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Molecular mechanism of ferricsiderophore passage through the outer membrane receptor proteins of *Escherichia coli*

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Abstract Iron is an essential nutrient for all microorganisms with a few exceptions. Microorganisms use a variety of systems to acquire iron from the surrounding environment. One such system includes production of an organic molecule known as a siderophore by many bacteria and fungi. Siderophores have the capacity to specifically chelate ferric ions. The ferricsiderophore complex is then transported into the cell via a specific receptor protein located in the outer membrane. This is an energy dependent process and is the subject of investigation in many research laboratories. The crystal structures of three outer membrane ferricsiderophore receptor proteins FepA, FhuA and FecA from *Escherichia coli* and two FpvA and FptA from *Pseudomonas aeruginosa* have recently been solved. Four of them, FhuA, FecA, FpvA and FptA have been solved in ligand-bound forms, which gave insight into the residues involved in ligand binding. The structures are similar and show the presence of similar domains; for example, all of them consist of a 22 strand-β-barrel formed by approximately 600 C-terminal residues while approximately 150 N-terminal residues fold inside the barrel to form a plug domain. The plug domain obstructs the passage through the barrel; therefore our research focuses on the mechanism through which the ferricsiderophore complex is transported across the receptor into the periplasm. There are two possibilities, one in which the plug domain is expelled into the periplasm making way for the ferricsiderophore complex and the second in which the plug domain undergoes structural rearrangement to form a channel through which the complex slides into the periplasm. Multiple alignment studies involving protein sequences of a large number of outer membrane receptor proteins that transport ferricsiderophores have identified several conserved residues. All of the conserved residues are located within the plug and barrel domain below the ligand binding site. We have substituted a number of these residues in FepA and FhuA with either alanine or glutamine resulting in substantial changes in the chemical properties of the residues. This was done to study the effect of the substitutions on the transport of ferricsiderophores. Another strategy used was to create a disulfide bond between the residues

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located on two adjacent β-strands of the plug domain or between the residues of the plug domain and the β-barrel in FhuA by substituting appropriate residues with cysteine. We have looked for the variants where the transport is affected without altering the binding. The data suggest a distinct role of these residues in the mechanism of transport. Our data also indicate that these transporters share a common mechanism of transport and that the plug remains within the barrel and possibly undergoes rearrangement to form a channel to transport the ferricsiderophore from the binding site to the periplasm.

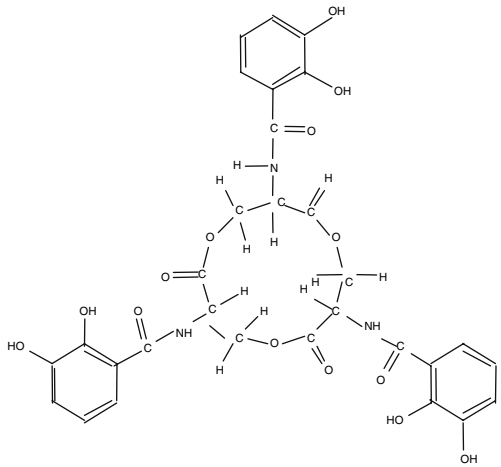
Keywords Iron · Siderophore · FhuA · Ferrichrome

Introduction

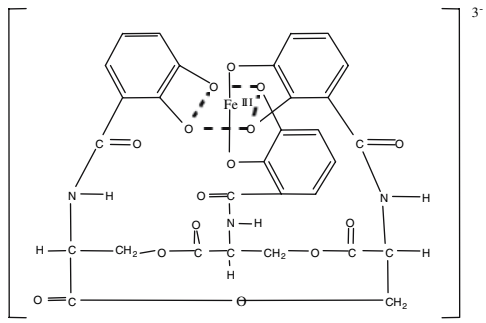
Iron is a major nutrient required by all living beings except a few microorganisms belonging to the genera *Lactobacillus* and *Streptococcus* (Neilands 1981; Guiseppi and Fridovich 1982). In an oxidizing environment ferric ion, in spite of being the fourth most abundant element in earth's crust remains unavailable to the microorganisms. This is because in the presence of oxygen at physiological pH ferric ions form polymers of iron oxyhydroxide with a concentration of soluble iron in the range of 10^{-18} M (Raymond et al. 2003). Thus soluble iron cannot get into the cell via diffusion because the concentration of iron in the cytoplasm of metabolically active bacteria is about 1 μM. A similar shortage of iron is created in the human body by iron binding proteins like transferrin and lactoferrin present in body fluids. Many gram-negative bacteria including some important human pathogens have overcome such iron unavailability by producing small organic molecules called siderophores (Braun and Killmann 1999; Schryvers and Stojilikovic 1999; Braun and Braun 2002a). Siderophores have high affinity specifically for ferric ions; they chelate ferric ions and transport them via an energy dependent, multicomponent transport system across the membranes into the cytoplasm. The components of such systems include a receptor protein located in the outer membrane (OM), a periplasmic binding protein and an ABC type transporter located in the inner membrane. The energy required for the transport via OM receptor is presumably provided by the interaction of the receptor with the TonB-ExbB-EXbD complex located in the inner membrane, which couples the proton motive force created by the inner membrane for energy transduction (Postle 1993; Braun 1995).

