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Identification and mutational studies of conserved amino acids in the outer membrane receptor protein, FepA, which affect transport but not binding of ferric-enterobactin in *Escherichia coli*

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Abstract

Many gram-negative bacteria produce and excrete siderophores, which complex iron with high affinity in the environment. The ferric siderophore complexes are transported across the outer membrane by receptor proteins. This process requires energy and is TonB dependent and must involve conformational changes in the receptor proteins to allow the transport of the ferric siderophores from the extracellular binding site to the periplasm. There is a large variety in the structures, molecular weights and charges among the siderophores. It was therefore realized that when the sequences of the many different receptor proteins were compared, simultaneously, all identities and close similarities, found in this manner, could only be due to residues involved in the conformational changes and transport mechanism, common to all the proteins, and not be due to the specificity of ligand recognition. Once the crystal structures of FepA, FhuA and FecA became available, it was immediately clear that the sequence similarities which were found in the simultaneous alignment, were all localized in a few structural domains, which are identical in the three structures and can therefore be expected to be maintained in all the proteins in this family. One of these domains, tentatively named the lock region, consists of 10 residues with a central quadrupole formed by two arginines and two glutamates, from the plug region and the beta barrel. We mutated several of these residues in FepA. All showed normal binding in quantitative binding studies. Some showed normal transport as well, however, the majority showed moderate to severe defective transport with ferric enterobactin. The results therefore show the validity of the hypothesis that the simultaneous sequence alignment will select the residues involved in the transport function of the receptor proteins. In addition the results allow to relate the severity of the transport deficiency to be correlated with the structure of the lock region while it is also possible to propose a function of this region in the conformational changes of the protein during the transport of the ligand from the binding site to the periplasm.

Introduction

Iron is an important growth-limiting factor for a majority of microorganisms barring a few Lactobacilli (Archibald 1983) and *Streptococcus sanguis* (Guiseppi & Fridovich 1982). Although iron is present in abundance, it is unavailable to microorganisms due to its presence as insoluble iron-oxyhydroxide under

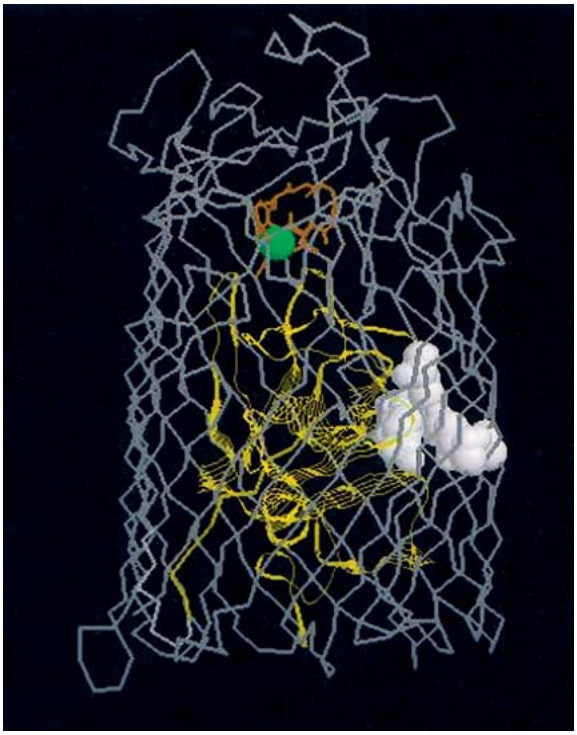

Fig. 1. The conserved cluster of amino acids (Arg 93 and Arg 133) is shown in space-filling model against the gray barrel, yellow plug and orange ferrichrome with green iron, in FhuA. This picture shows the relative location of the site of mutagenesis and the binding site of ligand in FhuA, which is structurally homologous to FepA. Residues Arg93 and 133 in FhuA are Arg 75 and 126 in FepA.

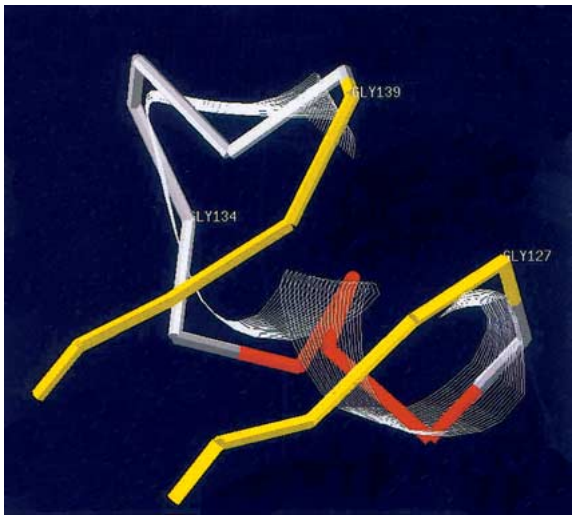

Fig. 6. The figure shows the relative location of Gly127 and Gly134 in the plug domain with respect to the β-strands 5 and 6 (yellow), in the central mixed beta sheet. A short alpha helix (red) is part of the loop connecting the two strands.

