Binding of Iron-free Siderophore, a Common Feature of Siderophore Outer Membrane Transporters of Escherichia coli and Pseudomonas aeruginosa*

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TonB-dependent iron transporters present in the outer membranes of Gram-negative bacteria transport ferric-siderophore complexes into the periplasm. This requires proton motive force and an integral inner membrane complex, TonB-ExbB-ExbD. Recognition of iron-free siderophores by TonB-dependent outer membrane transporters (OMT) has only been described for a subfamily called OMTN. These OMTN's have an additional domain at the N terminus, which interacts with an inner membrane regulatory protein to activate a cytoplasmic α factor. This induces transcription of iron transport genes. Here we showed that the ability to bind ap siderophores is not specific to the OMTN subfamily but may be a more general feature of OMTs. FhuA, the ferrichrome OMT in Escherichia coli, and FptA, the pyochelin (Pch) OMT in Pseudomonas aeruginosa, were both able to bind in vitro and in vivo the apo-forms and the ferric forms of their corresponding siderophore at a common binding site. FptA produced in P. aeruginosa cells grown in an iron-deficient medium copurifies with a ligand that, as characterized by fluorescence, is iron-free Pch. As described previously for the FpvA transporter (pyoverdine OMT in P. aeruginosa), it appears that in conditions of iron limitation all the FptA receptors at the cell surface are loaded with apoPch. This FptA-Pch complex is less stable in vitro than the previously described copurified FpvA-Pvd complex and can be loaded with iron in vitro in the presence of Pch-Fe, citrate-Fe, or ferrichrome-Fe. These findings improved our understanding of the iron uptake mechanism via siderophores in Gram-negative bacteria.

When grown in iron-deficient conditions, many bacteria syntehsize and release iron chelators, termed siderophores (1–3). Siderophores make iron available for use by the cells by solubilizing ferric ion, which otherwise forms insoluble complexes, under aerobic conditions at physiological pH. In the host, siderophores from infectious or parasitic bacteria are thought to sequester iron from iron-containing molecules such as transferrin and lactoferrin and then to deliver it to the microbial cells. Ferric uptake mediated by siderophores in Gram-negative bacteria involves a specific outer membrane transporter (OMT) and an inner membrane ABC transporter (4, 5). The energy required for transport across the inner membrane is provided by ATP hydrolysis. Transport across the outer membrane by the OMT is driven by the proton motive force of the inner membrane via an inner membrane complex composed of TonB, ExbB, and ExbD (6–8).

Most studies of iron uptake by Gram-negative bacteria focused on transport across the outer membrane via OMT. The crystal structures of the following five OMTs have been solved: FhuA, FepA, FecA, and BtuB from Escherichia coli and FpvA from Pseudomonas aeruginosa, the ferric-ferrichrome, ferric-ferrienterobactin, ferric-citrate, vitamin B$_12$, and ferric-pyoverdine OMTs, respectively (9–14). These five transporters are composed of a C-terminal β-barrel domain and an N-terminal cork domain that fills the barrel interior. The ferric-siderophore-binding site is located above the cork, well outside the membrane. It is composed of residues of the plug and of the β-barrel domains. The electrostatic properties of the siderophore-binding pocket are specific to each OMT and are related to the chemical features of the siderophore. The binding pocket of iron-free Pvd on FpvA is mainly composed of aromatic residues (14). In the FhuA-ferrichrome (9, 10), eight aromatic and two hydrophilic residues form the ferrichrome-binding site. The ferric-citrate-binding pocket of FecA is composed of 5 Arg residues, which interact with the negatively charged ferric-citrate (11, 12).

Recently, a subfamily of siderophore OMTs has been defined, OMTN (15). These transporters have an additional domain of about 70 amino acids at the N terminus, absent from all other OMTs (15, 16). This region is localized in the periplasm and is involved in the regulation of the transcription of the genes governing iron uptake by the bacteria (17–19). In addition, some OMTN's bind the iron-free and the iron-loaded form of their corresponding siderophore, with close affinities, to a common or overlapping binding site (15). This has been described for the pyoverdine (Pvd) outer membrane receptor FpvA of P. aeruginosa (20, 21), the ferric-citrate outer membrane receptor FecA of E. coli (12), and the hemophore outer membrane receptor HasR of Serratia marcescens (22). The crystal structures of FecA, loaded with apo-citrate or ferric-citrate, have

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‡ The abbreviations used are: OMT, outer membrane transporter; Pch, pyochelin; Pvd, pyoverdine; OMTN, a subfamily of outer membrane transporters; MEM, maximum entropy method; pmf, proton motive force; octyl-POE, n-octylpolyoxyethylene.

