The Role of FhuD2 in Iron(III)-Hydroxamate Transport in Staphylococcus aureus

DEMONSTRATION THAT FhuD2 BINDS IRON(III)-HYDROXAMATES BUT WITH MINIMAL CONFORMATIONAL CHANGE AND IMPLICATION OF MUTATIONS ON TRANSPORT*

The fhuD2 gene encodes a lipoprotein that has previously been shown to be important for the utilization of iron(III)-hydroxamates by Staphylococcus aureus. We have studied the function of the FhuD2 protein in greater detail, and demonstrate here that the protein binds several iron(III)-hydroxamates. Mutagenesis of FhuD2 identified several residues that were important for the ability of the protein to function in iron(III)-hydroxamate transport. Several residues, notably Tyr-191, Trp-197, and Glu-202, were found to be critical for ligand binding. Moreover, mutation of two highly conserved glutamate residues, Glu-97 and Glu-231, had no affect on ligand binding, but did impair iron(III)-hydroxamate transport. Interestingly, the transport defect was not equivalent for all iron(III)-hydroxamates. We modeled FhuD2 against the high resolution structures of Escherichia coli FhuD and BtuF, two structurally related proteins, and showed that the three proteins share a similar overall structure. FhuD2 Glu-97 and Glu-231 were positioned on the surface of the N and C domains, respectively. Characterization of E97A, E231A, or E97A/E231A mutants suggests that these residues, along with the ligand itself, play a cumulative role in recognition by the ABC transporter FhuBGC. In addition, small angle x-ray scattering was used to demonstrate that, in solution, FhuD2 does not undergo a detectable change in conformation upon binding iron(III)-hydroxamates. Therefore, the mechanism of binding and transport of ligands for binding proteins within this family is significantly different from that of other well studied binding protein families, such as that represented by maltose-binding protein.

Iron, the fourth most abundant element on the earth’s crust, is one of the most important micronutrients for almost all forms of life. The majority of that iron, however, exists in a biologically unavailable form as a result of the fact that ferric iron is highly insoluble at a physiological pH. To overcome this iron shortage, bacteria have developed a variety of mechanisms to sequester what little available iron may exist within their environment. In particular, one important iron acquisition strategy is through the production of small molecules, termed siderophores, which have an extremely high affinity for Fe(III) and which can interact with cognate transporters that effectively move Fe(III)-siderophore complexes across the bacterial envelope.

ABC transporters are protein conduits that use energy derived from the hydrolysis of ATP to move solutes across the cell membrane. Typically, the basic unit of an ABC transporter is (i) two transmembrane domains that span the membrane usually 6 times each for a total of 12 membrane-spanning domains, (ii) two cytoplasmic ATP-binding proteins that serve to energize the system, and (iii) a binding protein that interacts with the transmembrane domains (1). Binding proteins in Gram-negative bacteria are present within the periplasm, whereas those in Gram-positive bacteria are tethered to the cell membrane via the acylation of a cysteine residue that is an integral component of a lipoprotein signal sequence.

Solute binding proteins typically exhibit a common structural design in that they contain a cleft or groove region that serves as the ligand binding site, and this ligand binding site is surrounded by two globular domains typically consisting of a central β-sheet surrounded by α-helices (2). The canonical binding protein, maltose-binding protein (MBP), undergoes a significant structural rearrangement upon ligand binding. Indeed, the closure of the two domains around the ligand has been likened to the active motion of a Venus flytrap. Recent x-ray crystallographic studies have defined a new class of binding proteins that include Escherichia coli FhuD (3, 4), which binds iron(III)-hydroxamates; E. coli BtuF (5, 6), which binds vitamin B₁₂; and Treponema pallidum TraA (7), a zinc-binding protein. These proteins possess an architecture that differs from the sugar- and amino acid-binding proteins (represented by MBP) in that they have adopted a single, more inflexible backbone α-helix that connects two globular domains. This topology is not found within the interdomain connections of group I or group II binding proteins (2), and, as such, this class of proteins can be placed into a new group. Structural constraints imposed by the backbone α-helix have led to the suggestion that this new class of binding protein does not undergo a significant change in conformation upon ligand binding. Indeed, crystal structures of apo and holo forms of BtuF indicate a relatively minor conformational change upon ligand binding.

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FhuD2 Function in S. aureus

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Bacterial strains and plasmids used in this study are listed in Table I.

