric forms (bottom right; PDB ID [1XX3](#)). The dimer is shown in ribbon form beneath FepA (PDB no. [1FEP](#); green β -barrel and red N domain), also in ribbon form, modeled prior to binding of FeEnt (atomic spheres in elemental colors). The TonB box of FepA (cyan) associates with the wall of the FepA β -barrel until FeEnt enters its binding site in the exterior vestibule. When an LGP binds a metal complex, as seen for BtuB with bound B_{12} (PDB ID [2GSK](#); dark magenta β -barrel and sky blue N domain), its TonB box relocates to the center of the β -barrel, where it may be bound by the monomeric TonB CTD (PDB ID [1XX3](#); bottom right). FepA and BtuB are depicted in a frozen bilayer of phospholipid. Molecular graphics were developed and analyses were performed with the UCSF Chimera package.

trochemical PMF caused TonB to dislodge from the IM and physically relocate to the OM, where the dissipation of its energized state promoted the uptake of metal complexes through OM proteins. However, the postulated extraction of the TonB N terminus from the *E. coli* IM bilayer and its subsequent reinsertion had no precedent and were difficult to reconcile with membrane biochemistry and thermodynamics. It is now apparent that TonB does not relocate between membrane bilayers. The normal TonB-dependent physiology of green fluorescent protein (GFP)-TonB fusion proteins, despite the confinement of the fluorescent protein moiety in the cytoplasm, disproved the TonB shuttle idea (37), as confirmed by additional studies (36). Instead, the TonB-ExbBD IM complex may harvest electrochemical force from the electrochemical proton gradient ($\Delta\mu H^+$) created by the proton gradient across the IM and convert it into rotational motion (38). These phenomena are explained by the rotational surveillance and energy transfer (ROSET) model, described below.

Recent progress in the understanding of TonB action relates to the architecture of the Gram-negative bacterial cell envelope. Insight into its biochemical properties came from bioinformatic and structural data that revealed its general affinities for peptidoglycan (PG) and OM proteins (37), its unexpectedly restricted localization in the cell envelope (28, 38), and its rapid physical motion, driven by PMF (38).

PG and TonB. The 75-residue TonB CTD was initially crystallized as a dimer (22), but solution structures also revealed a monomeric form. NMR analyses of TonB residues 103 to 239 indicated that the polypeptide was monomeric, with residues 152 to 239 having a mix of $\alpha\beta$ -structure (23) (Fig. 1). The dimer has physiological relevance *in vivo* (39); monomeric forms (40) and monomer-dimer transitions (41) are also important to TonB ac-

tivity. Although the demonstration of the solution monomer led to arguments against the function of the dimer *in vivo* (42), the nature of its original crystallographic form, with three β -strands and a single α -helix per monomer intertwined into two three-stranded antiparallel β -sheets, left little doubt of its biological relevance. The affinity of the TonB CTD complex for PG underscored this conclusion (37). The realization that TonB binds PG came from its sequence and structural homologies to *E. coli* LdtC (formerly YcsF), a proline-rich (8.4%) protein that typifies a family of putative periplasmic proteins (YnhG, YbiS, and ErfK [43]). Each contains a hydrophobic potential IM anchor, a central proline-rich sequence, and a lysin (LysM) motif that confers affinity for PG. LdtC is a transpeptidase that removes D-Ala from the PG tetrapeptide stem and attaches Braun's lipoprotein, which explains its affinity for PG (44). LysM domains (Pfam family PF01476) occur in >27,000 proteins across all biological kingdoms: in PG-binding proteins and PG-degrading enzymes of bacteria, in about half of bacteriophage baseplate assemblies, in innate immunity proteins of both plants and humans, and in many other protein classes (45, 46). Like LdtC, TonB contains a hydrophobic N terminus anchored in the IM, a central and rigid proline-rich (16.7%) region, and a C terminus with lysin motifs. Analyses of the LysM motif in the context of the dimeric TonB C terminus found four sites of structural relatedness, with extensive superposition of TonB and LysM residues projecting from the β -sheets of the dimeric CTD (37). These data raised the possibility of functional interactions between TonB and the murein sacculus. Binding experiments verified the affinity of the TonB CTD for purified PG. It is noteworthy that the LysM homologies in TonB occur only in the context of a dimeric TonB CTD, because the PG-binding surfaces contain elements of both monomers. PG

binding does not involve the residues that recruit heterologous TonB box polypeptides. To summarize these findings, the monomeric TonB CTD binds and recruits the TonB box of OM iron transporters, whereas the dimeric CTD manifests affinity for PG. The activity of the dimeric form, which was unknown when Chang et al. (22) solved the TonB CTD structure and postulated its rotation, adds new dimensions to the mechanism of TonB action, giving a rationale for the localization of the CTD near the OM and the possibility of transmission of mechanical force (see below).

