X-ray Crystallographic Structures of the *Escherichia coli*
Periplasmic Protein FhuD Bound to Hydroxamate-type
Siderophores and the Antibiotic Albomycin*

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Siderophore-binding proteins play an essential role in the uptake of iron in many Gram-positive and Gram-negative bacteria. FhuD is an ATP-binding cassette-type (ABC-type) binding protein involved in the uptake of hydroxamate-type siderophores in *Escherichia coli*. Structures of FhuD complexed with the antibiotic albomycin, the fungal siderophore coprogen and the drug Desferal have been determined at high resolution by X-ray crystallography. FhuD has an unusual bilobal structure for a periplasmic ligand binding protein, with two mixed β/α domains connected by a long α-helix. The binding site for hydroxamate-type ligands is composed of a shallow pocket that lies between these two domains. Recognition of siderophores primarily occurs through interactions between the iron-hydroxamate centers of each siderophore and the side chains of several key residues in the binding pocket. Rearrangements of side chains within the binding pocket accommodate the unique structural features of each siderophore. The backbones of the siderophores are not involved in any direct interactions with the protein, demonstrating how siderophores with considerable chemical and structural diversity can be bound by FhuD. For albomycin, which consists of an antibiotic group attached to a hydroxamate siderophore, electron density for the antibiotic portion was not observed. Therefore, this study provides a basis for the rational design of novel bacteriostatic agents, in the form of siderophore-antibiotic conjugates that can act as “Trojan horses,” using the hydroxamate-type siderophore uptake system to actively deliver antibiotics directly into targeted pathogens.

Because the bioavailability of iron is very low (10⁻⁵⁻¹⁷ m), there is an intense competition between host and pathogen for soluble iron. Low molecular weight compounds called siderophores serve to scavenge iron from the environment for bacterial uptake. Two major classes of siderophores exist, hydroxamates and catecholates. Although *Escherichia coli* itself produces only one siderophore, enterobactin, it can utilize siderophores from a variety of sources. Several ferric siderophore uptake systems have been characterized from *E. coli*. Each system consists of a specific outer membrane receptor, a periplasmic protein, and several inner membrane-associated proteins. The energy for transport of the ferric siderophore across the outer membrane is provided by interaction of the receptor with the TonB complex (reviewed in Ref. 1). Subsequently, the energy for ferric siderophore transport across the inner membrane is provided by hydrolysis of ATP by the inner membrane-associated proteins. This arrangement of protein components and mechanism of transport is typical of systems for uptake of amino acids, sugars, and other nutrients in Gram-negative bacteria (2–4).

The uptake of hydroxamate-type siderophores in *E. coli* is the best characterized bacterial iron uptake system to date. *E. coli* has several distinct receptors for each different siderophore, including FhuA for ferrichrome, FhuE for coprogen, and FoxA for ferrioxamine B. However, a common periplasmic protein, FhuD, can bind and shuttle a variety of hydroxamate siderophores to the inner membrane-associated proteins FhuB and FhuC. Fungal hydroxamate siderophores generally consist of similar structural units (δ-N-hydroxy-ornithine, trans-anhydrovaleric acid, and acetic acid), but the number and arrangement of these units can vary. Ferrichrome, originally identified from the smut fungus *Ustilago maydis*, is likely the most studied hydroxamate-type siderophore thus far (5–7). The apo and holo structures of the *E. coli* receptor FhuA, which transports ferrichrome and the structurally related antibiotic albomycin, has recently been solved (8–10). The receptor forms a 22-stranded β-barrel in the bacterial membrane, with an

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Structures of FhuD Bound to Hydroxamate-type Siderophores

