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Transmembrane Signaling across the Ligand-Gated FhuA Receptor: Crystal Structures of Free and Ferrichrome-Bound States Reveal Allosteric Changes

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Summary

FhuA protein facilitates ligand-gated transport of ferrichrome-bound iron across *Escherichia coli* outer membranes. X-ray analysis at 2.7 Å resolution reveals two distinct conformations in the presence and absence of ferrichrome. The monomeric protein consists of a hollow, 22-stranded, antiparallel β barrel (residues 160–714), which is obstructed by a plug (residues 19–159). The binding site of ferrichrome, an aromatic pocket near the cell surface, undergoes minor changes upon association with the ligand. These are propagated and amplified across the plug, eventually resulting in substantially different protein conformations at the periplasmic face. Our findings reveal the mechanism of signal transmission and suggest how the energy-transducing TonB complex senses ligand binding.

Introduction

The crux of bacterial iron uptake is the low biological availability of this transition metal. To secure sufficient intracellular concentrations, bacteria have evolved two strategies to overcome this problem. Iron may be scavenged from eukaryotic host proteins, such as transferrin (Nau-Cornelissen and Sparling, 1994), or be acquired from the environment by the use of siderophores (Neilands, 1995). In Gram-negative bacteria, both pathways involve substrate-specific outer membrane receptors and an energy-transducing module (TonB complex), which is anchored in the cytoplasmic membrane (Klebba et al., 1993; Postle, 1993; Guerinot, 1994; Braun, 1995). Binding of substrate to the extracellular face of the receptor protein is signaled to the TonB complex (Moeck et al., 1997) and triggers the energy-consuming translocation across the outer membrane. This critical interaction with the energy-transducing TonB module involves a conserved sequence motif at the N terminus of the receptor (TonB box, Schramm et al., 1987), which is exposed at the periplasmic face and also present in bacterial toxins requiring the TonB system for uptake (B group colicins).

The TonB-dependent receptors are intimately related to virulence of pathogenic organisms such as *Haemophilus*, *Neisseria*, and *Yersinia* (Stojiljkovic et al., 1995; Stevens et al., 1996; Nau-Cornelissen et al., 1998). Their relevance to microbial growth is documented also by their multiplicity in the genomes of *Haemophilus influenza*, *Helicobacter pylori*, and *Escherichia coli* (Fleischmann et al., 1995; Blattner et al., 1997; Tomb et al., 1997). These prokaryotic systems are particularly intriguing because their signal transduction and regulated channel activities may be considered paradigms of gated proteins in eukaryotic organisms. Among these, well-studied examples are the ligand-gated nicotinic acetylcholine receptor and the K^+ channel. The structure of the former has been solved at 9 Å by electron crystallography (Unwin, 1998), while that of the active domain of a bacterial homolog of a K^+ channel, lacking the electric gating and the desensitization domains, was determined to 3.2 Å resolution by X-ray crystallography (Doyle et al., 1998).

For our study, we have chosen the full-length, multifunctional FhuA protein (*f*erric *h*ydroxamate *u*ptake protein component *A*, 714 residues) from *E. coli* (Coulton et al., 1986). Its physiological function is the uptake of the siderophore ferrichrome (Hantke and Braun, 1975). The protein, being exposed at the cell surface, is also subject to pathological usurpation. Thus, it constitutes the target of several bacteriophages (T1, T5, ϕ80, and UC-1) and bacterial toxins (colicin M and microcin 25), and it mediates the passage of antibiotics such as albomycin (Braun and Wolff, 1973). On the basis of its sequence and extensive genetic studies, a topological model of the FhuA protein was proposed (Koebnik and Braun, 1993). The model predicted a barrel of 32 β strands, analogous to porins, yet larger and with substantially longer extracellular loops. Almost complete deletion of the seemingly largest loop (34 out of 41 residues) resulted in nonspecific, TonB-independent substrate diffusion, leading to the notion of a "gating loop" that is bent into, and constricts, the channel lumen (Killmann et al., 1993). This model was further supported by the finding that phage T5, which is unique in not requiring energy transduction, is known to recognize sequences on the proposed "gating loop" (Killmann et al., 1995), thus converting the FhuA protein into a permanently open channel (Bonhivers et al., 1996). This uncoupling has been exploited for an in vitro functional assay that allows determination of ligand-gated ferrichrome translocation across the FhuA protein, as well as the passage of viral DNA across liposome membranes (Letellier et al., 1997; Plançon et al., 1997).

