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Ferrichrome Transport in *Escherichia coli* K-12: Altered Substrate Specificity of Mutated Periplasmic FhuD and Interaction of FhuD with the Integral Membrane Protein FhuB

MARTIN R. ROHRBACH, VOLKMAR BRAUN, AND WOLFGANG KÖSTER*

Mikrobiologie II, Universität Tübingen, D-72076 Tübingen, Germany

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FhuD is the periplasmic binding protein of the ferric hydroxamate transport system of *Escherichia coli*. FhuD was isolated and purified as a His-tag-labeled derivative on a Ni-chelate resin. The dissociation constants for ferric hydroxamates were estimated from the concentration-dependent decrease in the intrinsic fluorescence intensity of His-tag-FhuD and were found to be 0.4 μM for ferric aerobactin, 1.0 μM for ferrichrome, 0.3 μM for ferric coprogen, and 5.4 μM for the antibiotic albomycin. Ferrichrome A, ferrioxamine B, and ferrioxamine E, which are poorly taken up via the Fhu system, displayed dissociation constants of 79, 36, and 42 μM, respectively. These are the first estimated dissociation constants reported for a binding protein of a microbial iron transport system. Mutants impaired in the interaction of ferric hydroxamates with FhuD were isolated. One mutated FhuD, with a W-to-L mutation at position 68 [FhuD(W68L)], differed from wild-type FhuD in transport activity in that ferric coprogen supported promotion of growth of the mutant on iron-limited medium, while ferrichrome was nearly inactive. The dissociation constants of ferric hydroxamates were higher for FhuD(W68L) than for wild-type FhuD and lower for ferric coprogen (2.2 μM) than for ferrichrome (156 μM). Another mutated FhuD, FhuD(A150S, P175L), showed a weak response to ferrichrome and albomycin and exhibited dissociation constants two- to threefold higher than that of wild-type FhuD. Interaction of FhuD with the cytoplasmic membrane transport protein FhuB was studied by determining protection of FhuB degradation by trypsin and proteinase K and by cross-linking experiments. His-tag-FhuD and His-tag-FhuD loaded with aerobactin specifically prevented degradation of FhuB and were cross-linked to FhuB. FhuD loaded with substrate and also FhuD free of substrate were able to interact with FhuB.

Transport of ferric hydroxamates into *Escherichia coli* requires outer membrane receptor proteins for certain ferric hydroxamates (6) and a periplasmic binding-protein-dependent system of transport across the cytoplasmic membrane that is common for all ferric hydroxamates (6, 24). The latter consists of the FhuD protein in the periplasm, the integral transport protein FhuB in the cytoplasmic membrane, and the FhuC protein, which is associated with the cytoplasmic membrane (7, 8, 26, 27, 29, 42) and presumably energizes transport into the cytoplasm by ATP hydrolysis. Binding of substrates to the FhuD protein has been demonstrated by proteolysis experiments; only ferric hydroxamates that were transported, aerobactin, ferrichrome, and coprogen, prevented degradation of FhuD, while ferric hydroxamates that were not transported by the Fhu system of *E. coli* did not protect FhuD from being hydrolyzed (28). No data for the activity of periplasmic proteins for ferric siderophore transport systems other than those for FhuD exist. Studies of the interactions of ferric siderophores with their cognate periplasmic proteins are hampered by the very low concentrations of the binding proteins and by the difficulties in performing reliable binding studies via equilibrium dialysis, presumably because of the nonspecific adsorption of ferric siderophores to many cell components.

An important step towards biochemical characterization of FhuD properties has been achieved by improving FhuD expression by cloning of *fhuD* downstream of the gene 10 promoter of phage T7, transcription through the T7 RNA polymerase, and introduction of an ideal ribosome binding site in front of *fhuD* (38). A His-tag has been placed at the N terminus of FhuD, which allowed purification of His-tag-FhuD by chromatography on a Ni-chelate column. Although His-tag-FhuD remained in the cytoplasm because of the replacement of the signal sequence by the His-tag, His-tag-FhuD was still active, as shown by the inhibition of proteolysis by the substrates aerobactin, ferrichrome, and coprogen and by restoration of ferrichrome uptake by addition of His-tag-FhuD to calcium-treated cells of a *fhuD* deletion mutant (38). In this study, we used this technique to further characterize wild-type FhuD and newly isolated mutated FhuD derivatives. In addition, we used the intrinsic fluorescence of FhuD to determine changes in fluorescence intensity caused by interaction with its cognate substrates to arrive at dissociation constants for the substrates bound to wild-type FhuD and mutated FhuD. Promotion of growth of cells expressing wild-type and mutated FhuD proteins by various ferric hydroxamates was correlated with their ability to interact with FhuD, demonstrating an essential role of FhuD in the determination of the substrate specificity of the Fhu transport system. Furthermore, we obtained evidence that FhuD interacts with FhuB and that this interaction does not require loading of FhuD with one of its substrates.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used are listed in Table 1. Cells were grown in nutrient broth (NB) (13) supplemented as required with 0.1 mM 2,2′-dipyridyl (NBD) or in tryptone-yeast extract (13). Promotion of growth was determined with iron-limited NBD agar plates seeded with the strain to be tested. Filter paper disks loaded with 10 μl of a 1 mM solution of the ferric siderophores were placed on the NDB plates, and the growth zones (diameter and density) were determined after incubation overnight at 37°C (38).