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shown that it binds its apo- and ferric-siderophore, dictate, and diferric-dictrate at a common binding site, but the interactions are completely different (12). In the FecA-citrate-Fe complex, the two citrate ions are in a planar orientation, whereas in the FecA-citrate complex the two iron-free citrate ions are orthogonal. Only the interactions between the diferric dictrate to FecA is accompanied by significant conformational changes in two extracellular loops L7 and L8; these changes close the binding pocket, thereby sequestering the ligand in the binding site (12). When iron-free dictrate binds to FecA, no binding pocket closure is observed, and the iron-free dictrate remains accessible to the extracellular domain. For the FpvA transporter, only the structure of the FpvA-Pvd complex is known (14). However, the interactions between FpvA and each apo- and ferric-Pvd have been studied using the fluorescent properties of the siderophore. Consequently, a mechanism for the iron uptake cycle has been proposed (for a review see Refs. 15 and 23). Under iron limitation all FpvA receptors at the cell surface are loaded with iron-free Pvd (20, 21). This FpvA-Pvd complex is extremely stable and dissociates in vivo following activation of the transporter by the pmf via the TonB machinery (24). Ferric-Pvd then binds to the transporter. Time-resolved fluorescence spectroscopy studies show that Pvd is less mobile in its binding site and less solvent-accessible in the FpvA-Pvd-metal complex than in the FpvA-Pvd complex (25). Possibly, extracellular loops form a lid and trap the Pvd metal in its binding site as observed in FecA-Cit-Fe. The heme uptake cycle of HasR, the hemophore receptor in S. marcescens, shares common features with FpvA as follows: (i) binding of the holo- and apo-hemophore with high affinities to a common binding site on HasR (22) and (ii) activation of the release of the apo-hemophore from HasR by the pmf via the TonB machinery (26).

It is not known if the binding of iron-free siderophores to their corresponding OMT is a specific feature of the OMTN subfamily or if it is common to all OMTs. We investigated the binding properties of two transporters, which do not belong to the OMTN subfamily: FhuA, the ferrichrome OMT in E. coli, and FptA, the pyochelin (Pch) OMT in P. aeruginosa. FhuA is a typical example of iron uptake systems (4), but its ability to bind the apo-form of ferrichrome has not been rigorously described. The FptA-Pch system is one of the major iron uptake pathways in P. aeruginosa (27, 28), but its mechanism has never been investigated at the molecular level.

We report that these two receptors bind with close affinities and to a single binding site the apo- and ferric forms of their corresponding siderophores. Like FpvA, the normal state of FptA in the outer membranes of P. aeruginosa, under iron limitation conditions, seems to be the FptA-Pch complex. This in vitro complex is less stable than the FpvA-Pvd complex described previously. Finally, the mechanism of formation of iron-loaded FptA-Pch in vitro seems to be more efficient than that for the FpvA-Pvd system.

Bacterial Strains and Growth Media—The E. coli and P. aeruginosa strains used and their phenotypes are listed in Table I. P. aeruginosa strain K691 is an FpvA-deficient mutant and has been described previously (20). The Pvd-deficient P. aeruginosa strain, CDC5, was described by Ankenbauer et al. (32). The mutation has been mapped to the pdv locus that contains genes involved in the synthesis of the peptide moiety of Pvd. FpvA was overproduced in K691 and CDC5 strains by introduction of a plasmid, pPV2, carrying the fpvA gene (33). The Pch- and Pvd-deficient P. aeruginosa strain, PAD07, and the strain overexpressing FhuA, HO830(pHX405), have been described previously (34, 35).

Pseudomonas strains were grown overnight in succinate medium (31) in the presence of 150 μg/ml carbenicillin for strains CDC5 (pPV2) and K691 (pPV2) and 100 μg/ml streptomycin and 50 μg/ml tetracycline for strain PAD07. HO830 (pHX405) was grown in LB medium (Luria-Bertani broth) containing 100 μg/ml ampicillin and 0.1 mM 2,2′-bipyridyl.

Preparation of Outer Membranes and Purification of FpvA, FptA, and FhuA—Outer membranes and purified FpvA receptors were prepared as described previously (20). FptA was purified with the same protocol as the FpvA-Pvd-deficient strain CDC5(pPV2). Contaminant FpvA was separated from FptA by chromatofocusing. FhuA was purified as described previously (35).

Fluorescence Spectroscopy—Fluorescence intensity and anisotropy decays were obtained from the polarized components I(τ) and I(τ) by the time-correlated single photon-counting technique. A LDH370 diode laser from Picoquant GmbH (Berlin-Asheldorf, Germany) was used at a repetition rate of 10 MHz for excitation at 370 nm. Fluorescence emission was selected by a single monochromator (Jobin Yvon UV-H10, bandwidth 8 nm). A MCP-PMT Hamamatsu detector (model R3809U-02) was used. The instrument response function (~160 ps) was monitored with the reference compound decay of 4-dimethylamino-4′-cyanostilbene (τ = 41 ps in cyclohexane). Time resolution was ~20 ps, and the data were stored in 24 channels. Fluorescence intensity and anisotropy decays were fitted to multieponential functions by the maximum entropy method (MEM) (36, 37).

Excitation spectra were recorded with a Cary Eclipse spectrofluorometer. All other fluorescence experiments were performed with a Photon Technology International TimeMaster™ (Bioradtech) spectrofluorometer. For all experiments the sample was stirred at 29 °C in a 1 ml cuvette.

