The ferric hydroxamate uptake receptor FhuA and related TonB-dependent transporters in the outer membrane of gram-negative bacteria

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FUNCTIONAL CLASS

Active transporter; siderophore; integral outer membrane protein; ferric hydroxamate uptake receptor, known as FhuA;

chemiosmotic gradient; ABC transporter; bacteriophage infection; colicin import; siderophore-antibiotic conjugate and peptide antibiotic sensitivity; TonB-dependent receptor; structurally related to the ferric enterobactin receptor, and

3D Structure The three-dimensional structure of FhuA in complex with ferricrocin. (a) FhuA in ribbon representation. The view is perpendicular to the barrel axis. The strands that comprise the front of the barrel domain have been removed to provide an unobstructed view of the cork domain. (b) FhuA as viewed from the external environment along the barrel axis. In panels a and b, the barrel domain (residues 161–714) and the cork domain (residues 1–160) are colored blue and yellow, respectively. The ferricrocin molecule is shown as a bond model with carbon atoms white, oxygen atoms red, and nitrogen atoms blue. The ferric iron atom is shown as a large red sphere. These and all the following color figures were prepared using MOLSCRIPT⁹⁵ and RASTER3D.⁹⁴ PDB code: 1QFF.

Figure 1 Selected TonB-dependent siderophore uptake systems of *E. coli*. Abbreviations: T1, bacteriophage T1; T5, bacteriophage T5; ϕ 80, bacteriophage ϕ 80; UC-1, bacteriophage UC-1; Col M, colicin M; CGP 4832, rifamycin CGP 4832; Col B, colicin B; Col D, colicin D; Dhb, dihydroxybenzoate; Col Ia, colicin Ia; Col Ib, colicin Ib; Col V, colicin V; Dhs, dihydroxybenzoylserine; Col A, colicin A; Col E, colicin E. See Table 1 for the related TonB-dependent outer membrane receptors.

functionally related to the ferric citrate, coprogen and rhodoturulate, ferric aerobactin, and vitamin B₁₂ receptors from *Escherichia coli* K-12 and to the lactoferrin, transferrin, and heme receptors from *Neisseriaceae*.

FhuA is a member of a diverse family of integral outer membrane proteins, the TonB-dependent receptors, which together with the TonB-ExbB-ExbD complex actively pump siderophores, vitamin B₁₂ and hemes across the cell envelope (see 3D Structure).

In *Escherichia coli*, FhuA mediates the uptake of hydroxamate-type siderophores across the outer membrane of gram-negative bacteria.¹ In addition to transporting ferrichrome, FhuA acts as the primary receptor for several bacteriophages, the antibiotics albomycin and rifamycin CGP 4832, the cyclic peptide antibiotic microcin J25, and the bacterial toxin, colicin M. The focus of this review is an analysis of the relationship between the three-dimensional structure of FhuA and its biological function as an energy-dependent outer membrane transporter (Figure 1).

OCCURRENCE

To survive as free-living microbes, and to proliferate within the iron-restricted regions of host organisms, bacteria have developed extensive siderophore-mediated strategies to obtain iron.¹ The presence of these high-affinity uptake systems can be correlated with bacterial virulence *in vivo*,^{2,3} and the sequestration of iron is an extremely effective, nonspecific host response to bacterial infection. One strategy that most bacteria use to circumvent this

defensive mechanism is the secretion of siderophores.^{4,5} Siderophores are low-molecular weight, water-soluble substances which can be arranged into three broad classes depending on their chemical structure: (i) catechol-type (e.g. enterobactin); (ii) citrate-type (e.g. citrate); and (iii) hydroxamate-type siderophores, including aerobactin, coprogen, ferrichrome, and rhodoturulate. Ferricrocin and phenylferricrocin are structural analogs of ferrichrome; these siderophores are functionally identical. Of the approximately 200 known siderophores, most possess three bidentate iron-chelating groups that are derived from hydroxamate, catechols or oxazolines. Although these agents display considerable structural diversity, all form six coordinate octahedral complexes with Fe(III) of extraordinary affinity. Figure 2(a)–(c), show schematically the structures of ferricrocin, phenylferricrocin and albomycin together with the protein residues involved in binding. All contain the same iron-chelating structure (Figure 2(d)).

Siderophores avidly scavenge Fe(III) from the external environment or host iron-containing proteins, and are subsequently bound by distinct receptors located within the bacterial cell envelope. Siderophore-mediated uptake systems translocate iron-chelates across the outer membrane by utilizing the free energy derived from the chemiosmotic gradient. *E. coli* has multiple negatively regulated iron uptake operons; each composed of an outer membrane receptor, a periplasmic binding protein, one or two hydrophobic membrane proteins that span the cytoplasmic membrane, and an ATP-binding protein that is associated with the inner leaflet of the cytoplasmic membrane. That

Table 1 Representative members of the TonB-dependent family of outer membrane transporters

Substrate	Organism	Outer membrane receptor
Ferric aerobactin	<i>E. coli</i>	IutA
Ferrichrome, albomycin, rifamycin CGP 4832, microcin J25	<i>E. coli</i>	FhuA
Coprogen, rhodoturulate	<i>E. coli</i>	FhuE
Ferric enterobactin	<i>E. coli</i>	FepA
Dihydroxybenzoate, catechol-substituted cephalosporins	<i>E. coli</i>	Cir
Dihydroxybenzoylserine, dihydroxybenzoate	<i>E. coli</i>	Fiu
Ferric citrate	<i>E. coli</i>	FecA
Vitamin B ₁₂	<i>E. coli</i>	BtuB
Iron-loaded transferrin	<i>N. meningitidis</i> , <i>N. gonorrhoeae</i>	TbpA–TbpB
Iron-loaded lactoferrin	<i>N. meningitidis</i> , <i>N. gonorrhoeae</i>	LbpA–LbpB
Heme	<i>V. cholerae</i>	HutA–HutB
Heme–heme binding protein	<i>S. marcescens</i>	HasR
Heme	<i>Y. pestis</i>	HmuR
Heme	<i>Y. enterocolitica</i>	HemR
Hemoglobin	<i>N. meningitidis</i> , <i>N. gonorrhoeae</i>	HmbR
Hemoglobin–haptoglobin complexes	<i>H. influenzae</i>	HhuA
Hemoglobin–haptoglobin complexes	<i>N. meningitidis</i> , <i>N. gonorrhoeae</i>	HpuB–HpuA

