Ferric hydroxamate binding protein FhuD from *Escherichia coli*: mutants in conserved and non-conserved regions

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Abstract

Uptake of iron complexes into the Gram-negative bacterial cell requires highly specific outer membrane receptors and specific ATP-dependent (ATP-Binding-Cassette (ABC)) transport systems located in the inner membrane. The latter type of import system is characterized by a periplasmic binding protein (BP), integral membrane proteins, and membrane-associated ATP-hydrolyzing proteins. In Gram-positive bacteria lacking the periplasmic space, the binding proteins are lipoproteins tethered to the cytoplasmic membrane. To date, there is little structural information about the components of ABC transport systems involved in iron complex transport. The recently determined structure of the *Escherichia coli* periplasmic ferric siderophore binding protein FhuD is unique for an ABC transport system (Clarke *et al.* 2000). Unlike other BP's, FhuD has two domains connected by a long α -helix. The ligand binds in a shallow pocket between the two domains. *In vivo* and *in vitro* analysis of single amino acid mutants of FhuD identified several residues that are important for proper functioning of the protein. In this study, the mutated residues were mapped to the protein structure to define special areas and specific amino acid residues in *E. coli* FhuD that are vital for correct protein function. A number of these important residues were localized in conserved regions according to a multiple sequence alignment of *E. coli* FhuD with other BP's that transport siderophores, heme, and vitamin B₁₂. The alignment and structure prediction of these polypeptides indicate that they form a distinct family of periplasmic binding proteins.

Abbreviations: ABC – ATP-Binding Cassette; BP – binding protein.

Introduction

Iron is an essential nutrient for most living bacteria. Moreover, it has been shown for a number of bacterial pathogens that iron sufficiency contributes to their virulence (Cox 1982; Payne 1988, 1993; Byers & Arceneaux 1998), yet little is known about bacterial iron transport mechanisms at the molecular level. Since the bioavailability of iron is very low, bacteria have developed a number of strategies to procure

iron from their environment. Highly specific, TonB energy-dependent outer membrane receptors are essential for the acquisition of iron from transferrins, lactoferrins, hemoglobin, hemophores and for the uptake of siderophores and heme into the periplasm. The subsequent import of ferrous iron, ferric iron in its ionic form, and of complexed ferric iron is mediated by special uptake systems located in the cytoplasmic membrane. Some of these importers belong to the group of ATP-Binding-Cassette (ABC) transport

systems consisting of a periplasmic binding protein, two integral membrane proteins (or two domains of a single polypeptide chain) and an ATP-hydrolyzing protein, which supplies the energy for the translocation process (Braun *et al.* 1998, Braun 1998; Braun and Killmann 1999; Schryvers and Stojiljkovic 1999; Vasil & Ochsner 1999; Ratledge & Dover 2000; Wandersman & Stojiljkovic 2000; Clarke *et al.* 2001; Köster 2001).

Although a specific bacterial strain may produce only a few types of siderophores, uptake systems for several different siderophores may be expressed. For example, hydroxamate type siderophores (i.e., ferrichrome, aerobactin and coprogen) have individual receptors in the outer membrane of *Escherichia coli*. The structure of the ferrichrome outer membrane transport protein FhuA reveals that its N-terminal domain forms a plug inside the 22-strand β -barrel (Ferguson *et al.* 1998; Locher *et al.* 1998), similar to the enterobactin outer membrane receptor FepA (Buchanan *et al.* 1999). The ferrichrome and the structurally related antibiotic albomycin nestle in a hydrophobic binding pocket, stabilized by several hydrogen bonds (Ferguson *et al.* 1998; 2000b). Uptake of ferric hydroxamates across the periplasm and inner membrane is mediated by the common *fhu* system. FhuD, the periplasmic binding protein, accepts a number of structurally different hydroxamate-type siderophores as ligands and transports them to the inner membrane components FhuB and FhuC. The structure of FhuD forms two distinct domains linked by a long α -helix (Clarke *et al.* 2000). The ligand binding pocket lies in a shallow groove between these two domains and slight movements of the amino acid side chains allow a variety of hydroxamate type siderophores to bind (unpublished data). Although there are no crystallographic structures of the inner membrane proteins, analysis of FhuB mutant proteins (Böhm *et al.* 1996), FhuB topology studies (Groeger & Köster 1998), FhuB-FhuD cross-linking experiments (Rohrbach *et al.* 1995a), and peptide mapping studies (Mademidis *et al.* 1997) suggest that several external loops may exist in FhuB that are important for physical interaction with FhuD.