Formation of Iron-loaded FptA-Pch Complex and Binding of Pch to FptA—Outer membranes prepared from CDC5(pPV2) cells, at concentrations between 0.5 and 1.3 mg/ml, were incubated overnight in the presence of 1 μM Pch-Fe, in 200 μl of 50 mM Tris-Cl buffer, pH 8.0. According to the fluorescence emission spectrum of the outer membranes, the concentration of FptA-Pch in the experiment was ~20 μM. The same experiment was repeated in the absence of ferric-siderophore and also in the presence of Pvd-Fe, Pch-Fe or ferrichrome-Fe. After incubation, the membranes were pelleted at 20,000 × g, washed with 1 ml of 50 mM Tris-Cl buffer, pH 8.0, to eliminate free siderophores, and resuspended in 1 ml of the same Tris-Cl buffer containing 700 μM for each of the conditions presented in Fig. 5A and 250 μM for the experiments in Fig. 8. An emission scan was recorded for each experiment with the excitation wavelength set at 315 nm.

To study the binding of Pch to FptA in vitro, outer membranes prepared from PAD07 cells at a concentration of 1 mg/ml were incubated overnight in the presence of 200 μM Pch in 200 μl of 50 mM Tris-Cl buffer, pH 8.0. The excess Pch was then eliminated by centrifugation at 20,000 × g as described above, and the pellet was resuspended in 50 mM Tris-Cl buffer, pH 8.0, and an emission scan was monitored (excitation wavelength set at 315 nm).

Ligand Binding Assays Using 55Fe—To determine the apparent dissociation constants in vivo μM Pch-55Fe, Pch-55Fe, and ferrichrome-55Fe binding to their corresponding receptors, CDC5(pPV2), PAD07, and HO830(pHX405), cells were washed twice with an equal volume of fresh medium and resuspended in 50 mM Tris-Cl buffer, pH 8.0, at an A600 of 0.05 for CDC5(pPV2) and of 0.3 for PAD07 and HO830(pHX405). The cells were then incubated at 0 °C to avoid any iron uptake (24) for 1 h; in a final volume of 500 μl in the presence of various concentrations (0.1 to 80 nM) of siderophore-55Fe. Incubations were stopped by centrifugation at 12,000 × g (4 °C) for 2 min. The supernatant containing the unbound siderophore-55Fe was removed, and the tubes containing the cell pellet were counted for radioactivity in a scintillation mixture. The experiments were repeated in the absence of cells to estimate nonspecific interactions of siderophore-55Fe with the tubes.

For competition experiments, purified receptor (FpvA, FhuA, or

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erophores were calculated from IC50 values, determined in competition labeled) was separated from the cells in a different way; the mixtures of 0.3 for PAO1 15692 and PAD07, and 0.05 for CDC5(pPVR2) and HO830(pHX405). The experiment was also repeated in the absence of cells to estimate nonspecific interaction of siderophore-55Fe with the filter.

A second method was also used for the in vivo competition experiments. Cells were incubated in the presence of the siderophore as described above. However, the unbound siderophore (labeled or not labeled) was separated from the cells in a different way; the mixtures were centrifuged at 12,000 × g (4 °C) for 2 min, and the supernatant was removed. The tubes containing the cell pellet were counted for radioactivity in a scintillation mixture. Apparent binding affinity constants (K1) of siderophores were calculated from IC50 values, determined in competition experiments, according to the Cheng and Prusoff relation (38): 

\[ K_i = \frac{IC_{50}}{1 + [L]} \]

where L is the concentration of radiolabeled ligand, and K1 is the equilibrium dissociation constant determined experimentally.

The experiment was repeated in vivo with the cells prepared at an A600 of 0.3 for PAO1 15692 and PAD07, and 0.05 for CDC5(pPVR2) and HO830(pHX405). The experiment was also repeated in the absence of cells to estimate nonspecific interaction of siderophore-55Fe with the filter.

FptA at a concentration of 0.1 μM and in a final volume of 500 μl was incubated at room temperature for 1 h with siderophore-55Fe (0.5 nM) and various concentrations of unlabeled siderophore loaded or not loaded with iron (0–1000 nM). Incubations were stopped by filtering the assay mixtures over GF/B filters (Whatman, presoaked in 0.1% polyethyleneimine). The filters were then rapidly washed three times with 3 ml of 50 mM Tris-HCl, pH 8.0, and counted for radioactivity in a scintillation mixture. Apparent binding affinity constants (K1) of siderophores were calculated from IC50 values, determined in competition experiments, according to the Cheng and Prusoff relation (38): 

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FptA Copurifies with Iron-free Pch—FptA was purified in parallel with FpvA from P. aeruginosa strain CDC5(pPVR2) (Table 1), using the protocol described previously for FpvA (20). FptA was subsequently separated from FpvA by chromatofocusing. N-terminal sequencing clearly showed that the purified protein was FptA with no contamination by FpvA. Purified FptA (17 μg) was excised at the absorption maximum of iron-free Pch (315 nm, Fig. 1). The emission peak was at 450 nm (Fig. 1, dotted line), showing that FptA was copurified with a fluorescent molecule (FptA-X). Previous studies have shown that the FptA transporter of P. aeruginosa copurifies with its corresponding iron-free siderophore, Pvd, when expressed in a Pvd-producing strain (20). Unlike FpvA-Pvd, the molecule copurifying with FptA-X was not easily identified because of the small amounts of purified receptor available.