The results of our X-ray crystallographic studies at 2.7 Å resolution of the liganded and unliganded structures of the FhuA protein, presented here, reveal a domain structure that has been anticipated conceptually over

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Table 1. Summary of Data Collection and Refinement Statistics

Compound	Apo Native	Holo Native		Apo Selenomethionyl	
Space group	C2	C2		C2	
Unit cell[a] a (Å)	132.9	132.2		135.3	
b (Å)	89.9	89.4		89.5	
c (Å)	90.2	89.9		84.7	
β (°)	95.2	95.4		98.3	
X-Ray Data					
Beam line	LURE DW32	ESRF BM14		ESRF BM14	
Temperature	20°C	20°C		−170°C	
No. of crystals used	5	4		1	
Wavelength (Å)	1.0000	0.9998	0.97877	0.97893	0.8856
Max. resolution (Å)	2.74 Å	2.59 Å	2.95 Å	2.95 Å	2.70 Å
Unique reflections[b]	26534	30766	41160	41149	53186
Completeness (%)	95.6	92.8	99.5	99.4	98.4
Redundancy	3.5	5.3	2.4	2.4	2.3
R_{sym} (%)	5.5	6.0	3.8	3.7	3.8
R_{ano} (%)			5.2	3.9	3.6
Anomalous phasing power for acentric reflections[c]			3.3	2.6	2.8
Figure of merit					
After SHARP refinement				0.57	
After solvent flattening				0.80	
Refinement					
R factor (%)[d]	18.6	18.3			
R_{free} (%)[d]	24.3	23.0			
Ordered water molecules	151	143			
Ordered detergent molecules	7	8			
Rms dev. from ideal geometry					
Bond lengths (Å)	0.007	0.007			
Bond angles (°)	1.4	1.3			
Average B factor (Å^2)					
Of the protein	52	53			
Of the ligand		46			

[a] At the temperature of the X-ray measurements.
[b] In the case of the selenomethionyl MAD data, Bijvoot pairs I_+ and I_- are considered as different unique reflections. $R_{sym} = \Sigma|I_{avg} - I_i|/\Sigma I_i$, and $R_{ano} = \Sigma|I_+ - I_-|/\Sigma|I_+ + I_-|$.
[c] Average of the calculated anomalous contribution of the Se atoms to the structure factor, divided by the lack of closure.
[d] $R = \Sigma|F_{obs} - F_{cal}|/\Sigma F_{obs}$. R_{free} is calculated from about 5% of randomly chosen reflections, which were not used in the refinement.

20 years ago by the ball-and-chain model for the inactivation of sodium channels (Armstrong and Benzanilla, 1977). The conformational changes occurring upon ligand binding explain how the state of ligation of the receptor may be sensed by the energy-transducing TonB complex.

Results and Discussion

Structure Determination and Protein Architecture

Well-ordered crystals of the FhuA protein were obtained from detergent solution and belonged to the monoclinic space group C2 with one monomer in the asymmetric unit and a solvent content of 63%. The structure was solved using multiwavelength anomalous diffraction (MAD), with data collected at 100 K from a single selenomethionine crystal with a slightly altered unit cell (Table 1). Two refinements, both of the liganded and unliganded native forms, were then carried out in parallel, using room temperature diffraction data to resolutions of 2.6 Å and 2.75 Å. The final R (R_{free}) values are 18.4 (22.9%) and 18.9 (24.5%). The models lack the first 17 and 19 residues, respectively, of the 714-residue polypeptide chain.

The overall structure of the FhuA protein (Figure 1) reveals two domains. In the prominent, hollow β barrel, a plug is located (Figure 2), which exhibits significantly higher sequence conservation among TonB-dependent receptors (Figure 2A) than the barrel. The scaffold of the barrel consists of 22 β strands and has a bean-shaped cross section (35 × 24 Å). Two rings of aromatic residues delineate the face of the protein that is exposed to the hydrophobic membrane core (Figure 2E). The barrel architecture and the existence of disulfide bridges in loops L4 and L11 (Figure 2C) are reminiscent of the folds observed in nonspecific and specific porins (Weiss et al., 1991; Cowan et al., 1992; Schirmer et al., 1995). However, the barrel is larger, the β strands are longer (7–28 residues), and the loops (3–31 residues) extend substantially further into the extracellular medium. The farthest protruding loop (L4) roughly corresponds to the proposed "gating loop." It forms, in conjunction with L3, L5, and L11, a sturdy protuberance of about 35 Å height above the membrane surface, and it constricts the channel entrance to about half the area of the total cross section.