N-terminal domain, which fills the inside of the barrel. In addition, the periplasmic protein FhuD has also been solved as a complex with the ferrichrome analogue gallichrome, where the iron atom coordinated to ferrichrome is replaced with gallicium (11). The structure of the periplasmic protein is unusual for its function, forming a bilobate structure connected by a long α-helix, in contrast to the typical two domain structure connected by several β-sheets found in most other periplasmic ligand binding proteins (12). In FhuD, a shallow pocket is located between the two domains for ferrichrome binding. In both the FhuA receptor and FhuD periplasmic protein, ferrichrome is cradled in a pocket lined with aromatic residues, with a few specific hydrogen bonds from the hydroxamate portion of the siderophore to side chains in the binding site. In all the hydroxamate-type siderophores, six oxygen atoms of the hydroxamate portion coordinate a ferric iron, although the overall structures vary considerably. Unlike ferrichrome, coprogen is a linear chain of three \( \text{N}^2\)-hydroxy-\( \text{N}^6 \)-acylated ornithines (13). In this siderophore, two amino acids join to form a diketopiperazine ring, whereas the third is attached by an ester linkage. Two trans-anhydromevalonic acid groups are attached to either end of the molecule. Desferal, which is related to desferroxamine siderophores, has a very high affinity for Fe\(^{3+} \) to either end of the molecule. Desferal, which is related to transferrin patients (14) and has been shown to retard human skeletal transferrin (16), and can utilize iron loaded Desferal as an iron source. Presumably, the hydroxamate portion of each of these iron complexes is the common structural feature that is important for recognition by the bacterial proteins involved in iron uptake.

Conjugation of antibiotics to siderophores has shown promise for therapeutic control of bacterial infections (17). The naturally occurring antibiotic albomycin has a thioribosyl pyrimidine antibiotic group attached to an iron binding moiety similar to that of ferrichrome. This antibiotic enters the cell by the ferrichrome uptake system, then the antibiotic group is released from the remainder of the molecule by peptidase N (18, 19). Although the intracellular target of albomycin is unknown, the minimal inhibitory concentration (0.005 μg/ml) is very low compared with other antibiotics, such as ampicillin (0.1 μg/ml) (20). In recent years, development of synthetic conjugates of antibiotics and siderophores has been successful in limiting the growth of certain bacteria (17). The siderophore acts as a “Trojan horse,” actively carrying the antibiotic across the cell membrane via the specific ferric siderophore uptake system into the cell.

However, rational design of a novel, effective antibiotic by chemical conjugation is challenging. Many chemical and biological factors pertaining to the effect on the bacteria and impact on the host must be considered. Chemical properties important for drug design include structural similarity to siderophores, solubility, and lipophilicity. Biological considerations for development of novel bacteriostatics involve toxicity, the bacteria and host and repercussions from the metabolism of the conjugate. Criteria for broad-spectrum use of the antibiotic and for overcoming the resistance mechanisms of the bacteria must also be considered.

Identification of the important structural features for siderophore recognition by bacteria constitutes a key step in the design of novel bacteriostatic agents. In this study, the structures of different FhuD siderophore complexes have been solved by x-ray crystallography. The crystal structures of FhuD complexed to the antibiotic albomycin, the siderophore coprogen, and the drug Desferal are presented here. This study reveals how FhuD is able to bind, with high affinity, a structurally diverse series of hydroxamate-type siderophores.

MATERIALS AND METHODS

Purification of FhuD—The overexpression strain BL21(DE3) pLysS pMR21 was obtained from Dr. W. Küster (Swiss Federal Institute for Environmental Science and Technology, Switzerland). His-tag FhuD was purified by metal chelate chromatography (PORSOL 20MC) charged with nickel, similarly as previously described (21) except that the BioCad high performance liquid chromatography system (PerSeptive Biosystems) was used. After cell lysis in 50 mM HEPES, pH 7.6, 0.5 mM NaCl, the protein was bound to the column and washed with 10 volumes of 50 mM HEPES, pH 7.6, 0.5 mM NaCl to remove unbound proteins and eluted by a gradient of 0–0.5 M imidazole. The protein was then dialyzed extensively against 10 mM Tris, pH 7.5 at 4 °C. The His-tag of the protein was not cleaved off by enterokinase, because previous studies showed that the modified protein was functional (21).