Theories of PG architecture. The preceding discussion describes the association of the TonB CTD with PG. These interactions, with approximately micromolar affinity (X. Jiang, unpublished data), imply that the structural features of PG impact the activities of TonB. Many OM proteins tightly associate with PG in the cell envelope; even differential solubilizations with Triton X-100 (47) or SDS (48, 49; P. E. Klebba, unpublished data) do not completely extract OmpA, porins, FepA, Cir, and other OM proteins. Two contrasting models predict the *N*-acetylglucosamine-*N*-acetylmuramic acid (NAG-NAM) oligosaccharide of PG as a parallel (planar network [PN] model [50, 51]) or perpendicular (vertical scaffold [VS] model [52–55]) to the cell surface. Relevant to these theories, Meroueh et al. (55) synthesized and structurally solved (by NMR) the NAG-NAM oligosaccharide with oligopeptide chains attached to the NAM lactyl group. Their results showed a 3-fold symmetry of the pentapeptides on NAM, which when cross-linked create a hexagonal honeycomb-like matrix of PG. These findings potentially impact TonB-dependent physiology. With the glycan chains perpendicular to the cell surface, the resulting hexagonal cells form an underlying network capable of organizing and supporting OM proteins (55). Additional findings, including crystallographic data (56–58), support the VS model. One striking aspect is that the diameter of individual cells within its predicted matrix (~ 60 Å) is about the same as that of many OMP β -barrels, including those of OmpF, LamB, TolC, LGP, and the dimeric form of the TonB C terminus (Fig. 2). VS architecture provides a regular framework for the arrangement of LGP in cells that may allow facile interactions with the TonB CTD (37, 38) (Fig. 2). Although other data raise questions as to the validity of VS PG architecture in both Gram-negative (59) and Gram-positive (60) bacteria, the PN model does not suggest similar mechanistic advantages and raises some potential physical impediments to protein-protein interactions in the periplasm (see below).

Consideration of these theories hinges on NMR spectra showing 3-fold symmetry of the peptide stems (55) and the implications of those findings on PG architecture. The original NMR data demonstrating the 3-fold symmetry of groups attached to the NAG stems of the glycan polymer were unambiguous, but they do not resolve overall glycan strand orientation *in vivo*: glycan chains with 3-fold stem symmetry may still orient either vertically or horizontally. Furthermore, the orientations may differ in Gram-negative and Gram-positive cells and/or in various regions of the cell envelope within a single cell. Little is resolved about the organization of PG in a single organism, in different bacteria under different conditions, or in different stages of growth. PG is certainly not a single uniform structure in Gram-negative bacteria, as shown by its different attributes at the poles versus the body of the rods (61). Certain observations are pertinent to the models of PG organization.

(i) Micrographs of the PG polymer of Gram-positive bacteria (Fig. 2D) resemble a honeycomb of regularly arranged and sized cells, similar to VS models (55). This is best seen in Gram-positive cells, because they lack an OM (e.g., *Staphylococcus aureus* [Fig. 2D]), but no *a priori* reason exists to expect different PG polymerization in Gram-negative bacteria. When *E. coli* was extracted with 2% SDS at 60°C (Fig. 2C), which removes many OM proteins from the sacculus but not general porins, a hexagonal array of OmpF adsorbed to PG became visible (48). These data support the concept of PG in VS form in Gram-negative cells.

(ii) A perpendicular orientation of glycan strands more readily explains different thicknesses of PG layers in Gram-positive bacteria. At the least, it is easier to regulate thickness as the oligosaccharide grows outward from the cell surface.

