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Biphasic Binding Kinetics between FepA and Its Ligands*

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The *Escherichia coli* FepA protein is an energy- and TonB-dependent, ligand-binding porin that functions as a receptor for the siderophore ferric enterobactin and colicins B and D. We characterized the kinetic and thermodynamic parameters associated with the initial, energy-independent steps in ligand binding to FepA. *In vivo* experiments produced K_d values of 24, 185, and 560 nM for ferric enterobactin, colicin B, and colicin D, respectively. The siderophore and colicin B bound to FepA with a 1:1 stoichiometry, but colicin D bound to a maximum level that was 3-fold lower. Preincubation with ferric enterobactin prevented colicin B binding, and preincubation with colicin B prevented ferric enterobactin binding. Colicin B release from FepA was unexpectedly slow *in vivo*, about 10-fold slower than ferric enterobactin release. This slow dissociation of the colicin B·FepA complex facilitated the affinity purification of FepA and FepA mutants with colicin B-Sepharose. Analysis of a fluorescent FepA derivative showed that ferric enterobactin and colicin B adsorbed with biphasic kinetics, suggesting that both ligands bind in at least two distinct steps, an initial rapid stage and a subsequent slower step, that presumably establishes a transport-competent complex.

Like other TonB-dependent outer membrane proteins, FepA serves as a receptor for a metal chelate (ferric enterobactin (FeEnt))[1] and for noxious agents (colicin B (ColB) and colicin D (ColD)). Like other outer membrane (OM) porins (1–3), FepA contains a hydrophilic channel (4), but the FepA pore (5) is closed by cell surface loops that impart binding and translocation specificity. Thus FepA is a TonB-dependent, energy-dependent, ligand-gated porin: its surface loops open in response to ligand binding and TonB action, to internalize FeEnt (6).

The siderophore FeEnt and the cytotoxins ColB and ColD differ in size, structure, and uptake mechanism, yet all three require FepA and TonB for passage into the periplasm (7, 8). The fifth proposed surface loop of FepA (PL5), bounded by residues 255–336, interacts with all three ligands during their passage through the OM (9, 10). Deletions in this loop abolish FeEnt transport, render the bacteria ColB- and D-resistant (11), and convert FepA into a general porin through which sugars, antibiotics, and other small molecules may enter the cell (5).

Colicins are tripartite cytotoxins: their central, N- and C-terminal domains participate in ligand binding, translocation, and bacterial killing, respectively (13). ColB and D are homologous in their translocation and binding domains, but divergent in their C termini, consistent with their different killing modes: ColB kills by forming a pore in the *Escherichia coli* inner membrane, whereas ColD acts as a ribonuclease in the cytoplasm.

Colicins may traverse the OM through porin channels (14). This idea originates in part from experiments on a colicin-resistant mutant of OmpF (G119D) with an occluded channel (15). Colicins A and E1 bind to external loops of OmpF and BtuB, respectively, and contain N-terminal determinants that confer pore specificity (16), suggesting that after adsorption they interact with the underlying transmembrane channels. However, no direct physical evidence exists to prove the passage of colicin polypeptides through OM porins.

We examined the mechanism of ColB binding to FepA *in vivo* with an ^{125}I-colicin binding assay and *in vitro* with a flourescent derivative of FepA. In both conditions the siderophore and the colicins showed complex binding kinetics. A rapid phase occurred first, followed by a slower step, and both steps were independent of energy and TonB. FeEnt bound about 10-fold faster than ColB during the second stage. The high affinity binding equilibria between ColB and FepA facilitated purification of the receptor and its mutants, by affinity chromatography with immobilized ColB.

EXPERIMENTAL PROCEDURES

Colicin B and D Purification—We isolated ColB from *E. coli* DM1187/pCLB1 (obtained from M. A. McIntosh) and ColD from *E. coli* CA23 (17). The former strain constitutively expresses ColB, and we induced the latter to ColD overexpression by incubation of mid-log cultures with mitomycin C (2 μg/ml; Sigma) for 2 h in the dark. We purified both toxins by selective precipitations and chromatography (17, 18), using specific killing activity and SDS-PAGE as measures of purity. Bacteria (20 g) were harvested by centrifugation, washed with 100 mM potassium phosphate, pH 7.4, resuspended in 50 ml of 20 mM phosphate buffer, pH 7.4, and lysed by 4 min of sonication in 30-s bursts, with cooling below <10 °C. The cell lysate was clarified by centrifugation at 100,000 × *g* for 45 min, and nucleic acids were precipitated with 0.5% polyethyleneimine (Polymin P, Sigma) and removed by centrifugation at 7000 × *g* for 30 min. ColB or ColD in the supernatant was precipitated with ammonium sulfate (between 28 and 55% for ColB, and between 41 and 49% saturation for ColD) and collected by centrifugation at 10,000 × *g* for 30 min at 4 °C. The pellet was resuspended in, and dialyzed against, 50 mM Tris-Cl, pH 7.4, then chromatographed on DE52 cellulose (Whatman). Colicin was eluted with a 0.0 to 0.5 M gradient of NaCl. Peak fractions of interest were concentrated by ammonium sulfate precipitation (55% for ColB and 49% for ColD) and purified over Sephacryl S-300-HR (110 × 1.0 cm) in the same buffer.