Bacterial binding proteins of ABC systems can be grouped into distinct classes based on similarities in primary sequence (Tam & Saier 1993). The siderophore binding proteins are grouped in a different class than other ligand binding proteins involved in the uptake of other essential nutrients (Köster 1991). In general, the different classes of binding proteins of

ABC transport systems share relatively little sequence homology and recognize diverse ligands, however, most are closely related in structure (Quioco & Ledvina 1996). Many consist of two globular domains connected by short stretches of polypeptide chain. Each lobe consists of a central β -sheet surrounded by α -helices and molecular recognition of ligands arises from slight differences in the number and arrangement of the β -strands and α -helices in the two globular domains. As a result of the differences in domain structure and sequence of the periplasmic receptors, the structure of the binding site is highly variable. In most ligand binding proteins, two or three crossovers of the polypeptide chain between the two domains form the hinge region and the base of the binding cleft. Closure around the substrate in the deep binding cleft formed by the two domains is suggestive of a 'Venus fly trap' or 'Pac-man' motion (Mao *et al.* 1982; Ames 1986). This mode of binding allows many interactions to form between the protein and substrate, increasing ligand specificity. The variations between the structures of the prototypical bacterial binding protein and the *E. coli* periplasmic ferric siderophore binding protein FhuD reveal that different ligand binding proteins may exercise distinct mechanisms for ligand binding and release. Since the structure of FhuD is atypical for a bacterial binding protein, larger ligands such as ferric siderophores may require a different structure. The specificity of periplasmic ferric siderophore binding proteins is also broader, compared to other periplasmic ligand binding proteins, so the binding pocket is more solvent exposed and some movement of side chains is allowed.

Thus, for the ferric siderophore binding protein family, a distinct structure may be required for function. The use of homology modeling studies, in this case, is limited, since there is relatively little sequence homology between these proteins (< 20%). In lieu of other structures for this family, we have attempted to determine key residues among these proteins important for function. Point mutations in *E. coli* FhuD have been isolated which had an influence on the performance of the protein *in vivo* and *in vitro*. These have been mapped to the three dimensional structure of FhuD to identify disruptions to the secondary and tertiary structure. When these mutations are identified in a sequence alignment of the siderophore binding proteins, many are located in conserved regions typical of this family. Our data indicates that in the ferric siderophore binding family, there are a number of

Figure 1. Map of the mutations on the structure of the FhuD gallichrome complex. (a) Ribbon diagram showing the overall tertiary and secondary structures. β -strands are coloured blue, α -helices are red and random coil regions are gray. (b) Backbone trace of the protein complex showing the location of mutated residues in the FhuD structure. The side chains of the residues are colour coded, with red residues having a large effect on function *in vivo* and *in vitro* and lighter pink residues having less effect. This figure was created with Setor (Evans 1993).

residues that may play a key role maintaining the overall structures of the proteins.

Methods

Construction of the FhuD mutants

By varying the conditions for the polymerase chain reaction, the *fhuD* gene was amplified according to the procedure outlined by Rohrbach *et al.* (1995a), thereby introducing point mutations at various sites. After cloning in a suitable plasmid vector, the sequences of the *fhuD* mutants were determined by the dideoxy method, and FhuD mutant proteins were expressed and purified as previously described (Rohrbach *et al.* 1995a, b).