Siderophores

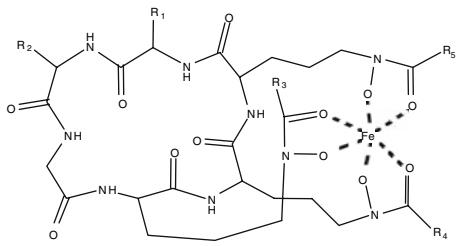
Siderophores are small organic molecules produced by many bacteria and fungi (van der Helm et al. 1987; Winkelmann 1991). Their molecular weight ranges from 500 to 1500 Da. Chemically, siderophores are comprised of very diverse structures but the majority of them can be classified under either catecholate or hydroxamate types, which use catechol and hydroxamate groups respectively to chelate iron. Enterobactin (Fig. 1) is a prototype of a catechol type siderophore produced by *E. coli*, which has a stability constant for ferric iron in the order of 10^{52} (Ecker et al. 1986). Ferrichrome (Fig. 1) is an example of a hydroxamate type siderophore produced by fungi *Ustilago sphaerogena* (van der Helm et al. 1980). Besides these two major types, siderophores with carboxylate groups or mixed groups also have been reported; schizokinen, pseudobactin (Fig. 1), and pyoverdine are the examples of such siderophores (Cobessi et al. 2005a; Storey et al. 2006). Many bacteria and fungi produce more than one type of siderophore. It is not unusual that the same siderophore is produced by more than one organism. Ferric siderophores form octahedral complexes with iron. Due to their capacity to specifically chelate ferric iron, they have been used for chelation therapy to treat iron overload diseases (Franchini 2006). While their role in bacterial pathogenicity is a subject of many reviews, they have also been investigated as potential drug delivering agents or antibiotics (Weinberg 1984; Miller and Malouin 1993; Ghosh and Miller 1996).



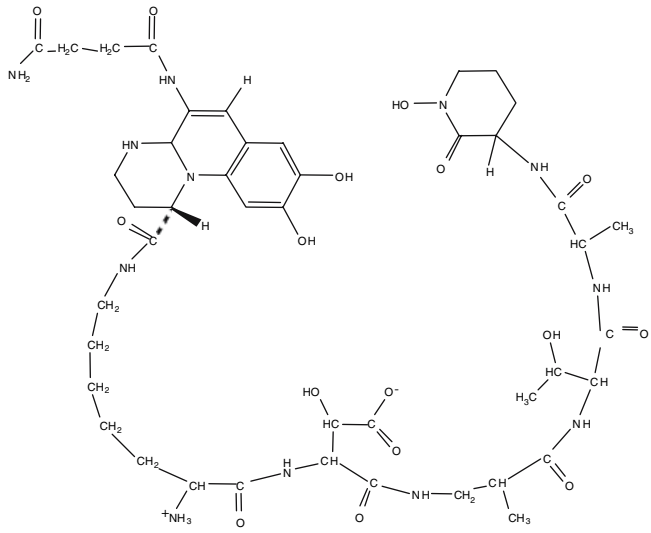
ENTEROBACTIN



FERRIC ENTEROBACTIN



FERRICHROME



PSEUDOBACTIN

Fig. 1 Few examples of typical siderophores

Outer membrane ferricsiderophore receptor proteins

Ferricsiderophores are specifically recognized by the receptors located in the outer membranes of gram negative bacteria. The protein sequences of more than 30 such outer membrane receptor proteins transporting chemically diverse ferricsiderophores are known (van der Helm and Chakraborty 2002). The crystal structures of five such receptors, FepA, FhuA, FecA, FpvA and FptA have recently been solved (Buchanan et al. 1999; Ferguson et al. 1998; Ferguson et al. 2002; Cobessi et al. 2005a; Cobessi et al. 2005b). The crystal structures of these receptors show remarkable similarity (Fig. 2). BtuB, the TonB dependent cobalamin transporter from *E.coli* also shares a common structure with these receptors (Chimento et al. 2003). All of them consist of a barrel made up of 22 anti-parallel β strands and a plug