Crystalization and Data Collection—Albomycin \( \delta_2 \) and coprogen were purified as previously described (10, 21). Desferal (desferrioxamine mesylate) was obtained from Ciba-Geigy Canada. For each siderophore, a 1 mM stock solution of the ferric siderophore complex was made using \( \text{Fe}_2(\text{NO}_3)_3 \) (99.9%, Aldrich Chemical Co.) at acidic pH. Albomycin, coprogen, and Desferal each bind iron in a 1:1 siderophore to iron molar ratio. The ferric siderophores were added to apo-FhuD in a 1:1 ratio in 10 mM Tris, pH 7.5. Crystals of His-tag FhuD complexed with coprogen and Desferal were grown at room temperature by hanging-drop vapor diffusion from 5-μl drops containing 8.3 mg ml\(^{-1} \) of the siderophore and 0.8 M disodium phosphate and 0.05 mM HEPES, pH 7.5. The drops were equilibrated against a 1-mL reservoir containing 16 mM sodium acetate, 5% polyethylene glycol, and 0.1 mM HEPES, pH 7.5. In a similar manner, crystals of His-tag FhuD complexed with albomycin were grown at room temperature in 5-μl drops containing 8.3 mg ml\(^{-1} \) of the FhuD-albomycin complex, 8% polyethylene glycol 4000, and 0.05 mM sodium acetate, pH 5.2 and equilibrated against a 1-mL reservoir containing 16% polyethylene glycol 4000 and 0.1 mM sodium acetate, pH 5.2. Large, diffraction quality crystals grew within 2 weeks. For cryo-crystallography experiments, crystals were soaked in a stabilizing solution identical to the reservoir solutions with a final concentration of 30% (v/v) glycerol.

Data on the FhuD-albomycin complex were collected on a Rigaku RU3HR rotating copper anode x-ray generator equipped with Osram confocal multilayer x-ray focusing optics and an MAR 345 image plate scanner. Data on the FhuD coprogen complex were collected at the X12-C x-ray beamline at the National Synchrotron Light Source (Brookhaven National Laboratories) using a Brandeis B4 CCD\(^1 \) detector. Data for the FhuD-Desferal complex were collected at the 7-1 x-ray beamline at the Stanford Synchrotron Radiation Laboratory (Stanford Linear Accelerator Center) using a Quantum 4R CCD detector. All data were collected on crystals frozen to 100 K in a cold gas stream generated by an Oxford Cryostream crystal-cooling device. Each FhuD ferric siderophore crystal was found to belong to the space group \( \text{P6}_3\text{mc} \), with small variations in unit cell dimensions. Data were indexed, integrated, and scaled using DENZO/SCALEPACK (22). Statistics are given in Table I.

Phasing and Model Refinement—All phasing calculations, density modification, and refinement were carried out using the CNS suite of programs (23). The structures of the complexes determined in this study are isomorphous with the recently solved structure of the FhuD gallicium complex (11) (Protein Data Bank (PDB) number 1EFD). The coordinates from this structure, minus the ligand and water molecules, were used as a starting model for refinement. Refinement was followed by refinement by maximum likelihood refinement against structure factors. The quality of the electron density maps was excellent, allowing for the unambiguous tracing of most of the polyepipeptide chain using TURBO-FRODO (24) for interactive model building. Five percent of the reflections were set aside for the calculation of a free \( \text{R} \)-factor, and the same set of reflections was maintained throughout refinement. The water molecules and the ferric siderophores could be incorporated into the model manually, using \( 2F_c - F \), \( F - F^\ast \), and \( F^\ast - F \), maps. The structures of similar ferric siderophores were found in the Cambridge Structural Data base (www.ccdc.cam.ac.uk/eds/csd/csd.html), and their structures were modified accordingly. For albomycin, the structure of ferrichrome from the FhuA-ferrichrome complex (PDB 1PFC(8)) was slightly modified. The model of coprogen was based on the structure of the neocoprogen I molecule (Cambridge Structural Database).
The siderophores in each case was partially complete, and only electron density describing the remainder of the protein is missing as well as the mesylate (OSO₂CH₃) group of the Desferal (Fig. 1).