A nearly globular plug clogs the barrel, the two domains being connected by a single hinge region. The

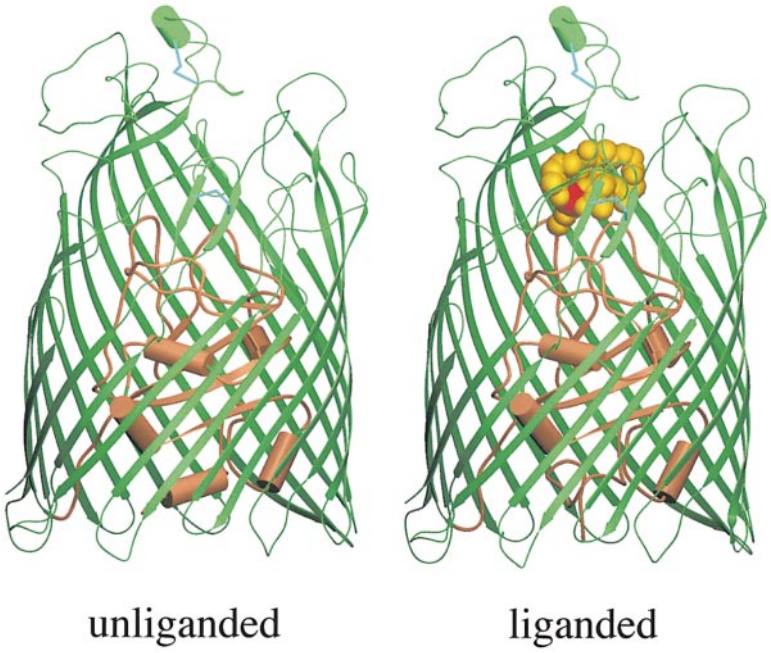

Figure 1. Overall Structure of the Free and Liganded FhuA Protein

In both conformations, a plug (brown) is located in and snugly fits a barrel formed by 22 antiparallel β strands (green). The top of the molecule is exposed at the cell surface, while the bottom faces the periplasm, as assigned on the basis of topological studies (Koebnik and Braun, 1993). The ligand, ferrichrome, is depicted in yellow, with the complexed iron atom shown in red. Note that in the liganded form, one α helix (bottom) is unfolded.

plug comprises five α helices and six β strands (Figure 2D). Three antiparallel and one parallel strand form a sheet that is slightly tilted with respect to the membrane plane. The overall fold of the plug has no precedent in the protein data bank. Its surface interacts extensively with the barrel lining, as witnessed by the presence of nine salt bridges and more than 60 hydrogen bonds (judged from the interatomic distances observed). The top of the plug is exposed to the extracellular medium and is thus accessible to solvent. The N-terminal residues are not visible in the electron density map, but six cycles of sequential Edman degradation showed unequivocally that they are present in the protein obtained from dissolved crystals (data not shown). This segment contains a conserved sequence motif (TonB box), which is an earmark of the interaction with the energy-transducing TonB complex.

Ferrichrome Binding

The binding site for ferrichrome is located at the apical interface of three turns of the plug domain and two loops (L3 and L11) of the barrel. The omit map, shown in Figure 3A, shows an excellent fit of the ferrichrome molecule in the electron density. We found no significant alteration of the conformation of bound siderophore when compared to the crystal structure of the free molecule (van der Helm et al., 1980), the rms difference after least-squares superposition being only 0.3 Å. The binding pocket in the protein is lined by aromatic side chains (Figure 3A), except for R81, which forms strong hydrogen bonds to the substrate (Figure 3B) and is conserved in ferrichrome-translocating outer membrane receptors (Figure 2A). The "head" of the siderophore, containing the complexed iron, is stuck in the pocket, whereas its "tail" remains solvent accessible. Contacts between ferrichrome and FhuA protein involve hydrogen bonds to three plug residues (R81, Y116, and backbone of G99) and two barrel residues (Y244 and Y315). These interactions (Figure 3B), in conjunction with the perfect fit of the substrate in the pocket, explain the strong binding (K_d of about 100 nM) of ferrichrome to FhuA protein (Locher and Rosenbusch, 1997).

