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MINIREVIEWS

FhuA (TonA), the Career of a Protein[▽]

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The interest in the TonA protein, now called FhuA, has endured since the beginnings of phage genetics and molecular biology. The functions of this energy-coupled transporter and receptor in the outer membrane of *Escherichia coli* cells are fascinating. The historical perspective of this protein presented here is not intended to provide a comprehensive account of the structure and function of FhuA but rather attempts to explain how this protein has attracted interest for so long.

The seminal work published by Salvador E. Luria and Max Delbrück (51) in 1943 is considered the beginning of modern bacterial genetics (12). In this publication, they reported their fluctuation test, which demonstrates that in bacteria, genetic mutations arise in the absence of selection rather than as a response to selection. They isolated virus-resistant mutants of *Escherichia coli* B, ascribed this virus resistance to mutations in the host cells prior to infection by the phage, and presented a means for quantitatively calculating mutation rates. The virus they used was later named phage T1, and the mutations that conferred resistance were subsequently designated *tonA* and *tonB* (*ton* derived from *T one*). It was later found that a phage morphologically and serologically distinct from T1, named T5, cannot infect *tonA* mutants but can infect *tonB* mutants; i.e., phage T5 requires only the *tonA*-encoded function but not the *tonB*-encoded function.

Members of the "phage group" surrounding Luria and Delbrück were interested in genetics, reproduction, and multiplication. They studied, as the most simple system, phages and *E. coli* host cells, mutants, phage morphology, serology, adsorption, lysis, and burst size, but not biochemistry (12, 14). The prejudice against biochemistry began to dissolve during the postdoctoral studies of Wolfhard Weidel, a biochemist by training, on phage adsorption and infection in Delbrück's laboratory. Delbrück's enthusiasm for Weidel's studies was evident in his report to the Caltech president, in which he referred to Weidel's work as "the most startling finding of the year" (66). Weidel's findings were included in the 1950 report on viruses of the Division of Biology of the California Institute of Technology. They were published in 1951 (68) after he was back in Germany at the Max Planck Institute of Biology in Tübingen, where he continued his work on phage adsorption, first with phages T2, T4, and T6 and then mainly with phage T5. His first achievement was the isolation of membrane fractions from *E. coli* B that bound the T phages with kinetics resembling those obtained with living bacteria. He could differentiate between receptors for T2, T4, and T6 on the one side and phage T5 on the other side. Electron microscopy revealed phage-induced degradation of the cell envelopes (68), which was later assigned to the T2 lysozyme (69).

The methods used at that time were rather crude. The T5 receptor was extracted from cells by treatment with 0.1 N NaOH, neutralized with CO_2, precipitated with 20% acetic acid, and then solubilized in phosphate buffer. The solution was treated with a mixture of pancreatic enzymes for 72 h, during which the solubilized components were removed by dialysis. After differential centrifugation, a solution of the T5 receptor of 8,000 U was obtained (1 U inactivates 3×10^3 ml^{-1} T5 particles in 20 min). Electron micrographs of the receptor solution revealed spheres of similar size. Phage T5 bound to the spheres by its tail tip, and DNA was released from the phage head (27, 70) (Fig. 1).

It was not until 1973 that the receptor activity was assigned to a single monomeric protein. The use of advanced methods, solubilization with detergents, gel filtration, ion-exchange chromatography, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis enabled the purification of the TonA protein to electrophoretic homogeneity (11). The TonA protein bound phage T5 and also pure colicin M, another ligand of TonA (see below). Preincubation of colicin M with the receptor prevented binding of phage T5, which suggested that the binding sites of the two ligands overlapped.

TonA was the second pure protein isolated from the *E. coli* cell envelope. The first protein isolated was the lipoprotein covalently bound to murein; its amino acid sequence and lipid and murein attachment sites were determined in 1972/1973 (5, 33). Research on the *E. coli* cell envelope at that time was focused mainly on the function, structure, and biosynthesis of murein and lipopolysaccharide (LPS). The time was ripe to study membrane protein function, structure, and secretion. These became and currently still are major research topics.