FepA Purification—FepA was purified by differential extraction with Triton X-100 (19) and ion exchange chromatography (20), or by affinity chromatography on ColB-Sepharose, synthesized by coupling ColB to

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[1] The abbreviations used are: FeEnt, ferric enterobactin; ColB, colicin B; ColD, colicin D; DM, dodecyl maltoside; OM, outer membrane; PL5, fifth proposed surface loop; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid.

cyanogen bromide (CNBr)-activated Sepharose 4B (21, 22). Crude or partially purified FepA was loaded on the column, which was washed with 10 volumes of TTE buffer (2% Triton X-100 in 50 mM Tris-Cl, 5 mM EDTA, pH 7.4), and FepA was eluted with a gradient of 0 to 2.5 M NaCl in TTE. Fractions were analyzed by SDS-PAGE. We regenerated the column with a reverse urea gradient (6.0 to 0.0 M in TTE).

α-FepA Sera—Rabbits were immunized weekly for 1 month with purified FepA, emulsified with complete (first immunization) or incomplete (subsequent immunizations) Freund's adjuvant. Rabbits were bled in week 5 and thereafter. The IgG fraction of the resulting α-FepA serum was purified and conjugated to CNBr-activated Sepharose 4B (22).

Protein Determinations—Protein concentrations were determined by the method of Lowry *et al.* (23), modified for accuracy in the presence of Triton X-100 (24, 25). The concentration of ColB was determined by the absorbance at 280 nm using a molar extinction coefficient of 62,160 M^{-1} cm^{-1}, calculated from its primary sequence (26).

FeEnt Purification—^{59}Fe-Enterobactin was prepared and purified as described previously (27).

ColB Killing Assays—Purified colicin was serially diluted in LB broth in microtiter plates, from 10^{-1} to 3.2×10^{-9}, and transferred with a sterile CloneMaster™ (Immusine Corp., San Leandro, CA) to an LB plate containing ampicillin (10 μg/ml), seeded with the tester strain. Colicin titers were measured after overnight incubation, as the inverse of the highest dilution that cleared the bacterial lawn.

Preparation of ^{125}I-ColB—ColB was iodinated with IODO-BEADs (Pierce). Six beads were rinsed with 500 μl of MOPS-buffered saline (MBS; 40 mM MOPS, 0.9% NaCl, pH 6.9), dried, and incubated with 1.0 mCi of $Na^{125}I$ in 300 μl of MBS for 5 min. 2.8 mg of ColB in 3.9 ml of MBS was added for 10 min at 25 °C. The reaction was stopped by removing the beads, and unreacted ^{125}I was eliminated by chromatography over Sephadex G-50 in MBS.

Fluorescence Measurements—The site-directed mutant FepA protein E280C was utilized for introduction of fluorescent probes onto the receptor. This mutant protein has been extensively studied and manifests a wild-type phenotype, even when covalently modified at the E280C site (6, 10, 12, 31). Its structural integrity *in vivo* was assessed with a battery of tests including accessibility to monoclonal antibodies, expression levels, binding and transport of ferric enterobactin, and binding and killing by colicins B and D. Purified FepAE280C-fluorescein bound ferric enterobactin with a K_d almost identical to the wild type FepA, providing good evidence that the overall structure of E280C-Fl was intact. We cannot completely rule out that fluorescent labeling may have some impact on the binding of ligands, but based on the above studies, we believe it is minimal. FepAE280C-Fl fluorescence was recorded with an SLM 8000C fluorimeter upgraded to 8100 functionality (SLM Instruments, Rochester, NY), equipped with a 450-watt xenon light source and a cooled photomultiplier tube housing and operated in photon-counting mode. The excitation and emission wavelengths were set to 490 and 520 nm, respectively. The ratio F/F_0 was calculated for each experimental point by dividing the net fluorescence (F) by the net fluorescence intensity at the beginning of an experiment (F_0).

Colicin B Binding Assays—Adsorption of purified ^{25}I-ColB to RWB18–60 (*fepA*) (11) and RWB18–60/pITS449 (*fepA*+) (11) was measured at 0 °C. Bacteria were grown to mid-log in minimal MOPS medium, and 2×10^8 cells were suspended in 50 μl of MBS. 10 μl of ^{125}I-ColB (varied from 0.075 to 2.0 μM, final concentration) were added and incubated 10 min, the reaction mixture was diluted to 560 μl, and the cells were pelleted at 14,000 rpm for 2 min and counted in a Beckman gamma counter.

Data Analysis—Statistical analyses were performed by nonlinear least squares methods using Grafit (28). For ColB binding, data were analyzed by the bound *versus* total equation. For fluorescence titrations, a form of the bound *versus* total equation was used that accounts for dilution of the fluorescent protein and the ligand due to sequential additions of ligand. Fluorescence time course data were fitted with both single and double exponential decay models, for increasing or decreasing functions.

