BioMetals 1995, **8**, 129–136

Binding characterization of the iron transport receptor from the outer membrane of *Escherichia coli* (FepA): differentiation between FepA and FecA

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Received 25 May 1994; accepted for publication 23 June 1994

The dissociation constants for the binding of ferric enterobactin with FepA and FecA are quantitated with displacement experiments. It is found that K_d for FepA is 12 times lower than the one for FecA. This indicates that FepA is an high-affinity receptor while FecA binds ferric enterobactin with a lower affinity. Monoclonal antibodies specific for binding epitopes of FepA inhibit the binding of ferric enterobactin with purified FepA. These same antibodies do not inhibit the binding of ferric enterobactin with purified FecA. This indicates that the binding epitopes in FecA and FepA are different.

Keywords: binding, FecA, FepA, iron, membrane proteins, transport receptors

Introduction

The outer membrane of Gram-negative organisms is composed of lipopolysaccharide, protein and phospholipid. For *Escherichia coli*, there are at least two mechanisms, both involving outer membrane proteins, by which small molecules are transported across the membrane. Hydrophilic molecules with molecular weights of 600 or less diffuse into the periplasm through gated non-specific porin pores (Decad & Nikaido 1976), whereas the transport of other solutes, such as vitamin B12 (DiMasi *et al.* 1973) and iron–siderophore complexes (Hancock *et al.* 1976), involves specific outer membrane receptor proteins.

In response to iron deprivation, *E. coli* produces the siderophore enterobactin and induces the expression of several outer membrane receptor proteins that function in the transport of ferric siderophores (Neilands *et al.* 1987). One of these, FepA, facilitates the uptake of ferric enterobactin. This has been shown by *in vivo* transport studies (McIntosh *et al.* 1979, Neilands *et al.* 1980, Ozenberger *et al.* 1987) and by *in vitro* binding experiments (Hollifield & Neilands 1978, Fiss *et al.* 1982, Zhou *et al.* 1993) using partially purified or purified FepA receptor proteins. The binding sites of FepA for ferric enterobactin have been mapped by using different monoclonal antibodies (Murphy *et al.* 1990). A report by Rutz *et al.* (1992), using deletion mutagenesis techniques, shows that FepA, without the peptide recognizing the ferric siderophore, is not capable of high-affinity binding and, instead, forms a non-specific passive channel in the outer membrane.

FecA is another outer membrane receptor in *E. coli* which transports iron citrate from the environment into the cell periplasm (Wagegg & Braun 1981). This has been proven by *in vivo* transport studies using different mutant strains (Frost & Rosenberg 1973, Wagegg & Braun 1981). However, the binding properties of FecA are not clearly identified. In our earlier report (Zhou *et al.* 1993), the results of the binding behavior of FepA and FecA with ferric enterobactin appeared to be similar, although weaker for FecA. In the earlier report FecA was separated from FepA with difficulty due to the fact that both need to be solubilized in detergent while both proteins have closely similar physical properties. The purity of FecA was determined by N-sequence determination and no contamination was found. However, possible contamination by FepA would not easily be found, because the N-terminus of FepA is glutamine, blocking the sequencing of that protein. Therefore, in the second report (Zhou & van der Helm 1993), the *fepA*$^-$ mutant cell line UT5600 was used to produce FecA, and its binding behavior and specificity with ferric enterobactin was confirmed but with a lower affinity than was found in the earlier experiments (Zhou *et*

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al. 1993). It also was found that UT5600 showed uptake for ferric enterobactin at a lower rate than would be expected for cells producing FepA.

It seems therefore important to quantitate the binding behavior of FepA and FecA. Also, since the binding of FepA with ferric enterobactin is stronger than that of FecA, it is important to eliminate any possible FepA contamination in the FecA preparation.

Materials and methods

Materials

[^{55}Fe]$FeCl_3$, specific activity 40–45 mCi mg^{-1}, in 0.5 M HCl was from Dupont NEN (Boston, MA); DE-52 (DEAE-cellulose) from Whatman biosystems (Maidstone, Kent, UK); MonoP, Superose-12, Sephadex G-10, Sephadex G-25, Sephadex LH-20 and polybuffer 74 from Pharmacia LKB (Sollentura, Sweden); PVDF membranes (Immobilon transfer), 0.45 μm pore size, from Millipore (Bedford, MA). Ascitic fluids containing monoclonal antibody 12, 34, 44 and 45, respectively, were kindly provided by Dr P. Klebba, University of Oklahoma.