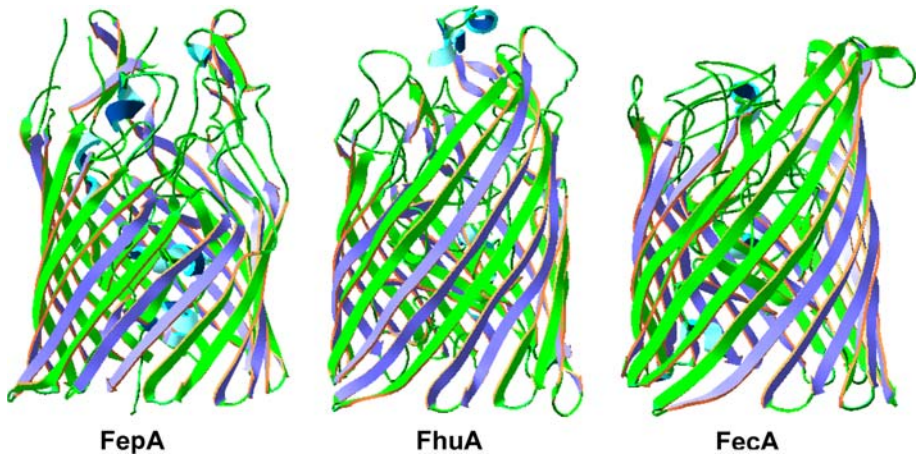


Fig. 2 Crystal structures of ferricsiderophore outer membrane receptors FhuA, FepA and FecA from *E.coli*

domain formed by approximately 150 N-terminal residues. The beta strands are connected on the extracellular side by loops of variable length while on the periplasmic side they are connected with shorter loops. The plug domain consists of multiple short beta strands connected via loops and a few alpha helices. The structures of four receptors except FepA have been solved with bound ligand, revealing their binding site. The binding site is formed by the residues contributed by the extracellular loops, as well as the loops formed by the plug domain.

OM receptors of *Escherichia coli*

Three out of the five receptors whose crystal structures have been solved are expressed in *E.coli*; these are FepA, FhuA and FecA, which transport ferric enterobactin, ferrichrome and diferric dicitrate respectively. The β barrel formed by the beta strands in all the three receptors is ellipsoidal with dimensions of 38–47 Å, 37–48 Å and 35–47 Å across the barrel at the top for FepA, FhuA and FecA respectively (Buchanan et al. 1999; Ferguson et al. 1998; Ferguson et al. 2002). The lengths of the external loops vary in the three receptors ranging from 4 to 50 residues while periplasmic loops are shorter in length. The binding pocket is formed with residues of several extracellular loops and the apices of the plug domain. In FepA and FhuA this pocket is lined mostly by aromatic and/or hydrophilic residues while in FecA it is lined predominantly by positively charged residues. This is expected as the ligand for FecA, the diferric dicitrate is negatively charged (Ferguson et al. 2002; van der Helm et al. 2002). FecA also has an extended N-terminus consisting of residues 1 to 79 and the plug domain is formed by residues 80 to 221 while in FepA and FhuA the plug domain is formed by residues 1 to 154 and 1 to 160 respectively.

The plug domains in the three receptor proteins have similar structure but the same topology (van der Helm 2004). All have a central beta sheet, which is in the same location and orientation in all structures. In addition each has three apices (A, B and C), which are involved in ligand binding. Their location and height differ but topologically they are in the same location in the sequence. There are two small beta strands (2 and 3) to and from apex A (Fig. 5B). They contain in FhuA the conserved residues Pro 74 and Arg 93. The topology of the core beta sheet is strands: 1(down), 5(up), 6(down), 4(down), 4(cont.) (up) (Fig. 5B). In FhuA the conserved Arg133 is at the top of strand 5 and the β5–β6 loop of 10–12 residues contain the conserved Gly's. There is short turn between strand 4 and its continuation. The TonB box is located close to the N-terminus (in FecA, however, it starts at residue 80). The switch helix is directly after the TonB box (van der Helm and Chakraborty 2001; van der Helm 2004). The loops or apices formed

by the plug domain also show structural differences and reach almost 20–25 Å above the membrane surface while the extracellular loops reach even higher above the membrane surface leaving enough space to form a binding pocket. The structure of FecA showed considerable movement (11–15 Å) of extracellular loops (7 and 8) to close the pocket after the ligand was bound. Such changes were not observed in FhuA (Ferguson et al. 1998; Locher et al. 1998), possibly due to crystal packing. The ligand binding also induced changes in the plug domain, including, in FhuA and FecA, the unwinding of the 'switch helix' located just after the 'TonB box' and an extension of the N-terminus towards the periplasm (Ferguson et al. 1998; Locher et al. 1998; Ferguson et al. 2002; van der Helm et al. 2002) possibly to signal the ligand occupied status.