**Table I.** Bacterial strains and plasmids

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| **S. aureus**     |             |                     |
| RN2564            | (80a) pE25 | J. Iandolo          |
| RN4220            |             |                     |
| RN6390            | Prophage-cured wild type strain |                     |
| H431              | RN6390 fhuD1::Km fhuD2::Tet; Km′Tc′ |                     |
| **E. coli**       |             |                     |
| DH5α              | d808lacZAM15 recA1 endA1 gvrA96 thi-1 hasR17 (r μ-r μ) supE44 relA1 deoR2 [lacZYA-argF-U169 | Promega |
| BL21 (DE3)        | F′ ompT [lon] hasSB (r μ-m ; an E. coli B strain) with a DE3 A prophage carrying the T7 RNA polymerase gene |                     |
| H584              | DH5α containing plMTS57; Ap′ | This study |
| **Plasmids**      |             |                     |
| pET-28A (+)       | 5.3-kb E. coli expression vector; Km′ | Novagen |
| pGEX-2T-TEV       | 4.9-kb E. coli expression vector, modified to contain a tobacco etch virus (TEV) protease cleavage sequence; Ap′ | F. Sicheri |
| pMT50             | 5.2-kb E. coli-S. aureus shuttle vector; Ap′Cm′ |                     |
| pMT507            | pL50, containing the S. aureus fhuD2 gene; Ap′ Cm′ | 32 |
| pMT59             | pGEX-2T-TEV, digested with BamHI/EcoRI containing the S. aureus fhuD2 gene minus the first 24 codons; Ap′ | 8 |
| pET28a(+)         | pET28a(+) carrying the E. coli fhuD gene inserted into the BamHI/EcoRI sites. Expresses FhuD with an N-terminal His tag; Km′ | This study |

**Expression and Purification of FhuD2 and Derivatives**—The FhuD2 gene was PCR-amplified as a 1.1-kb fragment that was cloned into the high-resolution structure of E. coli FhuD bound to different hydroxamate siderophores have been determined (3, 4). These structures identify a shallow binding pocket that exists between the two protein domains; siderophores are recognized by the side chains of a few key residues lining the binding pocket. The structures of different hydroxamate siderophores are accommodated in the binding pocket by subtle rearrangements in the positions of the side chains. In a recent study of the high-resolution structure of BtuF, Borths et al. (6) highlight two highly conserved glutamate residues that are located on the surface of each of the two lobes of the protein. When they aligned the BtuF structure above the structure of BtuCD, the ABC transporter, it was found that the BtuF glutamates align with the position of positively charged pockets of arginines present on the periplasmic surface of BtuCD. This has led to the suggestion that interprotein Glu-Arg salt bridges might form important docking contacts between the binding protein and the ABC transporter. Notably, both the glutamates and the arginines are conserved in a number of iron(III)-siderophore transport systems in different bacteria (6).

**Staphylococcus aureus** is a Gram-positive human pathogen that possesses an ABC transporter (FhuCBG, FhuD1, FhuD2) for the import of iron(III)-hydroxamates (8, 9). FhuB and FhuG constitute the membrane-spanning components, whereas FhuC is the ATP-binding protein and two acylated proteins, FhuD1 and FhuD2, are thought to serve as the binding proteins for the system. FhuD2 participates in the transport of a wider range of substrates than FhuD1 (8), and is the subject of the investigations presented herein. FhuD2, representative of a large family of putative iron-binding proteins in Gram-positive bacteria (10), was used as a model protein to characterize the interactions of E. coli FhuD protein. Moreover, solution x-ray scattering experiments, which are sensitive to protein conformation, indicated that, in solution, little or no conformational change is associated with ligand binding, the first such demonstration for this class of ligand-binding proteins. This result, in combination with mutations affecting ligand binding and transport, suggests a novel mode of recognition between the binding protein-ligand complex and the ABC transporter (FhuBCG′).

(5). High resolution structures of E. coli FhuD bound to different hydroxamate siderophores have been determined (3, 4). These structures identify a shallow binding pocket that exists between the two protein domains; siderophores are recognized by the side chains of a few key residues lining the binding pocket. The structures of different hydroxamate siderophores are accommodated in the binding pocket by subtle rearrangements in the positions of the side chains. In a recent study of the high-resolution structure of BtuF, Borths et al. (6) highlight two highly conserved glutamate residues that are located on the surface of each of the two lobes of the protein. When they aligned the BtuF structure above the structure of BtuCD, the ABC transporter, it was found that the BtuF glutamates align with the position of positively charged pockets of arginines present on the periplasmic surface of BtuCD. This has led to the suggestion that interprotein Glu-Arg salt bridges might form important docking contacts between the binding protein and the ABC transporter. Notably, both the glutamates and the arginines are conserved in a number of iron(III)-siderophore transport systems in different bacteria (6).

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The solver function in Microsoft Excel was used to fit the data to the equations and obtain values for the relevant parameters. All titration experiments were done in triplicate for each ligand.

**Proteinase K Digests—**Samples of FhuD2 (2 μg) were incubated with proteinase K (2 μg added to each reaction) in 30 mM sodium phosphate buffer, pH 7.5, for 30 min in the presence or absence of ferric siderophores at 55 °C. The resulting proteins were resolved on SDS-polyacrylamide gels as previously described (15).

**Computer Analysis—DNA sequence analysis,** oligonucleotide primer design, and sequence alignments were performed using the Vector NTI Suite software package (Informax, Inc., Bethesda, MD).

**Small Angle X-ray Scattering—**Initial low angle measurements and some high angle measurements were made at BioCAT (beamline 18ID) of the Advanced Photon Source (APS). A second collection was conducted at the European Molecular Biology Laboratory Outstation at the Deutsches Elektronen-Synchrotron (DESY, Hamburg, Germany), beamline X33 (16). Note that \( Q = 2 \pi \sin(\theta)/\lambda \) and \( S = \sin(\theta)/\lambda \).