In 1973, two more membrane proteins were partially purified from *E. coli*: the LamB receptor of phage λ (61) and the BtuB receptor of colicin E3 (64). In 1973, the BtuB protein was shown to be involved in vitamin B_{12} transport and to serve as a receptor for phage BF23 and colicins E1 and E3 (16). In 1975, it was shown that LamB facilitates the uptake of maltose (67). In the same year, TonA was shown to transport ferrichrome, an iron-complexing siderophore of the hydroxamate type (34, 50); this later prompted the coining of the new name, FhuA, for *f*erric *h*ydroxamate *u*ptake (42). Characterization of the matrix protein of *E. coli* B in 1974 (63), now named OmpF,

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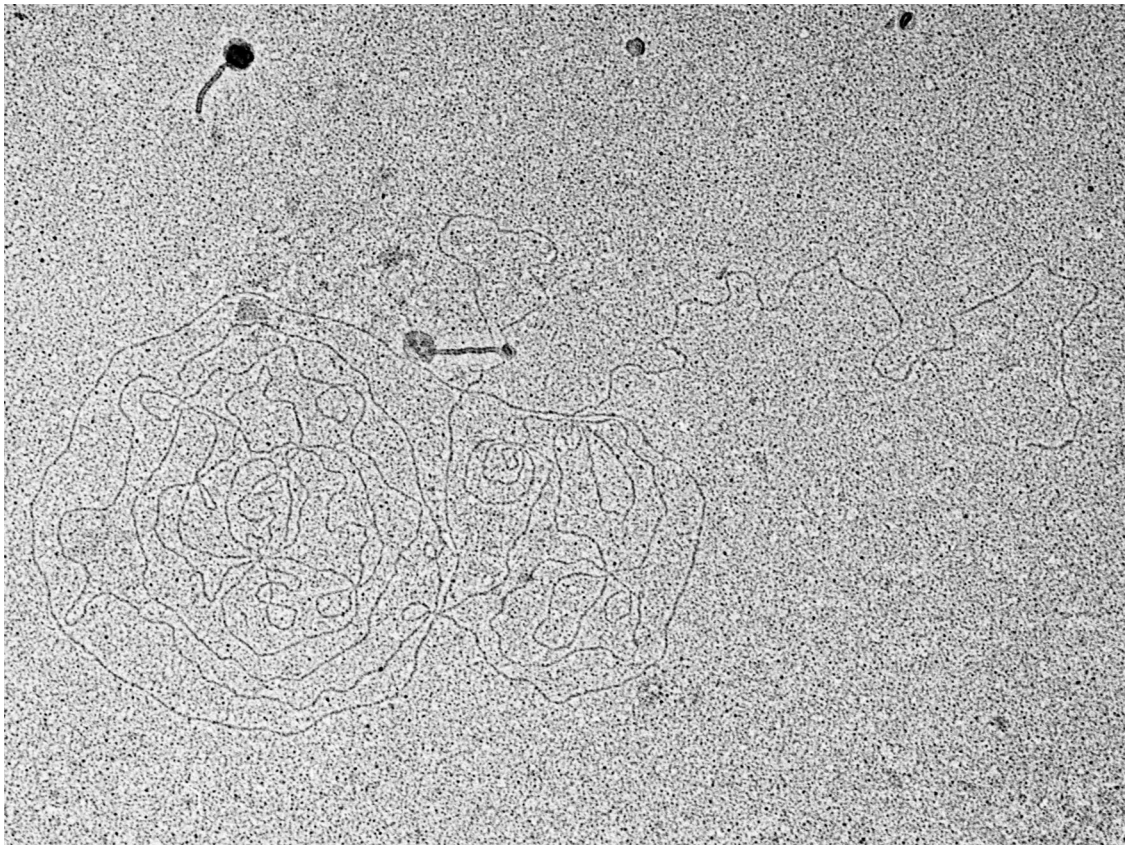

FIG. 1. Phage T5 with released DNA, an empty phage head bound through its tail to a receptor particle (center), and the intact phage not bound to a receptor particle (top) (27).

paved the way for studies of porins, which were discovered in 1976 (53). Porins mediate diffusion of hydrophilic molecules up to a certain size; in *E. coli*, the size limit is approximately 600 Da (54).