Signal Transduction by Local and Allosteric Transitions

Binding of ferrichrome triggers the critical interactions of the TonB complex with the periplasmic face of the FhuA protein (Moeck et al., 1997). How is ligand binding signaled across the membrane? The comparison of the apo- and the liganded structures reveals perfect superposition of the barrel and its loops, but small yet distinct changes in the binding site (Figure 4A). Thus, two turns of the plug (at T80/R81 and at G99/Q100) are shifted toward the ligand, with significant backbone displacements of about 1–2 Å. Displacements of other residues lining the binding site are smaller than the experimental errors. While the shift at G99/Q100 does not appear to affect the neighboring protein segments, the alterations at R81 do. The changes are asymmetrically propagated across the plug (left side in Figure 4B), and they are amplified such that a drastically changed conformation of the near N-terminal sequence results at the periplasmic surface (Figure 4C). Signal propagation involves a vertical shift (~2 Å) of the backbone of two helices (H3 and H4) with respect to the membrane plane and toward the ligand, combined with a slight twist. In the new conformation, the pattern of noncovalent bonds within the plug as well as with the barrel is altered. The changes result in the unfolding of H1 (Figure 1 and 4C), and the corresponding segment swings to the opposite side of the barrel wall, with the largest displacement observed for W22 (17 Å for the Cα atom). This finding is matched by the decreased tryptophane fluorescence of solubilized