Pch is a fluorescent siderophore (Fig. 1), and therefore like FpvA, FptA may copurify with apoPch. Pch is a 2-[2-((2-hydroxyxphenyl)-2-thiazolin-4-yl]-3-methyl-4-thiazolidinediacarbonylic acid; it has a low affinity for Fe(III) (2 × 105 M−1 in ethanol), binds the cation in a 2:1 (Pch:Fe(III)) stoichiometry ratio, and is poorly water-soluble (28). Pch3−Fe is not fluorescent (Fig. 1). As in the Pvd-Fe complex, iron quenches the fluorescence of the siderophore. The photophysical properties of Pch are complex and extremely sensitive to pH (39). It undergoes an excited state reaction, favored at basic pH, leading to ionization of the Pch phenolic group and producing a highly fluorescent phenolate ion. The phenolate displays absorption and excitation features, different from those of the protonated phenol form. The phenolate ion exhibits a new band at 350–360 nm, not observed with the phenol form. To characterize the fluorescent ligand copurified with FptA, it was therefore necessary to study in more detail the fluorescence properties of FptA-X with respect to Pch.

The fluorescence emission spectrum of free Pch in 50 mM Tris-HCl buffer, pH 8.0, differs from that of FptA-X in both the intensity and the maximum of fluorescence emission: 430 nm for Pch and 450 nm for FptA-X (Fig. 1). Pch is, however, not very soluble in water, and aggregate was detected by fluorescence anisotropy. The addition of methanol (10% v/v) as cosolvent completely solubilized the material, which emitted at 450 nm. Thus, the emission maxima of FptA-X and the free Pch were not significantly different. Nevertheless, the intensity of FptA-X was higher than that of Pch in solution, similar to observations for Pch at basic pH (39). This could be due to a change of the excitation spectrum and/or to an increase of the excited state lifetime. To distinguish between these two possibilities, the excitation spectra of Pch dissolved in water and of the FptA-X complex were recorded (Fig. 2), and the fluorescent intensity decays of Pch in solution and of FptA-X were monitored (Fig. 3).

The fluorescence excitation spectra of the FptA-X complex (Fig. 2) exhibited a band centered around 385 nm, characteristic of the phenolate ion (39). This band is almost absent from FptA-X in both the intensity and the maximum of fluorescence emission: 430 nm for Pch and 450 nm for FptA-X (Fig. 1). Pch is, however, not very soluble in water, and aggregate was detected by fluorescence anisotropy. The addition of methanol (10% v/v) as cosolvent completely solubilized the material, which emitted at 450 nm. Thus, the emission maxima of FptA-X and the free Pch were not significantly different. Nevertheless, the intensity of FptA-X was higher than that of Pch in solution, similar to observations for Pch at basic pH (39). This could be due to a change of the excitation spectrum and/or to an increase of the excited state lifetime. To distinguish between these two possibilities, the excitation spectra of Pch dissolved in water and of the FptA-X complex were recorded (Fig. 2), and the fluorescent intensity decays of Pch in solution and of FptA-X were monitored (Fig. 3).

FIG. 1. Fluorescence emission spectra of Pch, Pch2-Fe, and purified FptA from P. aeruginosa CDC5(pPVR2). The excitation wavelength was set at 315 nm (maximum absorption of Pch). Purified FptA (dashed line), Pch (solid line), and Pch-Fe ( ● ) were dissolved in 50 mM Tris-HCl, pH 8.0, and 1% (v/v) octyl-POE. The same spectrum was monitored for Pch ( ○ ) in the same buffer at pH 7.0.

RESULTS

FptA Copurifies with Iron-free Pch—FptA was purified in parallel with FpvA from P. aeruginosa strain CDC5(pPVR2) (Table 1), using the protocol described previously for FpvA (20). FptA was subsequently separated from FpvA by chromatofocusing. N-terminal sequencing clearly showed that the purified protein was FptA with no contamination by FpvA. Purified FptA (17 μg) was excised at the absorption maximum of iron-free Pch (315 nm, Fig. 1). The emission peak was at 450 nm (Fig. 1, dotted line), showing that FptA was copurified with a fluorescent molecule (FptA-X). Previous studies have shown that the FptA transporter of P. aeruginosa copurifies with its corresponding iron-free siderophore, Pvd, when expressed in a Pvd-producing strain (20). Unlike FpvA-Pvd, the molecule copurifying with FptA-X could not be easily identified because of the small amounts of purified receptor available.