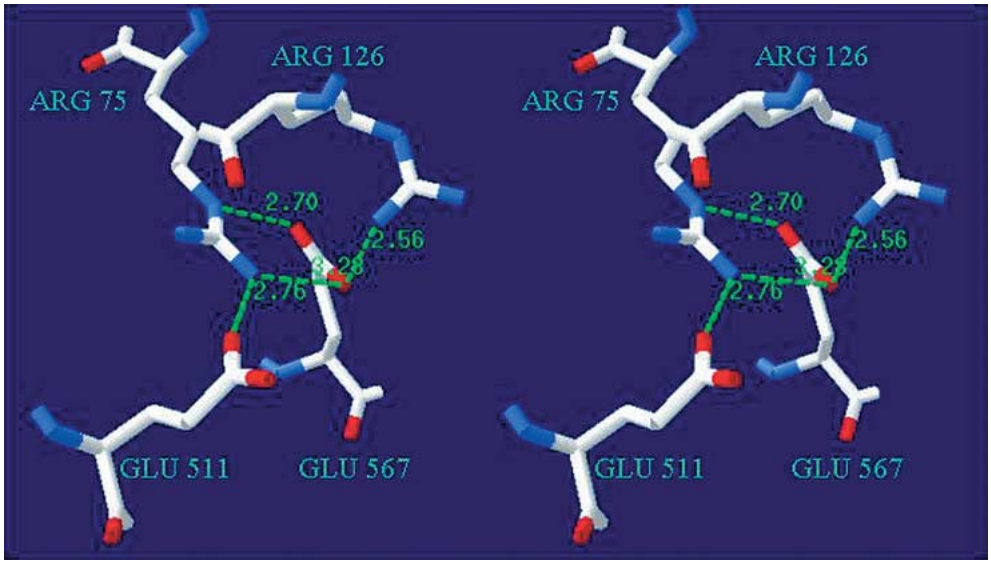

Fig. 5. The quadrupole formed by Arg75, Arg126, Glu511, and Glu567 and the hydrogen bonds between the four residues.

aerobic conditions. Many gram-negative bacteria have overcome the problem of iron deficiency by expressing high affinity iron transport systems (Braun *et al.* 1998; Braun & Killmann 1999; Schryvers & Stojiljkovic 1999). For example, there are six different iron-transporting systems reported in *E. coli*, which supply iron to the cell (Bagg & Neilands 1987).

The high affinity iron transport systems are energy requiring multicomponent system consist of iron-chelating organic molecules called siderophores, the corresponding specific outer membrane receptor protein, a periplasmic binding protein, an ATP dependent inner membrane ABC type transporter, and the TonB-ExbB-ExbD protein complex located in the inner membrane (Braun *et al.* 1998). The iron-siderophore complexes cannot be transported through water filled porin channels due to their size, which ranges from 600–1500 Da. Instead, they are actively transported through specific outer membrane receptors. The sequences of more than 30 receptors, involved in transporting a variety of iron-siderophore complexes, have already been reported (van der Helm 1998; van der Helm & Chakraborty 2001).

In *E. coli* an outer membrane receptor protein FepA actively transports ferric-enterobactin (FeEnt). Enterobactin is a catechol type siderophore produced by *E. coli* which complexes ferric ion with high affinity (Ecker *et al.* 1986). Active transport is assisted by energy transduction possibly involving the TonB complex and the chemiosmotic potential of the inner membrane (Moeck & Coulton 1998). After the transport through FepA the ferricenterobactin is delivered to the periplasmic binding protein FepB. Finally the ferric siderophore is transported across the inner membrane by a specific ABC system (Shea & McIntosh 1991; Sprencel *et al.* 2000). Other receptor proteins in this family use similar systems for transport to the cytoplasm (Staudenmaier *et al.* 1989; Braun & Killmann 1999). The present communication focuses on the transport function of FepA.

The crystal structures of FepA (Buchanan *et al.* 1999), FhuA (Ferguson *et al.* 1998; Locher *et al.* 1998) and FecA (Ferguson *et al.* 2002) have been determined. The crystal structures of the proteins show many similarities, which can be exploited to study their transport functions. Each structure can be divided into two domains: a 22-stranded beta barrel, and a plug or cork domain formed by the approximately 150 N-terminal residues. The plug domain is located within the barrel in such a way that it blocks access to the periplasm (Ferguson *et al.* 1998, 2002; Locher *et al.* 1998, Buchanan *et al.* 1999). The beta-strands are connected by solvent-accessible extracellular loops of variable length (Ferguson *et al.* 1998, 2002; Locher *et al.* 1998; Buchanan *et al.* 1999; van der Helm & Chakraborty 2001), and short periplasmic loops. The extracellular loops extend far above the outer leaflet of the membrane and allow entrance for the ferric-siderophore complex. The top of these loops plays a role in extracting the siderophore complexes from the environment and forms an initial binding site (Payne *et al.* 1997, Buchanan *et al.* 1999). Subsequently the ligand moves down to the second binding site as indicated in the ligand bound structures of FhuA and FecA (Ferguson *et al.* 1998, 2002; Locher *et al.* 1998; Thulasiraman *et al.* 1998). The second, specific site is formed by residues at the apices of three loops from the plug domain and residues from several extracellular loops (Figure 1) (Ferguson *et al.* 1998, 2002; Locher *et al.*, 1998). A four-stranded mixed beta sheet, inclined by about 45 deg with respect to the membrane plane, forms the core of the plug. The topology for the plug region is the same in all three crystal structures, determined so far, and the apices, involved in binding, form the top of this domain (Figure 1). In the liganded FecA structure it is observed that the specific binding site is closed off from the environment by the movement of several extracellular loops. This however, was not observed in the FhuA structures, possibly due to crystal packing or another solid-state effect. Binding of the ligand to the second site also induces significant conformational changes in the plug domain. These changes extend to the periplasmic side of the plug domain and may signal the initiation for TonB interactions and facilitating transport of the bound complex to the periplasm (Skare *et al.* 1993; Cadieux *et al.* 1999; Howard *et al.* 2001; Moeck *et al.* 2001).