To ensure that the protein preparations were free of aggregates, they were subjected to gel filtration chromatography several days prior to small angle x-ray scattering (SAXS) measurements. Protein samples (0.5 ml, 20–25 mg/ml) were applied to a Superdex 200 HR 10/30 gel filtration column (Amersham Biosciences), equilibrated and developed with 100 mM KCl, 20 mM Tris-HCl, 1 mM EDTA, pH 8.5. Samples were then dialyzed against 50 mM Tris-HCl, 100 mM KCl, 10 mM MgCl₂, 10 mM β-mercaptoethanol, pH 8.0. The dialysis buffers were reserved for buffer and measurement changes for SAXS in an FhuD complex with iron-loaded siderophores was obtained by adding iron-loaded siderophores directly to the protein solutions, which resulted in a 1.5:1 ratio of ligand:protein. For experiments at DESY, the samples (0.5 ml) were dialyzed against a 50-ml solution containing 20 μM iron-loaded Deferryl(II).

**Measurements at BioCAT (beamline 18ID) at APS were made as follows.** The sample temperature was 20 °C, and the protein solution was moved continuously through a 1-mm quartz capillary during the course of the measurement to minimize the effects of radiation damage. For each sample, five 10-s exposures were recorded, consisting of three measurements from the protein solution bracketed by two measurements of the buffer solution. Data were integrated using the Vector NTI program O (20); an initial superposition of residues 130–166 of FhuD and BtuF (5, 6). The structural alignment was carried out with the SMA/alignment and sequence alignments were performed using the Vector NTI Suite software package (Informax, Inc., Bethesda, MD).

**Molecular Modeling of S. aureus FhuD2—**To model the structure of FhuD2, we began with a structural alignment between FhuD2 and BtuF4 (5, 6). The structural alignment was carried out using the program O (20); an initial superposition of residues 130–166 of FhuD and 111–147 of BtuF2 (the two long α-helices on the underside of the proteins) was refined using the lsq,improve function in O, matching CA atoms with a cutoff of 3 Å. The final alignment yielded matches for 148 CA atoms throughout the FhuD and BtuF polypeptides. The root mean square fit was 2.2 Å. The structures diverged mainly in loop regions between secondary structure elements. We aligned the sequences of six iron-siderophore-binding proteins from Gram-positive bacteria (FhuD1 and FhuD2 from S. aureus, and FhuD proteins from Clostridium acetobutylicum, Bacillus halodurans, Streptococcus pyogenes, and Bacillus subtilis) as well as FhuD and BtuF from E. coli. The sequence alignment was manually adjusted using the three-dimensional structural alignment between E. coli FhuD and BtuF proteins as a guide. We reasoned that the FhuD structures from Gram-positive organisms would diverge in the same regions as the E. coli FhuD and BtuF
structures, and therefore gaps that had been inserted into structurally conserved regions of *E. coli* BtuF and FhuD were moved into regions where the two structures diverged. The final alignment is shown in Fig. 1.

The folds of *E. coli* BtuF and FhuD are similar, but we found that SAXS data from FhuD2 showed a better agreement with *E. coli* FhuD than with BtuF. Therefore, we used the coordinates of *E. coli* FhuD (4) to model the structure of FhuD2. Based on the alignment in Fig. 1, the sequence of FhuD2 was threaded through the coordinates of *E. coli* FhuD. This initial model was submitted to Swiss-Model (21) for optimization. Manual adjustments were made to the model using the program O (20), and it was subjected to one round of conjugant gradient minimization in CNS (22), with harmonically restrained CA positions. The final model has good stereochemistry, with only one residue falling just outside of allowed Ramachandran regions.

**RESULTS**

**Functional Characterization of Conserved Residues in FhuD2**—Our previous molecular genetic studies showed that the *fhuD2* gene product participates in the transport of a variety of iron(III)-hydroxamate complexes in *S. aureus* (8). Searches of the data bases, which now include many completed genome sequences, indicate that FhuD2 is representative of a large family of conserved proteins that are present in both Gram-positive and Gram-negative bacteria. Of the homologs in Gram-positive bacteria, the vast majority possess a consensus lipoprotein signal sequence and are, therefore, likely anchored in the cytoplasmic membrane. Not surprisingly, the proteins showing the highest overall similarity to FhuD2 were of Gram-positive bacterial origin. An alignment of the four best matches to FhuD2, *S. aureus* FhuD1, and FhuD homologs from *C. acetobutylicum*, *S. pyogenes*, and *B. halodurans*, is shown in Fig. 1, as well as alignments to *B. subtilis* FhuD, *E. coli* FhuD, and *E. coli* BtuF proteins. Readily apparent are highly conserved clusters of residues that appear throughout the length of the proteins.
To identify and characterize residues that are important for the iron(III)-hydroxamate transport function of FhuD2, several highly conserved residues were targeted for site-directed mutagenesis (identified in Fig. 1 by an asterisk), and the functional consequences on iron(III)-hydroxamate transport were assessed using a siderophore plate bioassay (9). Table III summarizes the mutations and the resultant growth phenotypes, which can be divided into three general groups: (i) mutations that drastically affect transport of all iron(III)-hydroxamates, (ii) mutations that have a differential effect on the transport of the various iron(III)-hydroxamates, and (iii) mutations that do not affect iron(III)-hydroxamate transport.