Pch is a fluorescent siderophore (Fig. 1), and therefore like FpvA, FptA may copurify with apoPch. Pch is a 2-[2-((2-hydroxyxphenyl)-2-thiazolin-4-yl]-3-methyl-4-thiazolidinediacarbonylic acid; it has a low affinity for Fe(III) (2 × 105 M−1 in ethanol), binds the cation in a 2:1 (Pch:Fe(III)) stoichiometry ratio, and is poorly water-soluble (28). Pch3−Fe is not fluorescent (Fig. 1). As in the Pvd-Fe complex, iron quenches the fluorescence of the siderophore. The photophysical properties of Pch are complex and extremely sensitive to pH (39). It undergoes an excited state reaction, favored at basic pH, leading to ionization of the Pch phenolic group and producing a highly fluorescent phenolate ion. The phenolate displays absorption and excitation features, different from those of the protonated

| Strains   | Phenotype | Reference |
|-----------|-----------|-----------|
| PAO1 15692 | Wild type | 40        |
| CDC5(pPVR2) | fpuA A− ΔPvd | 33        |
| K691(pPVR2) | fpuA A− | 20        |
| PAD07 | fpuA ΔPch ΔPvd | 34        |
| HO830(pHX405) | fhuA A− | 35        |
MEM recovered excited state lifetime distribution. The lifetime values mean excited state lifetime, Pch concentration in 1% octyl-POE: 0.6 mg/ml. Experiments were performed at room temperature. FptA-Pch complex is in agreement with the data are consistent with a single Pch molecule bound to FptA in intensity of the FptA-Pch complex than of the free Pch. This length excitation band at 350–390 nm explain the greater bound Pch, respectively, and the appearance of a long wave—excited state lifetime, from 2.54 to 4.12 ns, for the free and the excitation spectrum (Fig. 2). The increase of the main excited state lifetime, from 2.54 to 4.12 ns, for the free and bound Pch, respectively, and the appearance of a long wavelength excitation band at 350–390 nm explain the greater bound Pch, respectively, and the appearance of a long wave—

existence of a large mobility of the bound Pch, in contrast to Pvd bound to FpvA (25).

Outer membranes of CDC5(pPVR2) cells (Pvd-deficient and Pch-producing cells) display a maximum of emission of fluorescence at 450 nm like purified FptA-Pch (Fig. 5A). This fluorescent peak is absent from membrane preparations of Pch-deficient PAD07 cells (Fig. 5A), suggesting the presence in vivo of FptA-Pch complexes. No fluorescent peak at 450 nm was detected in living CDC5(pPVR2) cells, probably because of its low intensity. When Pch2-Fe (stoichiometry of 2 Pch for 1 iron ion) was added in excess to membrane preparations of CDC5(pPVR2), the fluorescent peak at 450 nm disappeared (Fig. 5A). Incubation of iron-free Pch with membrane preparations of PAD07 for 3 h at 37 °C resulted in the appearance of fluorescence at 450 nm (Fig. 5B), and similar treatment of CDC5 (pPVR2) had no effect on the fluorescence emission spectrum (data not shown). In binding experiments, unbound siderophore and ferric-siderophore were removed by centrifugation before monitoring the fluorescence emission spectra.

These experiments (Figs. 1–5) suggest the following: (i) FptA is able to bind apoPch with probably a 1:1 stoichiometry; (ii) the FptA-Pch complex is present in vivo; (iii) the FptA-Pch complex copurifies; and (iv) FptA-Pch can be transformed into FptA-Pch-Fe in the presence of Pch2-Fe. Unlike Pvd, Pch does not have the fluorescent properties to allow fluorescence resonance energy transfer with the Trp in the proteins; therefore, the fluorescence tools that can be used to study the FptA/Pch system are limited.

Iron-free Siderophore and Ferric-siderophore Bind with Close Affinities to the OMTs FptA and FhuA in Vivo and in Vitro—The dissociation constants of FptA, FhuA, and FpvA for their corresponding ferric-siderophore were determined using

2 D. Cobessi, H. Celia, and F. Pattus, manuscript in preparation.
This value is of the same order of magnitude as the centrifuged and resuspended in 50 mM Tris-HCl, pH 8.0, and the fluorescence spectra of PAD07 outer membranes after incubation with Pch. After incubation, the membranes were centrifuged and resuspended in 50 mM Tris-HCl, pH 8.0, and the excitation wavelength was set at 315 nm. Note that the emission maximum at 450 nm with outer membranes of CDC5(pPVR2) (solid line) is absent from outer membranes of PAD07 (dashed line). For the last spectrum (○), outer membranes of CDC5(pPVR2) cells were incubated for 24 h with excess of Pch-Fe. After incubation, the membranes were centrifuged and resuspended in Tris buffer, and the fluorescence spectrum was monitored. B, fluorescence spectra of PAD07 outer membranes after incubation with Pch. Outer membranes of PAD07 were incubated with (solid line) or without (dashed line) excess of Pch. After incubation, the membranes were centrifuged and resuspended in 50 mM Tris-HCl, pH 8.0, and the fluorescence spectra were monitored.