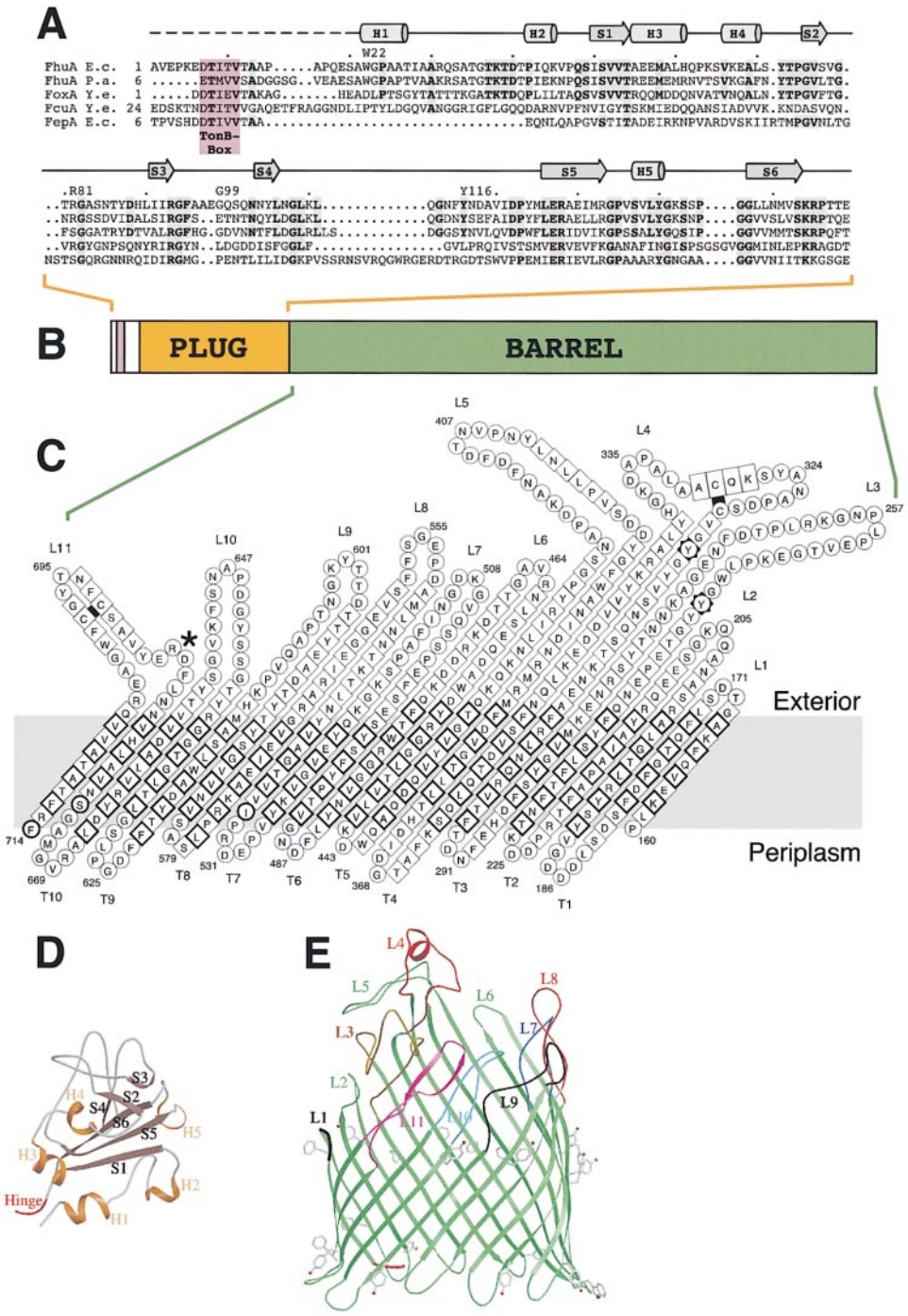

Figure 2. Schematic Domain Structure

(A) Sequence alignment of N termini and plug domains of five TonB-dependent receptors, with the first residue indicated. *E. coli* FhuA (EMBL accession code M12486), *Pantoea agglomerans* FhuA (Y14026), *Yersinia enterocolitica* FoxA (X60447), *Y. enterocolitica* FcuA (X67331), and *E. coli* FepA (M13748). Conserved residues are indicated in bold and are shaded. The assignments of helices (H1 to H5) and strands (S1 to S6) are based on our X-ray structure of the FhuA protein. The dashed line indicates disordered residues. Residues mentioned in the text (W22, R81, G99, and Y116) are labeled.

(B) Bar showing the three main regions of the FhuA protein: an N-terminal segment (white), containing the TonB-box (purple), a plug domain (orange), and a barrel domain (green).

(C) Topology of the barrel. L and T refer to extracellular loops and periplasmic turns, respectively. Residues are framed according to their secondary structure: β strands (diamonds), α helices (rectangles), loops or turns (circles); thick frames indicate residues that are exposed to the lipid bilayer (gray shading). The two disulfide bridges (in L4 and L11) are shown in black. Residues involved in ferrichrome binding are surrounded by spikes, and a highly conserved sequence motif near the C terminus is indicated by an asterisk.

(D–E) Folds of the plug (in the unliganded form) and the barrel domains. A belt of aromatic residues at the membrane boundaries is shown in the ball-and-stick representation, and the peptide segment connecting the plug to the barrel is indicated in red (lower left in [D] and [E]).

