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THREE PARADOXES OF FERRIC ENTEROBACTIN UPTAKE

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1. ABSTRACT

Bacteria elaborate iron chelators that scavenge iron from the environment, including their human and animal hosts, and iron acquisition is a determinant of pathogenicity. One such iron chelate, the siderophore ferric enterobactin, enters Gram-negative bacteria through the FepA protein of the outer membrane. The ferric enterobactin transport process is a high-affinity, multi-specific, multi-component, energy dependent reaction, that is a paradigm of ligand-gated transport: FeEnt binding activates FepA to transport competency. On the basis of the FepA, FhuA, FecA and BtuB crystal structures, and in light of recent molecular biological, biochemical, and biophysical findings, this review considers the mechanism of ferric enterobactin uptake. The discussion focuses on three preeminent questions about the transport reaction: the function of the N-terminal globular domain that resides within the FepA channel, the mechanistic contributions of TonB to the activities of ligand-gated porins, and the energy dependence of metal transport reactions through the OM bilayer. Available data points to the idea that the N-terminal globular domains of these receptor proteins dynamically exit their pores during transport, creating a suction-force that pulls ligands through the surface loops into the periplasm. The functions of TonB and energy in these processes remain unknown.

2. INTRODUCTION

Bacteria need iron for so many critical metabolic processes, including glycolysis, energy generation by electron transport, DNA synthesis, and defense against toxic reactive oxygen species, that the element is indispensable to their survival. Several decades ago this requirement was correlated to bacterial pathogenesis in animals and man, and research since then indelibly linked prokaryotic iron acquisition and infectious disease. Bacteria seek and acquire iron from their mammalian hosts, by secreting siderophores that capture the metal from iron-containing proteins in animal tissues, and by synthesizing elaborate cell envelope systems that transport either the bacterial ferric siderophores or the eukaryotic iron proteins themselves. Regardless of their method of iron accumulation, bacteria are susceptible to growth inhibition by iron deprivation, which, if it occurs *in vivo*, may prevent or reduce virulence. However, the multitude of specialized, sophisticated and efficient prokaryotic systems available to scavenge Fe^{+++}, combined with our limited knowledge of how they function, makes it difficult to use this strategy as a defense against pathogenesis. Hence the elucidation of the mechanism of iron transport through the outer membrane (OM) protein FepA directly pertains to efforts against bacterial disease. The delineation of how the receptor protein recognizes and transports the native *E. coli* siderophore, ferric enterobactin

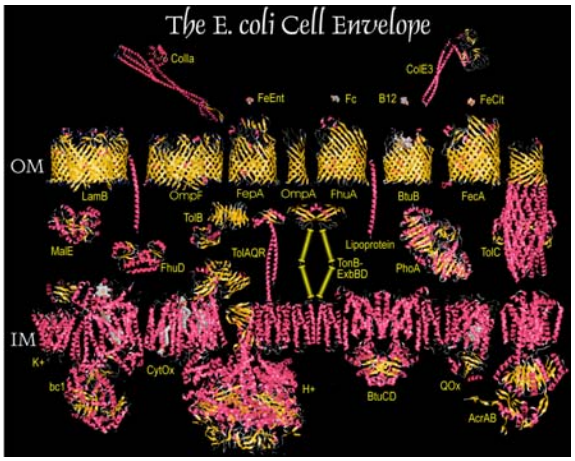

Figure 1. Proteins of the Gram-negative bacterial cell envelope. The figure depicts the structures that were crystallographically revealed in the past decade, including the ∃-barrel-containing transporters of the OM, the predominantly ∀-helical proteins of the IM, and the structurally-mixed forms of the periplasmic space. The majority of the TonB-ExbBD complex is modeled on the basis of current postulates: only the C-terminal domain of TonB was crystallographically solved. Molecular coordinates were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/) and modeled using Rasmol 2.6.

(FeEnt), furthermore, broadly relates to the other TonB-dependent iron acquisition systems of *E. coli* and other enteric bacteria, suggesting the possibility of new therapeutic strategies against bacterial pathogens.

3. LGP STRUCTURE

In the past few years, knowledge of metal transporters exploded (Figure 1), from the completion of the crystal structures of FepA (1), FhuA (2, 3), FecA (4) and BtuB (5), the receptors for FeEnt, ferrichrome (Fc), ferric citrate (FeCit), and vitamin B_{12} (B_{12}), as well as that of the C-terminal domain of the protein that they all require for functionality, TonB. During many preceding years geneticists microbiologists, molecular biologists and biochemists described the multiple protein components of cell envelope iron uptake systems, their energetic requirements, the dichotomy of beneficial and toxic ligands that enter the cell via their OM receptor proteins, the unique high-affinity nature of their uptake mechanism, their dependence on another cell envelope protein, TonB, their channel-forming properties, and their conformational dynamics in response to ligand binding. This research provided a conceptual foundation for the structures that crystallography revealed.

The first siderophore receptor that was crystallized and solved, FepA (1), contained, as expected and previously demonstrated, the largest known ∃-barrel of the OM, decorated on the exterior surface by large loops that bind ligands, and closed on the periplasmic side by its own N-terminus. In a fundamental sense receptors like FepA fulfill the definition of a porin: they contain a transmembrane pore through which solutes pass into the cell. However, they are ligand-gated in that ferric siderophore binding stimulates conformational changes that activate ligand internalization through the transmembrane channel. Further-more, the TonB- and energy-dependence of their transport reaction distinguishes such ligand gated porins (LGP) from general and specific porins: they use cellular energy to accumulate iron chelates against a concentration gradient, and the TonB-ExbB-ExbD complex in the inner membrane (IM) facilitates their transport process through the OM. In contrast to the typical trimeric arrangement found in porins, FepA, FhuA and FecA were isolated and crystallized as monomers, that contained two distinct domains: a C-terminal, 22-stranded anti-parallel ∃-barrel (C-domain) that spans the outer membrane and projects extracellular loops that function in ligand binding, and a globular N-terminal domain that folds into the barrel interior, blocking access to the periplasm (N-domain).

3.1. C-domain

General and specific porins contain anti-parallel, amphiphilic ∃-sheets that circumscribe an aqueous, open transmembrane channel. Short reverse turns on their periplasmic surfaces, and large loops on their external surfaces, join the ∃-strands within the sheet. FepA contains a comparable trans-membrane ∃-barrel, exclusively formed by its C-terminal 575 amino acids. The amphiphilicity of the component ∃-strands in the barrel, the nature of the loops and turns connecting them, and the delineation of position in the OM bilayer by aromatic residues at the internal and external interfaces, are conserved attributes among general, specific and ligand-gated porins. The most distinguishing differences between the LGP barrels and those of the other classes of porins are ∃-strand length and number: the longest strands in OmpF and LamB contain approximately 15 residues, but in the LGP ∃-strands may exceed twenty amino acids in length. The longer strands and larger surface loops project the pore vestibule higher above the cell surface, which explains the antibody recognition of epitopes within the loops in live bacteria. The LGP barrels contain 22 strands, whereas general porins have 16, and sugar-specific porins have 18. The increased number of ∃-strands creates a barrel with larger diameter, allowing passage of the larger siderophore ligands. Whereas the L3 (trans-verse) loops of general and specific porins fold inward and narrow the interior diameter of their channels, the N-domains of the LGP completely close their pores. This modified architecture of siderophore receptors demonstrates their distinctiveness among OM proteins, but also exemplifies further evolution on an existing theme: in other porins a structurally independent feature, the transverse loop, restricts channel permeability; in LGP, a novel globular assembly, the N-domain, fully regulates solute passage through the pore.