RESULTS

FeEnt and ColB Binding Equilibria in Vivo—-FepA in RWB1860/pITS449, cultured under iron stress in MOPS medium, bound FeEnt with a K_d of 24 ± 8 nM to a capacity 103,000 molecules/cell (Fig. 1). The siderophore reached equilibrium with FepA within 1 min. The affinity of the receptor for colicins was somewhat lower; it bound ColB with a K_d of 185 ± 45 nM, to a capacity of 96,000 molecules/cell (Fig. 1). Measurements of FepA expression in ^{125}I-protein A Western blots (75,000 monomers/cell; data not shown) were consistent with the observed siderophore and ColB binding capacities, indicating approximately 1:1 stoichiometry for the binding of either ligand to the FepA monomer. FepA bound ColD, under the same experimental conditions, with a K_d of 560 ± 130 nM, to a capacity of 33,000 molecules/cell, 3-fold lower than ColB (Fig. 1). Colicin binding experiments were incubated from 10 min to 3 h with no significant change in the parameters (data not shown), indicating that the reactions were at or near equilibrium.

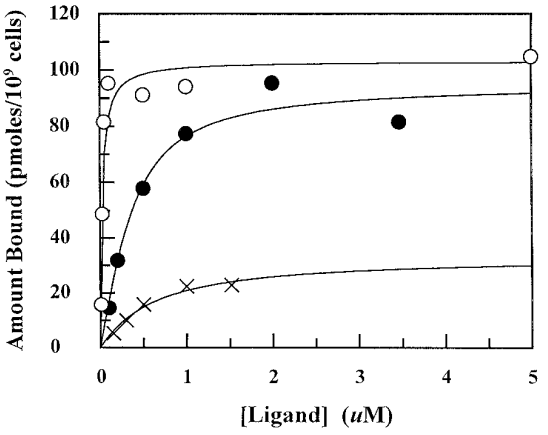


FIG. 1 **Ligand binding curves.** Data were obtained with RWB18–60 (*fepA*) (4) harboring pITS449 (*fepA*+) (11); binding to RWB18–60 was subtracted from each point. Theoretical binding curves are superimposed on the data. [^{59}Fe]Ent (○, average of three experiments), the K_d was 24 ± 8 nM and the capacity 103 ± 7 pmol/10^9 cells. ^{125}I-ColB (●, average of three experiments), the K_d was 185 ± 45 nM and the capacity 96 ± 3 pmol/10^9 cells. ^{125}I-ColD (×, average of two experiments), the K_d was 560 ± 130 nM and capacity 33 ± 3 pmol/10^9 cells.

In Vivo Competition between FeEnt and ColB for Binding to FepA—A 10-min preincubation of bacteria with saturating FeEnt (8.2 μM), or inclusion of saturating siderophore in the ^{125}I-ColB binding assay, prevented ColB binding (Fig. 2*A*). These data reiterate that FeEnt protects *E. coli* from ColB killing by occupancy of a common binding site on FepA (29, 30). The 10-fold lower affinity of ColB for FepA predicted a faster off-rate for the toxin, but if ColB was added first and excess FeEnt subsequently, almost no colicin dissociation occurred (Fig. 2*B*), despite the ability of the siderophore to exclude toxin binding. This discrepancy suggested an unaccountably slow dissociation of the ColB·FepA complex, and that ColB adsorption was effectively irreversible *in vivo*, even at 4 °C.

To further study the slow release of ColB from FepA, we used conditions that prevented its readsorption, saturating FeEnt. We equilibrated bacteria with 3.5 μM ^{125}I-ColB, then diluted, pelleted, and resuspended the cells in buffer with 10 μM FeEnt. From the radioactivity of the pellet, measured at intervals, we calculated k_{off} for ColB, 4.7×10^{-5} s^{-1}, a half-life ($t_{½}$) of 4 h. The rate of ^{59}FeEnt release under the same conditions was 6.9×10^{-4} s^{-1}, a half-life of about 20 min. While the slow off-rates explained the ligand competition results, they were inconsistent with a one-step binding mechanism; assuming single-step ColB binding and a k_{on} of 10^8 M^{-1} s^{-1} (an order of magnitude slower than the diffusion limit for small molecules), the measured K_d of 185 nM predicted a dissociation $t_{½}$ of less than 1 s. The slower observed ligand dissociation rates suggested that the measured K_d values contained rate constants for more than one binding step. The simplest explanation was that ColB bound in two steps, a rapid initial phase and a second phase that locked the receptor and ligand into a slow dissociating complex.