Bacterial strains, media and growth conditions

E. coli strains UT5600/pBB2 (*leu*$^-$, *proC*$^-$, *trpE*$^-$, *rpsL*$^-$, *entA*$^-$, Δ(*ompT-fepA*$^-$/*Ampr*, *fepA*)) and UT5600 (*leu*$^-$, *proC*$^-$, *trpE*$^-$, *rpsL*$^-$, *entA*$^-$, Δ(*ompT-fepA*)$^-$) were kindly provided by Dr M. D. Lundrigan, University of Mississippi Medical Center, Jackson, MS. *E. coli* strains AN102 and AN311 were provided by Dr J.B. Neilands, University of California at Berkeley and Dr G. Winkelmann, University of Tubingen, Germany, respectively.

Strain UT5600/pBB2 was grown in a trypton/yeast extract (TY) medium containing ampicillin to an optical density at 660 nm of 0.300 at 37 °C. The cell suspension in TY medium was stored frozen at −70 °C in 1 ml quantities as a 30% solution in glycerol until ready to use. Strain UT5600 was grown in the same medium, but with streptomycin and without ampicillin.

Strain 5600/pBB2 was used to produce FepA. Thawed cells at room temperature were inoculated in 50 ml of TY medium and grown at 37 °C to an OD of 0.15 at 660 nm. The cell suspension was transferred to 1000 ml of minimal growth medium in a 2 l Fernbach flask. The TY medium contained (per liter of distilled water): 10.5 g K_2HPO_4; 4.5 g KH_2PO_4; 1.0 g $(NH_4)_2SO_4$; 0.4 mg $FeSO_4$; 0.4 mg ampicillin; 0.25 g $MgSO_4 \cdot 7H_2O$; 0.01% L-proline; 0.01% L-leucine; 0.01% L-tryptophan; 0.2% glucose; 25 mg thiamine-HCl. Ampicillin was sterilized by filtering through a 0.2 mm disposable filter assembly. Other media components were prepared separately and sterilized by autoclaving. Cells were grown in the minimal medium at 37 °C and shaken at 200 r.p.m. for 16–18 h after which the cells were chilled to 4 °C with ice and harvested by centrifugation. The cells were washed once with buffer A (50 mM Tris-HCl buffer containing 10 mM benzamidine with pH of 7.5).

For FecA production, thawed *E. coli* UT5600 cells at room temperature were inoculated in 50 ml of TY medium and grown at 37 °C to an OD of 0.15 at 660 nm. The cell suspension was transferred to 1000 ml of minimal growth medium and grown at 37 °C for 16–18 h. Minimal culture medium contained (per liter of distilled water): 10.5 g KH_2PO_4; 4.5 g K_2HPO_4; 1.0 g $(NH_4)_2SO_4$; 0.5 g sodium citrate (dihydrate), 0.25 g $MgSO_4 \cdot 7H_2O$; 0.01% L-proline; 0.01% L-leucine; 0.01% L-tryptophan; 0.2% glucose; 25 mg thiamine-HCl (vitamin B1) and 20 mg streptomycin.

Preparation of crude extract

Procedure for the preparation of crude extract for FepA from UT5600/pBB2 and FecA from UT5600 was similar to that reported in our earlier paper (Zhou *et al.* 1993).

FepA purification

The FepA solution was dialyzed against buffer B (25 mM Tris-HCl buffer containing 10 mM benzamidine, 1% Triton X-100, pH 7.5), loaded to an FPLC anion-exchange column (Whatman DE-52, 1 × 28 cm) and washed with 500 ml of buffer B. Proteins were eluted at 0.4 ml min^{-1} with 700 min FPLC gradient from 0 to 10% (200 min), 10 to 20% (100 min), 20% (200 min), 20 to 40% (100 min) and then 40% (100 min) of 0.5 M NaCl in buffer B. Fractions containing FepA were pooled, dialyzed and rechromatographed on an anion-exchange column using the same procedure. The FepA fractions were then pooled and dialyzed against buffer C (25 mM Bis-Tris, 1% Triton X-100, 5 mM Benzamidine, pH 7.1 adjusted by saturated iminodiacetic acid). The partially purified FepA was applied to a Pharmacia MonoP column (5/20) pre-equilibrated with buffer C. FepA was eluted with buffer D (10% of polybuffer 74; 1% Triton X-100, 5 mM benzamidine, pH 4.0, adjusted by saturated iminodiacetic acid). For further purification of FepA and polybuffer removal, a FPLC-gel filtration column, Superose-12, pre-equilibrated with buffer E (25 mM Tris-HCl, 5 mM Benzamidine, 1.5 mM NaN_3, 1 mM EDTA-Na_2, pH 7.5) was used. The purity of FepA was examined by 7% SDS-PAGE by loading samples of various concentrations.