some bacteria express parallel iron acquisition systems, each specific for a single siderophore, underscores their biological significance. Despite the presence of multiple iron-responsive operons that target the structurally diverse siderophores, *E. coli* only carries the biosynthetic genes for the synthesis of a single endogenous siderophore, enterobactin. Plasmid-encoded genes may provide an additional siderophore and its cognate outer membrane receptor.⁶ Microbes that do not secrete their own siderophores can utilize those produced by neighboring bacteria and fungi. For example, the ferric hydroxamate uptake operon (the Fhu system) allows *E. coli* to utilize the fungal siderophore ferrichrome as an iron source. Similarly, although *Neisseria meningitidis* does not synthesize any known siderophore, functional homologs of the ferric enterobactin and ferric hydroxamate uptake receptors of *E. coli* has been identified for this pathogen.^{7,8}

BIOLOGICAL FUNCTION

Iron has played an essential role in the evolution of life on earth, and is required by all living organisms with the possible exception of a few *Lactobacillus* species. As a transition element, iron can reversibly modify its oxidation state [ferrous (Fe(II)) or ferric (Fe(III))], and as documented in this handbook is often utilized as a cofactor in central metabolic processes, including the RNA synthesis and

electron transport.⁹ Despite its relative abundance in nature, most iron in an aerobic environment is found as extremely insoluble ferric oxyhydroxide polymers. In fact, the concentration of biologically available iron may be as low as 10^{-18} M at physiological pH.¹⁰ To satisfy their iron requirement bacteria have evolved a diverse series of highly efficient iron acquisition strategies (Table 1).

Under iron-starved conditions bacteria increase the rate of transcription of genes involved in iron acquisition.¹¹ The transport of iron across the outer membrane and its intracellular storage must be tightly regulated to avoid the detrimental effects associated with iron toxicity.¹² To accomplish this, the Fur protein of gram-negative and some gram-positive bacteria,¹³ and the DtxR protein of the remaining gram-positive bacteria,¹⁴ control the expression of most iron-responsive genes and other virulence determinants. These regulatory proteins couple gene expression with the intracellular iron concentration, and thus provide a highly adaptive growth mechanism.

Bacterial siderophore-mediated iron acquisition pathways work by transporting iron-chelates across the outer membrane, periplasm, and the cytoplasmic membrane. The proteins needed for each phase of the transport process are localized to specific compartments within the cell envelope and have defined functions (3D Structure). In *E. coli*, the receptors for hydroxamate-type siderophores are: (i) IutA, which transports ferric aerobactin;¹⁵ (ii) FhuA, which transports ferrichrome and the structurally related

Figure 2 Chemical structures of siderophores and albomycin bound to FhuA: (a) ferricrocin, (b) phenylferricrocin, and (c) albomycin (extended conformational isomer) including the hydrogen bonding pattern and electrostatic interactions with side-chain residues in the ligand-binding site of FhuA. (d) Close-up view of the chemical environment and octahedral coordination of the ferric iron atom in these hydroxamate-type siderophores and albomycin. Hydrogen bonds and charge interactions to FhuA are indicated as dotted lines (distances are given in Å). These figures were prepared with ISIS DRAW and MOLSCRIPT.⁹⁵

antibiotic albomycin;¹⁶ and (iii) FhuE, which transports coprogen and rhodotorulate.¹⁷ The receptors for catechol-type siderophores are: (i) FepA, which transports ferric enterobactin;¹⁸ (ii) Cir, which transports dihydroxybenzoate and catechol-substituted cephalosporin antibiotics;^{19,20} and (iii) Fiu, which transports dihydroxybenzoylserine and dihydroxybenzoate.¹⁹ The sole receptor for citrate-type siderophores in *E. coli* is FecA. This receptor transports ferric citrate across the outer membrane.²¹ The vitamin B₁₂ uptake system is grouped with those for siderophores, as they share a similar organization of proteins and utilize an analogous mechanism of ligand transport. The vitamin B₁₂ receptor in the outer membrane of *E. coli* is BtuB.²² These TonB-dependent receptors are functionally different from the nonspecific and specific porins, which couple a concentration gradient across the outer membrane with diffusional transport.²³ The concentration of siderophores and vitamin B₁₂ in the external medium is exceedingly low and therefore, porin-mediated passive diffusion does not satisfy the cellular requirements for these essential metal-containing complexes. Thus, TonB-dependent receptors bind their cognate ligands with high-affinity ($K_D \sim 0.1 \mu\text{M}$) in order to concentrate them at the cell surface.

Pathogenic bacteria have evolved a class of additional host-iron utilizing systems based on roughly the same receptor structure and the TonB-dependent energy transduction mechanism.²⁴ Some receptors can acquire iron from host transporters for lactoferrin or transferrin; whereas others translocate the entire heme group.

AMINO ACID SEQUENCE INFORMATION

Presented here are the GenBank accession numbers for the *fhuA* gene from *E. coli* K-12, and *fhuA* homologs that have been identified to date.