It is apparent from the crystal structures that the transport of the ligand from the binding site to the periplasm will require major conformational changes in the plug domain in order to allow the complex to slide through the barrel to the periplasm. The information available from the three crystal structures together with the sequences of many proteins in this family, should make it possible to identify and study the amino acid residues, which are involved in these conformational changes and the transport process of these proteins, and to separate them from the amino acid residues important in the binding event.

In order to identify the amino acid residues involved in the transport process we performed amino acid sequence alignment studies on 19 different pro-

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FepA   IDIRGMGP---ENTLILIDGKPVSSRNSVRQGWRGERDTRGDTSWVPPEMIERIEVLRGP 128
PfeA   IDIRGMGP---ENTLILVDGKPVSSRNSVRYGWRGERDSRGDTNWVPADQVERIEVIRGP 130
BfeA   VDIRGMGP---ENTLILIDGKPVTSRNAVRYGWNGDRDTRGDTNWVPAEEVERIEVIRGP 134
RumA   VGIRGLPARLSPRSTILLDGIPLAAAPYGQPQLSMSPLSLG--------SISSIDVMRGA 126
FecA   FGIRGLNPRLASRSTVLMDGIPVPFAPYGQPQLSLAPVSLG--------NMDAIDVVRGG 198
FPVA   YYARGFSIN-----NFQYDGIPST---------ARNVGYSAGNTLSDMAIYDRVEVLKGA 209
PupB   YWSRGFAIQ-----NYEVDGVPTS---------TRLDNYSQS-----MAMFDRVEIVRGA 209
PupA   IYSRGSAIN-----IYQFDGVTTY---------QDNQTRNMPSTLMDVGLYDRIEIVRGA 210
FhuE   YYSRGFQID-----NYMVDGIPTY---------FESR-WNLGDALSDMALFERVEVVRGA 128
FptA   YYVRGFKVD-----SFELDGVPAL---------LGN----TASSPQDMAIYERVEILRGS 124
PbuA   FYSRGFRMSG----QYQYDGVPLD---------IGSSYVQADSFNSDMAIYDRVEVLRGA 208
FatA   FKIRGFS----------SDIGDVM---------FNGLYGIAPYYRSSPEMYQRIDVLKGP 132
FcuA   YRIRGYN----------LDGDDIS---------FGGLFGVLPRQIVSTSMVERVEVFKGA 157
FhuA   LIIRGFAAEG----QSQNNYLNGLK--------LQGN--FYNDAVIDPYMLERAEIMRGP 135
FoxA   VALRGFHG-G----DVNNTFLDGLR--------LLSDGGSYNVLQVDPWFLERIDVIKGP 129
FyuA   ISLRGVSSAQDFYNPAVTLYVDGVP-------------QLSTNTIQALTDVQSVELLRGP 114
AleB   QTLRGRGML------VLLDGIPLN----------TNRDSARNLANIDPALVERVEVLRGS 215
IutA   MNVRGRPLV------VLVDGVRLN----------SSRTDSRQLDSIDPFNMHHIEVIFGA 110
ViuA   PTIRGIDGSGPSVGGLASFAGTSPRLNMSIDG-RSLTYSEIAFGPRSLWDMQQVEIYLGP 128
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Fig. 2. An example of alignment analyses of 19 outer membrane sequences using CLUSTAL W multiple sequence alignment program (Thompson *et al.* 1994).

teins involved in iron transport. The alignment studies indicate that there is 20% similarity when the protein sequences are aligned in pairs. In the cases where they transport the same ferric siderophore, for instance FeEnt, the identity is about 40% (e.g. in cases of FepA, BfeA and PfeA). However, when all the protein sequences are aligned simultaneously much of the homology is lost and they only show 1.4% identity and 3.6% similarity (van der Helm 1998) (Figure 2). The reason, for the decrease is that, this type of comparison eliminates residues involved in recognition and specificity of binding of the diverse ligands. The results of the simultaneous alignment show two significant features. One is that no homology is found in the extracellular loops nor in the loops from the plug domain which form the apices involved in the specific binding site, indicating that homology due to binding has indeed been eliminated in this alignment. Secondly, all the homologies, which are found, occur below the binding site in the remaining part of the plug domain and in the barrel, in other words in the space between the specific binding site and the periplasm through which the ligand needs to be transported. This indicates that these are indeed residues, which are involved in the conformational changes in the common transport mechanism of the ferric siderophore through the receptor protein.