Energy transducing TonB-ExbB-ExbD complex

All ferricsiderophore outer membrane receptors transport the ligand in an energy dependent manner (Postle 1993; Braun 1995). There is no apparent energy source located in the outer membrane (OM) and the energy is presumably transduced by a physical interaction between the TonB-ExbB-ExbD complex located in the inner membrane and the OM receptor. TonB is anchored to the inner membrane by means of a single transmembrane domain. The C-terminus is located in the periplasmic region. Structurally TonB is divided into three distinct domains, amino terminal residues 1–33 are anchored to the inner membrane and are responsible for its interaction with ExbB-ExbD, residues 34–154 with Pro-Glu and Pro-Lys repeats and residues 155–239, both located in the periplasm. The third domain can contact the N-terminus of the ferricsiderophore OM receptors (Wiener 2005). All ferricsiderophore outer membrane receptor proteins have 8–10 conserved residues at their N-terminus known as the 'TonB box', which interacts with the C-terminus of TonB (Lundrigan and Kadner 1986; Gudmundsdottir et al. 1989; Sauer et al. 1990). ExbB and ExbD proteins play a role in energizing the TonB by coupling to the PMF of the cytoplasmic membrane; they are homologues of MotA and MotB proteins found in the bacterial flagellar motor (Braun and Braun 2002b). Efforts to isolate and purify the whole TonB protein have been unsuccessful due to its instability but recently several groups have determined structures of TonB with variable numbers of C-terminus residues. These structures include carboxyl terminal residues 155–239 as a tightly intertwined dimer (Chang et al. 2001), residues 148–239 (Ködding et al. 2005) as a loose dimer, however, an NMR study of residues 103–239 (Peacock et al. 2005, Fig. 3A) showed a monomeric structure. Their oligomeric state perhaps depends on the length of the recombinant constructs used for the structure determination (Pawelek et al. 2006). The monomeric structure consists of two alpha helices and four antiparallel beta strands β1–β4, where β-4 is the C-terminus. Two groups have recently solved structures of the C-terminus of TonB, complexed with ligand-bound FhuA and BtuB, respectively (Pawelek et al. 2006; Shultis et al. 2006). In both structures the C-terminus is swapped for the TonB box of the OM receptor protein, as shown in Fig. 3B. The interaction, however, does not result in a concerted change in the structures of the OM receptor proteins besides those which are involved directly with the motion of the TonB box (Fig.3B). Therefore the structures of the complexes do not answer the question in what manner the TonB complex transduces the energy to the OM receptor. The mechanism by which this is done is largely unknown and is a subject of investigation in many research laboratories.

Conformational rearrangement or dislodgement of plug domain during transport

The plug is oriented in all proteins as is shown in the Fig. 4. It blocks passage from the extracellular side to the periplasm. The plug is held in position by means of 50–60 hydrogen bonds between the barrel and the plug domain. The ligand bound structures of receptors, FhuA, FecA and recently solved structures of FpvA and FptA have shown

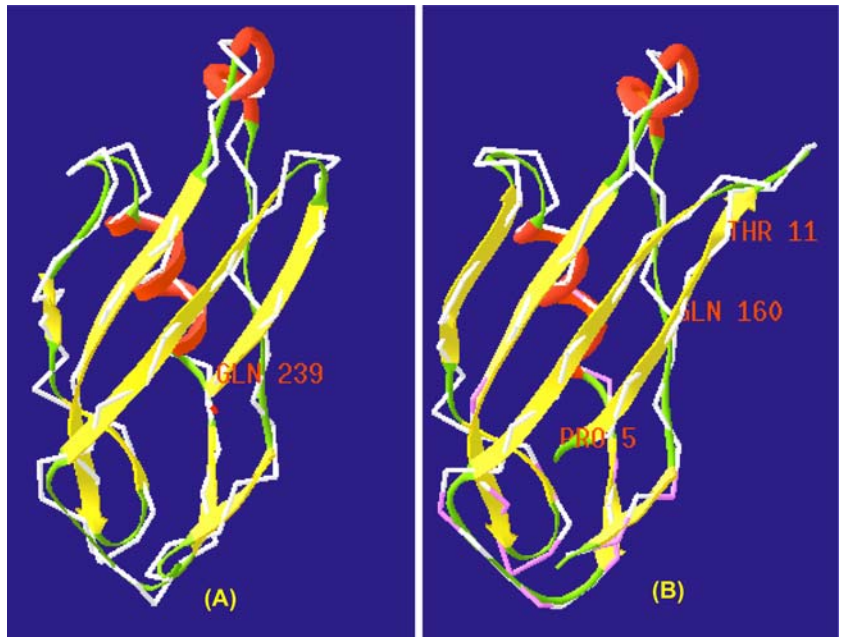


Fig. 3 Crystal structures of the C-terminus TonB, (**A**) structure solved in solution by NMR (Peacock et al. 2005), (**B**) TonB in complex with FhuA (Pawelek et al. 2006); only the TonB box of FhuA is shown; the TonB box (residues 5–11) has replaced the C-terminal strand of TonB in the beta sheet