* Corresponding author. Mailing address: Mikrobiologie/Membranphysiology, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany. Phone: 49 7071 294632. Fax: 49 7071 294634.

TABLE 1. *E. coli* strains and plasmids used

Strain or plasmid	Relevant genotype	Source or reference
Strains		
KO295	*aroB malT tsx zif*::Tn*10 metE fhuD*	38
BL21(DE3)	pLysS F^- *hsdS gal*; phage T7 polymerase under *lac*UV5 control; Cm^r	47
WM1576	K38 HfrC pGP1-2	48
Plasmids		
pGP1-2	T7 polymerase under *c*I857 control	48
pT7-6	ColE1 ori, T7 promoter, Amp^r	48
pWK346	pT7-6 *fhuB*$^+$	27
pWK480	pT7-6 *fhuD*$^+$	28
pBcKS$^+$	ColE1 ori, high copy number; Cm^r	45
pMRC16a	pBcKS$^+$ *fhuD*(A150S, P175L)	This study
pMRI51	pBcKS$^+$ *fhuD*(W68L)	This study
pET19-b	ColE1 ori; T7/*lac* promoter-operator; His-tag region; fusion cloning site; *lacI*$^+$ Amp^r	Novagen
pMRE	pET19-b with modified fusion cloning site	38
pMR21	pMRE *his-tag fhuD*$^+$	38
pMR26	pMRE *his-tag fhuD*(W68L)	This study
pMR27	pMRE *his-tag fhuD*(A150S, P175L)	This study

Recombinant DNA techniques. Isolation of plasmids, use of restriction enzymes, ligation, agarose gel electrophoresis, recovery of DNA fragments from agarose, and transformation were done by standard techniques (40). DNA was sequenced by the enzymatic dideoxy method (41), using ^{35}S-ATP for labeling. The genes encoding His-tag-FhuD derivatives were constructed by using vector pET19-b (Novagen, Madison, Wis.) as described previously for His-tag-labeled wild-type FhuD (38). The His-tag sequence reads Gly-$(His)_{10}$-$(Asp)_4$-Lys-His-Thr-$(Ala)_3$-Ile-Asp.

Mutagenesis by PCR was performed under two conditions aimed at increasing the mutagenesis rate (30, 32). The reaction mixture containing a suboptimal concentration of dATP consisted of 50 μM dATP and 250 μM (each) dTTP, dCTP, and dGTP, 0.0025 μg of template DNA of pWK480, 2.5 U of *Taq* polymerase, and 0.125 μg of the primers 5′-dGATTTATGGGATCCCGATG GG-3′ (nucleotides −69 to −49 of the *fhuD* sequence [7]) and 5′-dGAAAAGC GGAATTCGTTTACTC-3′ (nucleotides 889 to 910), except that a *Bam*HI site and an *Eco*RI site (replaced nucleotides are underlined) were introduced. The other reaction mixture used contained equal concentrations of all four deoxynucleotides and, in addition, 0.5 mM $MnCl_2$. Thirty cycles were performed in 0.1 ml of *Taq* reaction buffer (Promega, Madison, Wis.) at reaction intervals of 1 min at 94°C, 2 min at 58°C, and 3 min at 72°C.

Protein expression and protein separation. Proteins were overproduced in *E. coli* BL21(DE3), which contains the phage T7 RNA polymerase on the chromosome under the control of the *lac*UV5 promoter (47). Wild-type *fhuD* and mutated *fhuD* were cloned downstream of the T7 gene 10 promoter of plasmid pT7-7 (48), and transcription was initiated by addition of 1 mM of isopropyl-β-D-thiogalactoside (IPTG). Proteins were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (33). The His-tag-FhuD derivatives were purified on a Ni-chelate column (Diagen, Hilden, Germany) as described for wild-type His-tag-FhuD (38). Protection either of radiolabeled FhuD and mutated FhuD derivatives in osmotic shock fluids or of the isolated and purified proteins against trypsin and proteinase K by ferric siderophores was tested as described previously (28, 38).

Fluorescence spectroscopy. Fluorescence measurements were performed with an LS 50B luminescence spectrophotometer (Perkin-Elmer, Überlingen, Germany). Emission intensity was recorded with an excitation wavelength of 280 nm and an emission wavelength of 340 nm. Excitation and emission slits were between 2 and 4 nm. The measurements were done at a constant temperature of 23°C with 2 ml of a 10 mM sodium phosphate buffer, pH 7.5. Changes of fluorescence intensity as a function of ferric siderophore concentration were recorded by adding increasing amounts of the ferric siderophores to the protein solutions employed at an initial concentration of 50 μg ml^{-1}. Changes in fluorescence intensity caused by dilution of the protein solution were recorded separately. The fluorescence quenching was expressed as the percent decrease in fluorescence upon addition of the ligands. This method has been used previously for the determination of dissociation constants for the maltose-binding protein (43) and the C4-dicarboxylate-binding protein (50). The maximal quenching caused by fully saturated FhuD proteins was determined from the intercept of the straight line obtained by double-reciprocal plotting of the substrate concentration versus the fluorescence quenching with an axis representing the reciprocal of the quenching. The dissociation constants (half-maximal saturation) cannot simply be deduced from this saturation curve because not all of the substrate added is bound to the protein. Therefore, for each substrate concentration, the amount of protein-bound substrate was determined, and the saturation curve thus obtained was used to deduce the dissociation constants.