Group I mutations (Table III) yield the most drastic phenotype because they each severely diminish the ability of FhuD2 to function in transport of all iron(III)-hydroxamates. One of these mutants, Y57G, shows a severe deficiency in expression in S. aureus (as demonstrated by Western blots; data not shown) that likely accounts for the observed transport deficiency of this mutant. No other mutation described in this study resulted in any decrease in protein expression levels relative to wild type protein.

Group II mutations differentially alter iron(III)-hydroxamate transport function of FhuD2 (see Table III). Mutations E97A and E231A, highly conserved in this family of binding proteins, including FhuD and BtuF in E. coli, appear to have no significant global phenotype when mutated independently, although the charge on Glu-231 appears to be important in some cases, because E231Q resulted in a drastic loss in the ability of the protein to function in the transport of iron(III)-DesferalTM and iron(III)-rhodotorulic acid. Mutation of both conserved glutamates to alanine gave rise to a protein that was severely defective in transport of all siderophores except ferrichrome.

Finally, group III mutations, although occurring in highly conserved regions within this family of proteins, do not have a discernible affect on the function of iron(III)-hydroxamate transport in S. aureus, at least under our experimental conditions.

Characterization of Iron(III)-Hydroxamate Binding by FhuD2 and Mutants—To understand the relationship between ligand recognition by FhuD2 and the ability of the system to transport siderophores, we characterized the binding of various iron(III)-hydroxamates to wild type FhuD2 and a number of site-directed and randomly generated mutants. To obtain soluble FhuD2, a construct lacking the signal peptide (residues 1–18; all residues are numbered relative to the initiating methionine) was overexpressed and purified. We found, however, that FhuD2 18–302 (lacking simply the signal peptide) was rapidly degraded to a truncated form that began with residue Asn-25. Moreover, sequence conservation within the FhuD2 family of proteins begins at approximately residue 47 in FhuD2, suggesting that at least the first 7 residues after the signal peptide are not important for ligand binding, but may simply play a role as a spacer region that allows the globular part of the protein to function at a distance from the membrane anchor. Therefore, our in vitro analyses of FhuD2 and its derivatives were conducted with FhuD2 25–302, overexpressed in E. coli and purified as described under “Experimental Procedures.”

Binding affinities between FhuD2 and iron(III)-hydroxamates were measured using changes in the intrinsic fluorescence of the protein that occur upon ligand binding. Ligand-induced quenching of intrinsic fluorescence has been used extensively for the determination of dissociation constants for ligand-protein interactions (14). Interaction with saturating concentrations of iron(III)-ferrichrome resulted in a 60% decrease in fluorescence emission and a shift in emission maximum from 348 to 342 nm. The dissociation constants (KD) estimated from these experiments are shown in Table IV. Overall, iron(III)-ferrichrome and iron(III)-DesferalTM have the highest affinity for the FhuD2 protein with KD values of 0.02 and 0.05 μM, respectively, indicating that FhuD2 has a higher affinity for these compounds compared with the E. coli FhuD protein (13).

To verify our methodology, we overexpressed and purified E. coli FhuD and calculated the KD value of this protein for iron(III)-ferrichrome. The value we determined for E. coli FhuD was similar to that previously reported (13). Dissociation constants for iron(III)-hydroxamates of all FhuD2 mutants found to be impaired in iron(III)-hydroxamate transport were also determined (see Table IV). Even extremely high concentrations of iron(III)-hydroxamates were incapable of quenching the fluorescence of the two group I mutants, W197A and E202K, indicating that these two mutants do not associate with the siderophores. Therefore, the inability of these mutants to support iron(III)-hydroxamate uptake in the bioassays can be attributed to a severe defect in their ability to bind the siderophores.

The group II mutants, each of which could support iron(III)-

| Siderophore | Aerobactin | Coprogen | Desferal™ | Ferrichrome | Rhodotorulic acid |
|-------------|------------|----------|------------|-------------|------------------|
| Wild type   | +++++      | +++++    | +++++      | +++++       | +++++            |
| Y57G        | +/-        | +/-      | +/-        | +/-         | +/-              |
| W197A       | +/-        | +/-      | +/-        | +/-         | +/-              |
| E202K       | -          | -        | -          | -           | -                |
| E97A        | +++c       | +++c     | +++c       | +++c        | +                |
| E231A       | ++         | ++       | ++         | ++          | ++               |
| E231Q       | +          | +        | +          | +           | +                |
| E231Q       | +/−        | +/−      | +/−        | +/−         | +/−              |
| E97A/E231A  | ++         | ++       | ++         | ++          | ++               |
| K48A/R49A   | ++         | ++       | ++         | ++          | ++               |
| E97Q        | +++        | +++      | +++        | +++         | +++              |
| D105A       | +++        | +++      | +++        | +++         | +++              |
| T125A       | +++        | +++      | +++        | +++         | +++              |
| W155A       | +++        | +++      | +++        | +++         | +++              |
| D239A       | +++        | +++      | +++        | +++         | +++              |
| W259A       | +++        | +++      | +++        | +++         | +++              |

*See “Results” for explanation of mutation group.

a All phenotypes are reported as relative to the growth promotion of a particular siderophore on bacteria expressing wild type FhuD2. +++++, no change; ++++, slightly diminished; ++, diminished; +, poor; +/-, minimal; –, no growth.