Once the first crystal structure was determined, it was observed that the homologies found in the simultaneous alignment, proved to be located in four structural clusters, and when more crystal structures became available, it proved that these structural clusters were identical in all the proteins now known. The present communication deals with just one of these clusters. The center of the cluster consists of four charged amino acids, two arginines from the plug and two glutamates from the barrel, forming a quadrupole, which we will call the lock region. It is clear that this lock region is structurally the same in FepA, FhuA and FecA (Figure 3). It is supported by homologous serine (aspartate), proline and phenylalanine (tyrosine) residues. We propose that mutations in this cluster could thus affect transport but not the binding of the ligand. There is a previous report on mutations of some of the residues belonging to this cluster but the approach was based on random mutagenesis rather than their conservation (Barnard *et al.* 2001). Another interesting conservation includes two glycines, which are located at i and i+7, Gly127 and Gly134 in FepA. These residues are located in the loop, which connects the middle two strands of the central 4-stranded beta sheet in the lock or plug domain. Since glycines can exist in more conformations than other amino acid residues, they may play

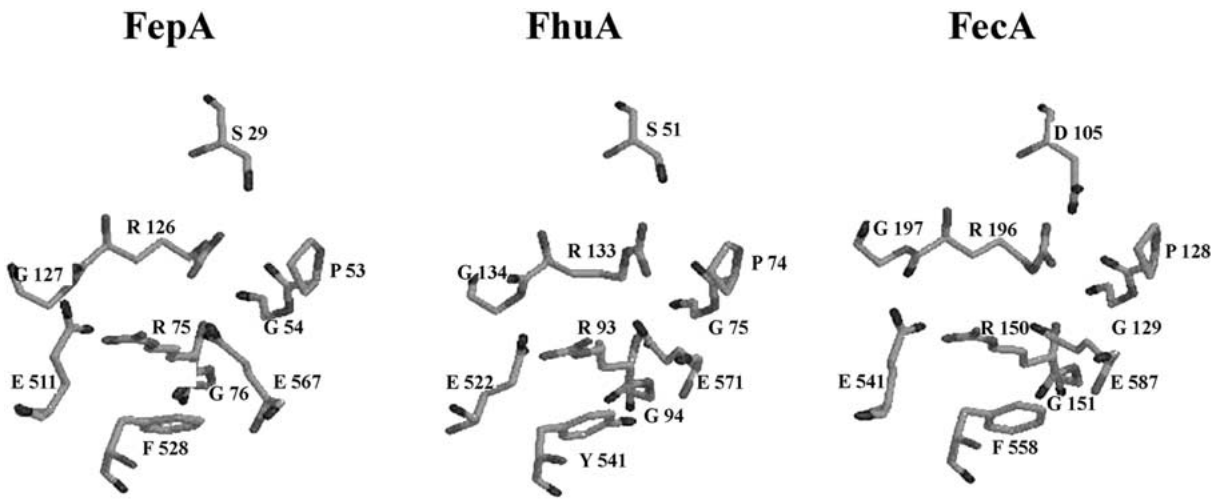

Fig. 3. Representation of the conserved amino acid cluster in the lock region for FepA, FhuA and FecA.

a role in facilitating required conformational changes by serving as a hinge. We believe that some of the conserved glycine residues play a specific role in affording the required conformational changes during the internalization of the iron-siderophore complex. Mutating these residues could therefore affect transport of ferric siderophores without affecting binding. We specifically mutated Glu511, Glu567, Arg75, and Phe528 belonging to the lock region, to non-polar alanine or polar but neutral glutamine, and Gly127, Gly134, and Gly76, adjacent to Arg75, to alanine. We expected to find mutants with affected transport but not the binding to establish the role of the structural cluster in the transport process. A siderophore and chromosomal iron-siderophore receptor protein deficient strain of *E. coli* KDF541 bearing a plasmid coding for wild type or mutated FepA protein was used to study concentration dependent binding and transport of FeEnt. All mutants we studied showed normal binding, but a number of them showed defective transport, which seems to support our hypothesis. The results also allow an evaluation of the relative importance of the mutated amino acid residues for the transport function of FepA, and by implication the homologous residues in the other proteins in this family.

Methods and materials

Bacterial strains, plasmids and media

Escherichia coli strains were grown in Luria-Bertani broth (Miller 1972) or MOPS media (Neidhardt *et al.* 1974) with 0.4% glycerol supplied as a carbon source instead of glucose. A 2.3 Kilobase pair (Kb) region of DNA containing the open reading frame of FepA was cloned in pUC18 under the control of the *lac* promoter and transformed into *E. coli* KDF541. Mutations in FepA were confirmed by sequencing the mutant FepA genes using appropriate primers.

Sequence alignments

The sequences of 19 outer membrane iron-siderophore receptor proteins from different organisms were aligned individually with FepA sequence as well as simultaneously using the protein sequence alignment program CLUSTAL W (Thompson *et al.* 1994). Large gaps were allowed in the alignment.

Site directed mutagenesis

For site directed mutagenesis the *Altered site II* mutagenesis kit was used (Promega corporation). The open reading frame of FepA without its iron promoter was introduced into XbaI/HindIII site of the pAlter plasmid provided in the kit. The procedure based on the site directed mutagenesis method of Hutchinson *et al.* (1978) described in the kit's manual, obtained on average about 90% efficiency of mutation. The plasmid pAlter carrying the mutant fepA gene was then transformed into *E. coli* ES1301 *mutS* for propagation. Using appropriate primers each mutation was confirmed by DNA sequencing and the mutant fepA gene was introduced into XbaI/HindIII site of pUC18 plasmid under the control of *lac* promoter. The plas-

mid pUC18 carrying wild type as well as mutant FepA genes were finally transformed into *E. coli* KDF541 (Rutz *et al.* 1992).