- *Escherichia coli* K-12 (748 residue unprocessed precursor), 33 residue signal sequence. GenBank accession number M12486.¹⁶
- *Haemophilus influenzae*: HI1466 at www.tigr.org
- *Neisseria meningitidis* (704 residues, *fhuA* pseudogene). GenBank accession number AJ391277.^{8,1}
- *Pantoea agglomerans* (733 residues). GenBank accession number Y14026.²⁵
- *Rhizobium leguminosarum* (727 residues unprocessed precursor), 38 residue signal sequence. GenBank accession number AJ238208.²⁶
- *Rhizobium leguminosarum* (*fhuA* pseudogene). GenBank accession number AJ007906.²⁷
- *Salmonella paratyphi* strain B (748 residues). GenBank accession number Y14067.²⁵
- *Salmonella typhimurium* (730 residues). GenBank accession number Y14025.²⁵

- *Synechocystis* species (864 residues, hypothetical *fhuA* gene). GenBank accession number D90899.²⁸
- *Vibrio cholerae* (701 residues), complete cDNA. GenBank accession number AF203702.²⁹

PROTEIN PRODUCTION, PURIFICATION AND MOLECULAR CHARACTERIZATION

For ease of purification, a recombinant FhuA protein was generated by inserting a hexahistidine tag plus five additional linker residues (SerA-SerB-HisC-HisD-HisE-HisF-HisG-HisH-GlyI-SerJ-SerK) into the *fhuA* gene after residue 405.³⁰ The recombinant protein was overexpressed from an *E. coli* K-12 background strain harboring the pHX405 plasmid³¹ and purified by the immobilized metal-affinity chromatography as described earlier.^{32,33} SDS-PAGE analysis of the purified protein, stained with silver or Coomassie-blue, and Western blotting using anti-FhuA monoclonal antibodies,³² revealed a single band and the absence of micellar LPS. Electrospray ionization mass spectroscopy also confirmed the expected molecular mass of the purified recombinant FhuA (M_r 79 960 Da). In accord with the previous sedimentation equilibrium and velocity data,³⁴ dynamic light scattering demonstrated that FhuA purified by this procedure is both monomeric and monodisperse. Alternate strategies are available for the purification of wild-type FhuA³⁴ or FepA,³⁵ and the isolation and subsequent refolding of FepA from inclusion bodies.³⁶

METAL CONTENT AND COFACTORS

The three-dimensional structure of FhuA in complex with the various siderophores or the antibiotic albomycin [ferricrocin,³³ ferrichrome,³⁷ phenylferricrocin,³⁸ and albomycin³⁸] shows one ligand molecule in the binding site of FhuA. Three bidentate iron-chelating hydroxamate groups coordinate a single Fe(III) ion in these metal-containing ligands (Figure 2(a)–(d)).

ACTIVITY TEST

Functional assays confirmed that the recombinant FhuA is fully active as a TonB-dependent transporter,³¹ and that the purified receptor does not form ion-channels when reconstituted into the planar lipid bilayers.³⁹ The irreversible adsorption of bacteriophage T5 to FhuA does not require an energized cytoplasmic membrane.⁴⁰ The binding of this bacteriophage to FhuA reconstituted in a lipid bilayer system also induces the formation of an ion-channel³⁹ and triggers DNA ejection. Accordingly, the binding of bacteriophage T5 triggers a conformational

change such that a high-conductance channel opens,³⁹ and radiolabeled ferrichrome is released into the external medium.^{41–43} Previous competitive peptide mapping studies also demonstrated that short hexapeptides, corresponding to residues 335–355 of FhuA, are also capable of triggering DNA ejection from the capsid of this bacteriophage.⁴⁴ The addition of bacteriophage ϕ 80 to proteoliposomes containing FhuA does not induce DNA ejection, because unlike bacteriophage T5, irreversible binding of bacteriophage ϕ 80 to FhuA requires an energized cytoplasmic membrane and the TonB–ExbB–ExbD energy-transducing module.^{42,43,45}

X-RAY STRUCTURE OF FHUA AND ITS COMPLEX WITH FERRICROCIN

Crystallization

By mixing 5 μ l of protein-containing solution (8 mg ml⁻¹ of FhuA, 10 mM ammonium acetate (pH 7.5), and 1% *cis*-inositol^{46,47}) with an equal volume of reservoir solution [11% PEG 2000 monomethyl ether, 100 mM sodium cacodylate (pH 6.4), 3% PEG 200, and 20% glycerol], well-ordered protein crystals were obtained by the hanging-drop vapor diffusion technique.^{32,33} Under these conditions, single crystals of FhuA grow within seven days to a final size of 350 $\times$ 350 $\times$ 300 μ m³ at 18 °C. Microseeding or macroseeding could accomplish no further increase in crystal volume. Alternate protocols for the crystallization of wild-type FhuA and FepA are described elsewhere.^{35,37} FhuA complexed with ferricrocin,³³ phenylferricrocin,³⁸ the antibiotic albomycin,³⁸ and a selenomethionine derivative of FhuA with bound ferricrocin³³ were all crystallized under similar conditions. X-ray diffraction analyses indicate that these crystals belong to the primitive hexagonal space group P6₁ with unit cell dimensions of $a = b = 171$ Å, $c = 86$ Å; $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$, with one molecule per asymmetric unit, a Matthews coefficient of 4.8 Å³ Da⁻¹, and a solvent content of 74%. These crystallographic statistics are in the range of the calculated values for other bacterial outer membrane proteins. Diffraction data sets were collected at approximately 100 K from flash-frozen crystals of unliganded FhuA (2.50 Å), and FhuA complexed with ferricrocin (2.70 Å), ferrichrome³⁷ (2.60 Å), phenylferricrocin (2.95 Å) or albomycin (3.10 Å). The structure of FhuA–ferricrocin complex was solved by multi-wavelength anomalous dispersion with the data collected from a single selenomethionine derivative.³³ All other liganded structures of FhuA were solved by molecular replacement. Specific interactions with the single LPS molecule noncovalently bound to the outer barrel surface have been described.⁴⁸

Overall description of the structure

The three-dimensional structure of FhuA and its complex with ferricrocin have been determined to have a resolution of 2.50 and 2.70 Å respectively.^{33,37} The crystallographic structure shows that FhuA is a monomeric integral membrane protein that is organized into two domains, which fold independently.⁴⁹ The C-terminal β -barrel domain (residues 161–714) is a 22-stranded antiparallel β -barrel β 1 through β 22) that spans the outer membrane (see Figure 3).