(iii) The VS model requires relatively short vertically oriented glycan strands in the periplasm. The mean length of glycan strands in Gram-negative bacteria is a complicated parameter that is difficult to interpret, as a result of limited data (62). For example, 75% of *E. coli* glycan strands had a mean length of <9 disaccharide units, but the remaining 25% averaged about 45 disaccharide units (62–64). Nevertheless, the distribution of *E. coli* glycan strands skews toward short lengths, with $\sim 50\%$ having a length of <6 disaccharides (65), which potentially accommodates a vertical orientation in much or most of the cell envelope.

(iv) The appropriate size and regular array of the hexagonal cells implied by 3-fold symmetry of peptides around the glycan strands are not likely coincidental. In the VS model, the dimensions of the individual PG hexagons underlying the OM are appropriate for harboring OM proteins, and images of OmpF on the *E. coli* cell surface (48) reflect such a regular hexagonal array (Fig. 2C). Different lengths of cross-linking peptides change the size of these cells; proteins more tightly bound within smaller cells may resist detergent solubilization. The cells are sufficiently large to precisely enclose the β -barrels of LGP (Fig. 2H); this stabilization has potential mechanistic relevance to the proposed rotational motion of TonB.

(v) Protein-protein interactions occur in the periplasm between TonB and LGP, between IM exporters (AcrAB and MdtABC [66]) and TolC, between TolAQR and Pal (67), and among other proteins that find each other and pair together. The PN and VS models have different implications for interactions between or among the components of TonB-dependent transport systems. In the PN model, both the hexagonal cells of cross-linked peptides (Fig. 2E) and the horizontal array of glycan strands (Fig. 2G) underlying the OM bilayer create physical and conceptual barriers to these essential interactions. A VS array is less restrictive to movement of the TonB CTD through the periplasm and to its binding of iron-loaded LGP (Fig. 2F and H). A lower extent of cross-linking in the VS model (e.g., $\sim 20\%$ in *E. coli* [63, 68]) creates PG that is more amenable to lateral movement of trans-cell envelope proteins, like TonB (see Fig. 3).

In summary, NMR structural determinations showing 3-fold symmetry of peptide stems on the NAG-NAM strands suggest the logic of the VS PG model, a regular framework that potentially organizes the ongoing biochemical and physiological activities of the cell envelope. Still, the individual residues involved in binding between TonB and PG are not yet known, and overall PG organization remains an open question. Both the PN and VS models potentially support PG-affiliated TonB

FIG 2 PG architecture. The PN (A) and VS (B) models have parallel and perpendicular NAG-NAM strands (rectangles) in the PG polymer, respectively, relative to the cell envelope plane (91). (C) Pentapeptides (green) project from NAM (orange) with 3-fold symmetry (republished from reference 55), implying hexagonal cells in PG that create a framework underlying OM and/or periplasmic proteins, like TolC (purple). The bar represents 100 Å. The electron micrograph shows a regular hexagonal array of OmpF adsorbed to the *E. coli* PG matrix (magnified from and with permission from reference 48); the scale bar is 1,000 Å. (D) Tomographic image of PG. (Republished from *Molecular Microbiology* [60].) The scale bar is 100 Å; the bottom right image originates from the coordinates (courtesy of Shahriar Mobashery) (55) of the glycan-pentapeptide polymer, rendered in VS orientation by Chimera. The circular inset is the 6th century rosette from cathedral San Giovanni Evangelista, Sicily. (E to H) PG glycan strands (depicted as brown wireframe) in PN form (seen in cross-section [E] and from beneath the OM [G]) or in VS form (F and H) imply different accessibility to OM proteins for trans-envelope IM-anchored proteins, like TonB (light blue and pale yellow), and different freedom of lateral movement. (G and H) The β -barrels and N domains of LGP Cir (gold and purple), FepA (green and red), FhuA (cornflower blue and yellow), BtuB (purple and cornflower blue), and FecA (orange and green) are more accessible in the VS model. Other OM proteins (OmpA, OmpF, OmpT, LamB, and TolC) are depicted in shades of gray. All coordinates from PDB were rendered by Chimera.

action, so whether the sacculus is arranged as PN or VS is not crucial to the ROSET model.