The overall structures of the FhuD proteins in all complexes are very similar to the protein found in the gallichrome complex (Fig. 2). In each complex, the overall quality of the protein structure is excellent, with over 90% of the amino acid residues occupying the most favorable region of a Ramachandran plot (data not shown). The protein is bilobate, with a kidney bean shape, with the two domains connected by a 23-residue kinked α-helix (residues 142–165) (11). The N-terminal domain has a twisted five-stranded parallel β-sheet whereas the C-terminal domain has a mixed five-stranded β-sheet, with both surrounded by α-helices. The binding site for the siderophores lies in the shallow cleft between these two domains, and several side chain residues form hydrogen bonds with each siderophore (Fig. 3). An overlay of the Cα backbone of FhuD from the gallichrome complex with the proteins in the other siderophore complexes gives root mean square deviations of 0.25 Å² with the albomycin complex, 0.27 Å² with the coprogen complex, and 0.20 Å² with the Desferal complex.

Siderophore Binding—The ligand binding site of FhuD is lined with hydrophobic residues, forming a depression large enough to accommodate the hydrophobic ornithyl linkers of each of the siderophores. Hydrogen bonds between several residues in the binding site and the siderophore also play a key role in stabilizing the complex. A comparison of the binding pockets in each of the complexes reveals significant differences in the positions of key ligand binding side chains, which alters the structural landscape of the binding pocket to accommodate the different ligands.

When albomycin binds (Fig. 3a), the tri-δ-N-hydroxy-δ-N-acetyl-l-ornithine peptide, which coordinates the ferric ion, is found in a similar orientation to ferrichrome. The coordination geometry around the iron atom is slightly distorted from octahedral symmetry with distances ranging from 1.9 to 2.1 Å from the oxygen atoms to the metal ion. Three hydrogen bonds form between the protein and the ligand, with two between the terminal amino groups of Arg-84 and the hydroxamate moiety of the siderophore and another between the remaining hydroxamate to the hydroxyl group of Tyr-106, similar to the interactions found between gallichrome and FhuD. However, the water-mediated hydrogen bond present in gallichrome between the peptide backbone of the siderophore and the protein is not found in the albomycin complex. Aromatic residues lining the binding pocket are placed in a similar position to those in the gallichrome complex. Interestingly, no electron density is visible for the thiobisyl pyrimidine antibiotic group, which is covalently attached to the peptide portion of the siderophore by an amino acetyl linker. This suggests that this group is unencumbered by protein or lattice contacts and is free to move in the crystal structure.

The binding mode of coprogen to FhuD is significantly different from the binding modes of gallichrome and albomycin (Fig. 3b). The iron atom is in a similar position relative to the protein, but the hydroxamate moieties are rotated ~10 degrees from those of gallichrome. The oxygen atoms coordinating the iron atom are distorted from octahedral symmetry, with distances ranging from 1.98 to 2.05 Å. Within the iron center, one hydrogen bond is formed between the hydroxamate oxygen opposite to the diketopiperazine ring and the terminal amine of Arg-84. The hydroxyl group of Tyr-106 makes a hydrogen bond to the other hydroxamate oxygens on the side of the ring system, and a water-mediated hydrogen bond to Tyr-275 is formed with the remaining hydroxamate oxygen. In addition, water-mediated hydrogen bonds form between a nitroxy oxygen of the coordinating group of the siderophore and an oxygen