The tilt of the β -strands relative to the barrel axis is $\sim 45^\circ$ and they range in length from 8–24 residues (Figure 3(b)). All extracellular loops protrude beyond the lipid bilayer, including L4, which extends 34 Å from the cell surface. Analogous to the nonspecific (16-stranded β -barrel^{50,51}) and sugar-specific (18-stranded β -barrel^{52,53}) porins, β -strands that compose the barrel domain of FhuA are connected by longer extracellular loops (L1 through L11) and shorter periplasmic turns (T1 through T10). The elliptical-shaped β -barrel of FhuA is 69 Å in height and has a cross-sectional diameter of 46 $\times$ 39 Å² (3D Structure). The crystal structure of FepA shows a similar fold and molecular dimensions.⁵⁴ Extending from residue 681–690 from β 21, is a β -bulge that protrudes outwards from the barrel surface of FhuA. This segment is highly conserved among all TonB-dependent receptors; its biological function, however, remains to be established. FhuA also contains a pair of cystine bonds. The first disulfide-bridge, Cys318–Cys329, connects the adjacent segments of L4 and is readily surface accessible. The second, Cys692–Cys698, connects strands β 21 and β 22, and is positioned close to the extracellular rim of the β -barrel. These results are in accord with the *in vivo* thiol-labeling experiments, which identified both the disulfide-bridges and the limited accessibility of the C-terminal cystine bond.^{55,56} As observed with other integral outer membrane proteins, two aromatic girdles form a ~ 25 Å hydrophobic zone on the membrane-embedded surface of FhuA (Figure 3(b)). They are positioned to extend into the lipid bilayer, and delineate the interface between the hydrocarbon acyl chains and the phosphorylated glucosamine moieties of the LPS monolayer.^{33,48}

Positioned within the β -barrel of FhuA is the N-terminal cork domain (residues 1–160). This domain is formed by a mixed four-stranded β -sheet with four interspersed α -helices and connecting segments (Figure 3(a)). The first 18 residues of the cork domain, including the TonB-box of FhuA (residues 7–11), are disordered in both the unliganded and liganded structures. Beginning with Glu19 in the ligand-free structure, helix α A is followed by a coil region which extends into the first of the four β -strands, β A, forming the periplasmic edge of the four-stranded β -sheet. After a pair of short helices, α B and α C, a coil region forms two apices, A and B, at Arg81 and Gln100, respectively. Apices A and B are located in the

Figure 4 The ferricrocin-binding site. The ferricrocin molecule is shown as a bond model with carbon atoms white, oxygen atoms red, and nitrogen atoms blue. The ferric iron atom is shown as a large red sphere. Those side-chain residues that form interactions with the siderophore are colored green. The cork domain is colored yellow; barrel β -strands and extracellular loops are shown in blue.

upper half of the barrel and contain side-chain residues directly involved in ligand binding. The polypeptide chain then leads into β B, forming the opposite edge of the β -sheet and positioned in the center of the barrel. From there a coil region angles towards the upper barrel rim forming apex C at Tyr116, which forms hydrogen bonds with ferricrocin, then sharply downwards forming another short helix, α D, leading into strand β C in the lower region of the barrel. A short coil leads into the antiparallel β -strand, β D, which leads into the N-terminal end of the first β -strand of the barrel.

A three-dimensional alignment with all structures deposited in the Protein Data Bank indicates that the fold of the cork domain is unique. The plane of the four-stranded β -sheet is tilted by $\sim 45^\circ$ relative to the membrane normal, such that the direct passage of the ligand and ions across the outer membrane is occluded. Accordingly, genetic excision of the cork domain (FhuA Δ 5–160) facilitates the nonspecific passive diffusion of ferrichrome, antibiotics, and maltodextrins into the periplasm.⁴⁹ In the wild-type receptor, the presence

of the cork domain within the β -barrel delineates a pair of pockets within FhuA. The larger extracellular pocket is restricted by β -strands and apices A, B and C of the cork domain, and is exposed to the external medium. The smaller periplasmic pocket is in contact with the periplasm.

The ligand-binding site

Located in the extracellular pocket of FhuA is a single ferricrocin molecule (Figure 4).

In its binding site the siderophore is positioned ~ 20 Å above the external outer membrane interface. Residues from apices A, B, and C of the cork domain, and the β -strands of the barrel domain form direct hydrogen bonds with the ligand. Additional van der Waals contacts are formed with aromatic residues that line the interior walls of the β -strands and the extracellular loops of the barrel. These side-chain residues may function to extract this, and other hydroxamate-type ligands from the external

solvent into the extracellular pocket by their low-affinity for aromatic residues. This proposal is supported by the observation that the deletion mutant FhuA Δ 5–160 is capable of capturing ferrichrome, albeit weakly, from the external medium;⁴⁹ apices A, B and C are essential for high-affinity binding (K_D 0.1 μ M).⁵⁷

Ligand-induced allosteric transitions and transmembrane signaling

Comparing the structure of FhuA–ferricrocin complex reveals two distinct conformations: unliganded and liganded (Figure 5(a)).

Within the barrel domain the coordinates of the α -carbons are essentially identical in both conformations (root-mean-square deviation of 0.40 Å). However, in the ferricrocin-binding site, an induced fit mechanism is observed. Specifically, in the unliganded conformation, Glu98, near apex B forms two hydrogen bonds with Ser504 and Gln505 from L7. The addition of ligand results in a 90° side-chain rotation and a $\sim$ 1.5 Å α -carbon translation towards the barrel wall. The α -carbon of Gln100 near apex B undergoes a $\sim$ 1.8 Å vertical shift towards the ferricrocin molecule, accompanied by a 50° side-chain rotation; multiple hydrogen bonds are formed with the siderophore. Apex A undergoes a similar vertical shift. All loops of the cork domain between apex A and the periplasmic pocket follow this vertical translation (Figure 5(a)). The position of the four-stranded β -sheet and the loops of the cork domain near the ‘putative channel-forming segment’ between apex B and the periplasmic pocket remain fixed. A thin layer of the side-chain residues occludes this segment of the barrel cross-section, such that a narrow water-filled channel connects the external with the periplasmic pocket of FhuA.