Cross-linking experiments. For cross-linking, 20 ml of cells of *E. coli* WM1576(pWK346 *fhuB*$^+$) in the exponential growth phase (3×10^8 cells per ml) was harvested, suspended in 1 ml, and labeled for 10 min at 37°C with 370 kBq of [^{35}S]methionine. The labeled cells were sedimented and suspended in 0.5 ml of ice-cold 0.2 M Tris-HCl–0.5 M sucrose (pH 8). Lysozyme (50 μl, 10 mg/ml) and 50 μl of 0.5 mM EDTA were added (51). His-tag-FhuD (0.4 mg), His-tag-FhuD together with 50 μM aerobactin (final concentration), 50 μM aerobactin, or 0.5 mg of carbonic anhydrase in 0.5 ml of 0.2 M Tris-HCl–0.5 mM EDTA (pH 8) was added. Formation of spheroplasts was inspected by microscopy. *N*-Ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide-9 sulfonic acid (sNHS) were added to the spheroplast suspension, each to a final concentration of 10 mM (3, 31). The reaction mixture was incubated for 1 h at 25°C. In addition, the samples contained the protease inhibitors *p*-aminobenzamidine (0.1 mM) and phenylmethylsulfonyl fluoride (0.2 mM). Spheroplasts were harvested by centrifugation and washed once with Tris-HCl–EDTA buffer. The proteins were separated by SDS-PAGE, and the gels were exposed to Kodak X-Omat S100 film for autoradiography.

Protection of proteolytic FhuB degradation by FhuD. *E. coli* WM1576 (pWK346 *fhuB*$^+$) was grown to an optical density at 578 nm of 0.6. Cells were collected by centrifugation, suspended in 0.5 ml of minimal medium, and labeled for 10 min at 37°C with 370 kBq of [^{35}S]methionine as described previously (26). Cells were converted to spheroplasts as described above, after which 200 μg of purified His-tag-FhuD, 200 μg of unpurified His-tag-FhuD, and 50 μM aerobactin or 200 μg of bovine serum albumin, dissolved in 0.5 ml of Tris-HCl–0.5 mM EDTA (pH 8.0), was added. After incubation of the samples for various periods with trypsin (final concentration, 20 μg/ml) or proteinase K (final concentration, 6 μg/ml), the spheroplasts were sedimented by centrifugation, dissolved in sample buffer, and subjected to SDS-PAGE and subsequent autoradiography.

RESULTS

***fhuD* mutants impaired in substrate binding.** *fhuD* was mutagenized by PCR using *Taq* polymerase and either a suboptimal concentration of dATP or added $MnCl_2$ to increase the mutation frequency. Mutated *fhuD* on plasmid pBcKS$^+$ was screened in *E. coli* KO295, which contains a deletion of two adenine residues at nucleotide 210 of chromosomal *fhuD*, resulting in an altered amino acid sequence that terminates after residue 86 (complete FhuD contains 295 residues) (7, 8). Mutated FhuD proteins that failed to restore growth of KO295 on ferrichrome and coprogen as the sole iron sources and that caused resistance to albomycin were analyzed by SDS-PAGE. Proteins of the size of wild-type FhuD were studied further. Plasmid pMRI51 encoded a FhuD protein that conferred growth on coprogen and only very weak growth on ferrichrome, whereas wild-type FhuD supported better growth on ferrichrome than on coprogen (Table 2). Sequencing of the mutated *fhuD* gene revealed a replacement of G by T at position 202 (7), which resulted in the substitution of leucine for tryptophan at residue 68 [FhuD(W68L)].

Previously, we have shown that binding of substrates to radiolabeled FhuD (28) and isolated His-tag-FhuD (38) prevents or diminishes degradation of FhuD by added proteases. Isolated His-tag-FhuD(W68L) (see below) subjected to proteolysis by proteinase K was not protected by aerobactin and fer-

TABLE 2. Growth promotion and sensitivity to albomycin of KO295 *fhuD* encoding wild-type FhuD or derivatives

Plasmid	FhuD protein	Sensitivity[a] to albomycin	Growth[b] on:	
			Ferrichrome	Coprogen
pWK480	Wild type	S	+++++	+++++
pMRI51	W68L	(S)	++	++++
pMRC16a	A150S, P175L	R	+	++++

[a] Degrees of sensitivity: S > (S) > R (S, sensitive; R, resistant).
[b] The number of crosses reflects the degree of growth promotion.