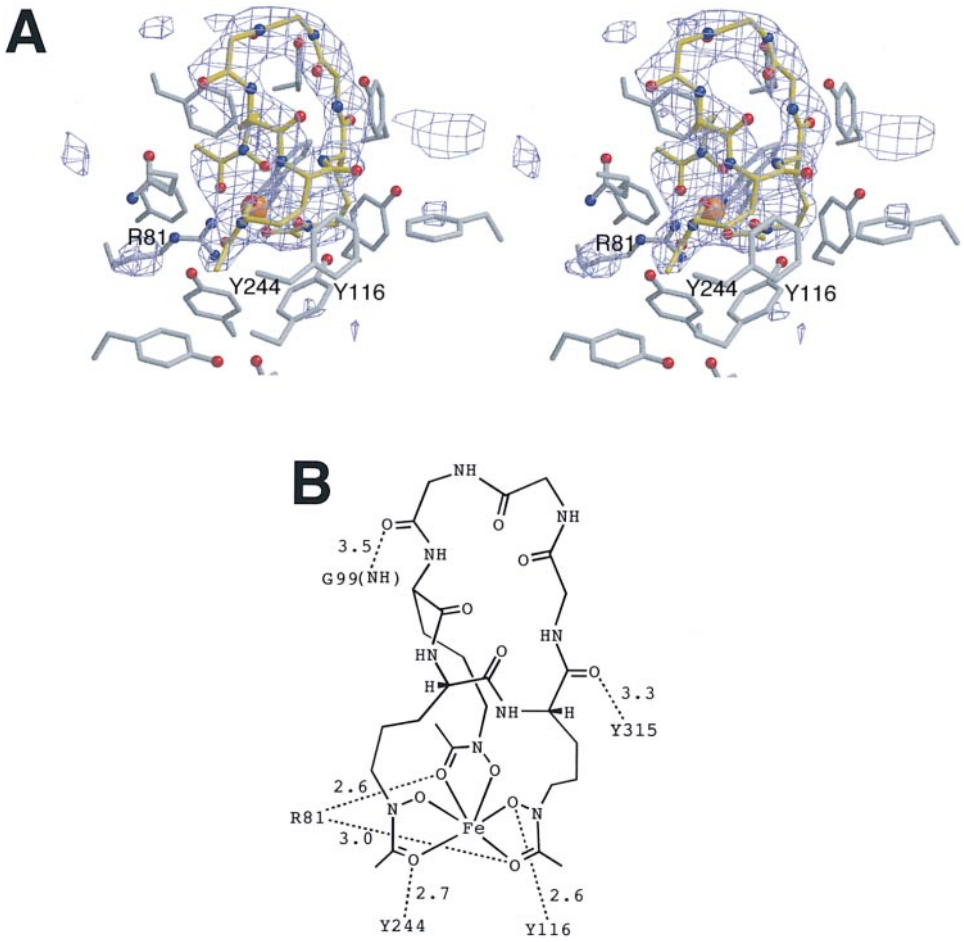

Figure 3. Ferrichrome in Its Binding Site

(A) Stereo view of the molecule (yellow) in the aromatic binding pocket. The electron density (blue net) is a ferrichrome omit map contoured at 3σ level.

(B) Chemical structure of ferrichrome. FhuA protein residues involved in hydrogen bonding are indicated, with distances in angstroms.

FhuA protein upon ferrichrome binding (Locher and Rosenbusch, 1997). The significantly altered protein surface is most likely the molecular basis of the signal transmitted to the TonB protein.

Translocation Pathways

After the signal has crossed the membrane, how is transport of the substrate accomplished? At first sight, removal of the plug from the barrel lumen would represent an attractive possibility of gating. Such a mechanism is reminiscent of the ball-and-chain model proposed for inactivation of sodium channels (Armstrong and Benzanilla, 1977). In this conceptual model, a ball domain (plug), linked to a channel domain by a peptide segment, was postulated to rapidly swing in and out of the channel for inactivation and recovery from inactivation. In FhuA protein, the plug obstructs almost the entire channel lumen and snugly fits the barrel wall. This appears inconsistent with a complete removal of the plug during ferrichrome translocation. Given the size of the siderophore, a channel large enough for translocation does not, in fact, require such a drastic dislocation, but it could be achieved by smaller conformational changes within the plug. Such alterations appear likely in view of the energy transduced by the TonB complex (Kadner, 1990). Tethering the plug to the barrel wall in various places by means of disulfide bridges designed on the basis of the atomic coordinates may serve as an experimental approach to detect major or minor movements occurring during transport. From a comparison of the liganded with the unliganded state, it is clear that the location of the N terminus, containing the TonB box, shifts considerably upon ferrichrome binding. Energy transduction from the TonB protein may thus be expected to cause the TonB box to assume a fixed conformation and to induce subsequent changes in the plug that are more drastic than those occurring during signaling. This can be investigated by determination of the structure of the two proteins in complex (in progress) and the identification of key residues by site-directed mutagenesis.

While ferrichrome does not require the total width of the channel lumen for translocation, injection of double-stranded virus DNA in B form (24 Å diameter, Drew et al., 1982), if it proceeds through the FhuA channel, would require the plug displaced completely. This appears