Localization of TonB in the cell envelope. Fluorescent GFP-TonB hybrids (28, 37, 38) allowed the visualization of something novel about its cellular distribution: TonB is present in much smaller amounts at the poles of the cell, to the point that it is undetectable in those regions. Confocal fluorescence microscopy showed that GFP-TonB was confined to the central parts of the cell and absent from the poles (see Fig. 3). The possibility that fusion of GFP to its N terminus influenced the distribution of TonB created some uncertainty about this observation, but similar GFP constructs that fused the fluorescent β -barrel to other IM proteins (e.g., LacY [38], Aer, YqjD, TnaA, and GroES [69]) did not similarly localize to the central part of the cell. The GFP-TonB constructs had wild-type levels of iron transport and sensitivity to TonB-dependent colicins and bacteriophages. Overall, these data imply that the fluorescent protein did not artifactually affect the localization of TonB but rather that TonB is largely confined to central regions of the bacterial cell envelope.

Energized motion of TonB. Several investigators equated the dual requirements of TonB action and bioenergetic force with a single biochemical activity associated with OM iron transport. They proposed that TonB-ExbBD mediated energy transduction from the IM to the OM (2, 35, 70–72) and that TonB disseminated the energy by direct contact to the OM LGP. To evaluate this theory, Jordan et al. (38) created a fluorescence system for analysis of the anisotropy of GFP-TonB fusion proteins in living bacteria. This approach found energized motion of TonB in the IM, coupled to the electrochemical proton gradient by ExbBD (38). The main result was that during the fluorescence lifetime of GFP (2.5 ns [73, 74]), the TonB protein reoriented such that light was emitted with a different directional vector than that of the polarized excitatory light. The motion of GFP-TonB was slower than that of free GFP in the cytoplasm, as expected when GFP is restricted by attachment to a membrane protein. However, the dissipation of PMF by exposure of the bacteria to the proton ionophores carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and 2,4-dinitrophenol (DNP) or other energy inhibitors further increased the anisotropy value (retarded the motion) of GFP-TonB. These data indicated that the movements of TonB are powered by electrochemical force. Last, the deletion of *exbBD* made TonB motion insensitive to the same inhibitors, suggesting that ExbBD creates the mechanical link to the electrochemical gradient. Thus, in a contrast of its activity in the absence and presence of inhibitors, the dependency of GFP-TonB motion on electrochemical force and the actions of ExbBD were apparent. TonB undergoes rapid energized movement, driven by an ExbBD-mediated connection to PMF.

The conclusion that the observed TonB motion is likely rotation comes from two considerations. First, the primary structure of ExbBD has homology to that of MotAB, the proposed stator element of the flagellar motor, within which the rotor (filament) turns in response to PMF energization (75). PMF also underlies the rotational mechanism of the proton ATP synthase. Second, the anisotropy data showed reorientation of the fluorescence transition dipole in a time frame that excludes most other types of membrane protein motion. These data do not explicitly demonstrate the rotation of TonB, but the reorientation of GFP-TonB within 2.5 ns (the fluorescence lifetime of GFP [73, 74]) excludes several other types of motion. Lateral translation of proteins in the *E. coli* IM, for example, occurs much more slowly (Tsr, $\sim 0.5 \mu\text{m}^2/\text{s}$ [76]; TonB, $\sim 3 \mu\text{m}^2/\text{s}$ [Y. Lill, L.

Jordan, S. M. Newton, P. E. Klebba, and K. Ritchie, unpublished data]). The lateral diffusion of GFP-TonB at this rate for 2.5 ns produces $\sim 0.75 \text{ \AA}^2$ of translational motion. Relative to the Stokes radius of the GFP β -barrel (28 $\text{\AA}$ [77]), this small amount of movement is insufficient to create the observed anisotropy. Motions of residue side chains (78) may occur on a nanosecond time scale, but rigid body motion in proteins is much slower, and in this case, the entire GFP β -barrel reoriented. The possibility that conformational change in the membrane regions of TonB-ExbBD rapidly reoriented the upstream cytoplasmic GFP also seems unlikely, because dynamics that bend or disrupt the N-terminal hydrophobic helix of TonB are inconsistent with the extensive H-bonding that stabilizes it. Having excluded other explanations, the most reasonable interpretation is that the rotation of TonB caused reorientation of GFP. The adage “when you hear hoofbeats, look for horses not zebras” probably applies: the sequence homology with components of the flagellar motor, combined with molecular reorientation in <2.5 ns, suggests rotational motion.