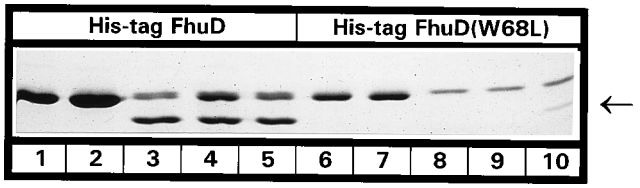

FIG. 1. Protection of purified His-tag-labeled wild-type FhuD and His-tag-FhuD(W68L) by proteinase K in the presence of aerobactin (lanes 3 and 8), ferrichrome (lanes 4 and 9), and coprogen (lanes 5 and 10). Control samples were incubated without proteinase K at 37°C (lanes 1 and 6) and at 56°C (lanes 2 and 7). The protected protein bands are indicated (arrow).

richrome (Fig. 1, lanes 8 and 9), and coprogen prevented degradation only slightly (lane 10), indicating no binding of aerobactin and ferrichrome and a reduced binding of coprogen. Comparison with isolated wild-type His-tag-FhuD, which was protected by all three ferric siderophores (Fig. 1, lanes 3 to 5), clearly demonstrates the altered binding properties of mutant FhuD.

Estimation of the dissociation constants of wild-type and mutated FhuD proteins for various substrates. To determine the dissociation constants, FhuD(W68L) had to be isolated and purified. Since the amount of FhuD per cell is very small (13, 27), FhuD(W68L) was cloned downstream of the phage T7 promoter and transcribed by the T7 polymerase. In addition, the vector used contained an ideal ribosome binding site which replaced the very weak ribosome binding site (7, 8) of FhuD. For purification, FhuD(W68L) was labeled with a His-tag at the N-terminal end and chromatographed on a Ni-chelate column, as described previously for wild-type FhuD (38). The His-tag replaced the signal sequence of the FhuD(W68L) precursor required for export across the cytoplasmic membrane. As a result, His-tag-FhuD(W68L) remained in the cytoplasm and caused no problems during protein overexpression. It has been shown for wild-type FhuD that the His-tag does not inactivate the protein. His-tag-FhuD restored ferrichrome transport of *E. coli* KO295 when applied to cells (38) whose outer membrane was made permeable by treatment with a high concentration (0.3 M) of Ca^{2+} (5).

Dissociation constants can be estimated from changes in the intrinsic fluorescence of proteins if substrate binding alters the fluorescence spectrum. The ferric siderophores used for testing fluorescence intensity alterations were purified by high-pressure liquid chromatography to avoid fluorescence of contaminants and to obtain reliable data for the dissociation constants. The fluorescence spectrum of wild-type FhuD changed in intensity and in maximum wavelength upon binding of substrates, as shown for ferrichrome in Fig. 2. The dependence of the fluorescence intensity on the concentration of the substrate (Fig. 3) was used to estimate the dissociation constants. FhuD was rapidly saturated at low concentrations of aerobactin, ferrichrome, and coprogen. The antibiotic albomycin, which is similar in the iron-binding center but otherwise substantially different from ferrichrome, exhibited slower binding kinetics than the ferric siderophores. Ferrioxamine B, which displays low iron transport rates, and ferrioxamine E and ferrichrome A, which support growth on iron-limited medium only very poorly (22), showed the lowest binding rates. The dissociation constants estimated from these curves are listed in Table 3.

Binding of coprogen to FhuD(W68L) occurred at lower concentrations than that of ferrichrome and aerobactin (Fig.