b Delayed growth promotion.
Siderophores were all used at a concentration of 10 mM. Proteinase K of no additions (lane 9) was incubated for 30 min in the presence of proteinase K at 55°C. The molecular mass markers (45 and 31 kDa shown); lane 1 shows the proteolytic degradation. Lane 2, iron(III)-Desferal™ (lane 3), iron(III)-ferriochrome (lane 4), iron(III)-Desferal™ (lane 5), iron(III)-ferrichrome (lane 6), iron(III)-rhodotorulic acid (lane 7), or iron(III)-pyoverdine (lane 8). Iron(III)-siderophores were all used at a concentration of 10 mM. Proteinase K alone was run in lane 9 to indicate its relative position throughout the gel. Samples were heated at 95°C in sample loading buffer for 5 min prior to electrophoresis. FhuD2 is identified with an arrowhead.

What is particularly intriguing about these results is that the E97A and E231A mutants show a dependence on the type of siderophore in the bioassay (Table III), despite the fact that they retain wild type binding affinities for all of the siderophores. Our conclusion is that the mutations affect the interaction between the binding protein and the membrane complex in a siderophore-dependent manner, and therefore the siderophore itself must also be playing a role in this interaction. These results provide evidence for a direct interaction between the siderophore and the ABC transporter complex, FhuBGC2, an interaction that appears to be critical for the function of the system.

Iron(III)-Siderophore Binding by FhuD2 Is Accompanied by Minimal Conformational Change—Upon ligand binding, many proteins become more resistant to proteolysis. When bound to different iron(III)-siderophore complexes, purified FhuD2 was protected from proteolytic degradation (Fig. 2, lanes 4–7). FhuD2 was not protected from digestion if incubated in the presence of FeCl₃ (Fig. 2, lane 3) or iron(III)-pyoverdine (Fig. 2, lane 8), a pseudomonal siderophore that does not promote the growth of S. aureus RN6390 in siderophore plate bioassays. The protection instilled upon FhuD2 in the presence of hydrox-
amate-type siderophores would suggest that there may be a ligand-specific conformational change occurring in the protein. To further address this possibility, we used small angle x-ray scattering (SAXS) to investigate the magnitude of the conformational change that occurs in FhuD2, in solution, when it binds its ligand. SAXS makes use of the same physical process, namely the scattering of X-rays by electrons that gives rise to crystal diffraction, and it therefore provides a direct measure of the size, shape, and conformation of a protein in solution. Low angle SAXS data can be plotted as a Guinier curve (23), the slope of which provides the radius of gyration ($R_g$), defined as the root mean square distance of all atoms from their common center of mass, and the intercept provides the molecular weight (Fig. 3). The linearity of the Guinier curve demonstrates that the protein was monodisperse. For the unliganded form of FhuD2, the calculated radius of gyration is 2.06 nm, whereas the molecular mass is 32.7 kDa, indicating that the protein is monomeric (see Table V). Addition of ligand had no significant effect on $R_g$ or the molecular weight (Table V). These results can be compared with those obtained with three other periplasmic binding proteins (those specific for maltose, ribose, or glucose/galactose), all of which undergo a significant decrease in $R_g$ of 0.1 nm upon binding ligand (24).

Conformational changes will affect the entire scattering curve, and changes that have no effect on the $R_g$ may still affect the structure of the protein such that a change in the solution scattering can be observed. To address this possibility, we have superimposed the SAXS curve derived from unliganded FhuD2 with that of the Desferal\textsuperscript{TM}-bound protein (Fig. 4A), and it is clear that the conformational change in the structure is very small, with the only significant differences occurring at relatively high scattering angles, in the region 0.25 < $S$ < 0.40 nm\textsuperscript{-1}. To put these results into context, solution x-ray scattering, both theoretical and experimental, is illustrated for three other binding proteins. First, experimental scattering from MBP is illustrated in Fig. 4B, in either the presence or absence of maltose. MBP undergoes a large conformational change upon binding ligand, with its two domains coming together to engulf the ligand, and the protein contracting to form a less extended, more globular structure (25). In the second example, the effect of simply removing the siderophore for E. coli FhuD is illustrated in Fig. 4C; here, SAXS from the