### Table I

| Summary of crystallographic data | FhuD-albomycin | FhuD-coprogen | FhuD-Desferal |
|---------------------------------|----------------|--------------|--------------|
| Data collection                 |                |              |              |
| Wavelength (Å)                  | 1.5418         | 0.9787       | 1.0800       |
| Resolution (Å)                  | 30–2.6         | 30–2.0       | 30–2.0       |
| Unit cell dimensions (Å)        | a = b = 85.48  | a = b = 86.09| a = b = 85.57|
| Complementarity                  | c = 92.47      | c = 91.94    | c = 91.60    |
| i(0)                            | 14.0 (1.6)     | 35.1 (6.6)   | 21.8 (3.8)   |
| R_m(F)                          | 0.0099 (0.646) | 0.043 (0.102)| 0.049 (0.205)|
| Refinement statistics           |                |              |              |
| Resolution (Å)                  | 30–2.6         | 30–2.0       | 30–2.0       |
| Number of reflections           | 11873          | 25292        | 22886        |
| Working set                     | 8900           | 22625        | 21769        |
| Free set                        | 484            | 1165         | 1097         |
| R-factor                        | 0.194          | 0.219        | 0.220        |
| Mean B-factors                  |                |              |              |
| Protein (Å²)                    | 42.63          | 28.69        | 33.26        |
| Ligand (Å²)                     | 50.67          | 31.46        | 44.74        |
| Metal ion (Å²)                  | 40.43          | 23.44        | 42.50        |
| Solvent (Å²)                    | 43.38          | 35.48        | 43.08        |
| Number of atoms                 |                |              |              |
| Protein                         | 2013           | 2017         | 2021         |
| Ligand                          | 40             | 54           | 28           |
| Water                           | 40             | 112          | 62           |

* Numbers in parentheses are the statistics for highest resolution shell.

† i ≥ 2.0 σ(i).
‡ R_m(F) = Σ_i-h|Fo,i|−|Fc,i|/Σ|Fo,i|, where h = set of Miller indices and j = set of observations of reflection h.
§ Fc = Fo - Fh

Data base number COFDIK10 (23), which was modified to include a trans-anhydromevalonic acid group attached to the ornithine backbone. Desferal, based on ferrioxamine (Cambridge Structural Data base number DUPJON (25)), was modeled into the binding site using TURBO-FRODO (24). Topology and parameter files for the ferric siderophores were generated using the Hic-Up server (x-ray.bmc.uu.se/hicup (26)) and adjusted appropriately. The structure of each complex was refined by several rounds of molecular dynamics and annealing using standard protocols by CNS (23). A final inspection of the different ligands of the coordinating group of the siderophore and an oxygen atom to the metal ion. Three hydrogen bonds form between the protein and the ligand, with two between the terminal amino groups of Arg-84 and the hydroxamate moieties of the siderophore and another between the remaining hydroxamate to the hydroxyl group of Tyr-106, similar to the interactions found between gallichrome and FhuD. However, the water-mediated hydrogen bond present in gallichrome between the peptide backbone of the siderophore and the protein is not found in the albomycin complex. Aromatic residues lining the binding pocket are placed in a similar position to those in the gallichrome complex. Interestingly, no electron density is visible for the thiobisyl pyrimidine antibiotic group, which is covalently attached to the peptide portion of the siderophore by an amino acetyl linker. This suggests that this group is unencumbered by protein or lattice contacts and is free to move in the crystal structure.

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of the peptide backbone of the siderophore to Ser-219, which in turn hydrogen bonds to Asn-215. The positions of most of the hydrophobic residues within the binding site shift slightly, compared with the FhuD-gallichrone complex, but the most dramatic change in position involves the reorientation of Trp-217. This movement allows the trans-anhydromeliac acid group to insert into the protein. The oxygen atom on the end of this group forms a hydrogen bond with Ser-103, as well as a water-mediated hydrogen bond to Trp-217 and the carbonyl of the peptide backbone of Ala-104, stabilizing the complex. Another hydrogen bond forms between Ser-103 and Glu-42, which bonds to the main-chain nitrogen of Leu-44 and has water-mediated hydrogen bonds to the side chain of Trp-273 and the carbonyl of the peptide bond of Tyr-275. The other trans-anhydromeliac acid group is hydrogen-bonded to Asn-64 through a water molecule.