The limiting step of this process in vitro is the dissociation of Pvd from the OMT (24). In vivo, this dissociation is strongly enhanced under the control of the pmf and the TonB machinery (21, 24). We looked for similarities between the mechanism of formation of FptA-Pch-Fe and FptA-Pch, in vitro. Outer membrane preparations from Pch-producing CDC5(pPVR2) cells were incubated with stoichiometric concentrations of Pch2-Fe, Pvd-Fe, or ferrichrome-Fe. As already established (Fig. 5A), a nonfluorescent iron-loaded FptA-Pch complex was formed (Fig. 8A), in the presence of Pch2-Fe (stoichiometry of 2 Pch for 1 iron cation), with a kinetic apparently faster than that of the formation of FptA-Pch-Fe (20). The equilibrium of the reaction seems to be reached in a few hours. Indeed, no differences in the fluorescence emission spectra were observed after 4 h and overnight incubation (Fig. 8). When incubated with Pvd-Fe, no decrease of fluorescence at 450 nm was observed, clearly showing that FptA-Pch-Fe complexes do not form (Fig. 7). Most surprisingly, a decrease of fluorescence at 450 nm was observed with ferrichrome-Fe, suggesting the formation of FptA-Pch complexes in the membranes (Fig. 7). Thus, the behavior of FptA-Pch differs from that of FptA-Pvd; no FpvA-Pvd-Fe formation was observed when copurified FpvA-Pvd was incubated in the presence of an excess of citrate-Fe (20). Our data show that FptA-Pch is apparently able to compete for iron with Pch2-Fe and ferrichrome-Fe but not with Pvd-Fe. Pch, free in solution, like FptA-Pch, is able to compete for iron with ferrichrome but not with Pvd (Fig. 8B).

The same experiment was repeated with purified FptA-Pch and stoichiometric concentrations of various siderophores (Pch, Pvd, citrate, and ferrichrome) loaded with 55Fe (Table IV). The unbound siderophore-55Fe was separated from the protein by filtration. The amount of FpvA-Pch-55Fe formed was determined precisely. Again, in the presence of Pvd-55Fe, no FptA-Pch-55Fe was formed. This complex was formed only in the presence of the other siderophore-55Fe complexes.

The in vitro mechanism of formation of the FptA-Pch-Fe complex thus appears to be faster and more efficient than that for the FpvA/Pvd system (Fig. 8A and Table IV). The presence of stoichiometric concentrations of ferric-Pch, ferric-ferrichrome, or ferric-citrate was sufficient.

DISCUSSION

Binding of apo-siderophores to OMTs was first demonstrated for the ferric-Pvd transporter, FpvA, in P. aeruginosa. This binding, which seems atypical at first sight, was visualized using the fluorescent properties of this siderophore. The fluorescent properties of Pvd and especially its ability to allow fluorescence resonance energy transfer with the protein Trps were exploited in a detailed study of the interaction of apoPvd with FpvA in vivo and in vitro (20, 21, 24, 25, 42). More...
of Pch-\[^{55}\text{Fe}\] to FptA

FhuA and FptA, two transporters not members of the OMTN (15). However, the binding of apo-siderophores to OMT devoid of terminal domain involved in a mechanism of signal transduction in vitro. Competition experiments with FptA and FhuA subfamily.

recently, the structure of FecA, loaded with apo-dicitrate, showed that this atypical binding property was not a specific feature of FpvA but also applied to FecA, the ferric-citate receptor in \(E. \text{coli}\) (12). The homophore receptor, HaaR, in \(S. \text{marcescens}\), binds also with close affinities and to a common binding site, its apo- and holo-hemophore (22, 26). These three OMTs belong to the OMT\(_N\) subfamily with an additional N-terminal domain involved in a mechanism of signal transduction (15). We suggested that this property of binding the apoferrichrome form was specific to the OMT\(_N\) subfamily (15). However, the binding of apo-siderophores to OMT devoid of this additional N-terminal end had not been rigorously investigated. We therefore studied the binding properties of FhuA and FptA, two transporters not members of the OMT\(_N\) subfamily.

**Binding of Apo-ferrichrome to FhuA and ApoPch to FptA**—Competition experiments with FptA and FhuA in \textit{vivo} and \textit{in vitro} with two different protocols (Table III and in Fig. 6) demonstrated binding of the apo- and the ferric form of the siderophore to a common or overlapping binding site on the OMT. Thus, the ability to bind the apoferrichrome form is around 1 order of magnitude lower (in the range of 1–20 nM according to Table III), the OMTs at the cell surface in an iron-limited environment will be loaded with apo-siderophore. Indeed, under iron-limited growth conditions, all FpvA receptors at the cell surface are loaded with iron-free Pvd (20, 21). Here we characterized purified FptA, produced in strain CDC5(pPVR2) (a Pch-producing and Pvd-deficient strain), by fluorescence spectroscopy and demonstrated copurification of FptA bound to iron-free Pch, probably with a stoichiometry of 1:1. The FptA-Pch complex is also present in outer membranes prepared from CDC5(pPVR2) cells (Pch-producing and Pvd-deficient) and absent from the outer membranes of PAD07 cells (a Pch-deficient strain; Fig. 4). Thus, the normal state of FptA in the outer membranes under iron limitation is probably the apo-siderophore-loaded form, as described previously for FpvA (20, 21). For the FhuA/ferrichrome system, the ferrichrome is a heterologous siderophore, not produced by \(E. \text{coli}\) and therefore not present in the growth medium. In this case the OMT is purified with its binding site empty.