3.2. N-domain

The structurally distinct N-domain within the C-domain consists of ∀- and ∃-structure and loops that rise to the top of the channel, directly beneath the ligand binding site, that provide a signaling pathway linking ligand recognition and transport. When Fc binds to FhuA, or FeCit binds to FecA, or B_{12} binds to BtuB, residues in the loops undergo minor changes that propagate through the N-domain,

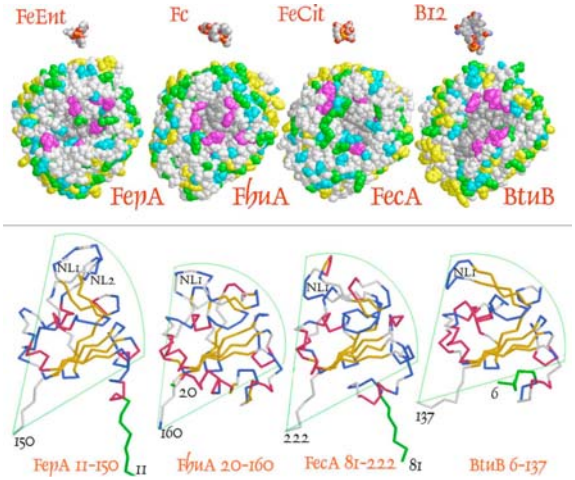

Figure 2. Top: Comparisons of FepA, FhuA, FecA and BtuB. The ligand-free receptors are shown from a top view in space-filling format. The underlying N-domains are colored grey. Basic and acidic residues in the C-domain are green and cyan, respectively; aromatic residues on the sides of the barrel are yellow, and those in the uppermost regions of the loops are magenta. Aromatic amino acids predominate around the vestibule entrances of FepA (Y217, Y260, Y272, F329 (not shown),Y481, Y495, Y553, Y540, Y638), FhuA (Y325, Y393, Y423, F557, F558, Y595, Y601, F693, Y696) and BtuB (Y328, F404, Y405, Y446, Y453, Y531, Y534, Y536, Y579), which recognize the hydrophobic metal complexes FeEnt, Fc and B_{12}. Conversely, mainly basic (R328, R373, R374, R419, R438, K525, K723) and acidic (E370, D437, D567, D662, D672, D719, D720) residues surround the opening of the FecA vestibule, which recognizes the more polar compound FeCit; aromatic residues (Y420, Y421, Y436, Y520, Y566, Y718) are less prominent Bottom: Backbone representations of the N-domains of the receptors reveal the central 4-stranded ∃-sheet (yellow), the short turns of ∀–helix (magenta), and ∃-turns (blue); the TonB-box region is green, and assumes a different position in BtuB than in FepA and FecA (here in the absence of ligands). In all four proteins the first loop of the N-domain (NL1) is large and projects toward the surface; only FepA has a substantial second loop (NL2), also directed to the surface. When considered from the perspective of their connections to the barrel (residues 150, 160, 222 and 137, respectively), the opposing surfaces of all four N-domains create the shape of an arc (outlined in green), with an appropriate curvature to permit the exit of the globular domain from the ∃-barrel.

changing the disposition of residues at the periplasmic interface of the OM. FepA was crystallized without full ligand occupancy, and comparable changes were not observed in its crystals. Although their overall topology is similar, differences occur in the folding and composition of the N-domains of FepA, FhuA and FecA, that sometimes localize analogous residues in different places. All the proteins contain four short, highly conserved sequences that assemble into four strands of a ∃-sheet and several short ∀-helices, or turns of helix. Two loops project up toward the opening of the pore vestibule. However, the N-domain the loops of FepA, FhuA and FecA fold differently, and are distinct in amino acid composition: those of FepA contain a preponderance of Arg residues at the top, whereas those of FhuA contain aromatic residues in the same relative position. The different folding patterns of the N-domain loops create individualized three-dimensional forms: that of FepA is more elongated, while those of FhuA, FecA and BtuB are more compact.

4. WHAT IS KNOWN: FeEnt BINDING TO FepA

4.1 The biphasic adsorption reaction

Eleven loops encircle the channel on the cell surface, forming an exterior vestibule through which ferric siderophores enter. Some are expansive and participate in binding of FeEnt and the toxins that pass through FepA. The 11 loops of LGP are not homologous; they may dramatically differ in length and composition. FepA, FhuA and BtuB contain aromatic amino acids in the loops that populate the mouth of the vestibule; FecA, on the contrary, does not (Figure 2). In FepA, this group of predominantly Tyr residues forms the initial adsorption site for ferric siderophores (6). Deeper in the FepA vestibule an abundance of basic residues group in a cluster at the top of the N-domain, presumably creating affinity for the triply-negatively charged catecholate siderophore.

FeEnt binding to FepA was thought to primarily involve the central region of the protein, because antibodies against surface epitopes in this region blocked FeEnt binding and transport. The dominant chemical properties of FeEnt, negative charge and aromaticity, suggested the involvement of basic and aromatic amino acids in its recognition. These predictions were verified by site-directed mutagenesis: alanine substitutions for R286, R316 and K483, Y260, Y272 and F329 impaired ligand binding.

The binding of FeEnt to FepA is a biphasic, high-affinity reaction. FeEnt initially adsorbs to aromatic and charged residues in the surface loops of FepA, in a site previously designated B1. The reaction is specific, in that it is not subject to competitive inhibition by other siderophores, except those mimicking the structure of the FeEnt iron complex. After initial adsorption, concomitant with conformational changes in the loops, the iron complex moves within the vestibule to a second site, designated B2. These binding reactions are TonB- and energy-independent events, in that they occur with indistinguishable high affinity (K_d = 0.2 *n*M) in *tonB* cells or *tonB*+ cells that are energy-starved or poisoned. In FhuA, Fc binding induces movement of the TonB-box, on the periplasmic surface, to the center of the ∃-barrel. Whether such movements occur in FepA is not known, but similar movements were inferred from ligand binding to both FecA (4) and BtuB (9, 5).

4.2. Ligand Selectivity

The ability of LGP to discriminate among different metal chelates is a striking feature of the transport process. FepA recognizes the metal center of the ferric catecholates it transports. FhuA interacts with ferrichrome in a site that complements the metal center of the chelate, lined with aromatic residues and defined by H-bonds from residues in

the N-domain and surface loops. However, FhuA shows extremely broad recognition of hydroxamates, including the dihydroxamate ferric rhodotorulate and the ferrichrome analogs ferrirubin and ferrirhodin, stereoisomers with bulky substitutions to the iron center of the chelate. This generality conflicts with the perfect complementarity between its ligand-binding site and ferrichrome. In a lock-and-key model of binding, such a perfect structural match excludes siderophores with diverse structures at the iron center. Thus another means of binding may occur, akin to induced fit of the binding site to the ligand, that accommodates molecules of different size or shape.

FepA has a more selective recognition pattern, accepting the tricatecholates FeEnt, FeTrencam, FeMecams and FeMyxochelin C, but rejecting the slightly different catecholates FeCorynebactin, FeAgro, and other analogs with chemical modifications to the catecholates around the iron. These results suggest a binding pocket restricted by size: all of the non-binding siderophores are larger, from their additional substituents, than FeEnt. Hence, although FhuA appears promiscuous in regard to ligand recognition, FepA seems opposite, fastidious to the structural nuances in its ligands.

On one hand, the similar size and coordination geometry of the iron centers of ferric siderophores, which provide the major determinants of binding, make it difficult to explain their selective adsorption to particular OM receptor proteins, whose surface structures are themselves at least superficially similar (Figure 2). On the other hand, for the most part each ferric siderophore has unique chemical properties. Consider for example, FeEnt, Fc, FeCit and B_{12}. The first has a fully aromatic character, in that the metal is chelated with three-fold symmetry and hexa-coordinate geometry, by three catecholate groups that impart a net charge around its iron center of -3. The second, ferrichrome, is not aromatic; its three hydroxamate chelation groups, derived from N-hydroxy ornithine, form an electrically neutral complex with Fe3+. The third compound, FeCit, is also a non-aromatic, neutral complex like Fc, and it achieves these properties by dimerization: two citrate moieties complex two Fe 3+ atoms. Finally, B_{12}, cyanocobalamin, contains a porphyrin-like corrinoid nucleus. Thus, the four metal complexes are chemically distinct from one another. In general, ferric siderophores are sufficiently structurally distinct so as to create unique chemical determinants, that siderophore receptors exploit in their specific recognition reactions.

On the other hand, In spite of their chemical differences, most ferric siderophores manifest a common hydrophobicity that undoubtedly plays a role in their binding to receptor proteins, and an important remaining question about the binding phase is how does ligand discrimination occur in the prokaryotic microenvironment? One possibility is that initial binding occurs by non-specific hydrophobic interactions with non-polar or aromatic amino acids in the surface loops. That is, hydrophobic side chains in the surface loops of siderophore receptor proteins may non-specifically sequester ferric siderophores, in a comparable manner to their extraction and purification from aqueous solution by organic solvents. In this case the selection of a correct ligand and the rejection of others occurs later, at a subsequent stage that precedes internalization. Alternatively, the discrimination of the correct siderophore may take place in the first stage of its adsorption process. The latter mechanism has more biochemical and physiological logic. If each receptor protein initially adsorbed several or many different classes of ferric siderophores with significant affinity, and only rejected inappropriate ligands at the secondary stage of the binding process, then the act of ligand selection would assume futile inefficiency in any environment populated with diverse organisms and siderophores (as for example, the vertebrate gut).