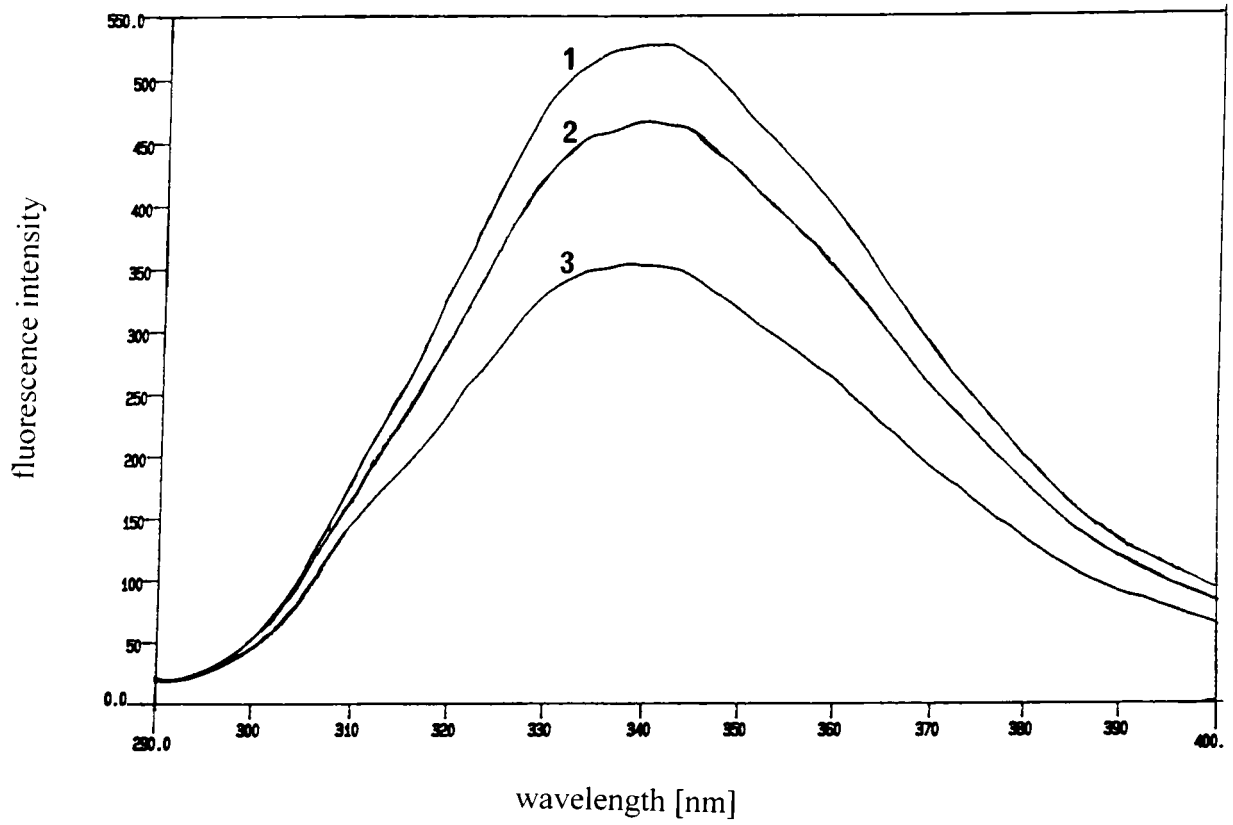

FIG. 2. Fluorescence spectrum of His-tag-FhuD in the absence of ferrichrome (curve 1) and in the presence of 0.8 (curve 2) and 11.5 (curve 3) μM ferrichrome.

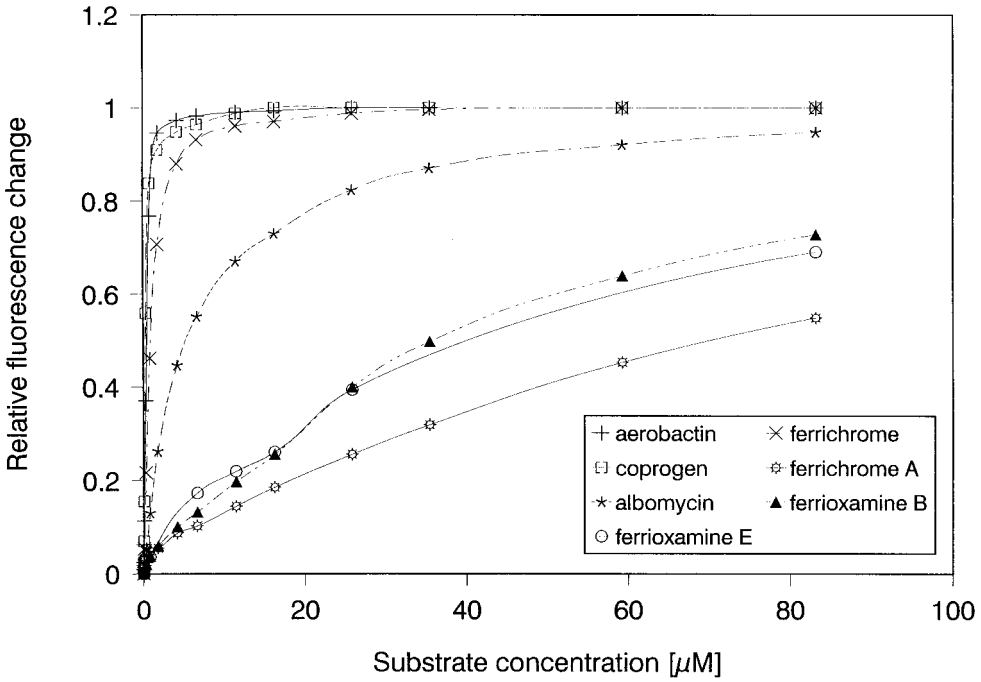

FIG. 3. Decrease in fluorescence intensity of wild-type His-tag-FhuD dependent on increasing concentrations of the indicated ferric siderophores and antibiotic. The values are related to the fluorescence intensity of His-tag-FhuD.

4). The same was true for albomycin. However, the binding rates of all substrates tested were lower than those of wild-type FhuD (note the higher concentrations of ligands used in the experiment whose results are shown in Fig. 4 than in that of Fig. 3). The dissociation constant derived from Fig. 4 for binding of coprogen to FhuD(W68L) was 7-fold higher than that of wild-type FhuD, and those for binding of aerobactin and ferrichrome to FhuD(W68L) were 330- and 156-fold higher than those of wild-type FhuD, respectively (Table 3). Since the dissociation constants qualitatively reflect the growth rates, it is concluded that the specificity of binding to FhuD determines to a large extent the uptake specificity for ferric hydroxamates.

A second mutant, FhuD(A150S, P175L), grew slowly on ferrichrome and coprogen, had a low sensitivity to albomycin, and was protected against proteolysis by aerobactin (Fig. 1). Fluorescence intensity changes were recorded (Fig. 5) and used to estimate the dissociation constants. They were two- to threefold higher than those for wild-type FhuD (Table 3), showing that binding to FhuD substantially contributes to the overall transport rates.

Interaction of FhuD with FhuB. According to the mechanism of periplasmic binding-protein-dependent transport (1, 2, 12, 19, 49), it is assumed that FhuD delivers the ferric hydroxamates to the FhuB transport protein in the cytoplasmic membrane. To examine interaction of FhuD with FhuB, two experimental approaches were used.