Desferal binds to FhuD with the ferrioxamine portion of the drug enveloped by the binding site to a greater extent than the other siderophores (Fig. 3c). This may be due to the absence of bulky functional groups on the peptide backbone, so that the smaller size can be accommodated. Coordination of the iron atom is slightly distorted, with distances to the oxygen atoms ranging from 1.95 to 2.00 Å. One ferrioxamine carbonyl oxygen is hydrogen-bonded to Arg-84, with the second ferrioxamine carbonyl sharing a hydrogen bond with Tyr-106. Unlike the other siderophores, the third ferrioxamine carbonyl oxygen does not appear to have any hydrogen bonds to FhuD. There are two additional hydrogen bonds to the peptide backbone of the ligand, one from a nitrogen to Tyr-275 and another from an oxygen via a water molecule to Asp-61. The orientation and position of the hydrophobic residues lining the binding pocket are similar to those in the gallichrone and albomycin complexes. The electron density for the mesylate (OSO₂CH₃) portion of the molecule is not present in this model, and it could be disordered in the crystal.

Comparison of the Binding Modes—An overlay of the binding site residues in each of the FhuD complexes shows that many of the side chains are in a similar position for siderophore binding, with the exception of Trp-217. With the exception of the coprogen complex, the shapes of the binding sites in the other complexes are very similar. The hydrogen bonds formed between each siderophore and the side chain residues of FhuD vary slightly in length and number (Fig. 4). In addition, not all of the hydrogen bonds are to similar oxygens of the iron-coordinating region of the siderophore.

A comparison between the binding modes of the outer membrane receptor FhuA and the periplasmic protein FhuD for albomycin is shown in Fig. 5. The interactions between the atoms of the ornithine backbone and iron coordination center of the extended conformation of albomycin in FhuA (10) are more numerous and involve more of the molecule compared with the interactions found in FhuD. The orientation of the coordinating Arg and Tyr residues in FhuA is not the same as in FhuD, and contacts form between different oxygens of the coordination sphere of the siderophore. As well, FhuA uses an additional Tyr side chain as well as a Trp and the backbone carbonyl of a Phe to hydrogen bond to the hydroxamate portion of the siderophore.

DISCUSSION

High affinity uptake of ferric siderophores in bacteria is aided by specific interactions with proteins along the uptake pathway. Although distinct receptors exist in the outer membrane to extract various siderophores from the environment, it appears to be evolutionarily advantageous to utilize a common ABC transport system that recognizes a certain class of siderophore to transport the siderophore from the periplasm through the inner membrane (1–5). Although the periplasmic protein FhuD is known to bind a number of hydroxamate-type ferric siderophores with relatively high affinity, as seen from the crystal structures of several of these complexes, there are few limitations on the variety of hydroxamate-type siderophores it could accommodate.