4.3. Discrepancies *in vitro* and *in vivo*

Experiments with a purified, fluorescently-labeled Cys mutant protein initially revealed two kinetically distinguishable stages of FeEnt binding, intimating that the ligand moves between two distinct binding sites in the surface loops. After rapid adsorption ($k = 0.02\ s^{-1}$) to the first site, FeEnt progresses more slowly ($k = 0.002\ s^{-1}$) to a second site. Crystallography supported the expectation of two potential sites in the vestibule, in that the FepA and FhuA crystals contained FeEnt and Fc in two different positions, likely corresponding to the proposed binding sites B1 and B2.

Conformational dynamics during ligand internalization are an inescapable feature of LGP-mediated transport, because their pores are completely occluded by their N-termini. Electron spin resonance (ESR) studies of nitroxides attached to four different sites (S271C, E280C, E310C, C493) in three different FepA loops, showed by three different methods (conventional, power saturation and time-domain ESR) that the purified protein undergoes loop movement when it binds FeEnt. Experiments with live *E. coli* expressing nitroxide-labeled residue E280C showed that additional, TonB- and energy-dependent conformational changes occur during FeEnt internalization. Results with both FepA (7, 8) and FecA (4) later confirmed that in the absence of ligand, surface loops L7 (FepA) and L8 adopt an open conformation, that closes when the appropriate ferric siderophore binds. Nevertheless, the crystallographic descriptions of FhuA did not show differences in the disposition of its loops with or without bound ferrichrome.

Comparisons of equilibrium binding data from purified FepA, studied by extrinsic fluorescence (K_d = 20 0M), and from live bacteria, studied by ^{59}FeEnt adsorption (K_d = 0.2 0M), disclosed a 100-fold difference in affinity of the siderophore-receptor interaction in the two conditions. The incongruity was even greater (250-fold) in binding assays utilizing FepA that was re-solubilized from crystals, and the affinity of FeEnt for the isolated N-terminus of FepA was 20,000-fold lower (10). Dissimilar behavior of proteins may occur in different environments, but disparities at this level do not likely result from experimental variation or methodological differences: it is apparent that FepA assumes different forms when resident in the OM bilayer, or when detergent-solubilized and purified. This difference was substantiated by measurement of the affinity of the FepA-

FeEnt interaction *in vivo,* using fluorescent methodologies (11), and likely stems from alterations of FepA tertiary structure that occur upon extraction from the OM bilayer. The crystallographic environment, replete with salts and/or precipitants, is distinct from that of the native membraneous state, in which the loops of FepA exist in an open conformation (8, 11). These environmental differences perhaps also explain the observation that solubilization by non-ionic detergents and crystallization produced monomeric forms of both FepA and FhuA, while evidence exists that *in vivo* LGP are oligomeric or trimeric.

4.4. The ligand-free state

The ligand-free open state *in vivo* is not likely a static conformation with loops spread like the petals of a flower. Instead, as was illustrated by crystallography, the loops are flexible in solution, imparting an overall form and motion akin to the tentacles of a sea anemone. During its diffusion near the membrane surface FeEnt encounters charged {e.g, K483; (8)} and aromatic {e.g., F329, Y272; (6)} residues at the loop extremities that entrap the siderophore in a network of non-covalent interactions comprising the first binding stage. As multiple determinants, within multiple loops, converge on the ferric siderophore, the natural affinities of the individual association reactions close the loops around metal complex, in effect creating the secondary interactions that occur with charged and aromatic residues deep within the now vestibule, W101, Y260 and R316. Hence the loops of the barrel ultimately select the correct ferric siderophore (7, 12), and the binding reactions require neither energy nor TonB to reach completion, or maximal affinity (11). Other ferric siderophores do not mistakenly adsorb to FepA because the chemistry of their metal complexes do not properly configure with the appropriate side chains in the loops of FepA. An implicit benefit of this selection mechanism is that the initial binding sites remain unoccupied, and unblocked after encounters with inappropriate metal complexes.

5. WHAT IS UNKNOWN: FeEnt UPTAKE THROUGH FepA

It is simplifying to consider transport of ferric enterobactin through FepA as a series of sequential steps, some of which are biochemically and genetically well defined, and some of which are comparatively obscure. The functions of the receptor's N-terminal globular domain, the TonB protein, and cellular energy fall into the latter category, creating three paradoxes of iron transport: how do solutes pass through a transmembrane ∃-barrel that is blocked by the globular N-terminal domain; how does TonB, a structurally simple protein that associates with the inner membrane, facilitate the transport activity of receptor proteins in the outer membrane; what are the energetic requirements of metal transporters like FepA, and how are they fulfilled?

The sub-reactions of FeEnt uptake, subsequent to binding, are less clearly resolved. The ferric siderophore begins the transport phase localized at the top of the N-domain, and through an unknown sequence of energy- and TonB-dependent events that involve further conformational changes in the loops (5), and unavoidably, movement of or in the N-domain as well, it traverses the FepA channel and enters the periplasm. Three preeminent questions remain about the mechanism of metal transport, that derive from three paradoxes of existing data:

1. *How does the N-domain regulate pore activity?*
2. *What is the function of TonB?*
3. *How is metal transport energized?*

The LGP crystal structures, and those of the C-terminal domains of TonB and TolA completely changed the study of OM metal transport, superceding the structural guessing games that preceded them. Nevertheless, mechanistic uncertainties persevere, as illustrated by the unexpected disposition of the N-domains within LGP channels. The latter two conundrums are historical (circa 1970) artifacts that still confound ferric siderophore transport mechanisms.

5.1. Paradox 1: Transport through a closed channel

The position of the N-domain inside the C-domain leaves no opening, gap, or pore through which FeEnt may pass, insisting that structural changes must occur in FepA during transport. FeEnt binds to FepA with a sub-nanomolar K_d, that translates into a dissociation half-life of over a minute, and this calculation conflicts with the receptor's experimentally observed, 20 second turnover time. LGP monomers apparently bind only a single molecule at a time, and transport it against a concentration gradient, so their uptake thermodynamics differ from those of general or specific porins, which transport solutes through open channels by mass action. Hence internalization of FeEnt requires a driving force. These points argue for protein conformational changes that undermine the affinity of the siderophore-receptor binding interaction, creating a pathway to the periplasm, and propelling the metal complex from the surface loops through the pore.

5.1.1. Insights from sequence

The N-domain contains approximately 150 amino acids that compactly fold into the periplasmic outlet of the barrel domain. Protein sequence and structure analyses (Figure 3) reveal several important features of LGP N-termini. They are predicated on a 4-stranded ∃-sheet that is remarkably similar among them. Sequence comparisons of FepA homologs, some of which transport FeEnt and some of which transport other ferric siderophores, or iron from lactoferrin or transferrin, show the conservation of individual ∃ strands within the N-domain sheet, as well as a variety of primarily basic residues that distribute on its surface, at the aqueous interface with the barrel walls (Figures 3, 4). The conservation of residues within the barrel and N-terminal domains of FepA and its relatives, juxtaposed against the diversity in their surface loops, suggests that once bound, siderophores pass through LGP channels by a common mechanism that involves the N-domain. The similarities further establish that the mechanism of iron acquisition from eukaryotic iron-binding proteins, by the transferrin and lactoferrin receptors of