TABLE 3. Dissociation constants of ferric siderophores bound to FhuD and FhuD derivatives

Siderophore or antibiotic	Dissociation constant (μM)		
	Wild-type FhuD	FhuD(W68L)	FhuD(A150, P175L)
Ferrichrome	1.0	156	2.4
Aerobactin	0.4	133	0.9
Coprogen	0.3	2.2	0.5
Albomycin	5.4	27.5	14
Ferrioxamine B	36	ND[a]	ND
Ferrioxamine E	42	ND	ND
Ferrichrome A	79	ND	ND

[a] ND, not determined.

To determine whether FhuD protected FhuB from being degraded, trypsin and proteinase K were used. Isolated FhuD was added to spheroplasts containing radioactively labeled overexpressed FhuB. After incubation with trypsin or proteinase K for 30 min at 25°C, the spheroplasts were dissolved in SDS-PAGE sample buffer without heating. Heating converts the very hydrophobic FhuB protein into an insoluble product that does not enter SDS gels (26, 27). Unheated FhuB forms rather diffuse bands, as has been observed for other hydrophobic integral membrane proteins (27). Trypsin (Fig. 6, lane 1) and proteinase K (lane 5) degraded most of the FhuB. Degradation was not prevented by added bovine serum albumin (Fig. 6, lanes 2 and 6). Protection of FhuB from degradation was obtained with His-tag-FhuD (Fig. 6, lanes 3 and 7) and with His-tag-FhuD loaded with aerobactin (lanes 4 and 8). In this experiment and others, aerobactin did not improve protection of FhuB by FhuD, which might be expected if only substrate-loaded FhuD bound to FhuB.

Physical interaction of FhuD with FhuB was also examined by cross-linking experiments. His-tag-FhuD was added to spheroplasts containing overexpressed [^{35}S]methionine-labeled FhuB. Only FhuB was labeled in notably large amounts under these conditions. After incubation with the cross-linkers EDC and sNHS for 1 h at 25°C, the proteins were dissolved in sample buffer without heating and subjected to SDS-PAGE. Of the cross-linking reagents tried [EDC, sNHS, formaldehyde (37), glutaraldehyde, and sulfosuccinimidyl 6-(4′-azido-nitrophenylamino)hexanoate], only EDC and EDC combined with sNHS yielded a cross-linked protein (Fig. 7, lanes 4 and 5). EDS activates carboxyl groups by forming *O*-acylurea derivatives that react with primary amino groups. In the presence of sNHS, more stable intermediary products that react exclusively

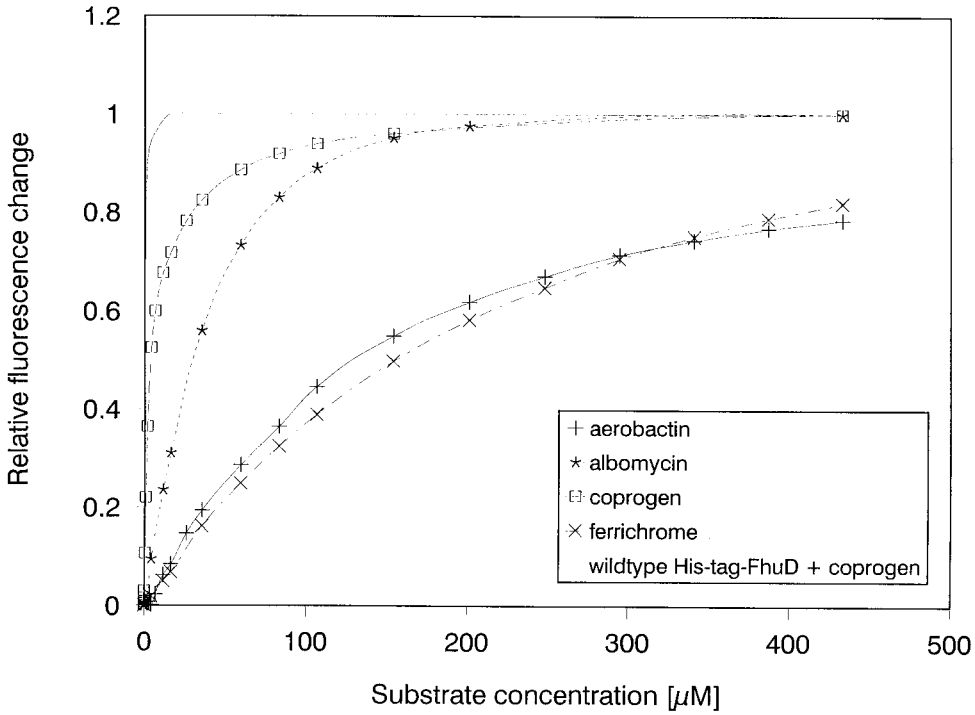

FIG. 4. Decrease in fluorescence intensity of His-tag-FhuD(W68L) dependent on increasing concentrations of the indicated ferric siderophores and antibiotic, relative to the fluorescence intensity of His-tag-FhuD. The curve obtained for wild-type FhuD in the presence of coprogen is shown for comparison.