FhuA DEFINES A NOVEL CLASS OF TRANSPORT AND RECEPTOR PROTEINS

FhuA is functionally a highly diverse protein in the outer membrane. It serves as receptor for the phages T1, T5, ϕ80, and UC-1, and in fact many similar—and not necessarily identical—*E. coli* phages that use FhuA as receptor can be isolated from nature (58). In addition, FhuA transports colicin M, microcin 25, ferrichrome, albomycin, and rifamycin CGP 4832.

In 1998, two groups published the crystal structure of FhuA, revealing a new type of protein structure (23, 49). Like the porin proteins, FhuA consists of a β barrel (22 instead of 16 antiparallel β strands), but unlike the porin proteins, it contains a plug (or cork or hatch) which fills the lumen of the barrel. The plug is a globular N-terminal domain that enters the β barrel from the periplasmic side and tightly closes the pore in the β barrel. The β barrel extends well above the lipid bilayer. The β strands are connected by short periplasmic turns and extracellular loops of up to 31 residues. Since then, the crystal structures of five other outer membrane transporters, three from *E. coli* and two from *Pseudomonas aeruginosa*, have been determined. Their overall design is very similar to the structure of FhuA, even though pair-wise comparison of the transporters reveals less than 20% sequence identity (22, 46).

One of the FhuA samples was cocrystallized with a rough LPS, as it occurs in *E. coli* K-12. For the first time, the crystal structure of LPS and the exact positioning of FhuA in the outer membrane were revealed (23). The fatty acid residues of LPS form part of the outer leaflet of the lipid bilayer. The FhuA-LPS structure supports the positioning of proteins within the outer membrane. Two girdles of aromatic residues 25 Å apart in FhuA are generally found and are predicted to delineate the interface between the membrane fatty acids and the polar head groups. A high number of charged and polar residues of FhuA interact with LPS; four of these are positively charged Lys and Arg residues that are found in similar three-dimensional arrangements in other known LPS-binding proteins (25).

Crystal structures of FhuA with and without bound substrates have also been determined. The substrates were ferricrocin, ferrichrome, albomycin, and rifamycin CGP 4832. Ferricrocin and ferrichrome are structurally closely related. They are iron chelators (siderophores) and considered to be the natural substrates for iron delivery to *E. coli*, although they are not synthesized by bacteria but rather by fungi. Albomycin is an antibiotic derived from ferrichrome. It is taken up via the ferrichrome transport system and kills cells through its thioribosyl pyrimidine moiety, which inhibits seryl-tRNA synthetase (65). The structure of a FhuA-albomycin cocrystal was deter-

mined to see how the bulky side chain of albomycin fits into the FhuA protein (21). Albomycin occupies the same position as ferrichrome. The antibiotic is positioned in the extracellular pocket, through which the iron carrier gains access to its binding site. It forms hydrogen bonds and van der Waals contacts with numerous side chain residues of the extracellular pocket, extracellular loops, and barrel strands. Rifamycin CGP 4832 is a synthetic rifamycin derivative that is transported by FhuA into the periplasm, even though chemically it is completely different from ferrichrome and does not bind iron. Active transport into the periplasm as opposed to diffusion of rifamycin across the outer membrane decreases the MIC of CGP 4832 by 200-fold. The structure of cocrystals of FhuA with rifamycin CGP 4832 was determined to see how rifamycin CGP 4832 is bound by FhuA and whether its three-dimensional structure displays common features with ferrichrome (24). Although the crystal structure of CGP 4832 is distinct from that of ferrichrome, it binds to 9 of the 10 amino acid side chains of FhuA to which ferrichrome binds and to 8 additional side chains. The FhuA transporter tolerates a large variety of substrates, provided essential structural conditions are fulfilled. FhuA substrate specificity is determined by the binding step and less so by the transport step. Whatever is tightly bound to the ferrichrome binding site is released upon structural transitions in FhuA and diffuses through the opened FhuA pore. Since energy-coupled transport strongly decreases the MIC of antibiotics, synthetic antibiotics with hydroxamates as carriers and linked antibiotics with high activity but poor uptake can be synthesized (10). Albomycin was clinically used in the former Soviet Union (29).