In the unliganded conformation, helix α A, which contains several hydrophobic residues, fits snugly into a complementary hydrophobic cavity formed by select side-chain residues from T8, T9, β A and helix α B (Figure 5(b)). On binding of ferricrocin, the upward translation of selected loops of the cork domain disrupts the interaction of this cavity with the hydrophobic face of helix α A, the switch helix, thereby promoting its destabilization. As a result of this helix–coil transition, all hydrogen bonds between residues of the switch helix and periplasmic turns T8 and T9 are lost. All residues N-terminal of Arg31 in our model assume an extended conformation, bending away from the previous helix axis within the plane of the periplasmic pocket by $\sim$ 180°. Further, Ser20 positioned near Arg128 from β D in the unliganded conformation, is translated by $\sim$ 17 Å from its former α -carbon position in the liganded structure. Arg128, which is strictly conserved among all TonB-dependent receptors, forms extensive hydrogen bonds

Figure 5 Conformational changes induced upon ferricrocin binding. (a) Superposition of the carbon coordinates of unliganded FhuA and its complex with ferricrocin. (b) Close-up view of the periplasmic pocket of FhuA illustrating the unwinding of the switch helix. In both panels the cork domains of FhuA and liganded FhuA are shown in purple and yellow, respectively. The β -strands of the barrel domain are represented as a wire frame. Apices A, B, and C and Glu19 are labeled.

with residues of the cork domain in both structures. This residue, in conjunction with Asp40, stabilizes the association of the N-terminal coil away from the periplasmic opening of the putative channel-forming segment in the liganded conformation. Previous genetic insertion studies have shown that insertion of a dipeptide into the *fhuA* gene after residue Arg128 results in a complete loss of the FhuA function.⁵⁸ Other dipeptide insertions after Ala59, Tyr82 and Pro135 led to diminished FhuA activities. These experiments affirm the importance of the topology of the cork domain. Given that TonB-dependent receptors must compete for a limited number of energized TonB molecules,⁵⁹ the unwinding of the switch helix and the associated altered protein surface, likely signals the ligand-loaded status of the receptor through the outer membrane.

Our crystallographic observations are in accord with the previous functional and antibody recognition studies. It has been shown that: (i) ferrichrome binding to viable bacterial cells decreases the fluorescence of a surface-exposed cysteine residue;⁵⁶ (ii) intrinsic tryptophan fluorescence measurements also show reduced levels of emitted fluorescence upon ferrichrome binding;⁴¹ (iii) monoclonal antibodies that bind to sequences between residues 21–59 can discriminate between the unliganded and liganded conformation of FhuA *in vitro*;³¹ and (iv) incubation of purified FhuA with ferricrocin enhances the complex formation between the receptor and TonB *in vivo*.⁶⁰ In light of the crystallographic structure of FhuA, these data may be re-interpreted as a measurement of the movement of Trp22. This side-chain residue is located at the base of the switch helix, and on ligand binding it is translocated $\sim$ 17 Å across the periplasmic pocket of the FhuA. We postulate that the inability of bacteriophage T5 and microcin J25 to promote the formation of the cross-linked FhuA–TonB complex *in vitro*,⁶⁰ suggests that allosteric transitions induced by these ligands are distinct from those observed with ferricrocin.

Transport of ligand across the periplasm and the cytoplasmic membrane

In *E. coli*, the transport of hydroxamate-type siderophores into the periplasm is mediated by three TonB-dependent receptors: FhuA, FhuE and IutA. With FhuA, ligand is passed through the outer membrane by an aqueous channel formed by the receptor following a physical interaction with TonB and the subsequent transfer of energy. Once present in the periplasm, all ferric hydroxamates are transported across the cytoplasmic membrane by a single periplasmic binding protein-dependent transport system composed of the gene products of *fhuBCD*.^{61–63} FhuD is a periplasmic binding protein that binds a diverse array of hydroxamates including ferric aerobactin, coprogen, ferri-

chrome, ferrioxamine B, schizokinen, rhodoturulate, and the antibiotic albomycin. When loaded with ligands that are uniquely transported by the Fhu system, FhuD is protected against proteolytic cleavage, indicating a conformational transition.^{64,65} Dissociation constants of ferric hydroxamates have been estimated from the concentration-dependent decrease in the intrinsic fluorescence intensity with an affinity tagged FhuD derivative (0.4 μ M for ferric aerobactin, 5.4 μ M for albomycin, 0.3 μ M for coprogen, and 1.0 μ M for ferrichrome).⁶⁶

FhuD shuttles hydroxamate-type siderophores and albomycin across the periplasm and delivers them to the FhuBC complex in the cytoplasmic membrane, a periplasmic binding protein-dependent ABC transporter. The FhuB protein is a pseudoheterodimer composed of two homologous transmembrane domains that are connected by a short hydrophilic linker. Both the domains are required for functional activity.⁶⁴ The docking of liganded FhuD generates a signal that is transmitted into the cytoplasm by FhuB, which triggers ATP hydrolysis. In the absence of ligand, FhuD molecules interact weakly with FhuB, thereby preventing unnecessary ATP hydrolysis. FhuC-catalyzed ATP hydrolysis precedes the passage of the ligand through FhuB into the cytoplasm. Once in the cytoplasm, the intracellular flavin reductases release the Fe(III) ion from hydroxamate-type siderophores by enzymatic reduction.⁶⁷ Whether this event occurs in the cytoplasm or within the cytoplasmic membrane remains to be established. In contrast, the removal of iron from enterobactin requires an esterase. In these studies, esterase cleavage of ferric enterobactin increases the dissociation constant from 10^{-52} to 10^{-8} M.⁶⁸