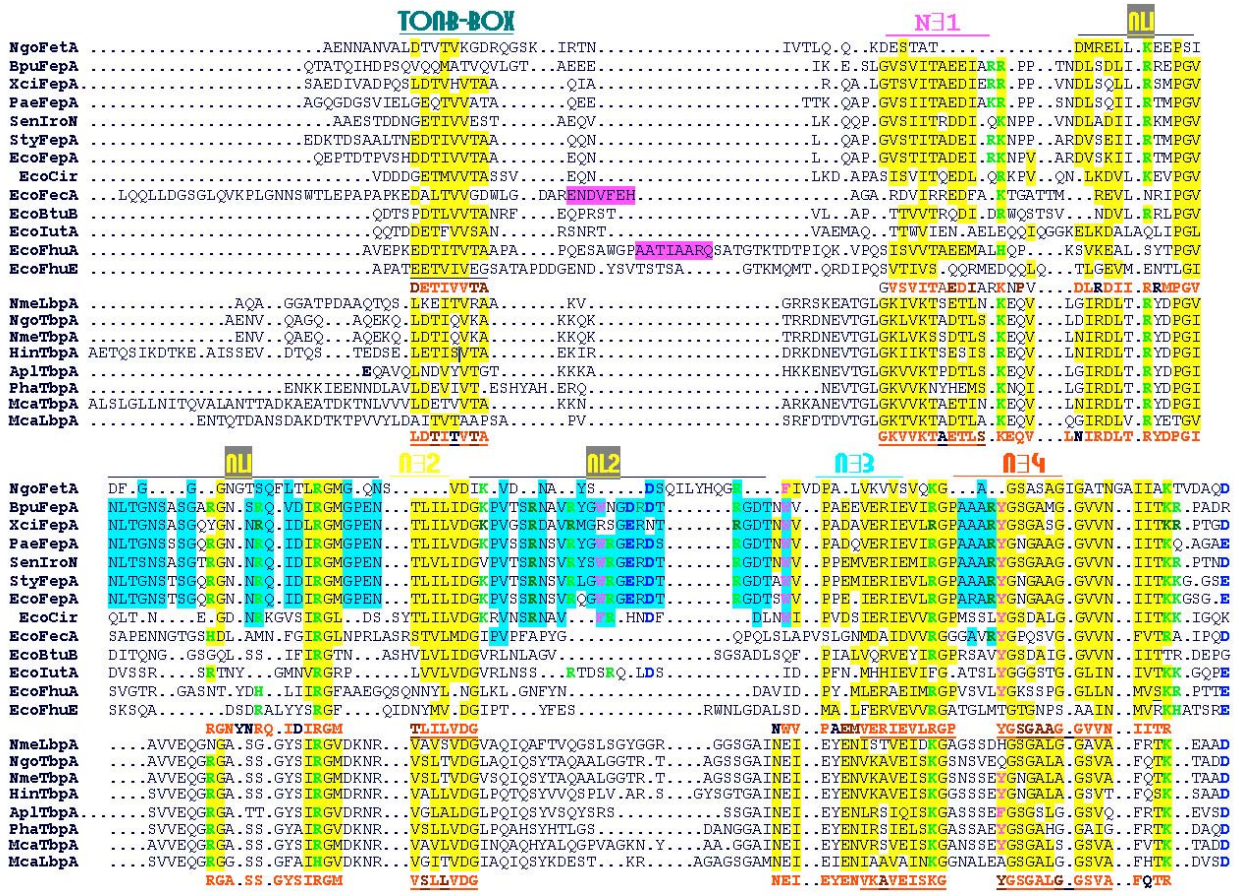

Figure 3. Sequence alignment of LGP N-termini. The top seven proteins, of *Neisseria gonorrhoea* (NgoFetA), *Bordetella pertussis* (BpuFepA), *Xanthomonas citri* (XciFepA), *Pseudomonas aeruginosa* (PaeFepA), *Salmonella typhimurium* (StyIroN, StyFepA), and *E. coli* (EcoFepA) are orthologs that transport FeEnt. The next six proteins (EcoCir, EcoFecA, EcoBtuB, EcoIutA, EcoFhuA, EcoFhuE) are *E. coli* LGP paralogs. The lactoferrin and transferrin binding proteins of *Neisseria meningitides* (NmeLbpA, NmeTbpA), *N. gonorrhoea* (NgoTbpA), *Haemophilus influenzae* (HinTbpA), *Actinobacillus pleuropheumoniae* (AplTbpA), *Pasteurella haemolytica* (PhaTbpA), and *Moraxella catarrhalis* (McalbpA, McaTbpA) are shown below. Basic residues are colored green, acidic residues blue, aromatic residues magenta. Significantly conserved regions among the 21 proteins are highlighted yellow; the consensus sequences below indicate moderately (black), highly (maroon) and very highly (red) conserved residue. Common residues in FepA orthologs are highlighted blue; the switch helix regions of FecA and FhuA are highlighted pink.

Gram-negative bacteria, is fundamentally similar to that of ferric siderophore receptors.

The identification of common amino acids in the transporters of different ligands is the primary rationale underlying the search for mechanistically important residues in LGP. FepA, Cir, FecA, BtuB, IutA, FhuA and FhuE (Figure 3) recognize two catecholate, one carboxylate, one corrinoid, and three hydroxamate metal complexes. Four of the chelates are uncharged, one has a net charge of -1, and two have net charges of -3. Therefore, conserved amino acids among the seven do not likely derive from ligand recognition properties, but they may originate from shared mechanistic features. Additionally, because different receptors transport ligands with different efficiencies, in any individual transporter particular residues may be more or less conserved than others in its homologs/paralogs. Thirdly, aligned residues in the sequences of the solved proteins do not always locate to comparable positions in their tertiary structures. In their N-domains, for example, diverse positions in sequence may fold to homologous locations in tertiary structure (FepA **147**, **148**, FhuA **154**, **155** *vs* FecA **109, 196**). Similarly, in the barrel, non-homologous amino acids sometimes localize to comparable sites (e.g., FepA **E248**, **E250** *vs* FhuA **E163**, **D179**). These considerations suggest that mechanistic residues may show less than complete identity in aligned LGP sequences (Figure 3).

5.1.2 Ion-pairs

Beneath the vestibule a common feature appears in FepA, FhuA and FecA: ionic interactions between the N-domain and the ∃ barrel wall. Basic residues in the N-domain and acidic and basic residues on the barrel

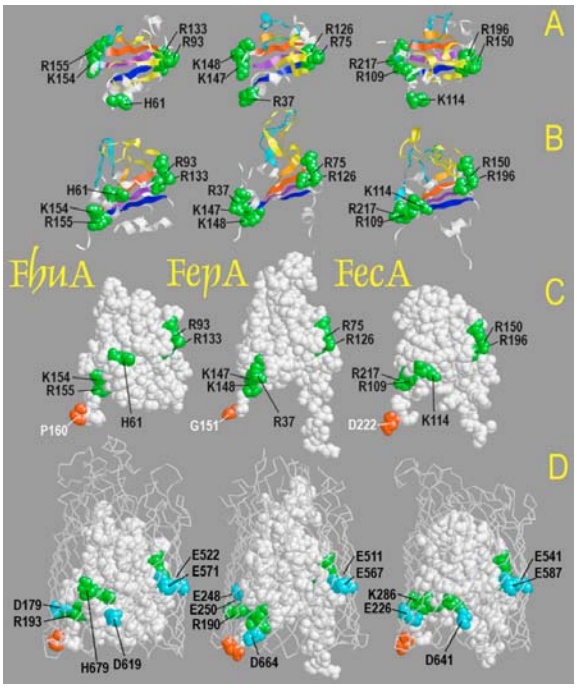

Figure 4. N-domain structure, and proposed Ion-Pair interactions. Top (A) and side (B) views of the ∃-sheets within the N-domains of FhuA, FepA and FecA, in ribbon format. The colors of the individual strands within the sheet correspond to those shown in Figure 3. Conserved basic residues (green) project from the sheet, shown in space filling format. These amino acids, on the surface of the N-domains (C), distribute around the ∃-sheet, within ion-pair distance to acidic (cyan) and basic residues (green) on the barrel wall (D; white, shown in backbone format). The last residues of the N-domains, that connect to the first ∃-strand of the barrel, are colored red.

appropriately converge in positions to form ionic bonds that stabilize the N-domain within the C-domain (Figure 4). The basic residues that exist on the N-domain surface are conserved in sequence, and derive from regions of structure within or at the extremities of the ∃-sheet strands. These pair with Asp and Glu residues on the interior barrel walls, also at equivalent positions in all three transporters.

The ion pairs are best observed by rendering the barrel transparent and viewing residues of interest from the exterior (Figure 4). In FepA, on the side of the protein where the N-domain connects to the barrel (the "hinge" side), basic residues in the N-domain **K147, K148**) exist within ionic bond distance (<3.5 Å) to two acidic residues **(E248, E250)** on the barrel wall. On the opposite side of the globular domain (the "lock" side) other potential ion pairs exist, joining **R75** and **R126** to **E511** and **E567**. These two sets of apposed charges portray the N-domain as a door, with a hinge and a lock. Analogous amino acids exist in comparable locations within FhuA and FecA (Figure 4). Flanking the hinge region are other charged residues **(R190, K37, D664)** The noted ion pairs fulfill a few conditions that suggest their indispensability to transport. They are conserved and comparably spaced in the sequence alignment. Certain of the charged residues are the most conserved residues in LGP sequence. From a mechanistic perspective, they reside on the exterior surface of the N-domain and internal surface of the barrel, matched with residues of opposite charge; any meaningful changes in N-domain structure probably require the dissolution of these interactions.