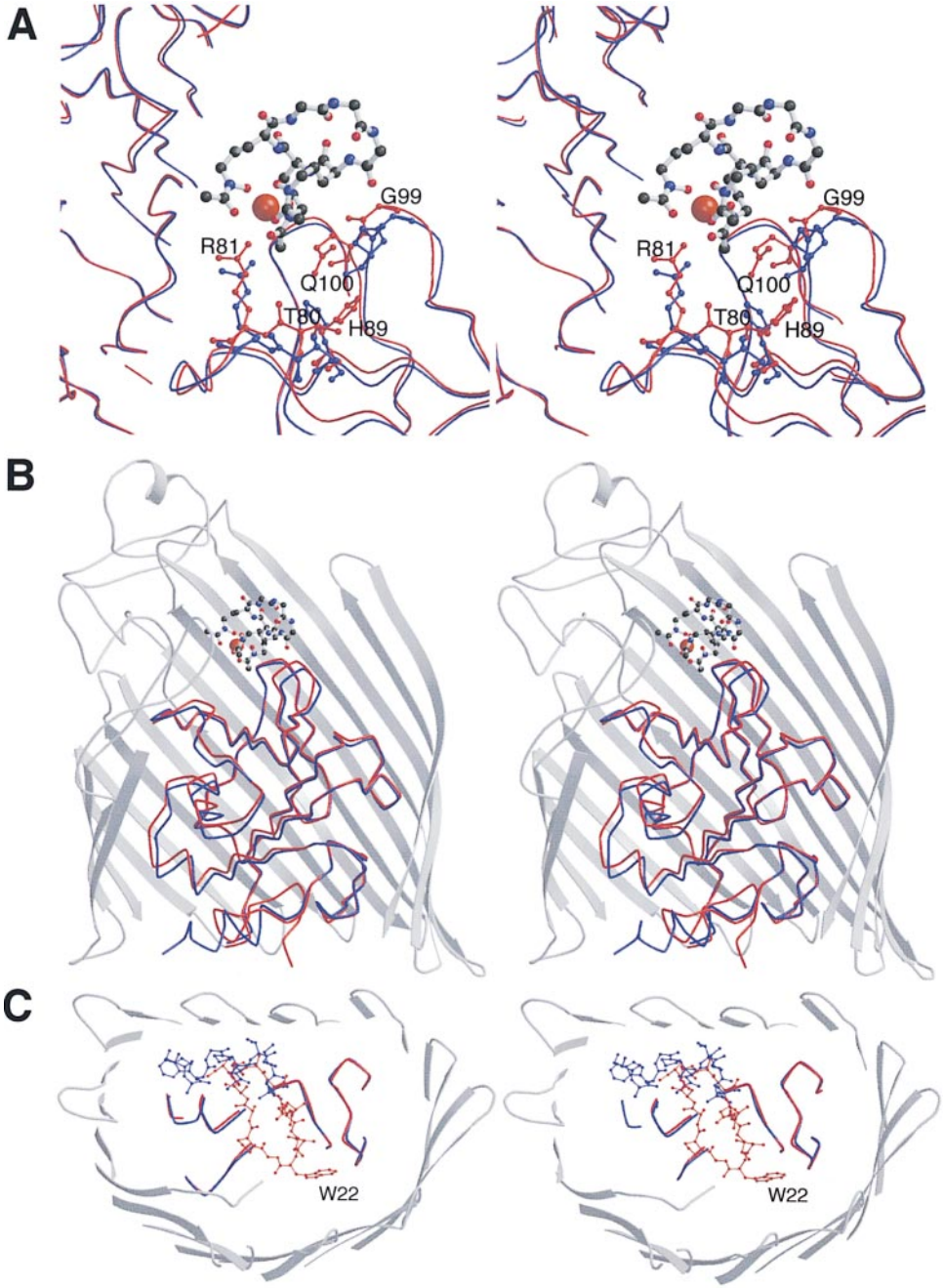

Figure 4. Stereo Diagrams of Bound Ligand and Signal Transduction

(A–C) Comparison of the structures of the apo-protein (blue) and of the complex (red) after superimposing the Cα atoms of the β barrel.

(A) Local conformational changes in the binding pocket upon binding of ferrichrome (shown in ball-and-stick).

(B) Conformational changes propagated through the plug domain. A side view of the FhuA protein, with a part of the barrel (residues 478–657) removed for clarity, shows that the resulting signal transmission is asymmetrical (more pronounced on the left side of the panel). The backbone displacements are about 2 Å.

(C) View onto the channel entrance from the periplasmic side, emphasizing the unfolding of helix 1. Residues 18–30 (red) and 20–30 (blue) are shown in ball-and-stick representation. The Cα atom of W22 is displaced by 17 Å.

plausible, considering the forces involved in DNA ejection from the phage capsid, and may be tested by examining the sensitivity of the plug to exogenous proteases. A different mechanism may apply to FhuA-mediated translocation of colicin M (29 kDa). If this protein has an elongated shape similar to that determined for another TonB-dependent colicin (colicin Ia, 69 kDa, Wiener et al., 1997), it could only slide through the barrel in folded state if the plug is ousted. At this time, it is not clear how this may be achieved, and a partial unfolding during translocation (Bénédetti et al., 1992) cannot be excluded.

What is the role of the proposed "gating loop" (Killmann et al., 1993), which corresponds to the largest of the extracellular loops (L4)? From its location, and from

the absense of conformational changes upon ligand binding, it is clear that this loop is not directly involved in gating. Examination of its surroundings shows that it interacts with three other loops (L3, L5, and L11) of the protuberance, which contacts the top of the plug near the ferrichrome-binding site. Deletion of L4, which results in a TonB-independent, permanently open channel (Killmann et al., 1993), may therefore not only prevent the assembly of the protuberance, but also the proper insertion of the plug into the barrel.