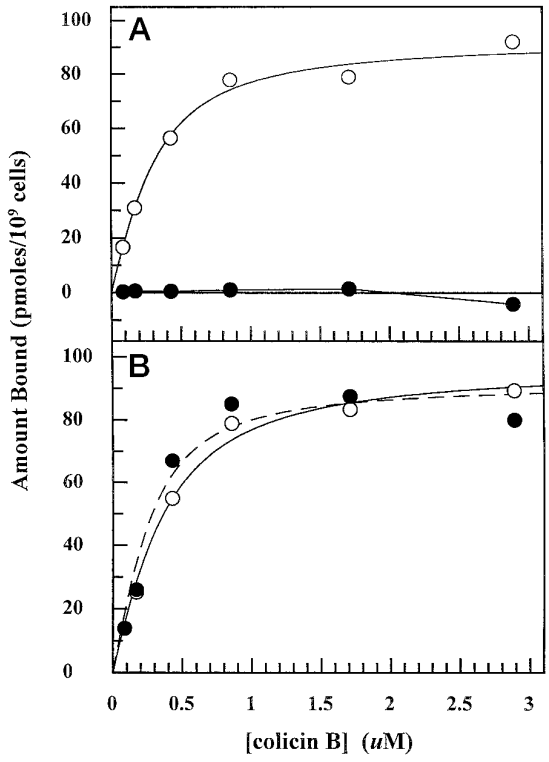


FIG. 2. ***In vivo* competition between ^{125}I-ColB and FeEnt.** Binding was measured as in Fig. 1 except that 8.2 μM FeEnt was included in the assays, creating a final volume of 70 μl. Binding to RWB18–60 was subtracted from each point. *A*, FeEnt was added and incubated for 10 min, followed by ^{125}I-ColB and further incubation for 1.0 h (●): the *lines* are a point by point connection of the data. Control binding curves were obtained by adding phosphate buffer in place of FeEnt (○): the *solid line* is the theoretical binding curve. The K_d was 163 ± 39 nM, and the binding capacity was 91 ± 3 pmol/10^9 cells. *B*, ^{125}I-ColB was added and incubated for 1.0 h, followed by FeEnt and further incubation for 10 min (●): the *dashed line* is the theoretical binding curve. The K_d was 118 ± 37 nM, and the capacity was 91 ± 3 pmol/10^9 cells. A control binding curve was obtained by adding phosphate buffer in place of FeEnt (○): the *solid line* shows the theoretical binding curve. The K_d was 212 ± 38 nM, and the capacity was 98 ± 3 pmol/10^9 cells.

In Vitro Ligand Binding in DM—The mutant protein FepAE280C (6, 10, 12, 31) binds and transports FeEnt, ColB, and ColD at wild type levels. Residue 280 lies in a proposed surface loop that participates in ligand binding (9, 10). We derivatized FepAE280C with the sulfhydryl-specific reagent 5-iodoacetamidofluorescein.[2] The fluorescence intensity of the labeled protein (FepAE280C-Fl) in DM micelles decreased approximately 40% upon the addition of FeEnt (Fig. 3); analysis of this quenching gave a K_d of 15 nM, in agreement with values derived from other measurements of FeEnt binding (10, 20). The fluorescence intensity of FepAE280C-Fl also decreased upon binding of ColB, but only about 15%, indicating that the siderophore and toxin interact differently with the labeled site (Figs. 3 and 4). As seen *in vivo*, ColB and FeEnt competed for binding to FepA *in vitro*; FeEnt excluded ColB, and vice versa (Fig. 3)

The reduction in FepAE280C-Fl fluorescence that occurred upon binding of FeEnt or ColB followed a double exponential decay (Fig. 4). Convergence did not occur with a single exponential decay model. Fluorescence decreased during FeEnt binding (Fig. 4*B*) with an initial rapid phase (k_1; $1.8 \times 10^{-2} \pm 8 \times 10^{-4}$ s^{-1}; see "Discussion" for rate constant nomenclature) and a second slower phase (k_3; $2.1 \times 10^{-3} \pm 2 \times 10^{-4}$ s^{-1}). ColB binding also followed biphasic kinetics (Fig. 4*A*) with faster initial (k_5; $2.1 \times 10^{-2} \pm 1 \times 10^{-3}$ s^{-1}) and a slower secondary

[2] Z. Cao, M. A. Payne, and P. E. Klebba, manuscript in preparation.

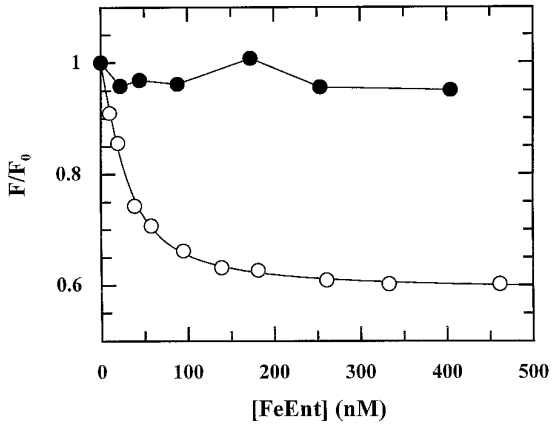


FIG. 3. **Fluorescence measurement of FeEnt binding to FepAE280C-Fl *in vitro*.** The decrease in fluorescence intensity after addition of FeEnt is plotted *versus* total input FeEnt. In the absence of ColB ○ the K_d was 15 ± 3 nM and the maximum fluorescence change (*M*) was 0.41 ± 0.01. The line shows the theoretical binding curve. A 15-min preincubation with 2.0 μM ColB (●) prevented FeEnt adsorption. The line is a point to point connection. Data points were corrected for dilution of the fluorophore by addition of titrant.