FUNCTIONAL ASPECTS

Mutant siderophore receptors form channels

The channel-forming ability of FhuA was first demonstrated by constructing deletion mutants of FhuA.⁶⁹ When residues 322–355 from L4 are removed (FhuA Δ 322–355), a nonspecific channel is formed through which ferrichrome passively diffuses at a rate proportional to the external siderophore concentration. FhuA Δ 322–355 incorporated into planar lipid bilayers forms permanently open ion-channels, with a single channel conductance three times larger than those observed with the porins. In contrast, the wild-type FhuA does not form channels under identical experimental conditions. The segment that is critical for channel formation was later restricted to residues 335 through 355. The deletion mutant FhuA Δ 335–355 supports the TonB-independent growth, albeit at a lower growth rate than cells which express the wild-type receptor, and when incorporated in artificial bilayers forms stable channels of defined size and conductance.⁷⁰ Bacterial cells that express FhuA Δ 322–355 or FhuA Δ 335–

355 become sensitive to sodium dodecyl sulfate and bacitracin. As expected, cells that express these deletion mutants or the mutant FhuAΔ5–160 are also capable of utilizing maltodextrins as a carbon source in the absence of maltoporin.^{49,69,70} These growth assays demonstrate that the membrane-impermeable agents can readily traverse the outer membrane through channels formed by FhuA. Considering the structure of FhuA, residues 335–355 compose portions of L4 (residues 315–342) and strand β8 (residues 343–367) of the barrel domain (Figure 3(b)). The structure suggests that the specific connections formed between the side-chain residues located on the inner barrel surface and apex C would be disrupted by such deletion mutations. As a result, certain coil segments of the cork domain may become more flexible, and thus facilitate opening of an aqueous diffusion channel.

Binding sites for bacteriophages and colicin M on FhuA

FhuA functions as the primary receptor for four bacteriophages (T1, T5, φ80, and UC-1) and the bacterial toxin, colicin M. Sequences between residues 333 and 355 have been shown to be important for the binding of FhuA-specific bacteriophages. Cells that synthesize and export the mutants FhuAΔ322–355 or FhuAΔ335–355 to the outer membrane are resistant to cell killing by bacteriophages T1, T5 and φ80, and the import of colicin M.^{69,70} In contrast, those cells which express FhuAΔ322–333 or FhuAΔ322–336 remain fully sensitive to bacteriophages, suggesting that residues 335–355 from L4 participate in the binding of bacteriophages and colicin M. Competitive peptide mapping has also shown that overlapping synthetic hexapeptides covering residues 316–356 reduced bacteriophage-mediated cell killing and the import of colicin M.⁴⁴ Considering that residues 335–355 comprise portions of L4 and strand β8, it is conceivable that FhuA-specific bacteriophages bind to the outer surface of the β-barrel above the external LPS monolayer. In the case of bacteriophage T5, this interaction is mediated by the straight tail fiber protein, pb5.

Primary structure comparisons of the FhuA proteins from *E. coli*, *P. agglomerans*, *S. paratyphi* strain B, and *S. typhimurium* further support the proposal that residues 335–355 are directly involved in bacteriophage binding.²⁵ The FhuA protein from *S. paratyphi* strain B shares significant homology with the equivalent segment of the FhuA protein from *E. coli*. When expressed in *E. coli*, the FhuA protein from *S. paratyphi* strain B confers full sensitivity to bacteriophages T1, T5, and φ80. The FhuA proteins from *S. typhimurium* and *P. agglomerans*, which do not have homologous 335–355 sequences, render the bacterium completely resistant to FhuA-specific bacteriophages. In contrast, cells that express the mutant FhuAΔ5–160 remain fully susceptible to the TonB-dependent

bacteriophages T1 and φ80, and colicin M. Thus, the cork domain of FhuA is not required for the binding of bacteriophages or the import of colicin M.⁴⁹

When FhuA is incorporated into planar lipid bilayers, the binding of bacteriophage T5 to FhuA triggers a conformational change in the receptor such that a stable high-conductance channel opens.³⁹ This channel is electrophysiologically indistinguishable from the permanently open channels formed by FhuAΔ322–355, suggesting that these channels resemble those formed *in vivo*.^{69,70} A structure composed of six copies of the pb2 protein, which is located at the distal end of the tail of bacteriophage T5, probably traverses the artificial bilayer as revealed by cryoelectron micrographs.⁴³ This structure contracts the following interaction of the bacteriophage with FhuA, and probably mediates the transfer of the DNA template across the outer and cytoplasmic membranes.^{71,72} Such a mechanism would eliminate the need for the cork domain to be completely removed and subsequently re-inserted into the β-barrel following the passage of the nucleic acid through FhuA.

TonB-receptor interactions are involved in energy transduction

Different from transport across the cytoplasmic membrane, where primary or secondary active transporters utilize hydrolysis of ATP or ion translocation for uptake, there is no available energy source in the outer membrane to drive the translocation of siderophores and vitamin B₁₂ into the periplasm. Energy is, however, needed to dissociate the bound ligand from its cognate receptor. Thus, gram-negative bacteria couple the chemiosmotic gradient of the cytoplasmic membrane with ligand transport across the outer membrane.⁷³ While the precise mechanism by which this process is accomplished remains to be determined, it is known that the TonB–ExbB–ExbD complex plays a key role in the energy coupling.^{1,74–76} TonB is a 273 residue periplasmic protein that is anchored, by an N-terminal hydrophobic segment, in the cytoplasmic membrane.^{76,77}