Functional analysis of the FhuD mutants

E. coli K-12 strain KO295 (*aroB*, *fhuD*) does not produce any siderophores and is unable to utilize ferric hydroxamates used in our study. The plasmids harboring the *fhuD* mutant genes were introduced into strain KO295. Then after protein expression, sensitivity to

albomycin and growth on ferrichrome or coprogen as the sole iron source was tested (Rohrbach *et al.* 1995a, b). The FhuD mutant proteins were analyzed with respect to processing and stability in a protease protection assay as previously described (Köster & Braun 1990; Rohrbach *et al.* 1995b).

Identification of the locations of point mutations in FhuD

Each of the point mutations in FhuD was identified in the X-ray crystal structure (Protein Data Bank accession code 1EFD (Clarke *et al.* 2000)) with respect to the location in the secondary and tertiary structure. The point mutations were colour coded according to the severity of the effect on the function of the protein in the functional assays and classified into regions of the proteins that are important for function.

Sequence analysis and multiple alignments

Sequence similarity searches to *E. coli* were initiated by the BLAST search tool at the NCBI web site (www.ncbi.nlm.nih.gov) (Altschul *et al.* 1997). The evolutionary tree was produced by the NJ method

Table 1. Phenotype of FhuD mutants

Site of mutation	Sensitivity to albomycin ^a	Growth on ferrichrome or coprogen ^b	Protease protection ^c
Wild type	S	+++++++	yes
T181A	(S)	++++	n.d.
F274L	S	++++	n.d.
W68L	R	cop»fer	no
Y65C	R	++	n.d.
I183T	S	++++	n.d.
N242S	(R)	+++++	reduced
V39A	R	+	no
T93N	(R)	++++	n.d.
F99S	R	+++	yes
E213G	(R)	+++	yes
W210R	(S)	+++++	n.d.
D234G	S	+++++	n.d.
L236P	(R)	+++	no
M258V	(S)	+++++	n.d.
F124L	R	+++	yes
F161L	R	++	no
S149G	(R)	++++	yes
N146D	(S)	+++++	n.d.
F169L	(R)	+++++	yes

^aDegrees of sensitivity: S > (S) > (R) > R (S, sensitive; R, resistant).

^bThe number of crosses reflects the degree of growth promotion (+++++, like wild type).

^cn.d., not determined.

from a distance matrix based on a multiple alignment obtained with CLUSTAL-X.

Results

Phenotype of the FhuD mutants

The conditions used for the PCR reaction produced a number of *fhuD* mutants with single point mutations in different positions along the polypeptide chain. The changes in sequence produced by this method resulted in amino acid conversions that did not necessarily maintain the original properties of the wild type residue. The single point mutations to *fhuD* and analysis of the function of the resulting proteins are listed in Table 1. Mutated FhuD proteins complemented the effect of the chromosomal mutation in the *fhuD* sequence of *E. coli* KO295 to varying levels. This is indicated by growth on ferrichrome or coprogen as the sole iron source, as well as sensitivity to the antibiotic albomycin (Table 1). Degradation of FhuD by proteases has been shown to be prevented or diminished by substrate binding (Köster & Braun 1990;

Rohrbach *et al.* 1995a, b). The amount of proteolysis of the FhuD mutant proteins when substrate is added provides an estimation of the ligand binding properties of the protein (Table 1).

Several single mutations to different positions on the polypeptide chain had a large effect on the ability of the protein expressing strain KO295 to grow on ferrichrome or coprogen as the sole iron source, and gave resistance to albomycin. On the other hand, other mutations had little effect on the function of the protein. No mutations were found that enhanced the activity of the protein. There were a number of mutants that were able to resist proteolysis similarly to the wild type protein. However, in some cases, the ability of the mutant to resist proteolytic degradation was diminished or absent.