Expression of mutant proteins

E. coli KDF541 strains harboring pUC18 with wild type or mutant FepA genes were grown in LB broth for 16 h at 37 °C and used as 1% inoculum into MOPS medium. The cultures in MOPS media were grown with vigorous shaking at 37 °C for 5 to 6 h. 5×10^8 bacterial cells were collected by centrifugation and lysed by boiling for 5 min with the SDS-PAGE sample buffer. Proteins in the samples were separated on 10% polyacrylamide gel and were transferred to nitrocellulose paper, which was analysed by immunoblotting with anti-FepA mAb 41 or 45 and [^{125}I]-protein A (Newton *et al.* 1997).

FeEnt binding assay

The ^{59}FeEnt binding assays were performed for the wild type and mutant strains using slightly modified previously described method (Newton *et al.* 1999). For binding assay bacterial cells were grown in LB broth for 16 h with vigorous aeration at 37 °C and used as 1% inoculum to inoculate 50 ml of MOPS medium with glycerol as a carbon source. The cells were grown in MOPS medium at 37 °C with vigorous aeration for 4 h and then induced with 0.1 mM of IPTG for 1.5 h. After induction the cells were incubated on ice-bath for 1 h and then assayed for 59FeEnt binding using the previously described method (Newton *et al.* 1999). Each experiment was done in triplicate and the Grafit 3.0 (Erithacus) program was used to calculate the K_d and capacity of binding.

FeEnt uptake assay

For the ^{59}FeEnt-uptake assay the wild type and the mutant strains were grown in the same way as described for the binding assay. During ^{59}FeEnt uptake experiment after induction, 0.1% glucose was added to the growing cells and cells were allowed to grow for 20 min under the same growth conditions and then assayed for the ^{59}FeEnt transport using the previously described method (Newton *et al.* 1999). Each experiment was done in triplicate and the Grafit 3.0 program was used to calculate the K_m and V_{max} for the transport.

Results and discussion

Mutants, expression and measurements

Nine single and double mutants of FepA are selected for this study. The mutations involve amino acid residues, which are part of the lock region and adjacent glycine residues. These residues are expected to be part of the transport function of FepA, but not involved in the ligand binding process as explained in the introduction.

A 2.3 Kb region of DNA containing the open reading frame of fepA digested with XbaI and Hind III is cloned in the plasmid pUC 18 and transformed into *E. coli* KDF541 (*pro, leu, trp, entA,* Δ*recA,* Δ*fepA, cir, fhuA*) (Rutz *et al.* 1992). The fepA gene is expressed under the regulation of 'lac' promoter. Thus the cultures are induced with the addition of IPTG (isopropyl-thio-β-D-galactoside) prior to each experiment for the optimum protein expression. The expression of the mutant proteins is checked with the help of western blot using the monoclonal antibodies against FepA. The western blot showed that the expression of the mutant proteins is comparable to the expression of the wild type FepA.

The effects of site directed mutagenesis are checked by studying binding and transport of the bound ^{59}FeEnt in the mutant strains as compared to the wild type strain. The concentration dependent binding of ^{59}FeEnt is carried out and K_d and capacity are determined by analyzing the mean values from three independent experiments using 'bound versus total' equation with GRAFIT 3 (Erithacus). Similarly, K_m and V_{max} are determined from the concentration dependent ^{59}FeEnt transport experiments by analyzing the mean values from three independent experiments using GRAFIT 3 with 'enzyme kinetics' equation.

The results in Table 1 show that all mutants have the same binding constant as wild type, within experimental error. Some of the mutants show normal transport (G134A and E511Q, Table 1), several may have some deficiency, while others have a distinct and sometimes large (G127/134A, Table 1, Figure 4) effect on the transport of ligand, even though the binding remains the same as for the wildtype. In addition even single mutants show transport deficiency (G127A, R75Q and E567A, Table 1, Figure 4), although the double mutant G127/134A has the largest effect.