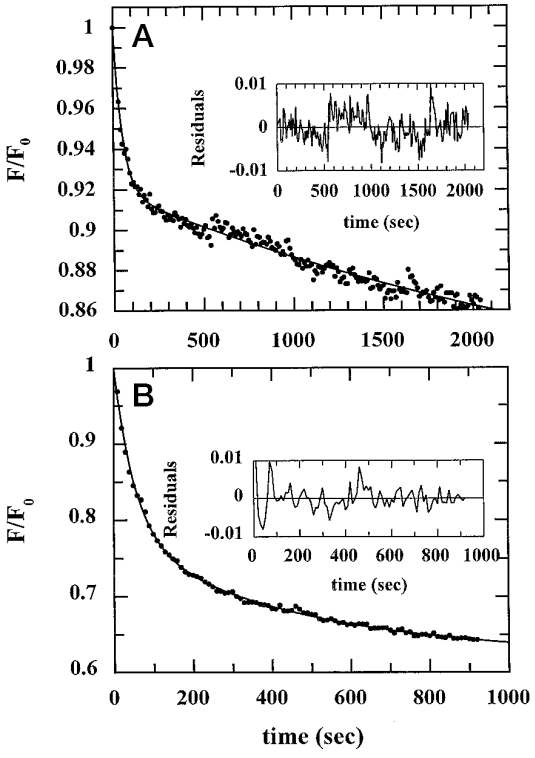


FIG. 4. **Time course of ligand binding *in vitro*.** FepAE280C-Fl was diluted to 50 nM in 50 mM Mops, pH 6.9, containing 60 mM NaCl and 1.0 mM dodecyl maltoside. *A*, fluorescence intensity was monitored to obtain a baseline, and ColB was added in the same buffer to 1.0 μM at t = 0. Raw data (●) were fitted to the theoretical time course for a two-component exponential model (*solid line*). The decay constants were (see Scheme 1): k_5, $2.1 \times 10^{-2} \pm 1 \times 10^{-3}$ s^{-1}; k_7, $3 \times 10^{-4} \pm 1 \times 10^{-4}$ s^{-1}. The *inset* plots the residuals from the fitting procedure. *B*, the experiment was performed as above except FeEnt (in 10 mM phosphate buffer, pH 6.9) was added to 500 nM. The decay constants were: k_1, $1.8 \times 10^{-2} \pm 8 \times 10^{-4}$ s^{-1}; k_3, $2.1 \times 10^{-3} \pm 2 \times 10^{-4}$ s^{-1}.

(k_7; $3 \times 10^{-4} \pm 1 \times 10^{-4}$ s^{-1}) components. Thus FeEnt and ColB both exhibited complex binding and a similar rate of initial adsorption to purified FepA in DM; their secondary, slower binding components differed by about 10-fold.

Ligand Dissociation in DM—-We also saw slow dissociation of ColB from purified FepA, demonstrating that the long lived

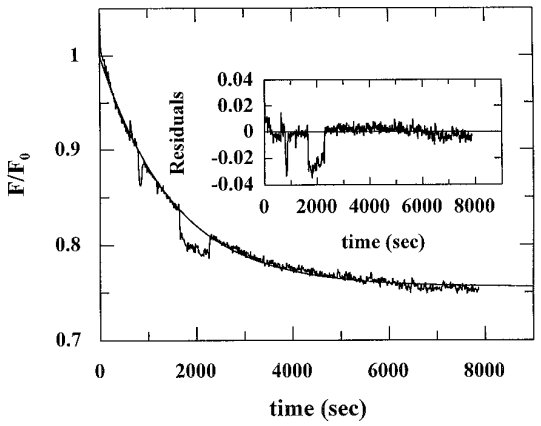


FIG. 5. **Ligand exchange; FeEnt binding to FepA·ColB.** The FepA·ColB complex was generated in DM, as in Fig. 4, by addition of ColB to 1.0 μM, and FeEnt was added to 4.0 μM at $t = 0$. Fluorescence intensity was monitored, and the raw data were fitted to the theoretical time course for a single exponential model (*solid line*). The first order decay constant (k_{10}) was $6.67 \times 10^{-4} \pm 4 \times 10^{-6}$ s^{-1}.

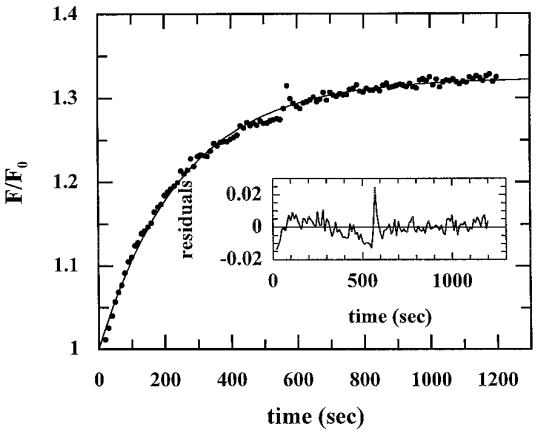


FIG. 6. **Ligand exchange; ColB binding to FepA·FeEnt.** The FepA·FeEnt complex was generated in DM, as in Fig. 4, by addition of FeEnt to 0.50 μM, and ColB was added to 2.0 μM at $t = 0$. Fluorescence intensity was monitored, and the raw data (●) were fitted to the theoretical time course for a single exponential model (*solid line*). The first order decay constant (k_9) was $3.9 \times 10^{-3} \pm 6 \times 10^{-5}$ s^{-1}.

complex depends solely on the receptor and ligand; other OM components or FepA localization in the OM were not required. When FepAE280C-Fl·ColB was incubated with excess FeEnt over an extended period, a further decrease in fluorescence gradually occurred (Fig. 5) that followed a simple exponential decay. It was slower (k_{10}; $6.7 \times 10^{-4} \pm 4 \times 10^{-6}$ s^{-1}, $t_{½} = 17$ min; Fig. 5) than the predicted off-rate, but 10-fold faster than ColB release in the absence of FeEnt *in vivo*. This decrease in fluorescence reflected the conversion of the FepA·ColB complex to the FepA·FeEnt complex. It occurred slower than the rate of FeEnt binding and probably approximates k_{off} for ColB *in vitro*. Likewise, addition of excess ColB to FepAE280C-Fl·FeEnt slowly increased fluorescence intensity (Fig. 6), again by a first order exponential process (k_9; $3.9 \times 10^{-3} \pm 6 \times 10^{-5}$ s^{-1}, $t_{½} =$ 3 min; Fig. 6) that reflected the conversion of FepA·FeEnt to FepA·ColB.