![Fig. 4. Conformational changes attendant upon ligand binding.](image-url)
non-hydrated crystal structure of *E. coli* FhuD (4) was calculated in the presence and absence of gallichrome, and it can be seen that the removal of the siderophore produces some minor changes in the higher angle data, not unlike the differences we observe between ligand-free and ligand-bound FhuD2. Finally, the calculated x-ray scattering for the non-hydrated structures of ligand-bound and ligand-free BtuF (5) is illustrated in panel D. Significant changes in the structure of BtuF were caused by ligand binding, which is reflected in changes in the calculated x-ray scattering, particularly in the low angle region where $0.08 < S < 0.2$ nm$^{-1}$. These changes are also reflected in the radius of gyration of the apo form of BtuF (2.04 nm), which is larger than that of the holo form (1.98 nm). The contraction of BtuF upon ligand binding is reminiscent of the changes in MBP, but the conformational change in BtuF is much less pronounced. With these three examples in mind, it appears that FhuD2 from *S. aureus* undergoes even less of a conformational change than BtuF and, in fact, the differences between ligand-bound and ligand-free FhuD2 can be attributed almost entirely to the presence or absence of siderophore, without invoking any structural change in the protein. Thus, the increased stability against proteolysis afforded by ligand binding must be the result of changes in the accessibility of vulnerable loop regions rather than significant movements of entire domains. Mutation to alanine at positions Glu-97 and Glu-231 caused changes in siderophore uptake in the bioassays without changing the affinity of FhuD2 for the various iron(III)-hydroxamate complexes. A simple explanation for this observation is that positions 97 and 231 mediate an interaction between siderophores and the membrane integral transporter complex. A simple explanation for this observation is that positions 97 and 231 mediate an interaction between FhuD2 and the membrane integral transporter complex FhuBGC2. Alternatively, it is possible that the E97A and E231A mutations affect the overall structure or conformation of FhuD2, or that these mutants undergo a conformational change upon binding ligand. We used SAXS to investigate these latter possibilities, and found that, in common with the wild type protein, ligand binding had only a very minor effect on the solution structure of the E97A mutant (data not shown) and E231A mutant (Fig. 5A). Furthermore, both the E97A and E231A mutants produced identical solution x-ray scattering as the wild type protein, in both the unliganded and liganded states, showing that the mutations have no effect on the overall structure of the protein (SAXS curves for the wild type protein and Glu-231 mutant, both in the presence of saturating Desferal™, are shown in Fig. 5B). Therefore, the E97A and E231A mutations act through a change in the surface properties of FhuD2, and not by altering the overall structure or conformation of the protein.

**Structural Modeling of FhuD2**—In contrast to the binding proteins in many other nutrient uptake systems, ligand binding by FhuD2 is accompanied by little, if any, conformational change, and therefore siderophore uptake by this system must be the result of changes in the accessibility of vulnerable loop regions rather than significant movements of entire domains. Mutation to alanine at positions Glu-97 and Glu-231 caused changes in siderophore uptake in the bioassays without changing the affinity of FhuD2 for the various iron(III)-hydroxamate complexes. A simple explanation for this observation is that positions 97 and 231 mediate an interaction between FhuD2 and the membrane integral transporter complex FhuBGC2. Alternatively, it is possible that the E97A and E231A mutations affect the overall structure or conformation of FhuD2, or that these mutants undergo a conformational change upon binding ligand. We used SAXS to investigate these latter possibilities, and found that, in common with the wild type protein, ligand binding had only a very minor effect on the solution structure of the E97A mutant (data not shown) and E231A mutant (Fig. 5A). Furthermore, both the E97A and E231A mutants produced identical solution x-ray scattering as the wild type protein, in both the unliganded and liganded states, showing that the mutations have no effect on the overall structure of the protein (SAXS curves for the wild type protein and Glu-231 mutant, both in the presence of saturating Desferal™, are shown in Fig. 5B). Therefore, the E97A and E231A mutations act through a change in the surface properties of FhuD2, and not by altering the overall structure or conformation of the protein.

**Structural Modeling of FhuD2**—In contrast to the binding proteins in many other nutrient uptake systems, ligand binding by FhuD2 is accompanied by little, if any, conformational change, and therefore siderophore uptake by this system must operate using a novel mode of recognition by the membrane integral ABC transporter complex (FhuBGC2). To understand the effect of the various mutations on the ability of FhuD2 to participate in siderophore uptake, we constructed a molecular model of FhuD2 to map the location of mutations on the protein.

Despite the low overall sequence similarity between *S. aureus* FhuD2 and *E. coli* FhuD (~25% total similarity), our SAXS data indicate that the solution structures are similar (Fig. 6), and therefore it is reasonable to model *S. aureus* FhuD2 using coordinates from the crystal structure of *E. coli* FhuD. For this type of modeling, an accurate sequence alignment is critical. Acidic residues that are conserved in this binding protein superfamily, and which are thought to be important for interaction with the integral membrane complex (6), are located at positions 97 and 231 in the FhuD2 sequence, and these conserved positions were used to “anchor” the alignment shown in Fig. 1. To refine the alignment, we superimposed the structures of *E. coli* FhuD (4) and BtuF (6), and manually adjusted the alignment so that insertions and deletions occurred in structurally variable regions. Only relatively minor changes in side chain conformations and some loop regions were required to produce a model with acceptable stereochemistry. Therefore, we are confident that our model of *S. aureus* FhuD2 will be close to the true structure.

The mutations shown in Table III were made on the basis of their conservation in the sequences of FhuD homologs from Gram-positive bacteria, but with a model for FhuD2 in hand, we can put these mutations in a structural context to help us understand why they affect or do not affect function. All of the group III mutations do not affect the biological function of the protein when mutated to alanine, and our model predicts that these residues are all located on, or close to, the surface of the...
protein, and away from the predicted ligand binding site. There are two residues, Glu-97 and Glu-231, that when mutated to alanines have a selective effect on the ability of the system to use particular siderophores. Our model indicates that these residues are surface-exposed and can be found opposite one another on either side of the ligand binding cleft. These residues are at a significant distance away from the binding site in our model and would not be able to interact with the ligand, consistent with our data demonstrating that mutation of these residues to alanine had no effect on ligand binding (Table IV), and also consistent with our SAXS data from FhuD2-E97A and FhuD2-E231A indicating that the mutations do not affect the structure of the protein (see Fig. 5). Nevertheless, alteration of either residue to alanine results in a protein that is reduced in its ability to utilize certain iron(III)-hydroxamates (Table III), and mutation of both residues to alanine in the same protein imparts a severe defect on the utilization of all iron(III)-hydroxamates except for iron(III)-ferrichrome (Table III). Their position on the protein and lack of an effect on iron(III)-hydroxamate binding indicate that these residues must be important for interactions with the membrane-integral FhuB and FhuG proteins.