5.1.3. Transport models: Sequential transport

The crystallographic data from FepA, FhuA and FecA resolved questions relating to their structural organization, but not those of their transport mechanisms. It is worthwhile to consider the implications of two mechanistic extremes of the transport process, "sequential" and "concerted" transport reactions. Ample precedent exists for the stepwise movement of molecules through membrane channels, including the transport of ions through the acetylcholine receptor, the potassium and chloride channels, bacteriorhodopsin and the proton ATPase, and the passage of various solutes through general porins, and sugars through specific porins. FeEnt passage through FepA may occur by similar sequential transport, that passes the acidic siderophore along a series of basic residues located on the interior of the ∃-barrel, and/or within the N-domain. Although LGP do not contain any pores or gaps in their interiors that might allow passage of a ferric siderophore, variations from these structures *in vivo,* combined with conformational changes, may transiently open a path to the periplasm. Maltodextrins traverse maltoporin through a very small pore, of 6 Å diameter at its constriction point, so narrow that it forces de-solvation of the sugars during transit. Ferric siderophores are considerably larger than a hexose, implying that a transient pore through FepA must acquire a minimum diameter of 15 - 20 Å. The existence of successive sites through the channel domain, with increasing affinity for the solute, is another prerequisite of the process. Potential basic candidate residues align across the FepA channel, but the high initial affinity of FeEnt binding in the surface loops creates a criterion of avidity that subsequent sites must overcome. Furthermore, mass action drives the movement of solutes through general and specific porin transport systems, a crucial difference relative to transport of ferric siderophores, which are present in small concentrations and actively accumulated: this requirement constitutes a primary argument against a sequential transport mechanism for FepA. In a sequential transport process the input of energy must accomplish several independent conformational rearrangements in FepA, the disruption of the initial binding sites, and the simultaneous creation or exposure of subsequent binding sites of increasing affinity within an appropriately-sized, nascent channel, that guide the ferric siderophore through the protein interior to the periplasm. The ability of FhuA to internalize a variety of ferric siderophores of with divergent chemical properties and masses (ferrichrome, ferrirhubin, ferrirhodin), and also the antibiotic rifamycin CGP-4832, is another argument against a sequential transport mechanism, because it presupposes that the mechanistic binding sites within the channel are themselves highly promiscuous.

5.1.4. Concerted transport: N-domain exit

The N-terminal domain is a unique folding pattern among all other solved protein structures, and its singularity raises the possibility that it also functions in an unprecedented way: an all-or-none transition from a structure that occludes the FepA pore, to a structure that promotes movement of FeEnt into the periplasm. The short ∃-sheet, ∀-helices and connecting loops within the N-domain may lend themselves to such a rearrangement According to this view the binding of FeEnt converts the N-domain into an activated form, that is then recognized and "triggered" to transport by cellular energy. At least two kinds of conformational rearrangements may accomplish transport, a global alteration in the N-domain that reduces its size and narrows its shape within the barrel, or dislodgement of the entire N-domain from the barrel. Either action achieves continuity with the periplasm, and accomplishes transport if the surface loops simultaneously close to prevent back-diffusion. However, it is difficult to envision a rearrangement of the N-domain to diminish its already densely compacted shape, and I will not further consider this mechanism.

The location, composition and arrangement of stabilizing ion-pairs insinuate a mechanism for motion of the N-domain. The susceptibility of FeEnt transport to PMF inhibitors raises the possibility that protons, routed to the barrel interior, disrupt the ionic bonds between these residues. If the aqueous milieu within the pore drops to a pH below 4, then protonation of acidic side chains on the inside of the barrel will neutralize the salt bridges. The existence of additional basic residues on the hinge-side of the barrel wall raises the possibility that in such conditions charge repulsions will occur with basic residues on the N-domain surface, causing movement, perhaps expulsion, of the N-domain from the pore. So the overall structure suggests that the N-domain acts as a door, hinged to the barrel on one side and locked closed by salt bridges around its circumference. A decrease in pH in the barrel may both unlock the door and actuate an electrostatic force that initiates its opening. The compact, conserved overall architecture of the globular domain, especially the arc-like surface that apposes its hinge-like connection to the barrel (Figure 2), is consistent with its concerted movement out of the channel.

When the FepA and FhuA crystal forms were solved, structuralists argued against the notion of N-domain exit from the ∃-barrel, because of the existence of over 50 potential hydrogen bonds that presumably hold it in place, between its surface residues and residues on the barrel walls (1, 2, 3). However, Scott *et al.* (7) argued for the concept of N-domain expulsion during transport. The unique structure of the N-domain may portend a novel mechanism, and data do not yet exist to assess the feasibility of its exit from the barrel. Presumably it enters the pore, because the ∃-barrel correctly assembles in its absence. Evidence does exist that the presence of the N-terminus in the channel optimizes or activates the motions of the surface loops for binding (7, 8, 12); charge interactions between the N– and C-domains may mediate this action. Pore closure in the absence of ligand is another potential function of the globular domain, that prevents influx of natural detergents that disrupt the inner membrane. This configuration maintains the physiological requirement of a selective OM permeability barrier.

The *a priori* bases for the N-domain-exit (Ball-and-Chain) mechanism are substantial. Regarding intra-protein H-bonds that may prevent movement of the globular domain, several points are germane. **(i)** The interior of the barrel is hydrophilic, a general porin characteristic that also occurs in siderophore receptors. Not only charged amino acids, but also polar, uncharged residues cover the interior walls of FepA, FhuA and FecA. So in biological environments the channel is water-filled. This layer of water separating the N– and C-domains, which was perhaps inadequately seen in the FepA crystal structure relative to the aqueous environment *in vivo*, showed that many of the potential H-bonds between the N-domain and the barrel are bridged by water molecules. **(ii)** The existence of 50 intra-protein H-bonds does not preclude exit of the N-terminus from the barrel, as long as a majority are re-formed as the opening occurs. The re-formation of the H-bonds may occur between the side-chains and water, with other side chains, or with H-bond acceptors/donors on the ferric siderophore, without any energetic penalty. **(iii)** The strength of H-bonds in proteins is 1.5-1.8 kCal/Mol, so even the energetic equivalent of hydrolysis of a single ATP (7.5 kCal/Mol) is sufficient to account for the breaking of 5 H-bonds without reformation. At present, the energy requirements of the transport reaction are unknown, but according to these considerations 10 ATP are needed to compensate the breakage of 50 H-bonds. This upper limit is probably not necessary because the N-domain departs into the aqueous periplasm, where H-bonds may immediately reform with water. Those that do not reform may justify the need for energy to internalize the solute. (iv) Finally, unstated in the argument against N-domain exit was the fact that a sequential mechanism also requires extensive dissolution of hydrogen bonds, in this case between the strands of the N-domain ¸-sheet, and presumably also between the N-domain surface and the barrel walls. The dissolution of a 4-stranded ¸-sheet will require a significant energy contribution, and no obvious mechanism exists to achieve it.

The strong conservation of charged residues on the interacting surfaces of the N– and C-domains suggests that not only the amino acids, but also the surfaces themselves, are intimately involved in the transport process. One potential driving force for N-domain expulsion is simple and readily rationalized. If protons enter the barrel from the periplasmic side, neutralizing negative charges and unmasking positive charges on the barrel walls, then electrostatic repulsive forces may arise that dislodge the N-domain from the channel. Movement of the N-domain out creates negative hydrostatic pressure in the pore, that pulls the surface loops closed, altering the ligand binding site to release the ferric siderophore into the channel, now continuous with the periplasmic space. TonB may participate in these reactions in several ways and at several stages (see following).

Some of the data supporting this so-called Ball-and-Chain mechanism derive from hybrid receptor proteins

that encode the N-domain of FepA, and the C-domain of FhuA (FepNFhu∃) (7). These constructs, which correctly bind and transport ferrichrome, demonstrate that the primary recognition specificity of the receptor protein resides in its loops, and that the N-domain plays a non-specific role in the transport process. Secondly, genetic constructs that completely delete the N-terminus, leaving only the empty ∃-barrels of FepA and FhuA (13, 7), still showed residual, TonB-dependent transport activity for their appropriate ferric siderophores. These data intimate that the empty barrel is, in fact, a transport intermediate. It was reported, nevertheless, that the activity of such constructs originates from complementation with a cryptic FepN-domain in the host bacterium (14). If they are valid, then these data provide further evidence for the N-domain expulsion model, because they demonstrate the feasibility of the independent movement of the isolated N-domain into the pore. Another attribute of the Ball-and Chain model is that it conceptually solves certain kinetic, thermodynamic and physiological problems. As a channel to the periplasm arises by N-domain exit, concomitant loop motion/closure collapses the ligand-receptor binding interaction and prevents solute escape to the exterior, eliminating the need for a driving force of transport. The ferric siderophore will enter the periplasm by diffusion, perhaps enhanced by low-level affinity for the N-domain itself, and accumulate therein by adsorption to its binding protein.