Recognition of Hydroxamate-type Ferric Siderophores by FhuD—The periplasmic binding protein FhuD appears to bind various hydroxamate-type siderophores with a similar binding mode. Many of the interactions between the siderophore and protein are contained within the iron coordinating components of the siderophore. These are mediated by a few hydrogen bonds with key amino acids residues in the binding pocket, namely Arg-84 and Tyr-106. Slight movement of these key residues involved in hydrogen binding permit a variety of different structures to bind. These hydrogen bonds are present for specificity and directing the siderophore into the binding site for correct fit. The number of hydrogen bonds to each siderophore also appears to be related to the relative affinity of FhuD for each siderophore. For example, there are more hydrogen bonds between FhuD and coprogen than there are for ferrichrome, and the binding constant for coprogen (0.3 μM) is lower than that for ferrichrome (1.0 μM) (21). It is interesting to note that FhuD can bind different geometrical isomers of the hydroxamate-type siderophore family. By themselves, ferrichromes crystallize in a Δ-cis coordination geometry (29), neocoprogen forms a Δ-trans geometry (23), and ferriferrioxamines can form racemic mixtures of Δ-cis and Δ-cis geometry.
When bound to FhuD, ferrichrome, albomycin, and Desferal crystallize as the $\Delta\text{-cis}$ complex and coprogen forms the $\Delta\text{-N-trans-cis}$ conformation. The large size and malleable shape of the binding site of FhuD allows either type of stereoisomer to bind. Because the charge of the residues surrounding the binding site is predominantly negative, the uncharged hydroxamic-type siderophores would be able to bind while the negatively charged catechol-type siderophores would not (11). Because Desferal has a negative charge on the sulfate group, this may account for the decreased affinity of FhuD for the drug (36 $\mu M$) compared with albomycin (5.4 $\mu M$) and coprogen (0.3 $\mu M$) (31).

Aromatic residues lining the binding pocket stack around the siderophores, providing a non-polar environment of the correct size and shape for these ligands. In FhuD, the positioning of aromatic groups, combined with the capacity to re-orientate Trp-217, appears to be the defining factor for allowing various siderophores to bind.

The binding of the siderophore-antibiotic conjugate would tolerate a much wider range of chemotypes in the periplasmic protein FhuD than in the receptor FhuA. In the latter, there
are several interactions between the thioribosyl ring and the protein, whereas no such interactions were found in the periplasmic protein (Fig. 5). In FhuA, the flexible amino acetyl linker group allows albomycin to be found in both a compact and an extended conformation (10). However, in the FhuD-albomycin crystal structure, the thioribosyl moiety is solvent-exposed and not visible in the electron density map. Likely, the antibiotic portion adopts a great number of conformations in the crystal structure of the FhuD-albomycin complex, due to the flexibility in the linker region, thus static electron density cannot be defined.

There are interesting parallels in the binding of coprogen and Desferal to FhuD compared with the binding of ferrichrome and albomycin. Coprogen, first isolated from Neurospora crassa (32), has a unique diketopiperazine ring not found in the other siderophores. Coprogen binds to FhuD with its iron hydroxamate center in a twisted conformation (Δ-N-trans-cis) compared with Δ-C-trans-trans configuration found in the crystal structure of neocoprogen alone (23). This allows the general shape of iron-bound coprogen to be very similar to linear ferrioxamines, especially in the orientation of the peptide backbone around the iron center (23). The two loops of peptide backbone in each of these structures are positioned in the binding site perpendicularly to the peptide backbone of ferrichrome and albomycin. In this way, the solvent exposure of the peptide backbone is reduced, although the number of hydrogen bonds between the siderophore and FhuD is not increased. The unusual insertion of the trans-anhydromevalonic acid group of coprogen into the interior of FhuD suggests that this functional group is important for recognition by the proteins of the coprogen uptake pathway. FhuD may recognize other hydroxamate-type siderophores such as aerobactin and rhodotorulinate in a similar manner. In all, FhuD seems to recognize the coordination type around the iron center of hydroxamate-type siderophores and moves amino acid side chains to accommodate the remainder of the molecule.

Relation to Other Periplasmic Ligand Binding Proteins—Although the overall structure of FhuD is distinctive for a periplasmic ligand binding protein, the characteristics of the binding pocket and binding mode of the ligands is not uncommon. Most periplasmic proteins from ABC transport systems form two domains, connected by several β-strands, with the binding pocket located in the deep cleft between these two domains. However, the binding pocket is larger and shallower in FhuD than in other periplasmic ligand binding proteins. This could be due to the larger size of siderophores compared with some of the other types of nutrients transported into the cell.