Many of the mutant FhuD proteins that mediated resistance to albomycin in strain KO295 (*fhuD*) also displayed limited growth on ferrichrome and coprogen. This indicates that these proteins have a decreased ability to function *in vivo*. These derivatives also tended not to be resistant to proteolysis, so the protein may not be able to bind the ligand in the same

Table 2. Location of FhuD mutant residues

Site of mutation ^a	Location	Possible effects on the protein structure
T181A	binding site	interactions with peptide backbone of albomycin, ferrichrome and coprogen
F274L	binding site	a hydrophobic group lining the binding pocket
W68L	binding site	a hydrophobic group lining the binding pocket; slight movement when coprogen is bound
Y65C	binding site	a hydrophobic group lining the binding pocket
I183T	binding site	contributes to the shape of the binding site
N242S	loop near binding site	side chain points into the C-domain
V39A	N domain	between β -strands in the interior of the domain
T93N	N domain	surface exposed side chain
F99S	N domain	important in domain packing
E213G	C domain	surface exposed side chain; charge important
W210R	C domain	between β -strands in the interior of the domain
D234G	C domain	between β -strands in the interior of the domain
L236P	C domain	part of a β -strand; the substituted proline could interfere with forming secondary structure
M258V	C domain	within the interior of the domain
F124L	interface between domains	hydrophobic interactions are important between the domains
F161L	centre of connecting helix	hydrophobic contacts between the helix and domains and between domains are present and could be important for stability
S149G	end of connecting helix	surface exposed side chain
N146D	end of connecting helix	surface exposed side chain
F169L	end of connecting helix	side chain interacts with the C-domain

^aIn addition to these single mutations, many multiple mutations of FhuD were also analyzed. These are not listed here, as their activity, in terms of the effects to the structure of FhuD is difficult to interpret.

manner as the wild type protein. The reverse is true for another set of mutants that complemented the *fhuD* mutation of KO295: those FhuD mutant proteins seem to be more resistant to proteolysis. In addition, there are several mutant proteins that did not follow this trend: they did not function *in vivo* but were able to resist proteolysis *in vitro*.

The majority of the mutations localized in or near the binding site had a large effect on the function of the protein. Although the residues that were mutated are not those implicated in hydrogen binding various ferri- hydroxamates (Clarke *et al.* 2000), many of these

amino acid side chains line the binding pocket, forming a surface complementary to the ligand. W68L and I183T are alterations to important residues for van der Waals interactions between FhuD and the siderophore. Since they stack close to the ligand, there is a significant loss of function compared to the wild type situation. Although the positions 181 and 274 are not immediately in the vicinity of the binding site, these amino acids are important for maintaining the structural integrity of the stacking interactions between the side chains. F274L and T181A clearly affect the properties of the protein. The mutation N242S is located