FecA purification

Procedure adopted for the purification of FecA was similar to that of FepA. The purity of purified FecA was determined by N-terminal sequence determination and 7% SDS-PAGE, respectively.

SDS–PAGE

SDS–PAGE was performed on 10 and 7% polyacrylamide slab gels, respectively, by the Laemmli method (Laemmli 1970). Coomassie brilliant blue R-250 was used for staining.

N-terminal sequence determination of receptor

This was performed by the previously reported modification (Zhou & van der Helm 1993) of Matsudaira (1987). Each purified protein sample was run for seven cycles for identification of the receptor proteins.

Protein determination

Protein content was determined by BCA method (Shihabi & Dyer 1988). Bovine serum albumin was used for the standard.

Preparation and purification of enterobactin

Enterobactin was prepared and purified using the procedure described before (Zhou *et al.* 1993).

Preparation and purification of ferric enterobactin

Purified enterobactin was dissolved in 80% methanol. The concentration of ligand was determined spectrophotometrically using molar absorbance at 319 nm (11 200) (Peterson & Neilands 1979). Ferric chloride was added in the molar ratio of 0.8. The pH of ferric enterobactin was carefully adjusted to 8 with KOH. The reaction mixture was stirred for a few minutes and the solution evaporated under vacuum. The residue was dissolved in a small amount of 20% methanol. The complexes were purified by high-voltage paper electrophoresis (Ecker *et al.* 1986) using 0.1 M HEPES buffer (pH 7.8) and eluted from the paper with distilled water. Labeled complexes were prepared similarly with [^{55}Fe] ferric chloride. The purity of ferric enterobactin was determined by silica gel thin layer chromatography using chloroform:methanol (6:5 v/v). The ferric complexes were freshly prepared for binding measurements.

Assay of receptor binding activity

Procedure for the assay of receptor binding activity of FepA used was the same as reported earlier (Zhou *et al.* 1993). Purified receptor and purified labeled ferric enterobactin were introduced to a reaction buffer (0.1 M Tris–HCl buffer with a pH of 7.4 containing 1% Triton X-100) in a total volume of 100 μl. A Sephadex G-25 column in a 1 ml hypodermic syringe was used to isolate the receptor ligand complex from the free ligand. Fourteen 120 μl fractions were collected following the application of the reaction mixture to the column and stepwise elution with 100 μl portion of reaction buffer. Blank control experiments were performed by determining the elution profile of [^{55}Fe]ferric enterobactin in a reaction mixture without protein. Radioactivity was measured by counting 60 μl aliquots of elution from each fraction in 4 ml of Eculome in a Beckmann liquid scintillation counter (Model LS5801).

Determination of the effects of monoclonal antibody on receptor binding to ferric enterobactin

The receptor protein (26 μg), FepA or FecA, various monoclonal antibodies (60 μl of 100-fold dilution of ascites containing monoclonal antibody), and labeled ferric enterobactin (25 000 d.p.m.) were added to a reaction buffer (0.1 M Tris–HCl buffer, containing 1% Triton X-100, pH 7.4) in a total volume of 140 μl. The reaction solution was incubated at room temperature for 30 min. [^{55}Fe]ferric enterobactin–receptor complex was isolated from free [^{55}Fe]ferric enterobactin by a 1 ml Sephadex G-25 column. The radioactive profile was determined by counting 60 ml aliquots of elution from each fraction in 4 ml of Eculome in a Beckmann liquid scintillation counter.