In FhuD2, Tyr-191 is predicted by our model to be close to the ligand binding site, and is probably able to make some contact with the bound ligand. Its position on the periphery of the binding site, coupled with our observation that mutation of Tyr-191 to alanine significantly affects the ability of the system to use iron(III) bound to rhodotorulic acid and aerobactin, slightly affects the uptake of Desferal™ and coprogen, and does not affect uptake of ferrichrome, could mean that this tyrosyl side chain is important for the binding of certain siderophores. Indeed, $K_d$ values for Y191A are in agreement with the suggestion that the observed deficiency of this protein to participate in transport is the result of its altered ability to interact with ligands.

There are two residues, Trp-197 and Glu-202, that, when mutated, result in a pronounced attenuation of the uptake of all iron(III)-hydroxamates. In our model of FhuD2, these residues are located in the "bottom" of the ligand binding pocket, suggesting that they do not interact with FhuB or FhuG in the membrane, but may be directly involved in binding of all hydroxamate-type siderophores. In agreement, $K_d$ values were dramatically higher for W197A and E202K. The values for these two mutants were actually so high that they were unable to be accurately measured by the fluorescence quenching method, because we observed that extremely high concentrations of several of the iron(III)-hydroxamate complexes (i.e. concentrations required to observe any decrease in fluorescence in these mutant proteins) absorb at 280 nm, the wavelength used to excite the proteins. A less severe change at position 202 to glutamine provided similar results except for iron(III)-ferrichrome. Although the E202Q mutant bound iron(III)-ferrichrome with lower affinity than the wild type protein, it was still able to support growth in the bioassay.

Trp-197 is part of an aromatic cluster consisting of residues Phe-186, Trp-197, Tyr-278, Tyr-280, and Trp-225; with the exception of Phe-186, this group of residues is conserved across the family of FhuD homologs in many other Gram-positive bacteria (Fig. 1).

Finally, mutation of Tyr-57 to glycine resulted in a strong attenuation of biological activity for all of the siderophores tested; this residue is located in the core of the protein, and the tyrosyl side chain is completely solvent-inaccessible. Mutation of Tyr-57 to glycine probably results in misfolding of the protein because of the destabilizing effect of glycine and the loss of tight hydrophobic packing in the protein core.

**DISCUSSION**

Previous studies in our laboratory showed that the FhuD2 lipoprotein is involved in the transport of iron(III)-hydroxamates in *S. aureus*. In this study, we have shown that FhuD2 binds a variety of hydroxamate siderophores, but binds iron(III)-ferrichrome and iron(III)-Desferal™ with much higher affinity than the *E. coli* FhuD homolog. Together with
other notable differences between FhuD2 and FhuD (e.g. lipid anchor, location on the cell surface versus the periplasm), FhuD2 serves as a prototype for a large and growing subfamily of iron(III)-siderophore-binding lipoproteins from Gram-positive bacteria. This subfamily of iron(III)-siderophore-binding proteins function as “high affinity” receptors at the external face of the cytoplasmic membrane in Gram-positive bacteria, as compared with the periplasmic location of FhuD2 homologs in Gram-negative bacteria.

Although the sequence alignment between FhuD2 from S. aureus and FhuD from E. coli indicates that the proteins share little homology, it is clear from our structural modeling and our SAXS data that the proteins possess the same overall fold. One important difference concerns the N-terminal region of the proteins and the fact that the protein in S. aureus must remain anchored to the membrane. The signal peptide of E. coli FhuD is 30 residues long, compared with 17 residues for S. aureus FhuD2; however, sequence similarity between the two does not start until approximately the 48th residue of FhuD2 (36th residue of FhuD). It is likely that the sequence from the acylated cysteine residue (18th amino acid) to at least Asn-25, if not also through to Pro-47, is not part of the folded FhuD2 protein, but rather is present as a “spacer” segment that helps maintain the functional regions of the protein at an optimal distance from the membrane anchor. Given an approximate distance of 3.3 Å per residue for extended polypeptides, this spacer region could span from as little as 24 Å to as much as ~96 Å from the membrane. The presence of such a spacer may better allow both lobes of the protein to interact with both of the transmembrane domains present within the FhuBGC₂ ABC transporter.