Ligand Binding in Triton X-100—Ligand binding kinetics in 2% Triton X-100 were similar to those observed in DM (Table I), except that in Triton X-100 the fluorescence of FepAE280C-Fl increased approximately 15% upon ColB addition. Nevertheless, the fluorescence of FepAE280C-Fl in Triton X-100 decreased approximately 30% upon the addition of FeEnt, consistent with the results in DM, and the antagonistic behavior of the two ligands in Triton X-100 was the same as was seen in DM. The rates of fluorescence changes in Triton X-100 were very similar to those measured in DM (Table I), and in both detergents ligand binding was clearly a two-component process (see Scheme 1).

Affinity Purification of FepA—The avidity of FepA for its ligands suggested affinity chromatography as a method for its purification. ColB-Sepharose purified FepA from Triton X-100-solubilized OM fractions of RWB18–60/pFepAE280C (Fig. 7*A*). It was more effective than immunoaffinity chromatography (Fig. 7*B*), because 81K* (32), the OmpT-generated degradation product of FepA, stuck tightly to anti-FepA-Sepharose, but not to ColB-Sepharose. Second, a mild salt gradient released FepA from ColB-Sepharose, while only high concentrations of the chaotropic agent trichloroacetate eluted the receptor from the immunoadsorbent, and these harsh conditions also released antibodies from the resin that contaminated the FepA product.

DISCUSSION

Affinity purification of siderophore receptors by binding to their antagonistic ligands, bacteriocins, was reported by Oudega *et al.* (21), who used cloacin DF13-Sepharose to purify an OM protein later recognized as the ferric aerobactin receptor, IutA (33). We reproduced this technique with ColB-Sepharose, permitting large scale, rapid purification of FepA or mutant FepA proteins; 20 ml of the affinity adsorbent isolated 5–10 mg of the receptor in 1–2 days.

Guterman (29, 34) discovered two mutations, *exbA* and *exbB*, that resulted in ColB resistance and hyperexcretion of a diffusible inhibitor of ColB killing. *exbA* was later recognized as *tonB* (35), which creates OM transport deficiencies resulting in ColB resistance. *exbB* also produces colicin uptake defects, and the soluble factor was identified as FeEnt. *tonB* and *exbB* mutants secrete enterobactin because they are unable to transport iron, making them chronically iron-deficient (36). Later experiments showed that metal chelates (30, 37, 38) block colicin (and bacteriophage) killing by competitive binding to common surface receptors. FeEnt, ColB, and ColD, for example, adsorb to a common site in FepA PL5 (10). We measured the affinities and kinetics of these binding reactions *in vivo* and *in vitro*. The siderophore bound more tightly to FepA than ColB or ColD, suggesting that its significant overall charge (−3) and aromaticity outweigh the potential for multivalent charge interactions between the colicins and the receptor. FeEnt may bind in a pocket of the receptor surface that is optimized to accept it in a complementary configuration. The colicins also use this site, but apparently as part of a broader surface area that creates weaker overall interactions. The lower capacity of FepA for ColD, relative to that observed for ColB and FeEnt, suggests that the larger colicin may bind as 1 ColD molecule per FepA trimer, as opposed to 1 molecule per FepA monomer, seen for ColB and FeEnt.

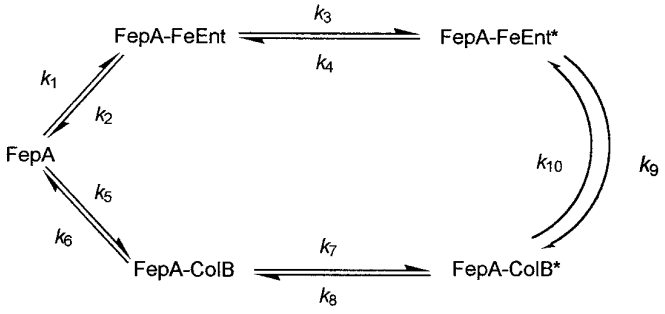
In the two nonionic detergents we employed FeEnt and ColB engendered spectroscopically distinct states in FepA upon binding, suggesting that the siderophore and colicins induce different effects when they complex FepA. Conformational changes in FepA were previously observed in the presence of FeEnt, by analysis of nitroxide probes attached at E280C (12). The close proximity of E280C to the FepA ligand binding domain (12) raises the possibility that the large reduction in intensity caused by the ferric siderophore results from chemical quenching instead of conformational changes in the receptor. However, both FeEnt and ColB exhibited biphasic binding kinetics to FepA, which strongly suggests a conformational change in the receptor during their adsorption. Analyses of the two-component pseudo-first order decay of fluorescent probes attached in PL5 (Table I) revealed several possible equilibria

TABLE I
Summary of rate constants

Rate constants determined from *in vitro* fluorescence time courses are summarized for the various interconversions in Scheme 1. Constants were determined at 25 °C in 50 mM MOPS, pH 6.9, containing 60 mM NaCl and either 1.0 mM DM or 2% Triton X-100. Standard errors represent the goodness of fit from a single determination. Replicates gave similar standard errors. The $t_{1/2}$ values were calculated from the respective constants using the relationships: $t_{1/2} = 0.693/k$. For comparison, k_{off} for both ligands determined *in vivo* are included.