5.2. Paradox 2: TonB-mediated transport facilitation

Despite intense interest, the mechanistic participation of the 239 residue TonB protein in metal transport remains unknown and enigmatic. Its connection to energy metabolism, postulated since the nearly simultaneous discovery of the TonB- and energy-dependence of iron transport by Wang and Newton, still has an inherent logic. Only a few exceptions exist to the almost ubiquitous need for TonB mediation of LGP transport; these include the penetration of T5 through FhuA, E-colicins through BtuB, and cloacin DF13 through IutA. The uptake of all metal complexes requires both TonB and energy, leading to the assumption that the two prerequisites are fulfilled by one molecular entity. This conclusion is not conceptually objectionable, but the idea that the concept is proven is incorrect (15, 16): although inferential data exist for the connection between TonB and energy, and for specific physical interactions between TonB and ferric siderophore receptors, no unequivocal proof exists for either theory. Nevertheless, considerable progress has occurred in the understanding of at least TonB, including the important demonstration that its C-terminus exists in close proximity to OM proteins in the cell envelope, as evinced by the crosslinking studies of Postle and colleagues.

Genetic and sequence data identified an approximately 7-residue, moderately conserved sequence, called the TonB-box, near the N-terminus of siderophore receptors, that was proposed to contact TonB residue Q160. The sequence conservation itself is merely tangential to a possible relationship with TonB, as illustrated by the fact that crystallography revealed proposed TonB-boxes 1.5 and 2, described by Phan *et al.* (17), as two ∃-strands of the N-domain. In fact, no true identity exists in the TonB-boxes of LGP (Figure 3) that might portend a specific biochemical interaction with a single site on a single protein. This realization argues against it's proposed binding to site Q160 in TonB. The crystal structure of the TonB C-domain reinforces this inference, because its overall form is smooth and contains no obvious binding clefts, and because Q160 is apparently blocked from access to the inner OM surface by the 70-residue C-domain dimer. Crosslinking studies involving TonB, either by activation with formaldehyde or by introduction of single Cys residues in the TonB-box region of the receptor proteins, at or near TonB residue 160, were interpreted as evidence that TonB acts as an energy transducer in metal transport reactions (18, 19). But proximity is not energy transduction. The experiments established that TonB is close enough to OM proteins in the cell envelope to form crosslinks with them. Similar interactions occur between TonB and FepA proteins lacking a TonB-box (12), and between TonB and the non-TonB-dependent OM protein OmpA (12, 20); TonB associates with the OM even when siderophore receptors are not present within it (20), underscoring the lack of specificity in the interaction. Furthermore, the TonB-dependent uptake of ferric siderophores by receptor proteins lacking the complete N-domain, including the TonB-box (13, 17), questions the proposed significance of this region to iron transport [footnote].

If the TonB-box of FepA does not specifically interact with TonB, then what is its function in FeEnt uptake? The region shows moderate conservation among iron transporters of Gram-negative bacteria, though less than that of strands in the N-domain ∃-sheet (Figure 3). The allegedly specific interaction between the proteins, which received support from the crystallographic finding that ligand binding relocates the TonB-box regions of FhuA and FecA from a position close to the barrel wall, to the center of the channel, neglects the existence of between 6 and 33 (82 if one includes the special case of FecA) residues upstream, that manifest an overall negative charge, flexibility, and intervene between the TonB-box and the TonB C-domain (Figure 3). Though movement of the TonB-box in response to ligand binding was seen as a signal of occupancy to TonB, it is conceivable that the interaction between the TonB-box and the barrel wall is itself the paramount association, because it physically holds the N-terminus in place in the barrel, maintaining the channels closed in the absence of ligands (Figure 5). In FepA and FecA these associations show nearly perfect complementarity between the non-polar surfaces of the TonB-box and the barrel wall. This explanation rationalizes the moderate overall conservation of TonB-box sequences: their true specificity may not embody interactions with a single, energy-transducing protein, but rather, hydrophobic bonds to residues on their individual barrel walls. It's relevant that in FepA and FecA this interaction between the TonB-box and the barrel physically shields the ion pairs in the lock region (in FepA, between **K75-R126** and **E511-E567**). Movement of the TonB box to the center of the channel permits access to this site. In this alternative model, ligand binding breaks the association between the

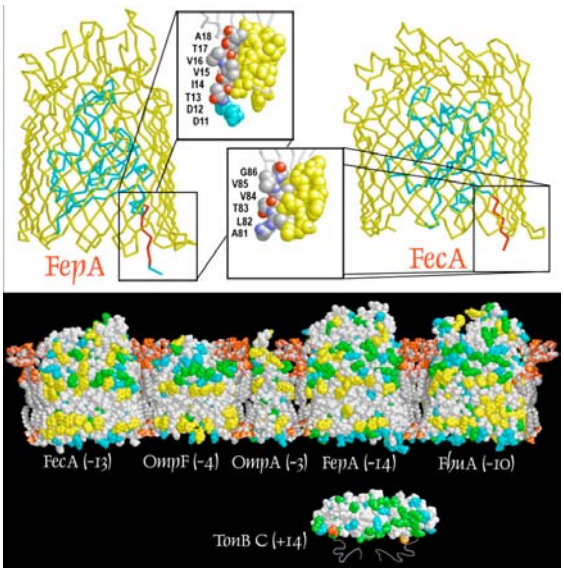

Figure 5. The TonB-box and TonB. (Top) A backbone representation of the N-termini of FepA and FecA (cyan), shown within their respective ∃-barrels (yellow), illustrates the disposition of the TonB-box (red) region. In both protein, the TonB-box (inset: seen in space filling form with CPK colors) packs tightly against the barrel wall, held by interactions between hydrophobic residues on both surfaces. (Bottom) The overall negative charge of the periplasmic surface of the OM, from phospholipid head groups (CPK colors) and acidic residues of OM proteins (cyan), likely explains the non-specific affinity of the TonB C-terminus for the inner surface of the OM bilayer: the TonB C-terminus shows an overall positive charge as a result of the presence of a preponderance of basic residues (green). The first solved residue of the domain (165) is red, and the last solved residue (238) is orange.

TonB-box and the barrel wall, not as a signal to TonB, but as a means of releasing the N-domain to swing out of the channel.

The biochemical function of TonB is the second major uncertainty of ferric enterobactin transport through FepA. The possibility exists that current perceptions of its role in transport facilitation, especially its ability to mechanically transmit energy, are fundamentally incorrect. Despite the intuitive connection between TonB and energy transfer, to date no experimental data directly implicate TonB in energy metabolism. Although purportedly involved in PMF-mediated facilitation of transport (18, 9, 19, 20), TonB is not known to either create a proton gradient or to utilize one. Although postulated to induce inner and outer membrane fusion, or to physically jump from the inner membrane to the outer membrane, carrying with it potential energy stored in a unique conformation, neither the origin of the protein's ability to leave one membrane and enter another, nor the nature of the energy-transducing conformation, are demonstrated. The most significant conceptual problem with current ideas is that they do not presuppose well demonstrated biochemical mechanisms. To summarize the major findings implicating TonB in an OM-associated activity: (i) genetic experiments show partial suppression between mutations in siderophore receptors and mutations in TonB, suggesting physical contact between the proteins; (ii) studies with crosslinking reagents indicate the proximity of TonB to proteins in the inner and outer membranes; (iii) TonB is an absolute requirement of OM iron transport, and such systems also require energy, for the *a priori* reason that they concentrate solutes against a concentration gradient, and the *a posteriori* reason that energy starvation and energy poisons eliminate iron transport. However, none of these observations and correlations constitute evidence that TonB transduces energy through the cell envelope. This is not to say that the participation of TonB in the energetic aspects of OM transport is inconceivable, but rather, that the conclusion that it is an energy transducer by some kind of mechanical process, based on existing crosslinking and genetic data, is inherently flawed. For example, FepA chemically crosslinks to OmpA and OmpF (8), but this does not imply that these proteins participate in the FepA transport process.

It is of interest to pose the question, if TonB does not itself charge with energy, diffuse across the periplasm, and discharge the energy to proteins in the OM (18), or achieve some approximation of this action, then what is its function in the cell envelope? Along these lines, new data are relevant.

1. TonB is present at low levels in the cell envelope, and forms a dimer (21), making the number of active, TonB-containing assemblies approximately 150 - 500 copies per cell (20).

2. As proposed (16) the TonB C-terminus forms a ∃∀∃ structure (21), that structurally resembles the C-terminus of TolA (22), a periplasmic protein that associates with PAL, a peptidoglycan-associated lipoprotein. Though the complete structure of TonB is unknown, its overall domain organization bears similarity to that of TolA. The latter protein, which associates with TolQ and TolR, performs a structural role in the cell envelope, and its integrity and ability to crosslink to peptidoglycan-associated lipoprotein (PAL) are apparently PMF-dependent (23). TolA was implicated in LPS biosynthesis (24).