All TonB-dependent receptors and group B colicins have one N-terminal segment of conserved primary structure, the ‘TonB-box’.⁷⁸ The consensus seven residue TonB-box sequence is: acidic residue (Asp or Glu)–Thr–(hydrophobic residue)₂–Val; polar residue (Ser or Thr)–Ala.⁷⁴ A functional role has been assigned to this highly conserved region. With the BtuB receptor, the introduction of a proline residue at positions 8 or 10, or a glycine residue at position 10, abolishes the TonB-dependent uptake of vitamin B₁₂.²² However, these and other point mutations do not impair the binding of vitamin B₁₂, the irreversible adsorption of the TonB-independent bacteriophage BF23, or the import of colicins E1 and E3.⁷⁹ By substituting an isoleucine residue at position 9 with a proline side-chain in FhuA, TonB-dependent uptake of ferrichrome is inhibited,

whereas Ile9Thr and Ile9Ser substitutions have no effect.⁴⁵ With the Cir receptor, replacing valine 8 with a glycine residue renders *E. coli* cells resistant to colicin Ia, without affecting the recognition and uptake of dihydroxybenzoate.⁸⁰ A similar substitution uncouples TonB-dependent uptake as mediated by the FhuE receptor.¹⁷

Initial evidence that TonB may associate with the TonB-boxes of the outer membrane receptors was provided by genetic suppresser mutation studies. Heller *et al.*⁸¹ isolated plasmid-encoded *tonB* mutants that partially restore the TonB-dependent activity in *E. coli* strains expressing the BtuB mutants mentioned above. The mutation was revealed to be a single amino acid substitution (leucine, lysine or proline) at position 160 of TonB.^{81,82} Analogous *tonB* suppressor mutations have been characterized for TonB-box mutants of FhuA,⁴⁵ Cir,^{45,80} and FhuE.¹⁷ The ability of *tonB* suppressor mutants to partially restore the activity may be explained if a segment of TonB at or near residue 160 physically interacts with TonB-dependent receptors.⁸² Considering that seemingly invariant TonB-box residues can be mutated without affecting the function, it is likely that it is the conformation rather than the primary structure of the TonB-box that is required for an efficient energy-transduction. Accordingly, point mutations may distort the conformation of the TonB-box or the adjacent regions of the TonB-dependent receptors, such that specific protein–protein interactions with TonB are impaired.

The direct physical interaction of TonB with its cognate receptors was first demonstrated by cross-linking studies. When treated with formaldehyde, FepA, embedded in the outer membrane of *E. coli* can be chemically cross-linked to TonB in the absence of ferric enterobactin.⁸³ The observation that a point mutation in the TonB-box of this receptor (Ile14Pro) prevents the TonB-dependent ligand transport and cross-linking to TonB⁸⁴ suggests that conformational changes in this region quite likely inhibit the protein–protein interactions with TonB. Similar cross-linking studies have been carried out with the FhuA receptor in the presence and absence of ligand.⁶⁰ In the absence of ferricrocin, the FhuA–TonB complex could not be detected *in vivo*. Other FhuA-specific ligands, bacteriophage φ80 and to a lesser extent colicin M, also enhance the formation of FhuA–TonB complexes *in vitro*. Cells that express certain *tonB* suppressor mutations are capable of restoring the partial activity to TonB-box mutants and the formation of cross-linked complexes.⁸⁴ Following the solution of the crystal structures of FhuA and FepA, and the observation that the TonB-boxes of these transporters are localized in the periplasm, site-directed disulfide cross-linking was used to unequivocally demonstrate the existence of direct physical interactions between outer membrane receptors and TonB.⁸⁵ Cysteine residues introduced at successive positions within the TonB-box of BtuB, which also resides in the periplasm,⁸⁶ form disulfide-bridges with a single cysteine residue

inserted at or near position 160 of TonB.⁸⁵ Only those BtuB mutants with engineered cysteines in their TonB-boxes were capable of forming a complex with TonB. As observed with FepA and FhuA, the efficiency of cross-linking was increased in the presence of ligand. This study also suggests that interactions between TonB and TonB-dependent receptors can be roughly described as a parallel N-to-C-terminal arrangement of both proteins. Site-directed spin labeling and EPR assays indicate that the TonB-box of BtuB, in the unliganded conformation, may form a helix that forms interactions with residues from the periplasmic turns of the β-barrel of the receptor.⁸⁶ The binding of vitamin B₁₂ to BtuB induces a helix-to-coil transition, such that the TonB-box assumes an extended conformation.

A proposed mechanism for TonB-dependent transport of ligand through the receptor

By integrating the available biochemical, genetic and structural data, a model of TonB-dependent energy-transduction can be proposed. TonB, embedded within the cytoplasmic membrane, is closely associated with the heterohexameric ExbB–ExbD complex.⁸⁷ The transfer of chemiosmotic energy from the cytoplasmic membrane to the outer membrane by the TonB–ExbB–ExbD complex might occur by an allosteric mechanism. The proton motive force likely drives a conformational transition of TonB from an energetically ‘uncharged’ to the ‘charged’ conformation, which in turn promotes its interaction with the outer membrane receptors.⁸⁸ As TonB is present in lower concentrations compared to the receptors which it services,⁵⁹ and given the possibility that most TonB-dependent receptors may not contain bound ligand, efficient use of the chemiosmotic gradient dictates that only ligand-loaded receptors are energized. The unwinding of the switch helix and the associated translocation of the TonB-box may serve to distinguish the liganded from the unliganded conformation of the transporter, and therefore fulfil this functionally important role.