Rate constant	Dodecyl maltoside			Triton X-100		
	Value	S.E.	$t_{1/2}$	Value	S.E.	$t_{1/2}$
	s^{-1}			s^{-1}		*min*
k_1	1.8×10^{-2}	8×10^{-4}	0.65 min	6.4×10^{-3}	3×10^{-4}	1.8
k_3	2.1×10^{-3}	2×10^{-4}	5.5 min	1.15×10^{-3}	7×10^{-6}	10.0
k_5	2.1×10^{-2}	1×10^{-3}	0.55 min	8.9×10^{-3}	5×10^{-4}	1.3
k_7	3×10^{-4}	1×10^{-4}	39 min	1.09×10^{-3}	3×10^{-5}	10.6
k_9	3.9×10^{-3}	6×10^{-5}	3 min	2.1×10^{-3}	6×10^{-6}	5.5
k_{10}	6.7×10^{-4}	4×10^{-6}	17 min	8.6×10^{-4}	2×10^{-6}	13.4
k_{off} FeEnt[a]	6.9×10^{-4}	ND[b]	17 min (3 min)[c]			
k_{off} ColB[d]	4.7×10^{-5}	ND[b]	4.1 hr (43 min)[c]			

[a] Determined in 40 mM MOPS, pH 6.9, containing 0.9% NaCl, 2.0 μM nonradioactive FeEnt, and no detergent, 0 °C.
[b] ND, not determined.
[c] Extrapolated to 25 °C.
[d] Determined in 40 mM MOPS, pH 6.9, containing 0.9% NaCl, 10 μM FeEnt, and no detergent, 0 °C.



SCHEME 1. **Kinetic model for the binding of ligands to FepA.** The equilibria represent a minimal kinetic binding model, derived from *in vivo* and *in vitro* binding data.

(Scheme 1). In DM the initial, fast binding component was similar for both ligands (approximately 0.02 s^{-1}), and the secondary, slow component was about 10-fold faster for FeEnt (2.1×10^{-3} s^{-1}) than ColB (3×10^{-4} s^{-1}). In Triton X-100 the binding of both ligands was also biphasic, but the initial stages were slower (approximately 0.006 s^{-1}), and we did not observe rate differences in the second stages of FeEnt and ColB binding (Table I). These data suggest a detergent effect on the binding reaction (see also below).

The mutual exclusion of one ligand by the binding of the other allowed us to measure their dissociation rates from FepA. In contrast to the biphasic association kinetics observed for FeEnt and ColB, their dissociation rates in the presence of the other followed single-component first order decays. The release of the siderophore and the binding of the colicin, or vice versa, may follow one of two pathways. In a microscopically reversible model, the two-stage FeEnt binding pathway may simply revert to release the siderophore and free FepA for ColB binding. In this case the rate constant for release of FeEnt in the presence of ColB (k_9) reflects the reversal of the steps leading to FeEnt binding (k_4 and k_2) followed by the forward progress of ColB binding (k_5 and k_7). Then k_9 reflects the rate-limiting step among k_4, k_2, k_5, and k_7. If the ligand exchange reaction proceeds along this sequential pathway, then the individual steps must occur as rapidly as the overall dissociation rate (*i.e.* k_4, k_2, k_5, and k_7 must be $\geq k_9$). This condition was met in Triton X-100, but not in DM (k_7 was $\ll k_9$). A possible explanation for this difference is that in DM the rapid release of FeEnt from FepA (k_9) results from an active solubilization of the siderophore off the surface of the receptor by the detergent. For release of ColB in the presence of FeEnt, k_{10} reflects the rate-limiting step among k_8, k_6, k_1, and k_3. In this case the individual steps occurred as rapidly as the observed dissociation rate (k_6, k_8, k_1, and k_3 were $> k_{10}$), in both DM and Triton X-100. Thus, the microscopically reversible pathway accounts for the dissociation kinetics of both ligands in Triton X-100, and the release of ColB in DM, and is the most likely explanation of the observed dissociation kinetics in both detergents. An alternative mechanism for the rapid release of FeEnt in the presence of ColB involves the binding of the toxin to FepA·FeEnt, creating a ternary complex (FepA·FeEnt·ColB) that dislodges the siderophore. Our data tends to exclude this mechanism, because no ColB binding was seen in the presence of FeEnt. Neither did we obtain evidence of a ternary complex *in vivo*, in [^{59}Fe]Ent binding experiments in the presence of saturating ColB (data not shown). Thus, our results are most consistent with full release of one ligand before binding of another.