that the binding of ligand induces conformational changes in the extracellular loops, barrel and the plug domain but it does not show the formation of any pore large enough to allow the passage of the bound ligand. For example, in case of FhuA, passage of ferrichrome will require a pore with a diameter of at least 10 Å. There are only two ways in which this is possible. In one, the plug undergoes conformational changes within the barrel to form a channel. In the other the plug is either partially or completely removed from the barrel. There are arguments either in favor or against each of these hypotheses. Dislodgement of the plug domain can easily explain transport of larger molecules like colicin via these receptors. On the other hand, due to the presence of considerable numbers of intramolecular polar contacts between the surfaces of the plug domain and the barrel, complete dislodgement of plug domain will not be energetically favorable. Recently, studies by Kurisu et al have shown that colicin E3 uses BtuB as a specific binding site but actually gains entry in to the periplasm through the porin OmpF (Kurisu et al. 2003). These receptors have also been known to function as receptors for bacteriophages. It has now been shown conclusively with the help of cryo-electron microscopy that bacteriophage T5 uses FhuA as an adhesion site for reversible binding but its DNA does not pass through the FhuA barrel (Böhm et al. 2001; Plancon et al. 2002).

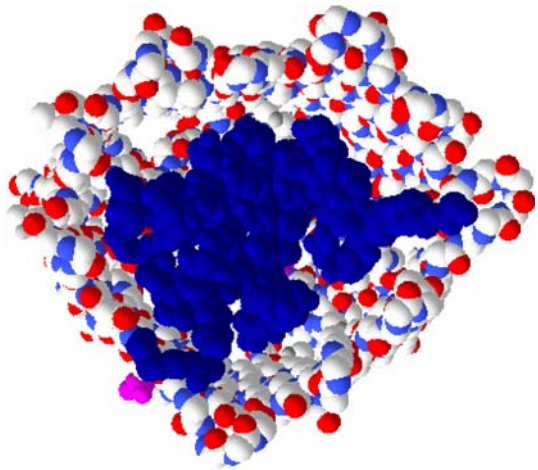


Fig. 4 Space filling model for FepA, a view from the periplasm. The plug residues are depicted in navy blue color

Multiple sequence alignment

Once the ligand is bound, one may expect a common mechanism for the transport of all

ferricsiderophores from the binding pocket to the periplasmic side of the OM. Multiple sequence alignments using various combinations of the sequences of ferricsiderophore receptor proteins revealed interesting facts about similarities and identities shared by these proteins (Lundrigan and Kadner 1986; Chakraborty et al. 2003; Chimento et al. 2005). When the sequences were aligned pair-wise with FepA 20% similarity was observed, while 40% identity was observed when protein sequences of the transporter transporting the same siderophores were aligned. The simultaneous alignment of all the protein sequences showed only 1.4% identity and 3.6% similarity, located predominantly in the first 150 residues, but also spread throughout the sequence. It was only after the first structures were determined that these small number of homologies started making sense (see below). There were no homologies observed in the extracellular loops or the apices formed by the plug domain. All the conserved residues were located below the binding site on both the barrel and the plug domain. Their location suggested that they are involved in a common property that is the mechanism of transport from the binding site to the periplasm. Some of them are depicted in Fig. 5A.

The conserved 'lock region'

Several conserved regions and motifs were identified but one such conserved region that we like to call the 'lock region' was found to be conserved in all proteins in this family. This region perhaps plays an important role in opening up the channel once the ferric siderophore is bound to the binding site. These residues, shown in Fig. 6, are not only conserved genetically but also in location and orientation with respect to the position of the barrel. The most important part of this region in FhuA is the quadrupole of two Arg93 and 133 from the plug which forms H bonds and electrostatic interactions with Glu522 and Glu571 located on strands 14 and 16 of the barrel. In FepA these residues are Arg75 and 126, which form hydrogen bonds with Glu511 and 567 of the barrel while in FecA Arg150 and 196 form hydrogen bonds with Glu541 and 587. In addition, there are also glycines, which are conserved; they are Gly127/134, Gly134/141 and Gly197/204 in FepA, FhuA and FecA respectively in the loop connecting the two middle strands (β-5, β-6) of the core beta sheet. There are also conserved prolines, serines (aspartate in FecA) and phenylalanines (tyrosine in FhuA), which are a part of the lock region, perhaps to support the quadrupole.