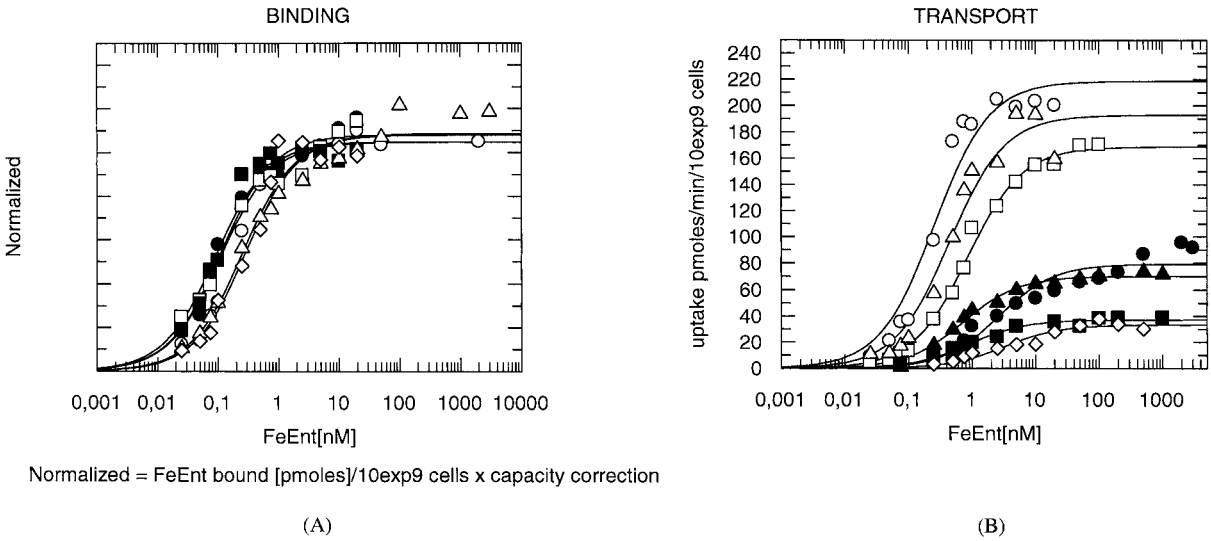

Fig. 4. (a) Concentration dependent binding of ^{59}FeEnt to FepA in the mutants with wild type binding and abnormal transport. The normalized binding data (Bound FeEnt/10^9 cells x capacity correction) is plotted versus concentration of ^{59}FeEnt. (b) Concentration dependent uptake of ^{59}FeEnt in transport affected mutants. The ^{59}FeEnt transported per minute per 10^9 cells versus concentration of ^{59}FeEnt. Wild type (◯), R75Q (●), G127A (□), E567A (■), G134A (△), E511/567Q (▲), G127/134A (◇).

Table 1. Kinetic constants for binding (K_d) and transport (K_m) for the mutants as compare to the wild type strain. The kinetic constants for binding and transport as well as the capacity and V_{max} are calculated by analyzing the mean values from three independent experiments with GRAFIT 3 (Erithacus), using 'bound versus total' and 'enzyme kinetics' equations for binding and transport experiments, respectively.

Strains	K_d	K_m	V_{max}	Capacity
Wildtype	0.19	0.26	219	108
F528A	0.15	0.27	240	103
G76A	0.16	0.25	196	85
G134A	0.29	0.44	193	72
E511Q	0.13	0.33	243	120
R75Q	0.11	2.59	79	36
G127A	0.11	0.85	169	37
E567A	0.09	0.91	37	52
E567/511Q	0.08	0.70	70	26
G127/134A	0.25	3.27	33	134

The lock region of FepA

In the N-terminal and barrel domains of FepA, the lock region includes a conserved cluster of ten amino acids (Figure 3). The central component are four charged residues, arginines at positions 75 and 126 and on strands 14 and 16 glutamates at 511 and 567, forming a quadrupole and phenylalanine at position 528 and serine at position 29. The Arg75, which is in the center of the cluster forms salt bridges with Glu511 and 567 while Arg126, also forms salt bridges with Glu567 (Figure 5). Phe528 supports the side chain of Arg75 while Ser29 positions Arg126 with an H-bond (Figure 3). Arg75 forms three hydrogen bonds (one with Glu511 and two with Glu567) while Arg126 forms only one hydrogen bond with Glu567 (Figure 5). Among glutamates, Glu567 forms three hydrogen bonds while Glu511 forms only one hydrogen bond (Figure 5). These hydrogen bonds play important role in maintaining the structural integrity of the lock region.

Mutations within the quadrupole

In the present studies we planned to mutate several of these residues to either neutral nonpolar alanine or polar but neutral glutamine to check the effect of mutation on binding and transport abilities of FepA. The substitution of glutamate with glutamine should at least allow the formation of a hydrogen bond, instead of a salt bridge and thus may allow full or partial functionality but an alanine substitution for a glutamate will not allow the formation of a hydrogen bond, and as a result should have a more pronounced effect. This is indeed observed, as shown in Table 1. The E511Q mutant has wildtype K_d and K_m values, but the mutant E567A shows a larger value for K_m (Table 1, Figure 4)

indicating the functional importance and the role of hydrogen bonding in order to maintain the structural integrity of this region. The double mutant E511/567Q has a similar large value for K_m, which may indicate, when one compares it to the effect of E511Q, that residue 567 is more important than 511. A substitution of arginine by glutamine will disturb the nature of the quadrupole, and the observation for R75Q shows a strong adverse effect on the transport without affecting the binding of substrate. We could not obtain data for the mutant R126A due to lower protein expression but previously published data indicate that the mutation of Arg75 showed greater effects on colicin sensitivity than the mutation of Arg126 (Barnard *et al.* 2001). This is in agreement with our structural analysis according to which Arg126 only forms a hydrogen bond with Glu567 while Arg75 forms hydrogen bonds both with Glu567 and Glu511. There is as well an indication that mutation of Glu567 has larger effect than the mutation of Glu511 (Table 1) in agreement with the fact that Glu567 forms hydrogen bonds both with Arg75 and Arg126 while Glu511 only forms a hydrogen bond with Arg75.

Mutation of the conserved glycines

The other area where we carried out mutations involved several conserved glycines. The amino acid glycine has a high freedom of rotation and therefore often serves as a hinge in complex protein structures. There are several glycines involved in this cluster, which may play a role in facilitating structural movement during the opening of the proposed second gate (van der Helm *et al.* 2002) involving the lock region. A mutation, which substitutes glycine with alanine might interfere with its speculated hinge function to facilitate the required conformational change to form a channel. Although, all the conserved glycines might not be important for the transport process, as some of them might be required to form structural bends.