3. TonB's reported affinity for OM proteins extends not only to LGP, but to the major OM protein OmpA, and lipoprotein as well. Immobilized OmpA adsorbs the TonB C-terminus from solution (12), and OmpA and Lpp are two of the cell envelope proteins that may crosslink to TonB after formaldehyde activation. Finally, TonB associates with the OM even in the absence of any siderophore receptors therein (20). This finding reiterates the non-specificity of the associations that TonB experiences in the cell envelope, which was previously seen in immunoblots of crosslinking reactions involving TonB, but was interpreted as a specific, ligand-potentiated affiliation between TonB and siderophore receptor proteins. Nevertheless under the conditions employed to study this

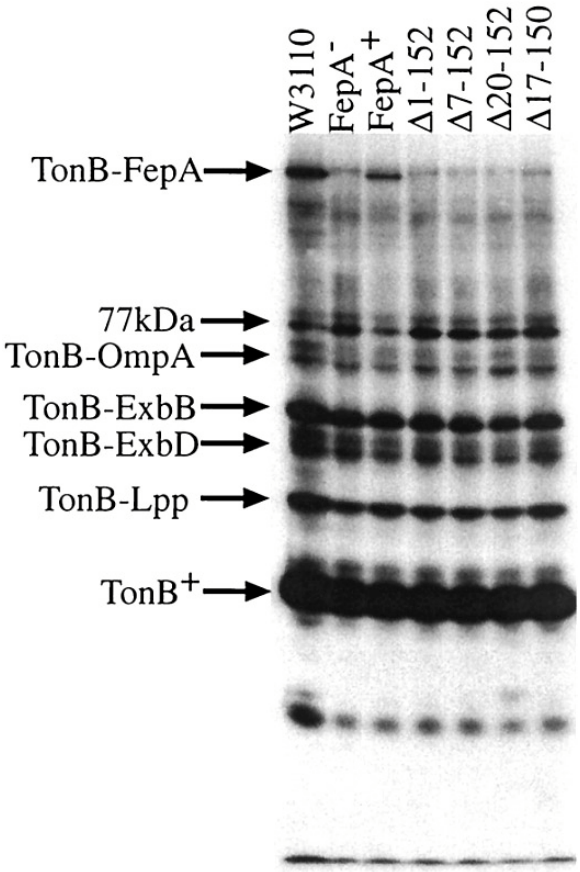

Figure 6. Non-specific crosslinking of TonB to other cell envelope proteins. Immunoblot analysis of formaldehyde-generated crosslinking, developed with anti-TonB serum. (From (27) reprinted with permission of the American Society for Microbiology). Approximately 50% of the total TonB present in the sample crosslinked to 15-18 cell envelope proteins, including lipoprotein and OmpA.

phenomenon, TonB crosslinks to at least over 15 cell envelope proteins ((14); Figure 6).

4. The TonB-C terminus is necessary for activity. Expression of the cloned C-terminal 69 residues of TonB inhibits the activity, and the isolated C-terminus shows the tendency to spontaneously insert into phosphatidyl choline liposomes (12).

From these and previous findings, the picture of the cell envelope that emerges portrays associations of OM proteins in complexes that include or are nucleated by the TolAQR, Pal and Lpp proteins, or the TonB, ExbBD and OM proteins. In the former case, existing evidence links the TolA system to the structural integrity of the cell envelope, LPS secretion and assembly. In the latter case, evidence links the TonB system to OM metal transporters. Together, these data suggest that both TonB and TolA span from the IM, where they associate with ExbBD and TolQR, respectively, to the OM, where they associate with integral or peripheral proteins, including lipoproteins, OmpA, FepA, etc. The exact natures of these affiliations are unknown, but they presumably involve non-covalent interactions with either the periplasmic interfaces of OM proteins, or the lipid bilayer itself. Thus the notion again arises that TonB (and TolA) bridge the two bilayers across the periplasm. A theoretical requirement likely exists for molecular trafficking between the two membranes, at least for biosynthetic reasons, and although the exact mechanisms of protein and lipid insertion into the OM are not known, evidence for zones of adhesion between inner and outer membranes does exist. Furthermore, studies of LPS biosynthesis revealed that export to the OM is blocked in TolA mutants (24). These data impart a previously lacking functionality to the inter-membrane assembly that TolA participates in: it acts in the movement of LPS molecules from the IM to the OM.

It is inaccurate to imply that TonB-mediated energy transduction is already demonstrated, or that its mechanism is understood. For example, the statement that "upon forming a complex with an outer membrane receptor, TonB releases stored energy, possibly in the form of mechanical force, and assumes the discharged conformation," (25) is not fact, but speculation. From the same data other consistent structural and mechanistic models may arise. One of these, for example, permanently anchors the N-termini of TonB and TolA in the IM by their trans-membrane hydrophobic helices, and non-covalently, *non-specifically* associates their C-termini with the OM. This affiliation with the OM is neither transient nor stimulated by energy, it is chemical in nature, involving ionic or hydrophobic bonds.

Although the FepA, FhuA and FecA ∃-barrels contain many Arg, Lys, His, Asp and Glu residues, they distribute with the same general pattern in all three proteins: the exterior surfaces of the vestibules are primarily basic, and the periplasmic surfaces of the barrels are acidic (Figure 5). The TonB C-domain, on the other hand, has a basic surface, suggesting its association with the acidic periplasmic surfaces of the lipids and proteins of the OM. In the case of the TolA C- terminus, the associations may primarily occur with peptidoglycan-associated, OM-imbedded lipoproteins. In this way TonB and TolA, complexed with their accessories ExbBD and TolQR, respectively, may span the space between the inner and outer membranes. Stoichiometric discrepancies (16, 14), and the much reduced fluidity of the OM make it unlikely that siderophore receptors permanently localize at these proposed, specialized transport zones containing the TonB and TolA complexes. However, preferential association with TonB is unnecessary in the model, because the proposed inter-membrane bridges have inherently random mobility, by virtue of their residence in the fluid IM bilayer, and their non-specific interactions with the internal surface of the OM. Such protein-mediated membrane connections may perform a variety of physiological functions, including biosynthesis (suggested by the requirement of TolA in the LPS export process) and metal transport (illustrated by the requirement for TonB in the FeEnt uptake reaction). In this context, one of their physiological roles is to provide a structural framework that may enable, in the case of TolA, a pathway for passage of the strongly hydrophilic LPS O-antigen through a

membrane bilayer, and in the case of TonB, a pathway for energy transfer that allows siderophore-mediated iron uptake. This postulate does not, however, resolve either the nature or the mode of delivery of the intra-membrane energetic currency.

5.3. Paradox 3: Active transport in an ion-permeable membrane

The biochemical constraints of metal transport, low extracellular concentrations of ferric siderophores, and high affinity between the metal complexes and their surface receptor proteins, create a convincing logic for active transport of iron across the OM. How the cell accomplishes this feat in the OM, which contains more than 10^5 10 Å holes, is a formidable question. Certainly, an unusual, undiscovered energetic system exists that acts on ligand-bound receptor proteins, stimulating them to ligand internalization. In this regard, it is difficult to conceive of TonB as a molecule that transduces the energy. Relative to other proteins involved in energy metabolism, TonB manifests stark differences. It is a small protein with undistinguished structural features (the sole exception is the central Glu-Pro, Lys-Pro rod region, that is reportedly dispensable to TonB function), present at low amounts in the cell envelope. The usual pathways of energy production and utilization, through oxidation of carbon sources, reduction of sequential electron carriers that pump protons, and dissipation of PMF to generate ATP, involve large multiprotein complexes that usually contain chromophores. The F_0F_1 ATPase, a relevant prototypic membrane protein that utilizes PMF to phosphorylate ADP, is thought to exist as a complex of 22 subunits of 8 different proteins. So if TonB-ExbBD delivers energy to the OM, it does so despite an undistinguished structure, and by an unprecedented mechanism. Therein lies the fascination with the energy transfer process, but also the need for conclusive proof.

5.3.1 Bioenergetics

Only a modest amount of data exists on the energetics of metal acquisition. Much of what is known originates from only a few papers on the transport of vitamin B_{12} (26), phages (27) and FeEnt (28). In the case of vitamin B_{12}, uptake of the metal complex by wild type *E. coli* is insensitive to cyanide. Inhibition of B_{12} uptake by cyanide only occurred if the bacteria were devoid of an ATPsynthase, suggesting that even if electron transport stops (and thereby also the normal production of PMF), transport still continues if ATP is utilized to generate a proton gradient. This result was observed in two separate sets of experiments, that considered both overall uptake to the cytoplasm, and uptake across the OM (26). The latter experiments also found susceptibility of the OM transport stage to CCCP, and that cyanide actually stimulated the B_{12} uptake process. For ferric siderophore transporters, however, a different pattern of inhibition occurs, in that their activities are blocked by both cyanide and inhibitors of phosphorylation. Hancock and Braun (27) reached this conclusion when studying the irreversible adsorption of bacteriophages T1 and N80 to FhuA. Furthermore, like FhuA activity, inhibitors of electron transport and phosphorylation blocked the uptake of FeEnt by *E. coli*, as did agents that deplete PMF (28). These initial experiments did not specifically isolate the OM transport stage of the ferric siderophore, only the overall uptake process into the cytoplasm. But experiments with an *in vivo* fluorescence system that focused on FeEnt uptake through FepA corroborated the inhibitory effect of both cyanide and arsenate, as well as susceptibility to PMF-depletion (11).