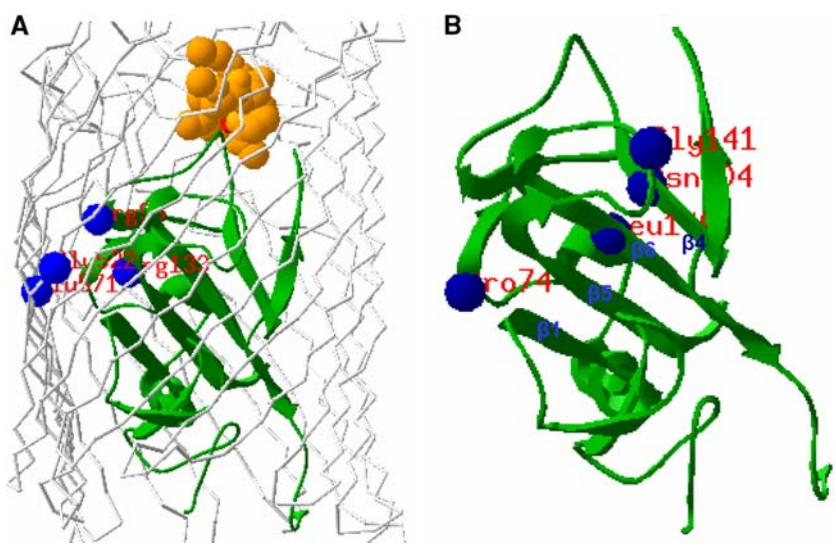


Fig. 5 (**A**) The location of the residues belonging to the 'lock region', bound ferrichrome in orange color and the residues Arg93/Arg133 and Glu522/Glu571 forming a quadrupole are shown as blue spheres, (**B**) Plug domain of FhuA showing the orientation of the beta strands and residues Pro74, Asn104/Leu149 and Gly141 marked also as blue spheres

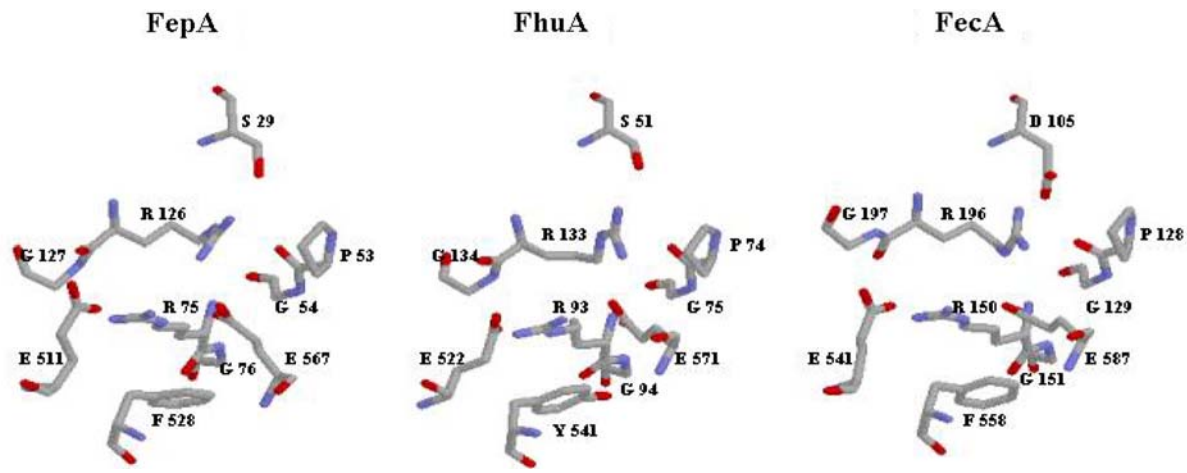


Fig. 6 Structural configuration of the residues of the 'lock region' in three OM receptors of *E.coli*

Site directed mutagenesis of the residues of the 'lock region'

The conserved residues of the lock region were mutated both in FhuA and FepA to investigate their possible role(s) in the transport process. Since the lock region as shown in the Fig. 5A is located well below the binding site, mutations of residues in this region were not expected to affect binding. The basic amino acid arginine and acidic amino acid glutamate were mutated to either neutral nonpolar alanine or neutral but polar glutamine. Mutation to neutral polar glutamine will not allow salt bridge formation but will possibly allow hydrogen bond formation. Mutation to neutral nonpolar alanine will not allow hydrogen bond formation and should have drastic effects on the integrity of the lock region. The residues were mutated individually as well as in pairs to alanine or glutamine. The conserved glycines located in the loop between strands 5 and 6 of the core beta sheets were mutated to alanine. Glycines, due to their conformational freedom are known to serve as hinges in tertiary structures of proteins; their mutation to alanine should prevent hinge function.