with primary amino groups are formed. A greater proportion of FhuB was cross-linked to FhuD (Fig. 7, lane 4) than to FhuD loaded with aerobactin (lane 5). Without addition of FhuD, no cross-linking of FhuB was observed (Fig. 7, lane 1), and carbonic anhydrase (lane 2), used as a control protein to test the specificity of the FhuD cross-linking, and aerobactin (lane 3) caused no cross-linking by EDC. The band corresponding to a protein of 66 kDa found in lanes 1 to 5 of Fig. 7 is of unknown origin. Obviously, it does not interfere with FhuD-FhuB cross-linking. The size of the cross-linked product (diffuse band, about 88 kDa) corresponds to the size of FhuB (diffuse band of about 50 kDa) plus His-tag-FhuD (33 kDa) and does not represent His-tag-FhuD linked to the 66-kDa protein (about 100 kDa).

DISCUSSION

Uptake of ferric hydroxamates into *E. coli* requires an energy-coupled transport system via receptor proteins across the outer membrane and an active transport system across the cytoplasmic membrane. Transport across the outer membrane is energized by the electrochemical potential of the cytoplas-

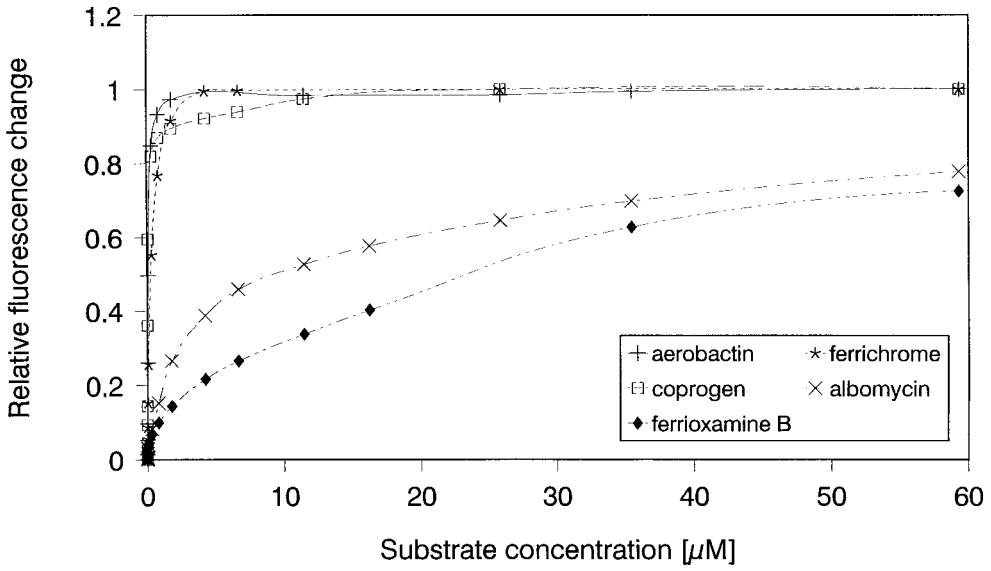

FIG. 5. Decrease in fluorescence intensity of His-tag-FhuD(A150S, P175L) dependent on increasing concentrations of the indicated ferric siderophores and antibiotic.

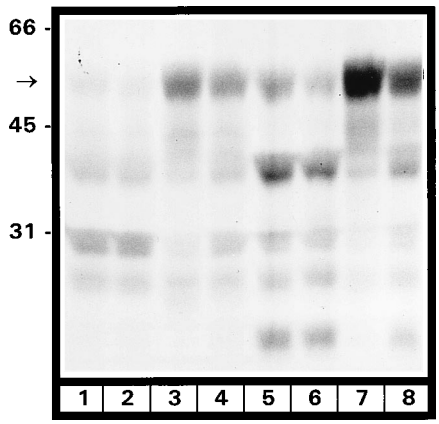

FIG. 6. Protection of FhuB contained in spheroplasts by FhuD against proteolytic degradation. Radiolabeled FhuB was incubated with trypsin alone (lane 1) or with trypsin and bovine serum albumin (lane 2) or trypsin and His-tag-FhuD in the absence (lane 3) and presence (lane 4) of aerobactin. FhuB was also incubated with proteinase K alone (lane 5) or with proteinase K and bovine serum albumin (lane 6) or proteinase K and His-tag-FhuD in the absence (lane 7) and presence (lane 8) of aerobactin. The FhuB protein band (arrow) and molecular masses (in kilodaltons) are shown on the left.

mic membrane (16), while transport across the cytoplasmic membrane is probably driven by ATP hydrolysis, as has been shown for maltose and histidine transport (4, 9–11). FhuC is the energy-providing protein that is localized at the inner side of the cytoplasmic membrane (42) and contains an ATP-binding motif (7, 8). The specificity for the uptake of ferric hydroxamates into *E. coli* K-12 is determined by the outer membrane proteins FhuA (ferrichrome-type siderophore), FhuE (ferric coprogen), and IutA (ferric aerobactin) (6). All the ferric hydroxamates are translocated across the cytoplasmic membrane by the same transport system, composed of FhuD, FhuB, and FhuC (6, 24). This implies a narrower substrate specificity of the transport system across the outer membrane than across the cytoplasmic membrane. Specificity of transport across the cytoplasmic membrane is determined by FhuD and FhuB. FhuD specifically interacts with the cognate ferric hydroxamates aerobactin, ferrichrome, and coprogen, as demonstrated by protection against proteolytic degradation (28, 38). In addition to mutants with point mutations in *fhuB*, which are albomycin resistant and fail to transport ferrichrome (25), derivatives that are impaired in the uptake of the different iron (III) hydroxamates to various extents were isolated. It is unlikely that FhuC recognizes ferric hydroxamates, since it has been demonstrated that the FhuC equivalent MalK of maltose transport complements a UgpC mutant of *sn*-glycerol-3-phosphate transport and vice versa (18), excluding substrate specificity of these energy-providing proteins.