Binding parameter determination

Binding parameters of FepA and FecA receptors to ferric enterobactin were determined by the displacement method. The typical binding assay consisted of incubating a mixture of the receptor protein (24 μg), non-labeled ferric enterobactin and labeled ferric enterobactin in a total volume of 100 μl of 0.1 M Tris–HCl buffer containing 1% Triton X-100 at pH 7.4 at room temperature for 30 min. Due to the high affinity of FepA to ferric enterobactin, low concentrations ranging from 0.05 to 2.5 μM of the non-labeled siderophore were used. Two concentrations, 50 and 105 nM, of the labeled ferric enterobactin, were used in the binding parameter determination for FepA. For the low-affinity receptor, FecA, a high concentration range of ferric enterobactin from 0.5 to 20 μM was used. In experiments to determine FecA binding constant, all mixtures contained 50 nM [^{55}Fe]ferric enterobactin. The data were analyzed by the method of Akera & Cheng (1977).

Results

Elimination of FepA from wild-type E. coli *in FecA preparation*

fepA$^-$ Strain UT5600 is a strain derived from RW193 (McIntosh *et al.* 1979), while the genotype of RW193 was demonstrated as *pro*$^-$, *leu*$^-$, *trp*$^-$, *lac*$^-$, *str*^{r}, *tonA*$^-$, *entA* . Therefore, the UT5600 cells are resistant to streptomycin while wild-type cells are not. Using streptomycin for the production of UT5600, therefore, eliminates contamination of FecA by FepA from spurious wild-type *E. coli*.

Elimination of FecA from FepA preparation

Previously (Zhou *et al.* 1993), a citrate containing medium was used in the growth of UT5600/pBB2 to overproduce FepA. The citrate induced contaminating chromosomal

FecA which was difficult to separate from FepA. A growth medium without citrate, as used in the present study, produces neglible amounts of FecA. During the solubilization of FepA a hydrolytic product is produced which is separated from FepA by anion exchange chromatography and chromatofocusing.

Accuracy of the membrane protein assay

In our purification, 1–2% of Triton X-100 was used for solubilization of the membrane receptors. The detergent makes an accurate determination of protein content difficult using the Lowry method, even while using deoxycholate to reduce the effects of the detergent. Instead, in this study the BCA method (Shihabi & Dyer 1988) was employed to assay the protein content. This method yields results which are not affected by the detergent.

Binding characterization of FepA

Figure 1(A) shows a typical elution profile of FepA with [^{55}Fe]ferric enterobactin using a 1 ml-Sephadex G-25 column. The sharp peak of FepA–[^{55}Fe]ferric enterobactin complex disappeared when a 100-fold higher concentration of cold ferric enterobactin was added into the reaction solution containing [^{55}Fe]ferric enterobactin and FepA (Figure 1B). The obvious self-competition shows that the binding of FepA with the siderophore is specific.

Several years ago a number of monoclonal antibodies (mAbs) were raised against FepA which were used to identify the regions of the protein on cell surface, that are involved in interaction with its ligand, ferric enterobactin (Murphy *et al.* 1990). Several of these as ascite solutions were made available to us for the present experiments. It was concluded (Murphy *et al.* 1990) that mAb-44 recognized the binding site of FepA (residues from 290 to 339)