Conclusions

The two structures of the FhuA protein, with and without bound ligand, provide the molecular basis of signal transmission at the atomic level. The unprecedented architecture of a membrane protein with an independent domain physically plugging a channel suggests a revised mechanism of ligand-gated receptor activity. If biochemical experiments can be designed to assess the position of the plug during translocation, the detailed investigation of the conformational changes involved requires the high-resolution structure of the FhuA and TonB proteins in complex. A prokaryotic system, whose complexity can easily be tackled by well-established genetic and biochemical methods, may thus serve once more as a paradigm of eukaryotic proteins with analogous functions. An immediate benefit may arise from exploiting the fact that the "tail" of the ferrichrome molecule is solvent accessible in the FhuA–ferrichrome complex, such that this siderophore could serve as a vehicle for antibiotic delivery.

Experimental Procedures

Protein Expression, Purification, and Crystallization

Wild-type protein was overexpressed and purified as described (Locher and Rosenbusch, 1997). Selenomethionyl FhuA protein was obtained by growing cells of *E. coli* B834(DE3) (Doherty et al., 1995) in M9 minimal medium containing D,L-selenomethionine at a concentration of 120 mg/ml. Cells were grown until the OD_{600nm} reached 0.6. Induction was achieved by addition of 2 mM IPTG for 8 hr. The purified protein had >95% substitution, as was judged by the disappearance of the methionine peak in amino acid analyses. Purification was as for wild-type protein. N-terminal sequence determination was performed according to Edman (1956). Crystallization was performed by vapor diffusion (sitting drops). FhuA protein (12.3 mg/ml) was mixed with a reservoir solution containing 33% PEG2000, 0.45 M NaCl, 0.15 M NaPi (pH 6.2), and 0.5% n-octyl-2-hydroxyethylsulfoxide, in a volume ratio of 1:2. To obtain the complex with ferrichrome, the latter was added at a concentration of 1 mM. Long crystals (maximum size 0.2 × 0.2 × 2 mm) appeared within 1 week. Crystals were either mounted in capillaries or frozen by immersion in liquid nitrogen.

Data Collection

Room-temperature X-ray diffraction data were collected from crystals of the apo-protein and of the FhuA–ferrichrome complex, using the synchrotron sources of LURE (Orsay) and ESRF (Grenoble). Low-temperature measurements were also made, but cryo-cooling always resulted in a large increase of the mosaic spread of the crystals. The search for heavy-atom derivatives was unsuccessful. A selenomethionyl derivative of uncomplexed FhuA was eventually used for multiwavelength anomalous diffraction (MAD) measurements (Hendrickson, 1991). All of the MAD measurements were performed on a single crystal, which was cooled at liquid nitrogen temperature. This crystal was first used for recording the X-ray fluorescence spectrum in the vicinity of the K absorption edge of selenium. Two of the three wavelengths used in subsequent data collection were chosen at the maximum and at the inflexion point of the absorption edge, respectively. All of the X-ray data were processed with DENZO (Otwinowski and Minor, 1997).

Structure Determination

All selenium sites of the ten selenomethionyl residues of FhuA were found using the program SOLVE (Terwilliger and Berendzen, 1997). The positions were refined and phases were calculated to 3 Å resolution using SHARP (de la Fortelle and Bricogne, 1997). Solvent flattening and phase extension to 2.7 Å were performed using SOLOMON (Abrahams, 1996), yielding an interpretable electron density map. Using the program O (Jones et al., 1991), the Cα trace and the side chains of 695 out of 714 amino acid residues were built into the map. At this point, the current model was placed in the room temperature cell of native FhuA, which exhibits slightly different cell parameters. This was done by using the rigid-body refinement procedure implemented in CNS (Brünger et al., 1998). The structure was then refined, in parallel with the room-temperature holo structure containing ferrichrome. A difference map calculated with the holo data and the apo model showed clearly the electron density of the ligand. The two refinements were made by molecular dynamics using CNS. Statistics are given in Table 1.

Figures

Figures were drawn using SETOR (Evans, 1993), MOLSCRIPT (Kraulis, 1991), RASTER3D (Merrit and Murphy, 1994), and MINIMAGE (Arnez, 1994).

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Brookhaven Protein Data Bank ID Codes

The ID codes for the coordinates of the structures reported in this paper are 1by3 (structure without ligand) and 1by5 (liganded structure).