Domain	N	C
Structure	-- HHHHHH SSSSSS	-- S HHHHHH SSSSSS
FhuD_Ecoli	87 PNLLELNMPKPSFVWVSAQYV	223 VSIDRLAAYKDVVPCFORDS
FhuD_Ecoli	-- PNLILTEMLKPSFVWVSAQYV	-- VSIDRLAAYKDVVPCFORDS
FhuD_Rhleg	-- VNFILVTLKPSFVLTTP-YL	-- GIEDSKITLPSFVLTTP-YL
FhuD_Rhcap	-- PSLTLALAPLKLSSSYYS	-- VPIELRLA-DEFTAVILVTGEI
FhuD_Cajej	-- PNLIAALRLKPSFVGVYA-YL	-- SLHRIDMRSTFAHRDCAYCS
Vc0202_Vicho	-- PNLIAALRLKPSFVLAAGPQQ	-- RLSELOHVEQGVVYVLPFAE
10G8.27c_Stcoe	-- PSMPTVASLPSFVLTATDCLA	-- TDVGLT-KLSEDTGPAFYN
MLR8228_Melot	-- PTLINLVAVPSEFFAGVYV	-- TDWTVASRNQFVLTLDYQD
BH1037_Bahal	-- PNLIAALRLKPSFVLTAKFRH	-- GYSQVNVKALPSFVLTANFYV
0872_Mejan	-- VNYGLVNLPSFVLTGDKWS	-- TFPGRMNAVDPVLTTPYGVV
Vyyv_Limon	-- PSIEKIANLKPDLITIS-DYQ	-- ETKAISSEALPSFVLTADRLF
XyeB_Basub	-- ISVQVMEVLKPSFVLTVM-NEE	-- EMKQSLSEVLPSEADYMFIT
FhuD_Basub	-- TSVEKVIDLNPDLITVMTTQG	-- GYTSISLEKPSFVLTADYIFAG
FhuD_Lalac	-- FNMKILIAQNPDLITVTDKQD	-- GYAVNTESLSEADYIFID
SPY0365_Stpyo	-- TULAVTTTLKPSFVLTGSTEE	-- GYLSLSEVLPSEADYIFVVA
SAV1025_Staur	-- PNMKIVSLKPSFVLTADVSRH	-- LNNELANINPVMILATDCK
SA1979_Staur	-- PNLKIVSLKPSFVLTADSSRH	-- LDTLHADLNPDMILMTDHA
SAV1115-Staur	-- PNMVAVKPKPSFVLTASVSTIK	-- SNTNLLNINPDLILPLPHGM
Z4382_Ecoli	-- IDIAVIAAALPSFVLTETPTIN	-- GRMDSVSAERLPSFVLTADFEAT
FeuA_Basub	-- PNLKILKPKPSFVLTASTKFP	-- SSLKILSEMPSEFVLTGFSDD
YfiY_Basub	-- PNLKIVSLKPSFVLTGKVRQ	-- TFSQDSKPSFVLTADVLYEF
ChrA_Erchr	-- PSLQDVLKPSFVLTASVSRFH	-- TNKQSPVVDALPSEFVLTFRSE
AP6168_Aeper	-- VNMKILKPSFVLTAVTTTGAQ	-- FMSQRTTWIYQPPERINRKF
SirA_Staur	-- PNLKIVSLKPSFVLTASVSRH	-- TSQSPILPMNAPSEFVLTGSDP
SidB2_Stepi	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
SidB22_Stepi	-- VNMKIVSLKPSFVLTASVSRH	-- INNVINKQNPSEFVLTADDRGS
SA0566_Staur	-- PNLKIVSLKPSFVLTASVSRH	-- VTKQSPILKPSFVLTGSDGK
BH3791-Bahal	-- PNLKIVSLKPSFVLTASVSRH	-- TDMSPILPMNAPSEFVLTGSDP
slr1319_Sycys	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
slr1491_Sycys	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
slr1492_Sycys	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
slr11202_Sycys	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
DR2588_Derad	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
DRB0125_Derad	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
YfmC_Basub	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
YhfQ_Basub	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
Vcub_Vicho	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FepB_Ecoli	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
PA4159_Psaer	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FecB_Ecoli	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FM0131_Pamul	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FxuD_Mysme	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
SPY1793_Stpyo	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
BH3297_Bahal	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FufA_Limon	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
BH329C_Bahal	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FecB_Myavi	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FecB_Myub	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FecB2_Myub	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
IrpL_Codip	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
VyrC_Basub	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
2480rf2_Stpyo	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
YclP_Basub	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
SstD_Staur	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
XXXX_Cajej	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
CeuB_Cacol	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FetB_Negon	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FatB_Viang	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
SCF34.13c_Stcoe	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
HP1561_Hepyl	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
HP1562_Hepyl	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
1472_Hainf	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
PM1149_Pamul	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
PAB049_Pyaby	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
PHAV036_Pyhor	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
PH1237_Pyhor	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
AF0432_Arful	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
0085_Mejan	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
TM0189_Thmar	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
Orf2_Mebar	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
RumB_Momor	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
Yadt_Ecoli	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
BtuF_Satyp	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
HmuT_Codip	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
VC2381_Vicho	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
TM0080_Thmar	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
HmuT_Yepes	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
HemT_Yeent	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
ChuD_Cajej	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
DRB007_Derad	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
HuB_Vicho	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
ShuT_Shdyt	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
CuWt_Ecoli	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
KuB_Flshi	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
FnuT_Psaer	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
HmcT_Brjap	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
ML1151_Melot	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK
CC1191_Cacre	-- PNLKIVSLKPSFVLTASVSRH	-- VSKQSPILKPSFVLTGSDGK

Figure 2. Sequence alignment of the ferric siderophore binding protein family. Only the segments of the N- and C-domains that are the most highly conserved are shown for clarity. The conserved residues are marked with a black or grey underlay. Above the sequence alignment, the secondary structure observed in *E. coli* FhuD is shown. Point mutations in the relevant regions of the *E. coli* FhuD protein are shown in bold print. Numbers indicate the positions in the sequence of the *E. coli* FhuD protein.

on a loop near the binding site, not directly involved in forming the binding pocket, so this exchange is tolerated well during protein function.