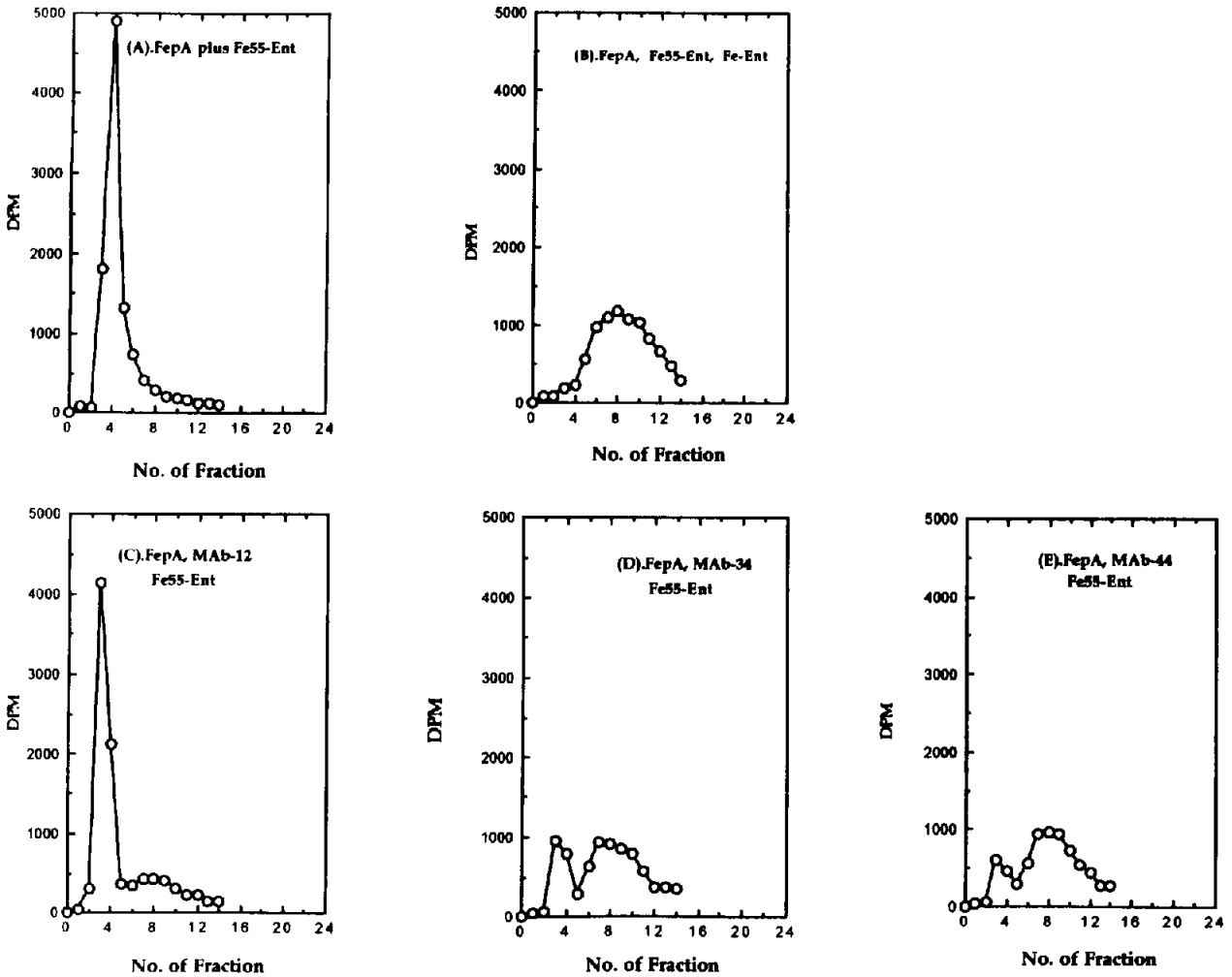

Figure 1. Determination of the effects of monoclonal antibodies on FepA binding to ferric enterobactin: (**A**) [^{55}Fe]enterobactin plus purified FepA; (**B**) [^{55}Fe]enterobactin plus purified FepA mixed with cold ferric enterobactin; (**C**) purified FepA plus mAb-12 mixed with [^{55}Fe]enterobactin; (**D**) purified FepA plus mAb-34 mixed with [^{55}Fe]enterobactin; (**E**) purified FepA plus mAb-44 mixed with [^{55}Fe]enterobactin. Full details are given in Results.

and that mAb-34 recognized the site immediately adjacent (residues 258–290). mAb-12 is a control that binds to an epitope which is buried. Figure 1(C) shows that mAb-12 does not affect the binding of [^{55}Fe]ferric enterobactin, while Figure 1(D and E) shows significant inhibition of this binding when either mAb-34 or -44 are present in the incubation mixture.

Binding characterization of FecA

Figure 2 shows the same experiments with FecA. Figure 2(A) shows the binding between FecA and [^{55}Fe]ferric enterobactin. The binding is weaker than for FepA (Figure 2A). Also in the case of FecA, however, the binding with [^{55}Fe]ferric enterobactin is specific (Figure 2B). In contrast with FepA, neither mAb-34 nor mAb-44 inhibits the binding of [^{55}Fe]ferric enterobactin with FecA (Figure 2D and E).

Estimation of the binding constant (K_d) by the displacement method

Figure 3 shows the results from the typical displacement binding experiments. When 105 and 50 nM concentrations of [^{55}Fe]ferric enterobactin were used to incubate a mixture of 24 μg FepA together with various concentrations of non-labeled ferric enterobactin (0–2.5 μM), the non-labeled ligand concentrations at which a 50% maximal specific binding activity was observed ($C_{0.5}$) for FepA were estimated to be 0.42 and 0.37 μM, respectively (Table 1). The dissociation constant

$$K_d = C_{0.5} - a$$

(Akera & Cheng 1977) in which a is the initial concentration of labeled ligand can therefore be calculated to be 0.32 μM for both experiments for the binding of ferric enterobactin with purified FepA receptor protein.