Following the unwinding of the switch helix upon transition to the liganded conformation, energy-charged TonB preferentially interacts with outer membrane receptors. However, portions of the available TonB molecules probably form complexes with the liganded TonB-dependent receptors whose switch helices have not unwound. This proposal is supported by the observation that some TonB copurifies with the outer membrane receptors during cell fractionation.⁸⁹ Moreover, bacterial cells that synthesize the deletion derivative FhuAΔ5–160, which does not have a TonB-box, also retains a diminished level of TonB-dependent activity.⁴⁹ In either case, the C-terminal domain of TonB likely forms direct physical contacts with the TonB-box and other side-chain residues found within the periplasmic turns of the β-barrel domain of TonB-

Figure 6 The putative channel-forming segment. A series of strictly conserved side-chain residues which line the inner barrel surface of the putative channel-forming segment extends from the external to the periplasmic pocket of FhuA, and may function as a series of low-affinity binding sites for ferricrocin and other ferric hydroxamates. (a) View along the barrel axis. (b) View from the membrane perpendicular to the barrel axis. The protein is shown in ribbon representation. The conserved residues are shown in green. The barrel and cork domains are shown in blue. The ferricrocin molecule is shown as a bond model with the carbon atoms white, oxygen atoms red, and nitrogen atoms blue. The ferric iron atom is shown as a red sphere.

dependent receptors,⁴⁹ as observed with the deletion mutant FhuAΔ21–128, which is capable of forming chemically cross-linked complexes with TonB,⁶⁰ and as observed with the BtuB receptor.⁸⁶ This physical association occurs independently of the energy state of TonB and the ExbB–ExbD complex,⁸⁹ but is enhanced by the presence of ligand.^{60,83,85,86}

Upon forming a complex with the outer membrane receptors, TonB transduces its stored conformational energy to the receptor. This event drives an energy-consuming conformational change that disrupts the ligand-binding site; the binding affinity is reduced or abolished. Because Tyr244 and Trp246 from L3 remain fixed whereas apices A and B of the cork domain are translated upward upon ligand binding, disruption of the binding site is likely effected by the shift of apices A, B and C towards the periplasm. Alternatively, the arrangement of the aromatic side-chain residues that line the extracellular pocket, specifically those found on L4, may alter their conformations.⁵⁶ We presume that the formation of a transient complex with an energized TonB molecule induces a high-conductance channel to open within the TonB-dependent receptors. When viewed along the barrel axis, the external pocket is connected with the periplasmic pocket of FhuA in one segment of the barrel cross-section by a ~10 Å aqueous channel, the putative channel-forming segment (Figure 6(a)). Subtle conformational changes in this region, specifically the loops of the cork domain between apex B and the periplasmic pocket of FhuA would suffice to open a channel. Following the disruption of its binding site, the ligand diffuses into the periplasm by utilizing a series of strictly conserved side-chain residues that line the interior barrel wall of the putative channel-forming segment from the ligand-binding site to the periplasmic pocket of FhuA (Figure 6(b)). By analogy to the ‘greasy-slide’ of the glycoporins LamB⁵² and ScrY,⁵³ the arrangement of these conserved side-chain residues might function as a series of low-affinity binding sites that mediate the surface diffusion of ligand through TonB-dependent receptors. Surface diffusion would decrease the amount of siderophore that is released back into the external medium following the disruption of the high-affinity binding site, and would increase the overall rate of flux of siderophores across the outer membrane.⁹⁰ Upon arrival in the periplasm these ligands are rapidly bound by specific, high-affinity periplasmic binding proteins and shuttled to distinct ABC importers embedded within the cytoplasmic membrane.

FUNCTIONAL DERIVATIVE

X-ray structure of FhuA in complex with the antibiotic albomycin

Albomycins are fungus-derived antibiotics that display a broad-spectrum bactericidal activity against the gram-negative bacteria. The potency of these ‘natural’ side-

rophore–antibiotic conjugates is derived from their ability to utilize the Fhu system for their uptake. The FhuA actively transports albomycin across the outer membrane by a TonB-dependent mechanism, whereas the FhuBC importer mediates uptake into the cytoplasm on the hydrolysis of ATP. Similar to other hydroxamates, the iron-chelating component of albomycin is a tri- δ -N-hydroxy- δ -N-acetyl-L-ornithine peptide (Figure 2(a)–(c)). Covalently linked to this segment, by an amino acetyl linker, is a thioribosyl pyrimidine antibiotic moiety.⁴⁸ Enzymatic cleavage of this group is required for albomycin to exert its antimicrobial activity.⁹¹ Although the uptake pathway and activation requirements are known, the intracellular target of albomycin remains to be determined.

The three-dimensional structure of FhuA in complex with albomycin has been solved to a 3, 10 and 2 Å resolution.³⁸ The characteristic side-chain residues from apices A, B and C of the cork domain and the extracellular loops and β -strands of the barrel domain which were identified to be involved in the siderophore-binding site are conserved in the albomycin-binding site (Figure 2(c)). Structural alignment of the α -carbon coordinates of the FhuA–siderophore and FhuA–albomycin complexes reveals perfect superposition (root-mean-square deviation of 0.25 Å) of the barrel domains. In the albomycin-binding site, an identical induced fit mechanism to that observed with siderophores occurs on the binding of the antibiotic. The vertical shift (1–2 Å) of apices A and B, and the other residues of the cork domain, is propagated to the periplasmic pocket of FhuA. Thus, the switch helix unwinds. This allosteric transition suggests that albomycin promotes the physical interaction between FhuA and TonB, thereby promoting its uptake into the periplasm.

The three-dimensional structure of FhuA in complex with albomycin provides a structural platform for the rational modular design of novel antibiotics. The proposal that antibiotics can be actively pumped across bacterial membranes is well supported.⁹² Siderophore–antibiotic conjugates are bactericidal agents that consist of three parts: an iron-chelating siderophore; a peptide linker; and an antibiotic group. The principle requirement in the design of these novel antibiotics is that the drug must be specifically recognized, and thus, transported across the cell envelope. In its binding site, the iron-chelating portion of albomycin is deeply buried within the extracellular pocket of FhuA, whereas the thioribosyl pyrimidine moiety remains surface-accessible. The composition of the binding site surrounding the iron-chelating component of albomycin is spatially restrictive, and provides for specific high-affinity binding. Accordingly, chemical or conformational alteration of this region inhibits the receptor-specific recognition.⁹³ The remaining portions of the drug are bound less tightly, with few apparent constraints on the chemical structure, conformation or the spatial requirements of the linker and the antibiotic group. By utilizing distinct receptors for their uptake, siderophore–antibiotic

conjugates designed to target bacterial pathogens with FhuA homologs in their outer membranes may be developed.

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