Substitutions in either domain of the protein have a range of effects on the function of the protein in the assays. The mutations in the N-domain localized on the exterior of the protein, T93N and F99S, affect the ability to grow on ferrichrome and coprogen to a different extent. Interestingly, both replacements are located in an α -helix – turn – β -strand region, which represents the most conserved signature motif (see Figure 3) among all siderophore family BP's. The residues corresponding to position 93 in FhuD are not highly conserved in the different BP's. This may explain that the alteration T93N is to a certain extent tolerated with respect to transport function. In contrast, the position equivalent to residue 99 in FhuD shows a high prevalence for a hydrophobic residue. Therefore the change from a bulky hydrophobic to a small polar residue (F99S) seems to be less acceptable thus resulting in a impaired growth with ferric hydroxamates and a significantly reduced sensitivity to albomycin.

The N-terminal sequence of FhuD, reading AAIDPNRIVALE, includes a short β -strand and is highly similar throughout the whole family of siderophore BP's (with RIV being the most conserved residues of the motif). Notably, the mutation V39A, which renders the FhuD protein more sensitive to proteases and drastically reduces its transport activity, is located in that β -strand in the interior of the N-domain, and may destabilize the secondary structure.

In the C-domain, the mutation E213G removes the charge and bulk of a surface exposed side chain, affecting the function of the protein *in vivo* but retaining the ability to protect the protein from proteolysis. The side chain of the replacement W210R does not significantly affect the formation of β -sheets in the interior of the C-domain, hence the proteins display almost wild type-like behavior. The mutation D234G and L236P are located in a β -strand in the C-domain, which represents a conserved region among the siderophore family BP's. The replacement at position 234 is largely tolerated, whereas the substitution to proline at position 236 may disrupt the formation of this secondary structure. The mutation M258V is located on a helix on the exterior of the C-domain, with the side chain pointing towards the interior of the protein. Changing this residue to a smaller side chain does not impair the function of the protein to a large extent. The substitution F124L is localized in the base of the binding

site, at the interface between the two domains of FhuD and has little effect concerning the ability to mediate growth on siderophores and sensitivity to albomycin.

The consequence of mutations along the α -helix connecting the two domains on the functionality of the protein increases as the center of the helix is reached. The replacements N146D, S149G and F169L located on either end of the helix, can be tolerated to a certain extent. However, F161L, which is near the center and the side chain of which interacts with the C-domain, causes a dramatic effect on the function of the protein.

Evolutionary relationships

The overall structure and the organization of the *E. coli* FhuD polypeptide chain are different from other known bacterial binding proteins from ABC transporters. A phylogenetic tree of the periplasmic binding proteins of the siderophore family from different bacteria is shown in Figure 2. An initial multiple alignment was used as a guide to identify related proteins and develop the family phylogeny. 47 genes were taken from a variety of bacterial species known to be involved with the transport of siderophores, heme or vitamin B₁₂. The periplasmic proteins are variable in size, between 25 and 40 kDa. There seems to be a common ancestral root and these bacterial proteins likely make up a large family of binding proteins. There appears to be at least five subfamilies of three members or more. Since the vast majority of these proteins have not been characterized with respect to binding and transport of the different siderophores, we cannot at this time absolutely cluster them according to their function. Nonetheless, it appears that all BP's for which participation in vitamin B₁₂ or heme transport has been demonstrated or predicted, cluster in a distinct subfamily. *E. coli* FhuD is grouped with other FhuD proteins from *Rhizobia* sp., which are also predicted to bind hydroxamate-type ferric siderophores.