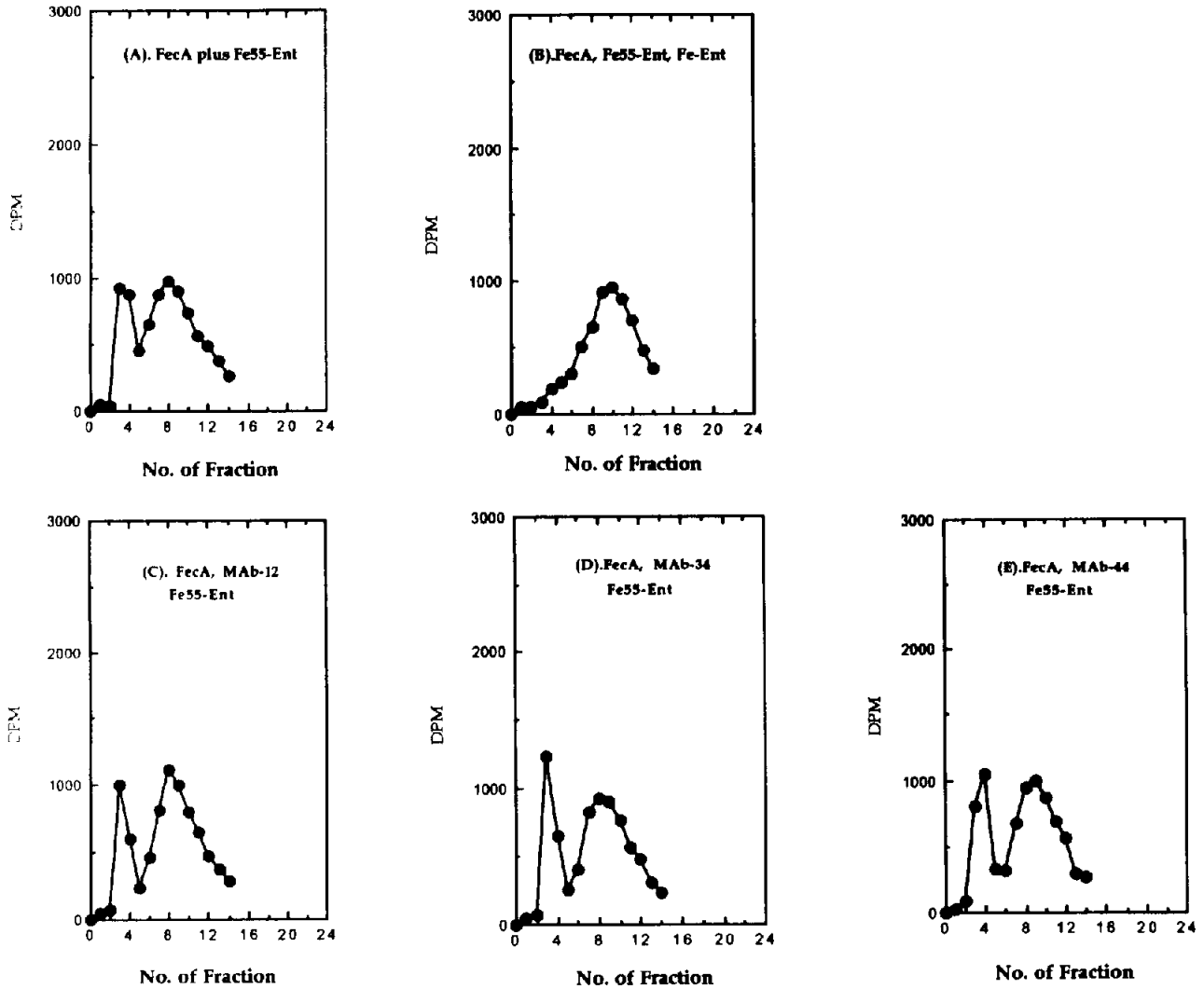

Figure 2. Determination of the effects of monoclonal antibodies on FecA binding to ferric enterobactin: (**A**) [^{55}Fe]enterobactin plus purified FecA; (**B**) [^{55}Fe]enterobactin plus purified FecA mixed with cold ferric enterobactin; (**C**) purified FecA plus mAb-12 mixed with [^{55}Fe]enterobactin; (**D**) purified FecA plus mAb-34 mixed with [^{55}Fe]enterobactin; (**E**) purified FecA plus mAb-44 mixed with [^{55}Fe]enterobactin. Full details are given in Results.