The mutants in FepA showed normal binding. However, the transport was affected to different degrees (Chakraborty et al. 2003). The transport in the double mutant of G127/134A was affected the most followed by R75Q, E567A and G127A. The corresponding residues were also mutated in FhuA. Figure 7 shows the time dependent transport at 1 nM concentration of labeled ferrichrome in the single mutants of arginine and glutamate compared to the strain carrying wild type FhuA. All of them showed variable extent of initial transport followed by drastic reduction in the rate of transport. The E522A and E571A showed diminished transport comparable to *fhuA*$^{-}$ strain (data not shown). The binding in the mutant strains was basically the same as the wild type strain (Table 1). The double mutant R93/133Q as shown in Fig. 8 showed about the same effect as the single mutants but the double mutants of E522/571Q and E522/571A both showed severely diminished transport. It is important to note that similar results were obtained for FepA mutants involving the corresponding residues E511/567A (Chakraborty et al. 2003). What is remarkable about this data is that in case of single mutants (specifically R93A) the initial rate of transport is as good as the wild type but is followed by severely diminished rates of transport. It is possible that the disruption of the 'lock region' allows the first cycle of transport after which the structure cannot resume the proper configuration of the lock region to support further transport cycles. In FhuA a single mutation of glycine 141 to alanine eliminated the time dependent transport (Fig. 7).

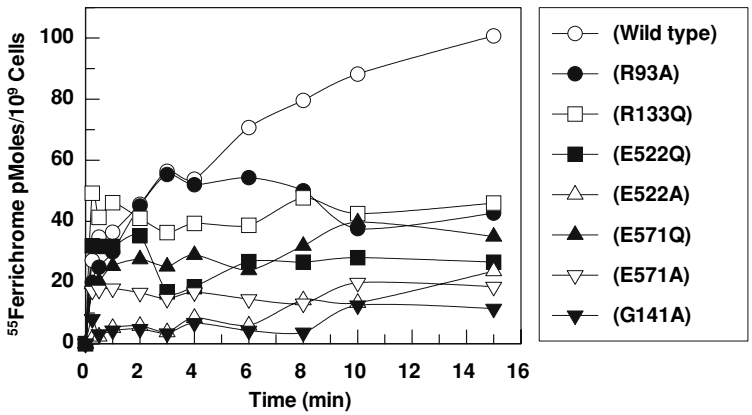


Fig. 7 Time dependent transport of 1 nM ^{55}Ferrichrome concentration in single mutants of FhuA

Table 1 Concentration dependent binding and transport in FhuA mutants

FhuA mutants	K_D (nmoles)	K_M (nmoles)
Wild type	0.5–5.0	0.1–1.0
R93Q	0.7	0.208
R133Q	6.8	0.545
E522Q	4.3	TDT
E522A	12.0	TDT
G141A	7.4	TDT
R93/133Q	1.2	TDT
E522/571Q	ND	TDT
E522/571A	ND	TDT
N104/L149C	1.5	287
Y72/S615C	17.2	0.300

ND = Not determined TDT = Time dependent transport

Double cysteine mutations

As discussed earlier, one of the possible mechanisms considered for the passage of bound ferricsiderophore through the receptor is the complete ejection of the plug domain from the barrel. In order to test whether tethering of the plug domain within the barrel will affect the transport or not, the plug domain in FhuA was tethered by means of covalent disulfide bonds between the selective residues from plug and barrel domains. The residues selected were Tyr72/Ser615 and Pro74/Ser587; these were mutated to cysteine. Care was taken to make sure that the selected residues are far removed from the 'TonB box' in order that their mutation should not affect the process of energy transduction. In order to check whether the core region of the plug underwent significant conformational change associated with the movement of the central β strands 4 and 6, a double cysteine mutant tying the β strands 4 and 6 was constructed by mutating residues Asn104/Leu149 in

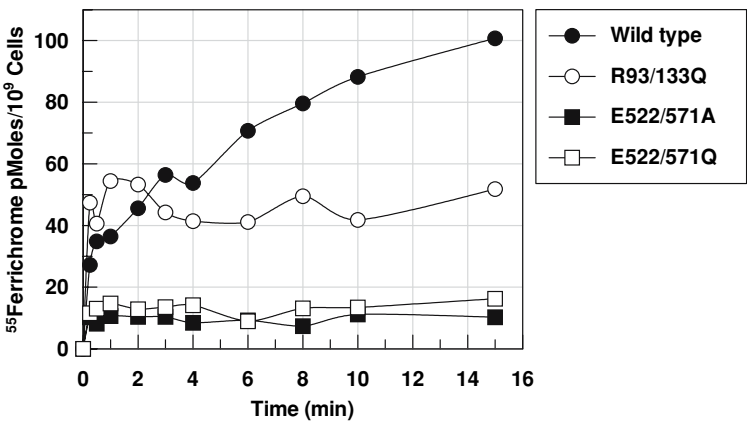


Fig. 8 Time dependent transport of 1 nM ^{55}Ferrichrome concentration in double mutants of FhuA