ENERGY-COUPLED TRANSPORT AND RECEPTOR FUNCTIONS OF FhuA

Energy dependence is the most interesting and elusive aspect of FhuA function. The finding in 1943 that resistance of *E. coli* to phage T1 was caused by two different mutations (51), later designated *tonA* and *tonB*, suggested a functional relationship between the two gene products. In 1951, it was shown that T1 infection occurs in two steps—one reversible and requiring *tonA* and the other irreversible and requiring *tonB*—and proposed that the irreversible adsorption of phage T1 requires energized cells (28). In 1976, it was demonstrated that this energy is provided by the transmembrane potential of the cytoplasmic membrane (32), thereby relating TonB with the energized cytoplasmic membrane. Phage T1 DNA uptake, in contrast, does not seem to require TonB, as host-range mutants of T1 transfer DNA into *tonB* mutants and multiply at a high rate. The same applies to phage ϕ80, of which the wild type requires TonB, but host-range mutants infect *tonB* mutants. Energized FhuA possibly assumes a conformation that allows wild-type T1 and ϕ80 adsorption such that it triggers DNA release from the phage head. This hypothesis is supported by the more efficient inhibition of phage T5 adsorption to FhuA by ferrichrome in unenergized rather than in energized cells (35). The alternative interpretation that ferrichrome is not transported into the *tonB* mutant and remains bound to FhuA, and for this reason inhibits T5 adsorption to *tonB* mutants more effectively than to *tonB* wild-type cells, probably does not apply for the following reason. A 10^6-fold molar excess of ferrichrome over FhuA inhibits T5 adsorption to the wild type by only 40%, whereas a 1,000-fold-lower ferrichrome concentration inhibits T5 adsorption to the *tonB* mutant by 100%. Despite the high ferrichrome concentration and the very high affinity of ferrichrome to FhuA in the range of 1 nM, inhibition of T5 adsorption to the *tonB* mutant by ferrichrome remains low. An energy flux from the cytoplasmic membrane into the outer membrane is required for the irreversible adsorption of phage T1 because there is no energy source in the outer membrane. The TonB protein is implicated in intermembrane energy transfer. In addition to TonB, the ExbB and ExbD proteins are required for TonB to react to the proton motive force of the cytoplasmic membrane (9, 46, 59, 60).

In FhuA, the plug must move within or out of the β barrel to open a pore through which the substrates pass into the periplasm. Does the binding of the substrate open the pore? No, this is not the case for FhuA and all other transporters whose crystal structures have been studied with and without bound substrates. Despite substantial conformational changes in the plug domain upon substrate binding, the pore remains closed. The largest change in FhuA occurs at the periplasmic side of the molecule, where a short helix is converted into a coil and residues E19, S20, and W22 move up to 17 Å. The functional meaning of these structural transitions is supported by the finding that prevention of the 17-Å translation through disulfide bridges between residues T27C (threonine replaced by cysteine) and P533C abolishes ferrichrome transport (20) and sensitivity to colicin M and phages T1 and ϕ80 (6). The TonB-independent infection by phage T5 remains unaffected. However, as discussed below, deletions in this region preserve all TonB-dependent FhuA activities. Therefore, the observed structural transitions may not be required for productive interaction of FhuA with TonB.