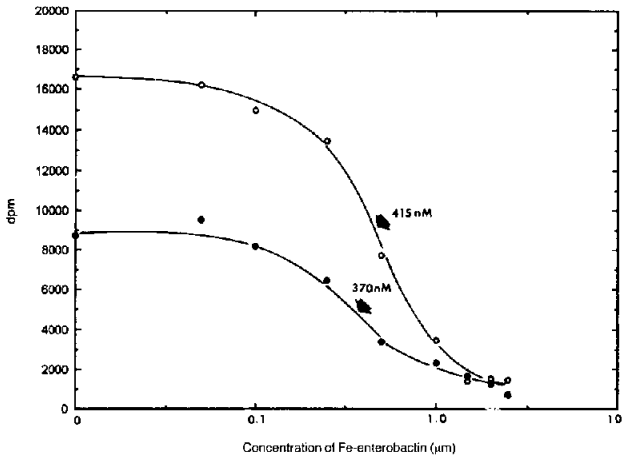

Figure 3. Estimation of the binding constant (K_d) of FepA by the displacement method.

Table 1. Determination of K_d values of FepA by the replacement method

Protein	$C_{0.5}$ (nM)	L (nM)	K_d (μM)
FepA	370	50	0.32
FepA	415	105	0.31
FecA	4000	105	3.9

$C_{0.5}$, the concentration of non-labeled ferric enterobactin which produces a 50% inhibition of labeled siderophore binding; a the concentration of the labeled drug used for replacement binding; K_d, $K_d = C_{0.5} - L$; L, the concentration of radiolabeled ligand.

The same procedure was repeated with FecA but with different concentrations of non-labeled ferric enterobactin (0–20 μM). The non-labeled ferric enterobactin concentration at which a 50% maximal specific binding activity was observed for FecA was estimated to be 4.0 μM (Figure 4) from which the apparent dissociation constant (K_d) for FecA with ferric enterobactin was calculated to be 3.9 μM

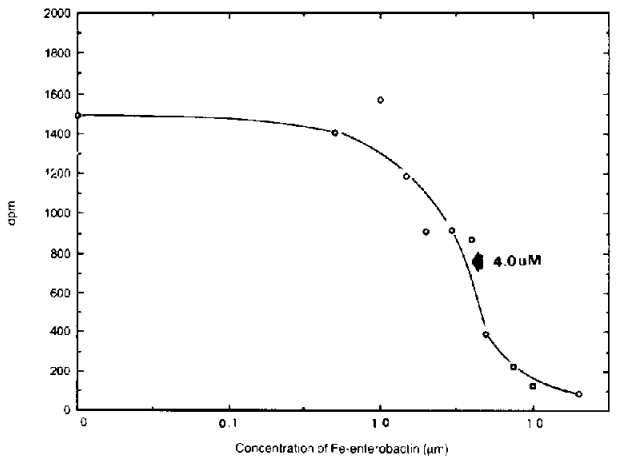

Figure 4. Determination of the FecA binding constant to ferric enterobactin by the displacement method.

(Table 1). Because of the low binding constant, no reliable data were obtained when 50 nM of labeled ferric enterobactin was used for the displacement study with the FecA receptor.