Conserved sequence patterns

Analysis of a multiple alignment of the primary sequence of the bacterial siderophore binding proteins reveals a number of residues that are conserved. The alignment was extended to 87 BP's from Gram-negative bacteria, Gram-positive bacteria, mycobacteria, and archaeae. There appears to be more sequence conservation in the N-domain region than in the C-domain region. Regions of similar sequence are also present in both halves of the protein. There

are a considerable number of amino acids, predominantly glycine and proline residues as well as charged residues, which are present at equivalent positions in all BP's of the siderophore family. Characteristic signatures were identified, most of them located in the β -strands and at the borders ('turning points') between different β -strands and α -helices. Two of the most similar regions found in the N-terminal and the C-terminal domains of all siderophore uptake-related BP's are shown in Figure 3.

Many of the residues, which were found to be important for the function of *E. coli* FhuD, as revealed by the analysis of point mutants, are present in a number of BP's throughout the whole family. The residues identified in the binding site of FhuD are from different sections of the polypeptide chain but tend to be aromatic side chains. The residues altered in the binding site in this study do not appear to be highly conserved amongst the family. However, other residues lining the binding pocket of *E. coli* FhuD, in particular Glu42, Trp43, Asp61 and Trp217, are present in other BP's of the group. Residues positioned to form hydrogen bonds between FhuD and its ligands, which include Arg84 and Tyr106 (Clarke *et al.* 2000), can be found in some of the other proteins. Interestingly, the positively charged guanidinium group from Arg84 is converted to a negatively charged glutamic acid in some proteins. Since substitution of Arg84 with uncharged residues eliminates the capacity of FhuD to bind hydroxamate siderophores (unpublished results), this substitution likely changes the ligand binding specificity of the protein. A considerable number (4–7) of those residues in *E. coli* FhuD that represent important contact sites to the hydroxamate ligands are located in only a few other BP's at equivalent positions. These are the so called 'FhuD' proteins from *Rhizobium leguminosarum*, *Rhodobacter capsulatus*, and *Campylobacter jejuni*, and the proteins BH1037 from *Bacillus halodurans*, Vc0202 from *Vibrio cholerae*, 10G8.27c from *Streptomyces coelicolor*, and Mlr8228 from *Mezorhizobium loti*. Interestingly, the FhuD protein from *Bacillus subtilis* does not show identity in any of the residues that form the contact sites with ferrichrome. The result is consistent with the finding that the FhuD proteins from *B. subtilis* and *E. coli* cluster in different subfamilies. Although there is no doubt, that all siderophore related BP's originally derived from a common ancestor our data suggests that the substrate specificity of these two BP's may have evolved independently starting from two different 'precursors'.

Figure 3. An unrooted evolutionary tree of the ferric siderophore binding proteins from different bacteria. The alignment and phylogenetic analysis was performed with CLUSTAL-X and the tree is drawn with NJPlot.

The residues that are found to be important for function in the N- and C-domains also tend to appear in some of the other proteins in the family. The residues forming the connecting helix between the two domains are obviously not conserved. Nevertheless, despite the low levels of identity in primary sequence, there might be a significant similarity in structure.

Discussion

The distinct architecture of the *E. coli* ferric siderophore binding protein FhuD compared to other

bacterial binding proteins immediately suggests that this type of fold is desired for binding iron complexes. The ligand is cradled in a shallow binding pocket between two domains, largely solvent exposed, with many interactions from aromatic residues lining the binding pocket. However, the importance of the long helix connecting the two domains is yet to be elucidated. Identification of regions and specific residues important for the function of the protein is possible by mapping point mutations along the polypeptide chain to the structure of the protein.

Certain regions of *E. coli* FhuD are more sensitive to point mutations than other areas. Substitutions in

the binding site of the protein alter the properties of the protein to a greater extent than mutations in other areas of the protein. Changes to the aromatic groups that line the binding pocket modify the structural complementarity of the protein to the ligand, decreasing the number of favourable interactions. Substitution of long chain amino acids will also sterically hinder ligand binding. Since there is some movement of the side chains when different hydroxamate-type siderophores are bound (unpublished results), substitution of side chains will interfere with the dynamic properties of the binding site.