All TonB-dependent transporters contain close to the N terminus a common sequence motif called the TonB box. Colicins that are taken up by TonB-dependent transporters also contain an N-terminal TonB box. The essential roles of the TonB box for TonB-dependent transport and receptor activities have been shown by genetic, biochemical, and crystallographic means. In FhuA, the point mutations I9P and V11D, which abolish all TonB-dependent FhuA activities but retain TonB-independent T5 sensitivity, are suppressed by mutations Q160L and Q160K in TonB. The I9P mutation is also partially suppressed by the R158L mutation in TonB (9). Restoration of inactive mutants in one protein by mutations in an interacting protein is taken as genetic evidence for functional interaction between the two mutated regions. The close physical proximity of the suppressor mutants was shown by the formation of spontaneous cystine cross-links between FhuA(I9C), FhuA(V11C), and TonB(Q160C) (6). In the crystal structure of FhuA with bound TonB residues 124 to 235 [TonB(124-235)] the FhuA sequence I9 to Q16 forms a β strand and aligns in parallel with β3 of the three-stranded TonB β sheet (55). A similar structure was derived from the nuclear magnetic resonance structure of TonB(152-239) and synthetic TonB box peptides (56). The TonB box serves as an energy-coupling motif. Electron paramagnetic resonance spectroscopy of the spin-labeled TonB box of isolated FhuA reveals that the TonB box is highly flexible, regardless of whether FhuA is substrate loaded or not (43). This stands in contrast to the TonB boxes of other transport-

ers, which undergo substrate-induced order-to-disorder transitions (43). Ferricrocin-induced enhancement of in vivo cross-linking between FhuA and TonB with formaldehyde (52) may not be caused by a stronger exposure of the TonB box but by a tighter binding to TonB.

The TonB-dependent activities of FhuA require TonB and the proton motive force of the cytoplasmic membrane. How TonB reacts to the proton motive force and how it transmits energy to FhuA are not known. Energized TonB assumes a structure that differs from unenergized TonB (30, 60). The energized conformation of TonB might induce conformational changes in FhuA so that the substrates are released from their binding sites and the plug moves within or out of the β barrel to open the pore. If the conformational transitions occur through interaction of TonB with the FhuA TonB box, subtle structural changes in the amino acid sequence that connects the TonB box with the plug are expected. However, deletion of residues 13 to 22 and 24 to 31 and duplication of residues 23 to 30 in the connecting region reduce FhuA activities only partially (20). Even deletion of residues 21 to 128 retains all FhuA activities except colicin M sensitivity, which is reduced eightfold (13). These results indicate that energy transfer from TonB to FhuA is mechanistically not understood.

Deletion of the entire plug (residues 5 to 160) converts FhuA into an open channel that facilitates diffusion of ferrichrome, antibiotics, and maltodextrins through the outer membrane. FhuA(Δ5-160) incorporated into artificial lipid bilayers increases the conductance for KCl and other solutes but does not form stable channels with clearly discernible single-channel conductance steps (7). In a complete chromosomal FhuA deletion strain, plasmid-encoded FhuA(Δ5-160) exerts none of the FhuA activities (7). A remarkable reconstitution of active FhuA occurs when FhuA(Δ5-160) is cosynthesized with a mutant FhuA with an intact plug but an inactive β barrel or with a FhuA N-proximal fragment of 357 residues. These cells display high transport and receptor activities (7). It is likely that active plugs are proteolytically excised from inactive FhuA and FhuA(1-357) and incorporated into FhuA(Δ5-160). Separate synthesis of the plug and FhuA(Δ5-160) results in active FhuA, provided both proteins are endowed with a signal sequence, showing that assembly of complete FhuA occurs in the periplasm or during incorporation into the outer membrane (7).