**Formation of an Iron-loaded FptA-Pch Complex**—Pch-loaded FptA therefore appears to be the normal state of this OMT. To load this FptA-Pch complex with iron, three different mechanisms can be imagined (Scheme 1). The first possibility (mechanism A in Scheme 1) involves the replacement of the OMT-bound siderophore by the free ferric-siderophore, as described previously for the FpvA/Pvd system. This mechanism is simplified in Scheme 1, and details are reported elsewhere (for a review see Refs. 15 and 23). Alternatively, the free siderophore-Fe complex acts as an iron donor for the OMT-bound siderophore (mechanism B in Scheme 1). This type of mechanism has been reported for \textit{Aeromonas hydrophila}, but the OMT concerned has not been described (43). This transporter is suggested to be loaded with an apo-siderophore under iron-limited conditions, and the ferric-siderophore binds to the OMT-siderophore complex and delivers the iron ion to it. This mechanism is unlikely in the FptA/Pch system, because no binding between FptA and ferrichrome-Fe, Pvd-Fe, or citrate-Fe could be demonstrated (data not shown). The third mechanism (mechanism C of Scheme 1) is that the OMT-siderophore complex competes directly for iron with the free siderophore-Fe complex. This mechanism is plausible, especially in the case of the FptA-Pch complex. Both the fluorescence excitation spectra and the excited state lifetime distributions of Pch bound to its receptor FptA and in water clearly showed changes in the fluorescence properties of Pch when bound to FptA (Figs. 2 and 3). They can be attributed to the presence of the phenolate anion form of Pch in the FptA-binding site (39). This phenolate anion is the form of Pch competent for metal binding, and therefore the FptA-Pch complex may have better iron-chelating properties than Pch in solution and could be involved in a mechanism as the third one described in Scheme 1. Note that for the FptA/Pch system, the true mechanism will probably be more complex than those presented in Scheme 1, because of the 1:2 stoichiometry of the chelation of iron by Pch.

We studied the formation of the FptA-Pch-Fe complex only \textit{in vitro}, and further studies will be necessary to clarify its mechanism and for detailed comparison with the FpvA/Pvd system.

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**Table II**

**Apparent dissociation constants \(K_{\text{d, app}}\) of siderophore-\[^{55}\text{Fe}\] to outer membrane receptors (FpvA, FptA, and FhuA) determined \textit{in vivo} at 0 °C**

| Siderophores-\[^{55}\text{Fe}\] | Strains | \(K_{\text{d, app}}\) \(\text{nM}\) |
|-------------------------------|---------|----------------|
| Pyoverdine-\[^{55}\text{Fe}\] | CDC5(pPVR2)(33) | 0.37 ± 0.11 |
| Pyochelin-\[^{55}\text{Fe}\] | PAD07 (34) | 0.54 ± 0.19 |
| Ferrichrome-\[^{55}\text{Fe}\] | HO830(pHX405) (48) | 0.65 ± 0.25 |
| Enterobactin-\[^{55}\text{Fe}\] | KDF541(pITS449) | 0.2 \(^3\) |

\(^a\) The constants were determined from a Scatchard representation and the errors from multiple Scatchard plots.

\(^b\) This dissociation constant was reported previously (41).

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\(^3\) F. Hoegy, H. Celia, G. L. Mislin, M. Vincent, J. Gallay, and I. J. Schalk, unpublished data.
Binding of Iron-free Siderophore

**TABLE III**

| Siderophore | Apo-Sid. | Ferric-Sid. | Apo-Sid. | Ferric-Sid. |
|-------------|----------|-------------|----------|-------------|
| Sid = Pyoverdine | 17 (20)  | 8 (20)      | ND       | ND          |
| Purified FpvA | 14.5 ± 2 | 5.0 ± 1.3   | 16.7 ± 0.2 | 3.0 ± 2.6  |
| CDC5(pPVR2) | ND       | ND          | ND       | ND          |
| PAD07       | ND       | ND          | 20.5 ± 5.3 | 2.5 ± 0.8   |
| Sid = Ferrichrome | 1.4 ± 4.9 | 0.5 ± 0.1    | ND       | ND          |
| Purified FhuA | 1.6 ± 0.1 | 0.4 ± 0.3    | 5.6 ± 1.7 | 0.8 ± 0.7   |
| HOS830(pHX405) | 1.7 ± 0.8 | 0.8 ± 0.5    | ND       | ND          |

*a* In this protocol, the unbound siderophores were separated from the cells by centrifugation.

*b* In this protocol, the unbound siderophores were separated from the cells by filtration on GF/B filters.