In addition, particular areas of the N- and C-domains, as well as the interconnecting helix, are important for protein function. In the two domains, these are residues involved in stabilizing the β -sheet central to the structure of the protein. Disruptions to the packing around the sheet by substituting different amino acids influence the hydrogen bonding network found in this area. In the helix area, hydrophobic interactions between the underside of the two domains and the hydrophobic surface of the amphipathic helix stabilize the structure. Hydrophobic groups on one side of the helix stack into the space between the helix and each of the two domains. A mutation in the centre of the helix, changing an aromatic group to a leucine (FhuD F161L) greatly alters the functionality of the protein by destabilizing the interaction between the helix and the C-domain.

In contrast, there are areas of the protein that appear to be unaffected by point mutations. Changes to many of the residues in the N- and C-domains of the protein produce less dramatic effects on the function. These substitutions of solvent exposed side chains seem to be tolerated, maintaining a functional protein. Furthermore, replacements at either end of the helix appear to be allowed. These areas form loops connecting the helix to the remainder of the protein and could be somewhat flexible. The side chains of these residues tend to point into the solvent surrounding the protein and are not involved with stacking interactions.

It is beneficial to distinguish between point mutations that affect the function of the protein as a part of the transport system and the ability of the protein to bind ligands. For example, mutants that did not function *in vivo* but functioned *in vitro* may be able to properly bind ligands but not function in the uptake pathway. This could be important in identifying regions of the protein that are used for recognition by the other components of the pathway. One interesting example is the FhuD derivative E213G, which has

reduced ability to grow on ferrichrome and coprogen and is partially resistant to albomycin, yet is protected from proteolytic degradation when ligand is bound. This suggests that the charge on the side chains that are solvent exposed may be important for the operation of the protein in the uptake pathway. This mutant FhuD might be impaired in the interaction with the integral membrane protein FhuB.

The mutations found to be important in the function of *E. coli* FhuD are not necessarily conserved in all the siderophore family members. The highest number of conserved residues occurs between *E. coli* FhuD and its closest neighbours, but conserved motifs are found throughout the family. Several of the conserved sequences found in the N-domains of the proteins are also similar to those found in the C-domains. This indicates that there may have been a gene duplication event early in the evolution of these binding proteins. The topology of the FhuD structure also reflects the homology in sequence between the two domains, with parallels in structure.

The residues found important in the binding site are very similar in the proteins that supposedly bind hydroxamate-type siderophores but differ in the other binding proteins. This is not surprising since the characteristics of the other ligands vary in size and charge. Any residues that form specific interactions, such as hydrogen bonds, between the protein and ligand would not necessarily be conserved since these would be complementary to each ligand. On the other hand, aromatic groups tend to be common in the binding site of other ligand binding proteins, including those that bind amino acids, sugars and peptides (Quiocho 1990; Quiocho & Ledvina 1996). There are several aromatic groups found in the binding site of FhuD that are conserved in most members of the family. Aromatic residues in the protein sequences in the vicinity of the sequence of the binding site residue of FhuD could also be orientated into the pocket with only slight variations in the overall structure.

Members of the siderophore family BP's are clearly distinguishable from all other periplasmic components involved in the uptake of metals and other nutrients (Winkelmann 2001). The other components of these ABC transport systems are also classified into their own families. The ATP binding proteins (e.g., FhuC, FepC, FecE and BtuD from *E. coli*) cluster in the same way and form subfamilies as well as the transmembrane proteins FhuB, FepG, FepD, FecC, FecD and BtuC (Groeger & Köster 1998; Saurin et al. 1999; Linton & Higgins 1999; Köster 2001). The fact

that sequences of all these components are quite different from those of other uptake systems suggests that the requirements for this type of high-affinity solute binding impose severe structural constraints on these proteins.

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