Implications of TonB rotation. (i) Transmission of force. How might TonB rotational kinetic energy transfer mechanical force that promotes conformational change in the OM LGP? For several reasons, OM proteins have different mobility than proteins in the more fluid IM bilayer, where the TonB N terminus resides. The tight association of OM proteins with lipopolysaccharide (LPS) is well known, so much so that it is difficult to purify them without LPS contamination (29, 79, 80). Therefore, in the context of the divalent cation-stabilized outer LPS leaflet of the OM (81), OM β -barrels are part of a cation-stabilized protein-LPS matrix. Perhaps more importantly, as noted above, the OM sits on and associates with the PG polymer; $>100,000$ OM-resident lipoproteins covalently attach to PG. Finally, the lipid A component of LPS in the outer leaflet of the OM has a higher transition temperature of about 40°C (82), so under physiological conditions, the OM is less fluid and potentially a frozen bilayer. These considerations suggest that a variety of forces retard the translational/rotational mobility of LGP, allowing receipt of mechanical force from the proposed rotational motion of TonB.

(ii) Lateral movement. In the context of a rotor (TonB) turning within a cylinder (ExbBD), if proton conduction generates a force that ratchets the rotor in one direction, an equal force will push the stator in the opposite direction. If the stator is anchored within the cell wall, as is the flagellar apparatus, then only the rotor (the flagellar filament) will turn. If, on the other hand, both the rotor and the cylinder containing it are capable of movement (as when located in a fluid bilayer), then their individual angular velocities (in opposite directions) will depend on their individual masses and inertias. The masses of *E. coli* TonB, ExbB, and ExbD are 26, 26, and 16 kDa, respectively. The stoichiometry of the TonB-ExbB-ExbD complex (1:6:1 [83], 1:4:1 [85], or 1:4:2 [41, 84]) implies that the TonB component, with about one-fifth of the mass and considerably less inertia, will spin faster than the ExbBD component. It is relevant that both ExbB and ExbD contain sequence or structural homology to the PG-binding LysM domain (see Fig. S1 in the supplemental material), suggesting their affinity for PG. An association of the exterior of the ExbBD complex with PG, even transient binding, will create frictional resistance that decreases the rate of its rotational motion, anchoring it relative to the rotation of TonB. Furthermore, if their LysM motifs confer transient binding to the static PG polymer, energized turning of the ExbBD complex will promote its lateral movement through the cell envelope. Therefore, en-

FIG 3 ROSET model of TonB action. TonB-ExbBD and TonB-dependent LGP are depicted in the same colors as in Fig. 2, in ribbon or space-filling formats. Other cell envelope proteins are shown in shades of gray, in ribbon format. PG appears in VS architecture, rendered in brown wireframe. FeEnt is shown in elemental colors and space-filling format. Affinity for PG localizes the TonB CTD dimer (light blue and pale yellow) at the periplasmic interface of the OM. It binds PG in low-affinity short-lived associations. The IM PMF-driven rotation of TonB and oligomeric ExbB (blue) in opposite directions moves the TonB-ExbBD complex laterally through the IM (green path) and the PG matrix, and it moves the TonB CTD complex along the underside of the OM, facilitating its survey of the OM. When it encounters an accessible TonB box of a (ligand-bound) LGP, monomeric TonB CTD recruits the polypeptide into its β -sheet. The energized motion of TonB allows this binding reaction to transfer kinetic energy to the OM, creating conformational motion in LGP that promotes the internalization of bound iron. The overall uptake pathway of FeEnt through FepA (89) in the OM and through FepCDG in the IM and its export through AcrAB-TolC to the exterior (3) are depicted by yellow arrows. FeEnt is also shown bound to FepB in the periplasm. Proton pathways through TonB-ExbBD are depicted with black arrows. The TonB-ExbBD complex is a cartoon model; it has not been structurally solved. Protein coordinates (RCSB) and PG coordinates (courtesy of Shahriar Mobashery) were rendered by Chimera. The inset shows the microscopic localization of TonB (green) and FepA (red) in *E. coli* (28, 38).

ergization by PMF provides mechanisms for both catalysis of metal transport through the OM (by TonB rotation) and lateral motion of TonB-ExbBD (by intermittent adsorption of rotating ExbBD to PG). The $\sim 20\%$ cross-linking of *E. coli* PG suggests the feasibility of protein motion among glycan strands arranged in VS architecture. Thus, in the ROSET model, electrochemically energized opposing rotational motion of the TonB and ExbBD components provides a means for the complex to laterally move through the cell envelope, allowing the TonB CTD to survey, identify, and convey mechanical force to individual ligand-bound LGP.