The conformational angles of these glycines, as observed in the crystal structure of FepA are important, for instance the conformational angles for Gly76 are (phi, psi) 74.9 and 14.0, which can accommodate the substitution by alanine. On the other hand angles for Gly127 and Gly134 are (phi/psi) 60.8/135.3 and 77.2/145.2 respectively, and these angles are far outside the allowed conformation for amino acids other than glycines. A glycine to alanine substitution will therefore force a conformational change for the residue. Similar conformational angles are observed for the equivalent residues in the FecA and FhuA structures. The mutant, G76A shows normal transport (Table 1), but G127A and the double mutant G127/134A show deficient transport and normal binding, more so in the latter case (Table 1, Figure 4). The almost normal behavior of G134A may not have been expected, although it is reasonable to think that since Gly134 is farther away from the cluster as compared to Gly127, its mutation to alanine might have caused less distortion in the cluster, because the structural change due to the new conformation of residue 134 may have been compensated by adjacent residues, while in contrast, residue 127 is adjacent to Arg126, an important component of the lock region.

Although the double mutant of G127/134A shows the largest effect, it is encouraging that also single mutants affect the transport function of FepA, without influencing the binding of the ligand. Our initial experiment was a mutation of Phe528, which supports the side chain of Arg75. In sequence alignments with all proteins this residue is either a Phe or Tyr, and in the structures of FhuA and FecA the equivalent residue, has the same structural function. However, the F528A mutant shows normal binding and transport. This is probably due to the secondary function of the residue in maintaining the lock region.

The data, which are presented, allow the conclusion that the structural integrity of the lock region is essential for the transport function of FepA, but not for the binding of ligand. Also glycines at 127 and 134 are a part of the transport function. Both residues are parts of a loop which connects the beta strands 5 and 6 (Figure 6), the middle two strands of the central mixed beta sheet of the plug domain. The two residues may allow a rotation of the two strands with respect to one another forming the transient channel through which the ligand moves to the periplasmic side of the protein.

V_{max} and capacity

The transport data has been treated and evaluated as those for an enzyme reaction. FepA is thus considered to be the enzyme. The data therefore yield values for K_m and V_{max} for each mutant and wild type protein. In all transport and binding experiments the number of cells are normalized to 10^9. The binding experiments yield values for K_d and capacity. All experiments were carried out at least three times (Table 1).

It can not be expected, primarily due to unknown effects of TonB, that the transport reaction follows a simple Michaelis-Menten mechanism, and this is

immediately clear from the results for K_d and K_m. Especially those mutants, which affect transport the most, do not follow the rule that K_m is approximately equal to K_d as would be expected for this type of mechanism. Instead the results indicate that failures in the transport do not affect the binding. Unexpectedly the capacity for about half of the mutants is lower than for wild type. This may be caused by lower expression of those mutant proteins, although Western blots do not show very large differences. It is possible that the mutations in the lock region affect the binding site, but the large distance between the two makes that unlikely. It seems therefore that the capacity differences may be due to differences in expression, and this correction is made in plotting the binding curves. It should also be noted that making this correction does not affect the values for K_d, which are listed. The Western blot reaction cannot be expected to note a difference for a mutation deep inside the protein.

A relationship which still may be applicable is $V_{max} = k_3[E_T]$, where k_3 is the rate by which the substrate is released into the putative channel, and $[E_T]$ is the total enzyme, i.e. FepA, concentration. For four of the five mutants, with a significantly high K_m value, V_{max} is very low, only 1/3 – 1/6 of the one for wild type (Table 1). However for the one with the highest value of K_m, the capacity is high. Equally important is the question if the lock region is in an active biological conformation. In other words it may be essential that the quadrupole of the lock region is intact for transport to occur, and affecting k_3. In addition the lock region of the proteins may consist of a mixture of active and inactive forms, either originally when expressed, or after the first ligand molecule is transported and the protein molecule is incapable to restore the quadrupole conformation and in this manner becomes biologically inactive, and affecting E_T. Both k_3 and the protein concentration seem to affect V_{max}, but it is not possible to ascertain which of these two is most important. Also the low initial rates of transport for those mutant proteins with the highest K_m value (Figure 4) do not allow this distinction to be made.

Role of conserved residues in the transport process of FepA

It is not possible to discuss the general mechanism of the transport process in these proteins as it involves several other factors including, the action of periplasmic binding proteins, and TonB. Therefore, we only focus on the possible roles of these conserved amino acids in the transport of FeEnt through FepA. Our approach is based on the identification of the functionally important residues on the basis of their genetic and structural conservation in the family of iron-transporting outer membrane proteins, and this approach allows a directed study of the transport process, involving mutations of carefully selected residues.

Significant conformational changes in the N-terminal domain are observed in liganded FhuA as well as FecA structures when compared to the unliganded structures (Ferguson *et al.* 1998, 2002; Locher *et al.* 1998). In FhuA on the periplasmic side of the plug the changes include the unwinding of an α-helix, switch helix, and displacement of Glu19 by 17.3 Å from its former α-carbon position (Ferguson *et al.* 1998; Locher *et al.*, 1998). Partial unwinding of the switch helix is observed in the liganded FecA structure (Ferguson *et al.* 2002). These conformational changes signal the ligand-occupied status of the receptor and allow an interaction between the protein and TonB. (Skare *et al.* 1993; Cadieux *et al.* 1999; Howard *et al.* 2001; Moeck *et al.* 2001). The possible result of the interaction is a subsequent step in which energy is transduced from the proton motive force across the inner membrane to the outer membrane protein, a process for which no details are available. The last part of the transport process involves, the changes in the outer membrane protein, which allow the formation of a transient channel through which the ligand is moved from the binding site to the periplasm.