Crystal structures indicate no movement of the loops upon substrate binding to FhuA(21-24, 49). However, the fluorescence of FhuA labeled in vivo with fluorescein maleimide at an introduced cysteine at position 336 of loop 4 is quenched in the presence of ferrichrome, indicating movement of loop 4 (3). This result is confirmed by in vitro fluorescence quenching of FhuA(D336C) labeled with MDCC [7-diethylamino-3-((((2-maleimidyl)-ethyl)amino)carbonyl) coumarin] in response to ferricrocin binding. Fluorescence quenching is also observed upon binding of unenergized TonB(32-239) in vitro (41), but quenching is not seen in vivo when TonB wild-type and TonB mutant cells are compared (3). In the in vitro experiment, TonB is not energized, which requires full-length TonB to be inserted into the cytoplasmic membrane of metabolizing cells. In addition, binding kinetics of monoclonal antibodies to FhuA surface loops 3, 4, and 5 change in response to ferricrocin and TonB (41). The results indicate that ferricrocin and TonB promote conformational changes in FhuA surface loops. Such changes were previously deduced from the energy- and TonB-dependent binding of phages T1 and ϕ80 (32). Lack of such changes in the crystal structures of siderophore-loaded versus unloaded FhuA and in TonB-FhuA cocrystals may be caused by crystal contacts that involve surface loops and by nonphysiological crystallization conditions (44).

INTERACTION OF PHAGE T5 WITH THE FhuA RECEPTOR PROTEIN

Binding of phage T5 to FhuA probably involves several regions of FhuA since no point or deletion mutants that specifically abolish T5 infection have been isolated. Only a deletion of loop 8 abolishes infection by phage T5, but this also abolishes infection by phages T1 and ϕ80. Since ferrichrome binding and transport are largely retained, the FhuA deletion derivative is properly inserted into the outer membrane (19).

Phage T5 binds poorly to *E. coli* K-12. Since it adsorbs much faster to *E. coli* F (47), this strain is the standard strain used for T5 propagation. The reason for the accelerated adsorption remained unclear until it was shown that *E. coli* F contains a particular LPS O antigen to which T5 binds reversibly via its L-shaped tail fibers (protein pb1) (37). Reversible binding to LPS accelerates adsorption by 15-fold. Adsorption-desorption movement of T5 along the cell surface reduces the search for FhuA from a three-dimensional diffusion to a two-dimensional diffusion and thus strongly increases the detection rate of FhuA. The O antigen of strain F consists of polymannose, and trimannoside is the strongest adsorption inhibitor (38).

The FhuA-triggered release of T5 DNA allows in vitro studies of FhuA-T5 interactions. Binding to FhuA is not mediated by the pb2 straight tail fiber protein of T5 but by pb5 (36, 39, 45). pb5 is not located at the tip of the tail (39), as one might expect, but rather 50 nm distant from the extremity of the straight tail fiber (1). Isolated pb5 forms a very stable equimolar complex with FhuA (57) and inhibits infection by phages T5 and ϕ80 as well as ferrichrome uptake. Binding of phage T5 to FhuA incorporated into planar lipid bilayer membranes results in a high conductance of KCl (2) and release of ferrichrome entrapped in liposomes (48). The apparent formation of a channel, however, does not mean that the phage DNA crosses the outer membrane through FhuA. Linear double-stranded DNA with a diameter of approximately 2 nm would hinder diffusion of ferrichrome and KCl through the channel, which has a diameter of approximately 3 by 2 nm when the complete plug has moved out of the β barrel. The tail fiber traverses the lipid bilayer of the proteoliposome, and the tip is observed within the proteoliposome (1). pb2 undergoes a major conformational change; it shrinks from 50 nm to 23 nm in length, and the diameter increases from 2 to 4 nm (1). The phage DNA probably passes through a channel formed by pb2 (26) and accumulates in the proteoliposome in a highly condensed structure (1). pb2 is enriched in contact sites between the outer membrane and the cytoplasmic membrane (31). It contains a predicted coiled-coil region, which is proposed to serve as a sensor for triggering the opening of the head-tail connector. The C-proximal region degrades murein and displays features of fusogenic membrane proteins. It thus could form fusions between the outer membrane and the cytoplasmic membrane, through which phage DNA enters the cells (4).