In this paper, it is shown that FhuD to a large extent contributes to the substrate specificity of transport through the cytoplasmic membrane. The dissociation constant for ferrichrome bound to wild-type FhuD, derived from the fluorescence quenching caused by binding of ferric hydroxamate, was similar to the Michaelis constant determined for ferrichrome uptake into wild-type cells (dissociation constant, 1 μM; Michaelis constants, 0.7 [52] and 0.5 [39] μM). The dissociation constants obtained for the various ferric hydroxamates correlated with the growth promotion data determined on iron-limited nutrient agar plates (22) and the transport rates obtained for aerobactin (15) and ferrichrome and albomycin (17). Although the strength of binding to FhuD does not necessarily determine the rate of transport, the qualitative agreement between the ability to bind and the ability to transport suggests an important role of FhuD in the selection of ferric hydroxamates that are transported by the Fhu system. The overall rate of ferrichrome transport in wild-type *E. coli* K-12 displays saturation kinetics that are abolished by conversion of the FhuA protein into an open channel (21). Cells expressing FhuA in which the largest surface loop (23) is deleted show a linear dependence of the transport rate on the ferrichrome concentration (21); therefore, in the concentration range tested (1 to 20 μM), the FhuD-FhuB-FhuC transport system was not rate limiting.

Interaction of FhuD with FhuB was tested by cross-linking the two proteins and by prevention of FhuB degradation by added His-tag-FhuD. In both experiments, unloaded FhuD was at least as effective as FhuD loaded with ferrichrome. In the absence of ferrichrome, FhuD may fluctuate between an open and a closed conformation so that the ferrichrome-free closed form interacts with FhuB. Alternatively, the two domains of FhuB (25, 29) may assume two conformations, one of which interacts with the closed form and the other of which interacts with the open form of FhuD. In the maltose system, it has been shown that the N-terminal lobe (46) of the binding protein binds to MalG and that the C-terminal lobe binds to MalF (20), suggesting a precise positioning of the binding protein at the two integral membrane proteins. All periplasmic binding proteins have similar three-dimensional structures (14, 34–36, 46, 53). X-ray analysis of maltose-binding protein loaded with maltodextrins and free of substrate reveals an open (unloaded) and a closed (loaded) conformation (44). However, unloaded glucose-galactose-binding protein could also be crystallized in the closed conformation (14), suggesting a dynamic equilibrium between the closed and the open conformations and stabilization of the closed conformation by the bound substrate.

FhuD(W68L) displayed a preference for coprogen. Coprogen was the only ferric hydroxamate that supported growth well under iron-limiting conditions. Although the dissociation constant for coprogen bound to FhuD(W68L) was higher than that for coprogen bound to wild-type FhuD, it was much lower than the dissociation constants for aerobactin and ferrichrome. Without knowledge of a three-dimensional structure of FhuD,

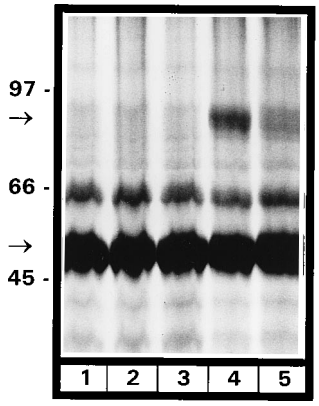

FIG. 7. Cross-linking of FhuD to radiolabeled FhuB by ECD-sNHS. Spheroplasts containing the radiolabeled, overexpressed FhuB protein were incubated with ECD-sNHS (lanes 1 to 5) in the presence of carbonic anhydrase (500 μg) (lane 2), aerobactin (100 μM) (lane 3), His-tag-FhuD (250 μg) (lane 4), and His-tag-FhuD with aerobactin (250 μg and 100 μM, respectively) (lane 5). The FhuB protein (approximately 50 kDa) and the FhuB-FhuD cross-linking product (approximately 88 kDa) (arrows) and molecular masses (in kilodaltons) are shown on the left.

it cannot be determined whether replacement of tryptophan by leucine directly affects the binding site of ferric hydroxamates or causes a conformational change that alters the geometry of the binding site located at some distance from residue 68. All 48 mutants isolated during this study contained one or more missense point mutations which, however, were not clustered at certain sites; therefore, no regions involved in substrate or FhuB binding could be identified by this means (data not shown). From the known conformations of binding proteins, it is clear that binding sites are composed of various regions along the polypeptide chains that come into close contact by folding of the polypeptides.

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