These data portray differences among the energy requirements of OM metal transporters, hinting that PMF may not play an exclusive central role. The main evidence for the energization of siderophore receptors by PMF derives from Bradbeer's studies of the isolated OM transport phase of B_{12} uptake: these data were extrapolated to iron uptake systems, under the assumption that they functioned identically. However, Bradbeer's characterizations of the cyanide-independence of BtuB reveals a critical difference to the FeEnt acquisition system. This discrepancy is unexpected for two transport proteins that are so similar in structure, but other differences also exist between them. For example, the transport of B_{12} by BtuB requires TonB, while the penetration of E colicins through BtuB requires the Tol system. In the case of FepA, the uptake of both FeEnt and colicins B and D is TonB-dependent.

5.3.2. Similarity between MotAB and ExbBD

Homology exists between ExbB and ExbD and two proteins of the bacterial flagellar system, MotA and MotB (29). The exact role of the former proteins, which presumably exist in complex with TonB, is uncertain, but the latter two proteins are cytoplasmic membrane components of the flagellar motor that are proposed to form a proton-conducting, $MotA_4MobB_2$ multimer; eight such complexes surround the flagellar rotor, and the MotAB complex is envisioned as a stator that imparts torque by proton conduction-driven conformational changes (29). The underlying explanation for the relationship between MotAB and ExbBD is sequence homology in proposed transmembrane strands 3 and 4 of MotA, and proposed transmembrane strands 2 and 3 of ExbB. The two potential hydrophobic helices manifest 20 per cent identity but extensive homology along their length. Furthermore, mutation of a conserved proline (P713) between MotA helices 3 and 4, presumably located at the cytoplasmic interface, has strong effects on the flagellar rotation, and ExbB contains a comparable conserved, functionally important Pro (P141) between its suggested helices 2 and 3. These and other similarities raise the possibility of a rotational mechanism of TonB action, in which proton passage through ExbBD turns TonB in the periplasm. On the other hand, the proposed stoichiometry of the *E. coli* TonB:ExbB:ExbD complex (1:8:2; (20)) differs from that of $MotA_4MotB_2$, and also unlike the Ton system, the rotational ability of the flagellar motor originates from a conglomeration of many proteins that create a rotor housed within a proteinaceous architecture spanning the bacterial cell envelope. No such architecture is known to exist and facilitate TonB action. Nevertheless, the relatedness between Mot and Exb is considerable, intimating the likelihood of their biochemical similarity.

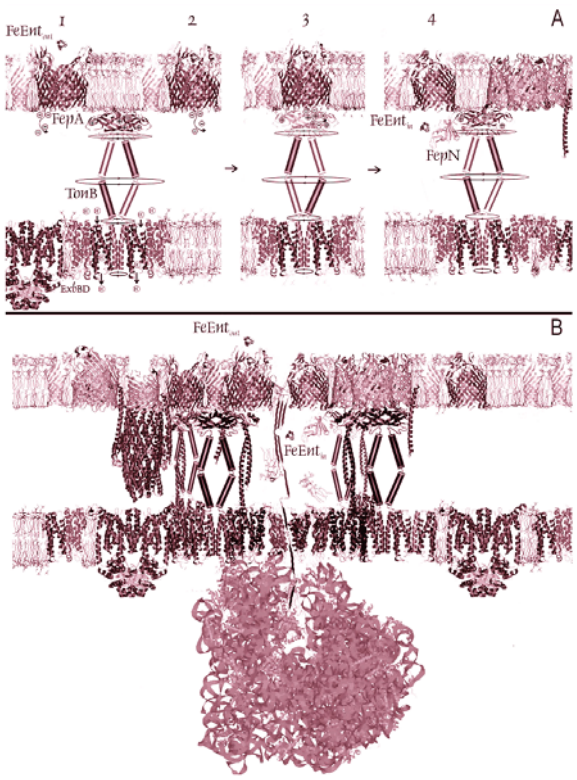

Figure 7. Two models of TonB association with the OM. In the top panel, the rotation of TonB moves it along the inner surface of the OM bilayer, where its positively-charged C-terminal domain adsorbs the negatively-charged N-termini of ligand-bound siderophore receptor proteins, pulling them from inside their ∃-barrels and concomitantly internalizing the ferric siderophores. In the bottom panel, TonB-ExbBD and TolA-TolQR form complexes that span the periplasmic space, creating a localized secretion zone that also manifests transport functions, by virtue of its ability to utilize energy sources in the IM.

In conclusion, I emphasize the legitimacy of alternative interpretations of existing data on TonB and energy transduction, and postulate two such contrasting mechanisms. These originate from the following findings on ferric siderophore transport: (i) ligand binding releases the TonB-box from its normal association with the interior of the ∃-barrel wall and relocalizes the negatively charged N-terminus; (ii) the N-domain is held in the barrel at least in part by a group of ionic bonds between its surface and the barrel wall, and evidence exists that the N-domain may exit the ∃-barrel as FeEnt traverses the channel; (iii) the C-terminus of TonB, which is required for its functionality, has a general affinity for the OM and its proteins, from ionic interactions between its predominantly basic surface, and the largely acidic inner surface of the OM lipids and proteins; (iv) TonB and TolA show structural similarity in their C-termini, and the latter protein, which is needed in LPS biogenesis, also spans the periplasm; (v) The sequence relatedness of MotAB and ExbBD suggests that the latter protein complex may cause the rotation of TonB in the IM.

6. MODELS OF TonB FUNCTION

A variety of interpretations may reconcile these observations, among which I will consider two (Figure 7).

6.1. Rotational motion

According to this view TonB, ExbB and ExbD form a PMF-utilizing rotational complex in the IM. TonB is the rotor, and the dissipation of the proton gradient across the IM, through a proton channel within ExbBD, the stator, promotes rotation. In light of the general affinity of the TonB--C-domain for OM bilayer, the model suggests that it crawls, twirls, or spins across the periplasmic surface of the OM, facilitating transport in the process. If, for example, the negatively charged residues upstream of the TonB box of ligand-bound receptors are sufficiently attracted to, or bind to the C-domain of TonB, then its motion may physically pull the N-domain from the channel, accomplishing transport in the process. Alternatively, a spinning action may create a flux of protons from IM to OM, that neutralizes acidic residues within on the interior barrel walls, releasing the ionic bonds that hold the N-domain within the C-domain. The rotational movement of the TonB C-domain may also randomly propel it across the inner OM surface. Encounters with ligand-free receptors will not interfere with the process, because their N-domains remain locked in place by the interactions of the TonB-box and ion pairs with the barrel wall. Exclusive energization by PMF is not a prerequisite of this proposal, since the rotatory mechanism is conceivably driven by other energy sources, perhaps explaining the susceptibility of FeEnt transport to inhibitors of phosphorylation and electron transport.

6.2. Structural continuities between IM and OM

In this postulate proteins that span the periplasm, including TonB and TolA, create or isolate a localized zone in which the energized state of the IM may directly impact upon the OM. Such inter-membrane connections may form a tunnel, analogous to that of TolC, between the two bilayers, which directs protons to the periplasmic surface of the OM. Whereas TolC anchors in the OM and floats above the surface of the IM, a TonB-TolA tunnel may anchor in the IM, and move along the underside of the OM. In this case the involvement of protons in the activation of transport is fundamental to the mechanism. Alternatively, the structure of the zone may intimately relate to the trafficking of molecules from IM to OM, and metal transporters may only utilize such connections between the bilayers as a fortuitous source of energization. Such transport zones likely contain other proteins that comprise the structure and contribute to the mechanism, whose essential role in cell membrane physiology prevents, under normal circumstances, isolation of spontaneous mutations in their structural genes.

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Footnote: Subsequent work raised the possibility that the *E. coli* strains harboring the N-terminal deletions of FhuA and FepA contain cryptic fragments of the FhuA and/or FhuA N-termini, that complemented the empty β-barrels by inserting within them and facilitating quasi-normal, TonB-dependent colicin sensitivity (14).

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