Hydrogen bonding between the binding protein and ligand are common. Hydrogen bonds are more directional than dispersion forces and are of sufficiently low strength to allow fast ligand dissociation (12, 33). Stacked aromatic residues lining the binding pocket are also found in other periplasmic proteins that bind amino acids and sugars (12, 33).

Other ferric siderophore binding proteins may have a similar structure to FhuD to make up a distinct class of ligand binding proteins. Most of these proteins share some sequence homology (34, 35), and previous secondary structure analysis (11) suggests that the E. coli periplasmic proteins FepB and FecB could possess a long α-helix in the middle of their sequence. Identification of the key residues involved in ligand binding would be difficult, because many of the residues belong to different regions of the polypeptide chain. However, several characteristics could be retained, including hydrophobicity and hydrogen bonding potential. When charged ligands are bound, complimentary charges may also exist in the binding site.

Proposed Mechanism for Ferric Siderophore Transport—Once the ferric siderophore has passed through the outer membrane receptor, it is transferred to the periplasmic protein. However, there is no evidence that the periplasmic protein FhuD actually interacts with the outer membrane receptor FhuA. Presumably, the high affinity of the periplasmic protein for ferric siderophores is sufficient for sequestering the ligand and transporting it to the inner membrane-associated proteins. Rearrangement of the hydrogen bonding and aromatic residues lining the binding pocket occurs to accommodate each siderophore and is likely accompanied by a global conformational change in the protein structure. Recognition of the holo form of

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**Fig. 5.** A comparison of the binding modes of albomycin in (a) the outer membrane receptor FhuA and (b) the periplasmic protein FhuD. The hydrogen bonds from the side-chain residues to the chemical structures are shown.
FhuD by the inner membrane-associated complex would also require a unique liganded conformation. Release of the ligand to the inner membrane-associated proteins would involve breaking the hydrogen bonds between the ligand and periplasmic protein.

Several periplasmic ligand binding proteins have been found to exist in an open apo form and as a closed holo form. Small changes in the β-strands connecting the two domains allow this conformational change to occur (12). However, the long α-helix and extensive domain interface in FhuD may preclude a significant conformational change. Because there are different crystal conditions for the apo form of FhuD and dynamic light scattering shows very small differences in the hydrodynamic radius of the two forms of the protein, there may be some flexibility in the structure. Because the structure of holo FhuD, nor the B factors throughout the protein, do not immediately suggest a mechanism by which domain opening could occur, we can only speculate at this time where a possible hinge would be located.

**Rational Design of Siderophore-Antibiotic Conjugates**—Structural analysis of the factors involved in recognition of siderophores by the outer membrane receptors and ABC transport proteins aids in the rational design of siderophore-antibiotic conjugates. For hydroxamate-type siderophore uptake systems, it appears that the common structural basis for ligand recognition is the iron coordination moieties. In lieu of structures of the entire repertoire of outer membrane receptors in *E. coli*, the structures of the FhuD complexes show that the mode of binding hydroxamate-type siderophores, including those not of the ferrihemochrome family, is very similar. Of course, the outer membrane receptors make more specific contacts to the siderophores and are more spatially restrictive, but it appears that a variety of structures can be accommodated in the receptor binding site (8–10). Requirements of the periplasmic binding protein for transport are less confining and the vast amount of solvent-exposed regions of the siderophore may be a good target for antibiotic conjugation. Similarly, in other iron transport systems, it also seems that it is the iron-chelating component of the siderophore that is recognized by the uptake proteins. In the crystal structure of the outer membrane receptor FepA, the putative enterobactin binding site shows possible interactions with the iron center (36).

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2. T. E. Clarke, L. W. Tari, and H. J. Vogel, unpublished results.
X-ray Crystallographic Structures of the *Escherichia coli* Periplasmic Protein FhuD Bound to Hydroxamate-type Siderophores and the Antibiotic Albomycin

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