Our results demonstrate that S. aureus FhuD2 has a 50- and 700-fold higher affinity for iron(III)-ferrichrome and iron(III)-Desferal™, respectively, when compared with E. coli FhuD. One fundamental difference between the uptake systems in Gram-negative bacteria versus Gram-positive bacteria is the lack of an outer membrane in the latter. In general, the outer membrane of Gram-negative bacteria is home to high affinity iron(III)-siderophore receptors, whereas periplasmic components need not possess as high an affinity for substrates because substrates transported across the outer membrane are “concentrated” in the periplasm. The higher affinity of FhuD2 for ferrichrome and desferrioxamine B (used in this study as Desferal™), potentially the natural substrates for this receptor, may have evolved as a function of the location of FhuD2 at the cell surface, as opposed to a periplasmic location in Gram-negative bacteria. In agreement, the low $K_D$ value of FhuD2 more closely resembles those of the outer membrane iron-siderophore receptors in Gram-negative bacteria (26). Further research is required to characterize substrate binding parameters for additional iron(III)-siderophore-binding proteins from Gram-positive bacteria to gain better insight into whether this is a general phenomenon for this family of proteins in this group of bacteria.

Differences in ligand binding affinities of FhuD2 versus FhuD undoubtedly result from differences within the binding pocket of the two proteins, because the overall fold of each of the proteins appears to be very similar. Through mutagenesis and structural modeling, we have located residues that form the ligand binding site, and it is clear that there are significant differences between FhuD2 and E. coli FhuD. One clear difference is that, whereas Arg-84 of FhuD is a key residue in interactions with hydroxamate siderophores, there are no arginine residues present within the predicted binding pocket in FhuD2. FhuD2 also lacks a homologous segment of amino acids that includes Trp-68 in E. coli FhuD, a residue shown to be important for iron(III)-hydroxamate binding in this protein (4, 13). A more detailed analysis of ligand binding by FhuD2 will require high resolution structural data.

An important aspect of these studies is the demonstration that FhuD2, a member of a large superfamily of iron(III)-siderophore-binding proteins, does not undergo significant conformational change upon binding ligand in solution. A muted conformational change was predicted from the high resolution structures of liganded FhuD and crystal structure data from the related proteins BtuF (E. coli) and TroA (T. pallidum) both crystallized in their liganded and unliganded forms. However, crystallization itself may select for a particular conformation, as it did in the case of unliganded ribose-binding protein, in which the “closed” conformation was present in the crystal despite the fact that, in solution, the predominant conformation of the unliganded protein is “open” (24, 27). Our SAXS analysis provides the first demonstration that, in solution, the conformation of FhuD2 does not change when it binds its ligand. The attenuated conformational change would then appear to be characteristic for this family of binding proteins and differentiates them from “canonical” periplasmic binding proteins, such as MBP, where ligand binding results in a dramatic conformational change that is detected both in solution and in high resolution crystal structures.

Another important result from our study is the evidence that the siderophore itself is recognized by the membrane integral
subunits FhuB and FhuG. We observed that mutation of Glu-97 and/or Glu-231 to alanine resulted in siderophore-dependent changes in biological uptake, despite the fact that neither of these mutations has any effect on ligand binding. We conclude that interactions between the various siderophores and the membrane integral subunits must be responsible for the differences in growth observed in the bioassays. This conclusion, coupled with the lack of a conformational change in FhuD2, leads to a new model for molecular recognition between the binding protein/ligand complex and the membrane-integral subunits.

In the case of the maltose transporter and related transport systems, recognition between the binding protein/ligand complex and membrane integral subunits is thought to originate from the ligand-induced conformational change in the binding protein. This conformational change effectively buries the ligand, brings together residues on either side of the ligand binding cleft, and leads to the formation of a surface that is not present in the open, unliganded conformation (Fig. 7). This surface is recognized by the membrane-integral subunits, which leads to ATP binding, hydrolysis, and coupled transport of the substrate through the membrane. Thus, for these systems the membrane-integral subunits recognize the combined presence of binding protein and substrate by productive engagement of only the closed conformation of the binding protein.

In contrast, our results indicate that molecular recognition of ligand-bound FhuD2 by the membrane-integral subunits FhuB and FhuG takes place as a result of at least two separate interactions; the first is between FhuBFhuG and residues Glu-97 and Glu-231 of the binding protein, and the second is between FhuBFhuG and the iron-siderophore complex itself (Fig. 7). Other interactions may also take place between the ABC transporter and less well conserved regions of the surface of the binding protein. In fact, given the lack of conformational change in FhuD2, a tight coupling between ATP hydrolysis and substrate transport necessitates direct recognition of the siderophore by FhuB/FhuG, because, in its absence, the binding protein alone would be able to trigger ATP hydrolysis by FhuB/FhuG.

The results we have presented here provide novel insights into the iron(III)-siderophore uptake process. Furthermore, analysis of this system has provided insight into the mode of recognition between the binding protein (the primary receptor for these uptake systems) and the membrane-integral complex. In common with other binding protein-dependent uptake systems, such as the maltose transporter, the membrane complex must recognize the presence of substrate-loaded binding protein on the exterior surface of the cell; this recognition is undoubtedly important for proper coupling of ATP hydrolysis with substrate transport. The maltose transporter (and related systems) recognize the combined presence of substrate and binding protein through a large, ligand-induced conformational change in the binding protein. In contrast, the iron-siderophore uptake system of S. aureus appears to recognize directly both the binding protein and substrate, without the requirement for a ligand-induced conformational change in the binding protein.

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