In this study we concentrate on the last aspect of the mechanism and these are the changes occurring in the receptor protein during the transport process. It is obvious, when one looks at the crystal structures of these proteins (Ferguson *et al.* 1998, 2002; Locher *et al.* 1998; Buchanan *et al.* 1999), that there is no space through which the bound ligand can pass through the receptor into the periplasm. The movement of the bound ferric siderophore towards the periplasm thus will require either dislodgment or structural rearrangement of the N-terminal domain to form a channel. Dislodgment as proposed (Scott *et al.* 2001) is not impossible, and it easily explains the entry of colicins and phage DNA through these receptors. However, it is difficult to envision, as it requires the breakage of more than 50 possible hydrogen bonds that hold the globular domain in its place. Therefore we (RC, EL, DvdH) believe that conformational change and a structural rearrangement is the likely mechanism by which transient channel for-

mation takes place within the domain with the help of energy transduction, possibly involving the TonB complex, facilitating the movement of bound ligand into the periplasm.

The residues that we have identified in our experiments possibly play structural role in the TonB assisted channel formation, in other words opening of the lock region. In addition there seems to be a structural role for some of the conserved glycine residues in this region, which may be crucial in the opening of the gate, and forming a transient channel. The arginines being positively charged can attract triple negatively charged FeEnt towards the cluster. The hydrogen bonds (Figure 5) in the cluster can then be diminished by the change in the localized pH assisted by TonB interaction making way for the ligand to move further in the tunnel. Once the ligand passes through, the cluster can regain its original conformation to receive the next incoming ligand. Thus mutating some of these residues like Arg75 or Glu567 to neutral glutamine or neutral nonpolar alanine can distort the cluster. The distortion can then block the restoration of the original conformation by preventing the formation of proper H-bonds between these residues (Figure 5). In other words the cycle of breakage of H-bonds to form a transient channel and formation of H-bond to restore the original conformation can be disrupted by mutation. This is supported by our observation that the single mutation of Glu567 to alanine shows larger effect than the double mutant E511/567Q and E511Q (Table 1). The results also indicate the importance of Gly127 and Gly134. In fact all proteins in this family show this feature in which two glycine residues are located at i and i+7. In the three known crystal structures these two glycine residues are in the loop connecting the two middle beta strands of the mixed central beta sheet of the plug region (Figure 6). The double mutant G127/134A shows a significant deficiency in transport, which may indicate that a hinge motion around these two residues, disrupting the mixed beta sheet, is indeed important for the formation of the transient channel. Once in the periplasm, the ferric-siderophore is received and delivered by periplasmic binding proteins to the ABC type transporter located in the inner membrane for further transport to the cytoplasm (Clarke *et al* 2000, 2002; Sprencel *et al.* 2000).

The lock region in FhuA and FecA

The amino acid residues identified in the lock region of FepA are conserved genetically in FhuA and FecA (Figure 3). The corresponding amino acids in FhuA include arginines 93 and 133, glutamates 522 and 571, tyrosine 541 and serine 51 while in FecA they are arginines 150 and 196, glutamates 541 and 587, phenylalanine 558, and aspartic acid 105 (Figures 2 & 3). It is significant that these residues are not only conserved genetically but also structurally in FepA, FhuA and FecA (Figure 3). The genetic and structural conservation suggests a functional role of this region in the mechanism of transport. It would be interesting to check the effect of mutations by performing similar experiments involving these residues in FhuA and FecA to further support our results.

Structural results

The structure of the G127A mutant was determined at a relatively low resolution (3.2A), by single crystal X-Ray diffraction (Lemke 2001). Gly127 in the native structure has a conformation (phi/psi: 60.8/135.3), which cannot accommodate an alanine residue. A local distortion of the structure can therefore be expected. Residue 127 is adjacent to Arg126, which is an integral part of the lock region. The structural results show that the H-bonding between the arginines and glutamates of the quadrupole of the lock region has weakened significantly due to the conformational changes around the alanine residue at location 127, in comparison with the native structure. The G127A mutant is defective in transport, as was discussed earlier. This appears to be a proof that the H-bonding in the lock region has to be intact for normal wild type transport. It will be important, however, to determine additional structures of transport deficient mutants, and at higher resolution, to confirm this observation.

Conclusions

The present study is based on the information available from sequences of many proteins, which transport ferric siderophores in a TonB dependent mechanism and on the results of three crystal structures in this family of proteins. The simultaneous alignment of many sequences allows the identification of several homologous clusters. These clusters are presumed to be involved in the transport function of the proteins.

This presumption is supported by the fact that the clusters form structures, which are maintained in FepA, FhuA and FecA, the three proteins for which structures have been determined by X-Ray diffraction. One of these clusters, the lock region, was investigated in this study, by constructing single and double mutants. All the mutants show normal binding, but a large fraction shows defective transport. It is thus possible to distinguish these two functions of the proteins. The extent of the transport deficiency can be related to the importance of the mutated residues for the H-bonding within the quadrupole, which is central to the lock region. It was not possible to define the precise function of the lock region, however the evidence indicates that its structural integrity and interactions within the quadrupole are essential for the transport of the ferric enterobactin ligand. In addition it is likely that the opening and proper closing of the lock region is an integral part for the proper transport of the ligand from the binding site to the periplasm in the outer membrane receptor protein. The lock region, however, is only one of four clusters in the outer membrane receptors for which there are indications that they are involved in the transport function of the proteins.

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