One or more intermediate states must exist in the transport mechanism of a ligand-gated channel like FepA, in which the solute initially binds to an external region of the receptor. Our results show the existence of a second phase in the initial adsorption stage, that is energy- and TonB-independent, and precedes the TonB-dependent internalization of ligands. The likely explanation is that a conformational change occurs in FepA as a second step of binding, as previously suggested by site-directed spin labeling of FepA *in vitro* (12). Such an intermediate complex also explains the discrepancy between the K_d of FeEnt binding (20 nM) and the K_m of FeEnt transport (200 nM) (10); when an initial rapid binding occurs, followed by other intermediate steps, the K_m deviates from the K_d according to the rate constants for formation and collapse of the intermediates. In subsequent stages the conformation of FepA surface loops change to "open" the receptor, and the ligand enters the underlying channel (6). This internalization reaction, which may itself contain numerous distinct steps, ultimately requires energy expenditure and the participation of TonB to reach completion.

Like the siderophore, transport of the colicin occurs in two stages: binding to the cell surface, and passage through the OM bilayer. Again, our data address the energy-independent, biphasic first stage of adsorption to FepA. The steps after binding remain ill defined. FepA·ColB dissociates more slowly than FepA·FeEnt; this slow dissociating complex likely results from conformational changes in the receptor and/or the ligand that hold the proteins together. The progression of ligand binding to

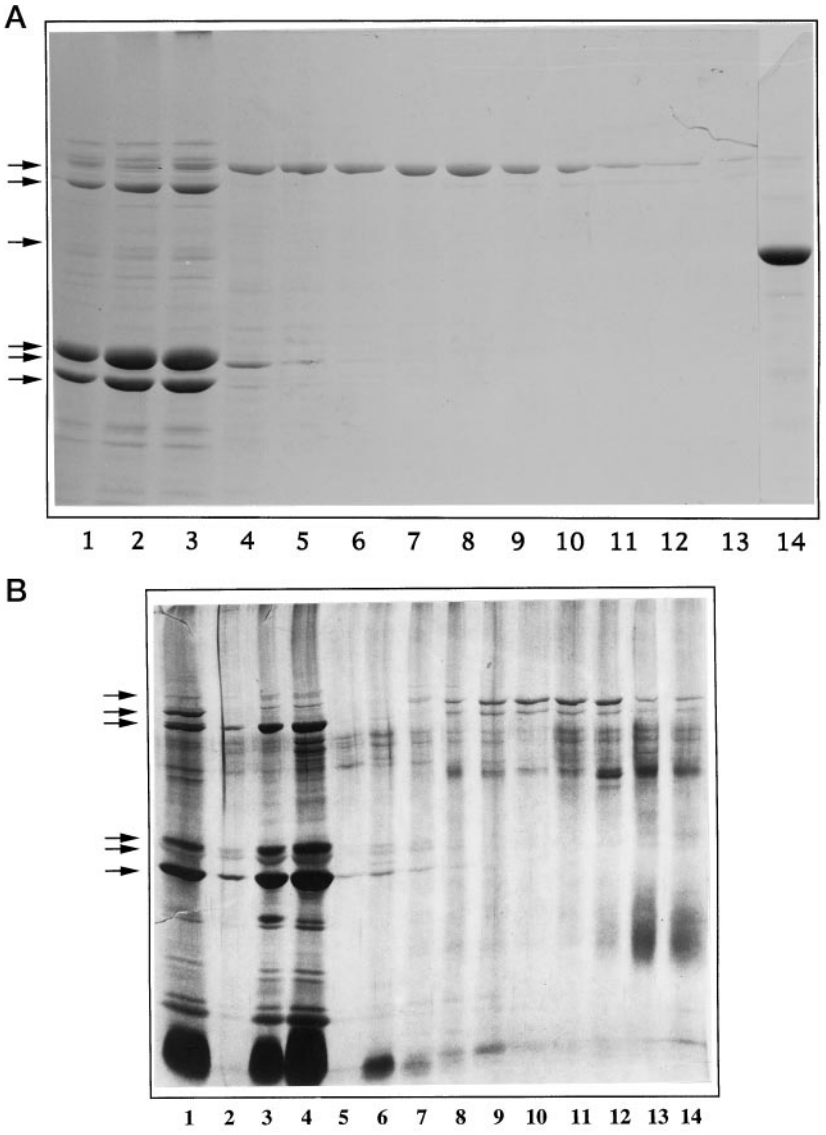


FIG. 7. **SDS-PAGE of affinity purification of FepA.** *A*, affinity chromatography of crude FepA on ColB-Sepharose. OM fractions from KDF541 (4)/pFepAE280C were solubilized with TTE buffer (*lane 1*) and adsorbed to ColB-Sepharose in TTE. The column was washed with 10 volumes of buffer (*lanes 2* and *3*) and eluted with a gradient of 0.0 to 2.5 M NaCl in TTE (*lanes 4–13*). Purified ColB (*lane 14*) was utilized for preparation of ColB-Sepharose, and ^{125}I-ColB. *B*, affinity chromatography of crude FepA on rabbit α-FepA-Sepharose. OM fractions from BN1071 (*fepA+*) (9) were solubilized with TTE (*lane 1*) and adsorbed to the resin. The column was washed (*lanes 2–4*) and eluted with a gradient of 0.0 to 2.0 M neutralized trichloroacetic acid in TTE (*lanes 5–14*). *Arrows* mark the positions, in descending order, of FepA, 81K*, Cir, OmpF/C, and OmpA.

this tight complex did not require TonB or other OM components. Subsequent, TonB-dependent conformational changes in FepA likely trigger uptake after binding, by initiating colicin passage through the FepA channel (6).

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