From in vivo and in vitro data, the following scenario can be derived for T5 infection of cells: phage T5 binds reversibly by pb1 of the L-shaped tail fibers to the polymannose O antigen and enhances the rate of pb5 binding to FhuA. pb2 of the straight tail fiber contacts the bacterial surface without the involvement of a pb2-specific bacterial receptor. The straight tail fiber inserts into the bacterial outer membrane until pb5 contacts FhuA, which triggers the release of the DNA from the phage head. The phage DNA passes through a channel formed by pb2 across the outer membrane, the periplasm, and the cytoplasmic membrane. This channel protects the phage DNA from being degraded by periplasmic nucleases.

A T5 LIPOPROTEIN INACTIVATES FhuA

Adjacent to the phage T5 gene that encodes pb5 is a gene that encodes a lipoprotein (15). Infected *E. coli* cells producing the lipoprotein are resistant to phages T5 and ϕ80 and to colicin M and albomycin, which suggests that the lipoprotein inactivates FhuA (8, 15). Indeed, outer membranes prepared from cells producing lipoprotein contain inactive FhuA (17). FhuA reconstituted with lipoprotein in liposomes fails to inactivate phage T5 and to release phage DNA. Most mutants in FhuA that are no longer inactivated by the lipoprotein have mutations located in periplasmic turns, but some are also located in surface loops (8). Fatty acylation of lipoprotein is essential for its association with the outer membrane and protection against phage T5 and colicin M (62). The lipoprotein enters the outer membrane with its lipid from the periplasmic side. Therefore, it is unlikely that the lipoprotein covers cell surface-exposed regions of FhuA that serve as ligand binding sites and thus prevents their adsorption. Rather, it is proposed that binding of the lipoprotein to FhuA triggers structural changes in FhuA such that it no longer binds the ligands. This conclusion is supported by two FhuA mutants that display a 10-fold-enhanced colicin M sensitivity when they synthesize the lipoprotein (8). Inactivation of FhuA by the lipoprotein develops slowly during infection (26). It is therefore unlikely that the lipoprotein excludes superinfection. Instead it prevents inactivation of phage T5 by FhuA when cells lyse and phages are released.

IMPORT OF COLICIN M THROUGH FhuA

Colicins are the only proteins which are imported by *E. coli* cells. Other proteins enter cells bound to DNA during phage infection and conjugation. The crystal structure of colicin M (molecular mass, 29 kDa) reveals a compact molecule (71) with a diameter of 3 nm by 4 nm that cannot pass through FhuA even when the entire plug moves out of the β barrel. Colicin M must unfold so that the phosphatase domain enters the periplasm, where it inhibits murein biosynthesis by cleaving between bactoprenol and 1-pyrophospho-MurNAc-(pentapeptide)-GlcNAc (18). Refolding in the periplasm specifically requires the FkpA chaperone (40). Colicin M becomes trypsin sensitive when it binds to FhuA, which indicates a conformational change. It is not known whether only the phosphatase domain is imported into the periplasm and most parts of the receptor binding and translocation domains remain bound to FhuA within the outer membrane or whether the entire protein is imported. Only the TonB box (residues 2 to 8) is known to be exposed to the periplasm, where it most likely interacts with β3 of the C-proximal TonB domain (71).

CONCLUDING REMARKS

In science, a few proteins serve as model proteins to answer central questions and to unravel basic principles. FhuA is such a protein, whose unusually long career spans from 1943 to 2009 with no end in sight. The numerous ligands with very diverse functions facilitate the study of the intricate mode of FhuA action. The in vivo assays are simple; in vitro studies are more demanding. An in vitro proteoliposome with FhuA in one membrane and TonB, ExbB, and ExbD in an adjacent membrane are required but difficult to establish. A challenging task is the isolation of the TonB/ExbB/ExbD protein complex needed to unravel its reaction to the proton motive force of the cytoplasmic membrane and its transmission of energy to FhuA. A membrane potential across the inner membrane of the two-layered proteoliposome must be established to simulate in vivo energization. The major questions of how FhuA and the other energy-coupled outer membrane transporters receive energy from the cytoplasmic membrane and how they react still await solution.

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