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**FIG. 7.** A, competition by unlabeled Pch2-Fe and iron-free Pch for the binding of Pch-55Fe to FptA in *vivo*. Experiments were performed as described under “Materials and Methods” in the presence of PAD07 cells at an A590 of 0.05, 0.5 nM Pch-55Fe, and various concentrations of Pch (●) or Pch2-Fe (○). B, competition by unlabeled ferrichrome-Fe and iron-free ferrichrome for the binding of ferrichrome-55Fe to FptA in *vivo*. Experiments were performed as described under “Materials and Methods” in the presence of HOS830 cells at an A590 of 0.05, 0.5 nM ferrichrome-55Fe, and various concentrations of ferrichrome (●) or ferrichrome-Fe (○). For both A and B, the unbound siderophores were separated from the cells by centrifugation.

However, there are already some indications of possible mechanisms. We show one major difference to the FpvA/Pvd system; the FptA/Pch complex loads with iron with a faster kinetic than the FpvA/Pvd complex loads with iron with a faster kinetic than Fe(III) in less than 4 h. For the FpvA/Pvd system, the t1/2 is 25 h in the presence of an excess of Pvd-Fe (20). This difference in the behavior of FpvA-Pvd and FptA-Pch may be due to differences in the stability of the two complexes. Fluorescence anisotropy decay of Pch bound to FptA (Fig. 4) evidenced substantial mobility of the bound Pch, unlike Pvd bound to FpvA (25). Moreover, Pch can be removed from FptA-Pch in *vivo* by dilution and separated by filtration (data not shown). In contrast, the copurified FpvA-Pvd complex is extremely stable and dissociates extremely slowly (20).

FptA-Pch can become loaded with iron in the presence of Pch2-Fe and also in the presence of other siderophores, including ferrichrome-Fe and citrate-Fe, but not in the presence of Pvd-Fe (Table IV and Fig. 8A). When FptA-Pch is incubated in *vivo*, in the presence of no siderophore other than Pch2-Fe, the free Pch2-Fe can bind to FptA, after dissociation of the bound apoPch, as described for the FpvA/Pvd system (mechanism A in Scheme 1). In the presence of ferrichrome-Fe, citrate-F, and Pvd-Fe, Pch may dissociate from FptA and compete with the ferrisiderophores present (citrate-Fe, ferrichrome-Fe, and Pvd-Fe) to form the FptA-Pch2-Fe complex. Free Pch is able to compete for iron with ferrichrome (Fig. 8B), although its stability constant for iron is lower (2 × 10^5 M^-1 for Pch and 10^10 M^-1 for ferrichrome). Pvd has a higher stability constant for iron, 10^12 M^-1, and it seems to be unable to give its iron to Pch. It is difficult to conclude which mechanism is most likely, but the mechanism described for the FpvA/Pvd system (mechanism A, Scheme 1) may fit the data. However, mechanism C may be adapted also for the formation of iron-loaded FptA-Pch. In this case, the presence of the phenolate ion in the binding site may add a new step to the mechanism of iron loading. Future work will be directed to establish which of these mechanisms is correct.
a mechanism where FptA-Pch itself is able to chelate directly to iron. More work is necessary to elucidate the FptA/Pch system and the formation of the iron-loaded FptA-Pch complex in vivo.

Biological Function of the Binding of Apor-siderophores to OMTs—We show here that the binding of iron-free siderophores is not characteristic of the OMT$_{N}$ subfamily but is a general feature of TonB-dependent OMTs. Therefore, it seems unlikely that the binding of apo-siderophore to its OMT is an activator of the signal transduction cascade described for the FpvA/Pvd and FecA/citrate systems (23, 45). Indeed, no such a regulation of the gene expression has been reported for the FhuA/ferrichrome and the FptA/Pch systems. The biological significance of the binding of iron-free siderophores to their corresponding OMT is unknown. In the case of FpvA, binding of iron-free Pvd to FpvA does not seem to play a key role in the iron uptake mechanism. The loading status of FpvA, with or without Pvd, has no effect on the rate of iron transport in _P. aeruginosa_, and this is consistent with the mechanism proposed previously (15, 23). This binding of apo-siderophores could be just a way to increase or limit the concentration of apo-siderophore around the bacteria. Indeed, under iron limitation, the expression of the OMTs is not down-regulated by the Fur protein (46), and an excess of OMTs is synthesized and exported into the outer membranes. At the cell surface, these OMTs bind the apo-form of the siderophore and thereby maintain locally a certain concentration of apo-siderophores. Less TonB proteins are produced than OMTs (47); therefore, not all OMTs are activated by the TonB machinery to transport iron into the cells (24). However, further studies will be necessary to confirm this hypothesis and to understand the function of the binding of apo-siderophore to the TonB-dependent OMTs.

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**TABLE IV**

Formation of 55Fe-loaded FptA-Pch complex

| Siderophore          | FptA-Pch-55Fe formed |
|----------------------|----------------------|
| Pch-55Fe (100 nm)    | 3.1                  |
| Pch-55Fe (1 µmol)    | 8.3                  |
| Pvd-55Fe (100 nm)    | 0.0                  |
| Ferrichrome-55Fe (100 nm) | 5.0              |
| Citrate-55Fe (100 nm) | 5.0               |

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Binding of Iron-free Siderophore, a Common Feature of Siderophore Outer Membrane Transporters of *Escherichia coli* and *Pseudomonas aeruginosa*

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