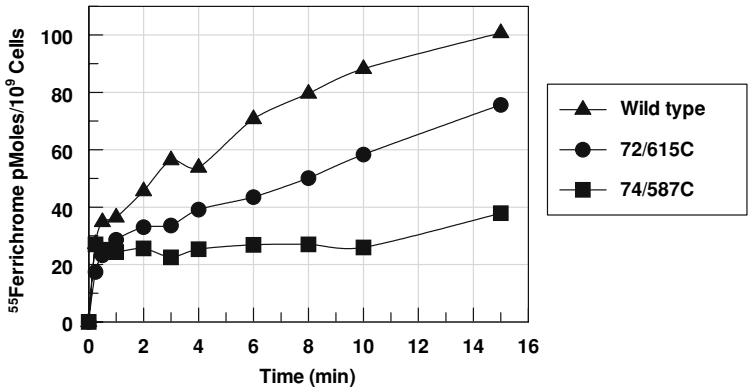


Fig. 9 Time dependent transport of 1 nM ^{55}Ferrichrome concentration in double cysteine mutants of FhuA

FhuA. The double cysteine mutant of Tyr72/Ser615 connecting the plug to the barrel showed close to normal time dependent transport (Fig. 9) and also normal concentration dependent binding and transport with similar K_D and K_M to the wild type (Table 1). Similar results have also been obtained by Eisenhauer et al. using ferricrocin transport with FhuA (Eisenhauer et al. 2005). The mutant Pro74/Ser587 also transported ferrichrome; however, the rate of transport was slower than both the wild type and mutant Tyr72/Ser615 strains. This might have been expected because Pro74 (see Fig. 5) is conserved in FhuA and the residue perhaps plays role in positioning the ferrichrome binding loop A (van der Helm and Chakraborty 2001; Chimento et al. 2005). The concentration dependent binding was not carried out for Pro74/Ser587 mutant.

The elimination of transport for the G141A mutant (Fig. 7) indicates that it hinders a conformational change in the $\beta5$–$\beta6$ loop required for transport. It may even indicate that transport requires a partial disassembly of the core beta sheet. This idea is reinforced by the observation that the double cysteine mutant Asn104/Leu149, in which the S–S bond connects strand 4 and 6 shows severely diminished transport with normal binding (Table 1). Only partial disassembly of the core beta sheet is indicated, because when the turn after the β 4 strand or the continuation of this beta strand ($\beta4$ cont.) is attached to the barrel with an S–S bond, normal transport is observed (Eisenhauer 2005).

Conclusion

The observations made on binding and transport activities in the mutants indicate that the conserved residues in the lock region play an important role in the transport cycle by maintaining structural integrity or facilitating the necessary conformational changes to form a channel. The electrostatic interactions within the lock region are crucial for the proper functioning of this region. It is possible that the physical interaction of the 'TonB box' of the ligand-occupied receptor with the carboxyl terminus of the TonB leads to pumping of protons from the periplasmic side into the barrel weakening the electrostatic interactions and thereby allowing the conformational change for the formation of the beginning of a channel. During this process, the glycines perhaps play a role of hinge to assist the movement of the beta strands and to facilitate partial or complete separation of the strands of the core beta sheet in order to form the complete channel allowing transport. Chimento et al. have recently analyzed the interface area and the nature of protein–protein interaction between the barrel and the plug domain of TonB dependent receptors (Chimento et al. 2005). According to their studies, the interfaces are very large and contain large numbers of water molecules. The fraction of bridging water molecules which form hydrogen bonds with both the barrel and plug domain is about one-third of the interfacial water. The average number of hydrogen bonds per water molecule is 3.1, which is lower than the typical value of 3.8 for interfacial water. Their analyses

also indicate that the nature of the protein–protein interaction between the barrel and the plug domain is like transient protein complexes that undergo conformational changes or domain movement during their function. Just like transient protein complexes barrel-plug domain interface contains nearly 40% polar residues (Lo Conte et al. 1999; Tsai et al. 1997). These data indicate that the plug domain has flexibility to undergo conformational changes or movement at relatively low energy cost. The binding of the TonB box to TonB may be important to apply the physical force for the temporary disassembly of the central beta sheet allowing the formation of a channel between the plug and the barrel through which the ferricsiderophore is transported from the binding site to the periplasm.

Close to normal transport is observed in cases of double cysteine mutants between plug and barrel. This is observed by us and also Eisenhauer et al (Eisenhauer et al. 2005). This indicates that the plug does not need to be totally removed from the barrel. In addition, the severe effects on transport, both by the glycine mutants connecting the middle strands (5 and 6) of the core beta sheet and by the formation of an S–S bridge between strands 4 and 6 of that sheet, indicate that drastic changes occur in the structure of the plug domain during transport. These observations indicate the formation of a channel rather than the removal of the whole plug domain during transport from the binding site to the periplasm. More biochemical and structural experimentations are still needed to validate this hypothesis further.

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