Besides the paper by Jordan et al. (38), two subsequent papers considered the *E. coli* TonB-ExbBD complex. Gresock et al. (41) focused on its stoichiometry (1:4:2) and endorsed the idea (37) of dimer-monomer interconversions of the TonB CTD. The authors did not address the nature of molecular motions in these processes nor their rates. Sverzhinsky et al. (85) purified TonB-ExbBD with an apparent stoichiometry of 1:4:1. Considering that Pramanik et al. (83) previously determined the TonB-ExbBD ratios to be 1:6:1, the exact composition of TonB-ExbBD remains uncertain, but it is likely that multiple copies of ExbB exist in the complex. Neither

article (reference 41 or 85) considered TonB's affinity for PG nor the energy-dependent nanosecond-scale anisotropy of TonB-GFP (38), so the mechanisms they propose are difficult to reconcile with these aspects of TonB biochemistry.

In summary, the existing data portray TonB as an energized entity beneath the OM bilayer, in complex with ExbBD in the IM/periplasm, which promotes metal uptake through OM transporters by a rotational mechanism.

The ROSET mechanism. A model of TonB action must reconcile the following. (i) The TonB N terminus anchors it in the IM (37), its central rigid domain (20, 86) crosses the periplasm, and its C terminus, which equilibrates between dimeric (22) and monomeric forms (23, 41), associates with PG (37) and binds to metal-loaded LGP (24, 25). (ii) OM proteins assemble on the PG underlying their periplasmic interface (48, 49, 55, 87). (iii) Fluorescence microscopy shows GFP-TonB localized within the central regions of the bacterial cell and unobservable at the poles of the cell (28, 38). (iv) LGP send a trans-OM signal (relocation of the TonB box) when iron binds (29, 88); the monomeric TonB CTD binds and recruits the TonB box into a 4-stranded β -sheet (24, 25). (v) Last,

TonB undergoes energized motion in the IM while associated with ExbBD (38), two IM-localized MotAB homologs that connect TonB action to the electrochemical proton gradient.

In the ROSET model, the affinity of the TonB C-terminal dimer for PG localizes it at the periplasmic interface of the OM. The relatively low (micromolar) affinity of the C terminus for PG results in short-lived associations that, in conjunction with PMF-driven rotational motion of TonB N termini in the IM, cause the dimer to turn and wend through the PG polymer, consequently moving the TonB-ExbBD complex laterally in the IM. This process allows the C-terminal dimer to survey the underside of the OM bilayer, until, when it encounters the pendant TonB box of a metal-bound LGP, the affinity of the monomeric CTD for the accessible TonB box recruits the polypeptide into its β -sheet. Recruitment of a TonB box by monomeric TonB precludes the formation of the (PG-binding) TonB dimer, in favor of the ternary complex of the ligand-receptor-TonB monomer. Because TonB is in constant energized rotational motion and ExbBD motion is retarded by its affinity for PG, this binding reaction transfers rotational kinetic energy to the OM protein, triggering conformational dynamics that promote the internalization of its bound metal complex (Fig. 3).

In more intuitive and general terms, the overall action of TonB is analogous to pulling a string hanging from a ceiling light in a dark room. To do so, one must first find the pull-string near the ceiling. In the ROSET model, the general affinity of the dimeric TonB CTD for PG localizes it near the underside of the OM (the ceiling of the periplasm), and the specific affinity of the monomeric TonB CTD for the TonB box of LGP allows its recruitment into a β -sheet (grabbing the string). All that remains for iron uptake is to pull the string: alter the conformation of the TonB box to unblock the LGP channel, allowing the passage of iron into the periplasm. PMF-driven rotation of TonB provides the force (pulls the string) that promotes conformational change in the N domain of LGP, which is consistent with existing data on the transport mechanism of FeEnt through FepA (28, 89, 90).

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