Discussion

Both outer membrane receptor proteins FepA and FecA are produced when citrate is added to the growth medium of UT5600/pBB$_2$ (Zhou *et al.* 1993). The separation of FepA and FecA is difficult due to the fact that both proteins have similar molecular weights (Lundrigan & Kadner 1986, Pressler *et al.* 1988) and similar isoelectric points (Fiss *et al.* 1988, Jalal & van der Helm 1989, Zhou & van der Helm 1993). In addition using N-terminal sequencing to prove the purity of FecA and absence of FepA is not a proper indication in this particular case because glutamine is at the N-terminus of FepA (Lundrigan & Kadner 1986), preventing N-sequencing due to cyclization. Possible contamination of FecA by FepA will, therefore, not be readily detected. Also, if the binding constant of FecA for ferric enterobactin is considerably lower than the one for FepA with this siderophore (*vide infra*) and FecA is produced by chromatographic separation from FepA, a small amount of contaminating FepA, not noticeable by N-sequence determination, can affect the observed binding behavior of FecA. This is the probable reason for the qualitative observation (Zhou *et al.* 1993) that FecA binding with ferric enterobactin was about 50% of that for FepA with that ligand. This possible problem was recognized and FecA was, therefore, produced from the *fepA* mutant, UT5600, in which FecA production was induced by citrate addition to the medium (Zhou & van der Helm 1993). Any contamination by FepA from wild-type *E. coli* was prevented by the addition of streptomycin to the growth medium of UT5600. The BCA method was used to assay protein content since detergents interfere with the Lowry method. The purity of FepA was improved by eliminating citrate from the growth medium for this plasmid, preventing simultaneous FecA production from UT5600/pBB2. Freshly prepared [^{55}Fe]ferric enterobactin and the unlabeled complex were used because of the instability of the compound. These precautions allowed us to quantitate the binding of ferric enterobactin with both FepA and FecA.

A displacement method was used for receptor binding parameter determination. When 24 μg of purified FepA and FecA are incubated with 105 nM of [^{55}Fe]ferric enterobactin, a significant difference in the specific binding activity for ferric enterobactin is found between these two receptor proteins. The K_d for FepA is determined to be 0.32 μM using two different concentrations of [^{55}Fe]ferric enterobactin. The K_d value for FecA is determined to be 3.9 μM. This is 12 times larger than the K_d value for FepA, indicating that FepA has more than 10 times higher binding activity with ferric enterobactin than FecA.

The K_d values for FepA obtained by the displacement method in this report (0.32 μM) are comparable to those

determined for whole *E. coli* RW-193 cells (0.2 μM) by Ecker *et al.* (1986) and the value determined by Rutz *et al.* (1992) using wild-type *E. coli* RWB 18-60 cells (0.2 μM). Our data are also in agreement with the value estimated by Hollifield & Neilands (1978) (0.2–0.5 μM) for a Triton X-100 solubilized, partially purified FepA from *E. coli*. They are, however, in conflict with the binding parameter for purified FepA from *E. coli* BN 3040 (10 nM) obtained by the same research group 4 years later (Fiss *et al.* 1982). No explanation for this difference can be given at this moment.

Monoclonal antibodies raised against FepA have been shown to identify the binding regions in the FepA receptor protein for ferric enterobactin (Murphy *et al.* 1990). MAb-44 and -34 recognize the binding site for ferric enterobactin and the peptide adjacent to this binding site, respectively. In order to qualitatively differentiate binding characteristics of FepA from FecA, these monoclonal antibodies are used in our studies. It is found that these monoclonal antibodies significantly block the binding of FepA with [^{55}Fe]ferric enterobactin. However, these agents (MAb-34 and -44) are not effective in blocking the binding of FecA to [^{55}Fe]ferric enterobactin. This suggests that the molecular characteristics of the FecA binding site for ferric enterobactin are significantly different from those of FepA.

The results confirm our earlier reports about the specific binding of ferric enterobactin with FepA (Zhou *et al.* 1993) and FecA (Zhou & van der Helm 1993). The specific binding of FepA to ferric enterobactin can be differentiated from that of FecA by K_d estimation. K_d values demonstrate that FepA is a high-affinity receptor for ferric enterobactin while FecA binds ferric enterobactin with a relatively low affinity (about 12 times lower). When ferric enterobactin is used as the sole iron source, UT5600 shows uptake of the ferric siderophore, although at a considerable lower rate, compared with UT5600/pBB2 (Zhou & van der Helm 1993). Since UT5600 strain is a *fepA*$^-$ mutant, there must be another receptor protein on the membrane surface of the cells, allowing ferric enterobactin transport at a relatively low rate. This protein most likely is FecA. Our evidence also is supported by previous results. Mutant UT2300 (*fepA* , *ent*$^-$), a derivative from RW193 (*ent*$^-$), shows specific uptake of [^{55}Fe]ferric enterobactin (McIntosh *et al.* 1978) at a low transport rate, while no iron transport is observed when the media contains [^{55}Fe]$FeCl_3$ and nitrilotriacetic acid without enterobactin. This indicates that FecA may play a different role in ferric enterobactin uptake from that of FepA. FecA has a low-affinity constant for ferric enterobactin, although the binding is specific. It may imply that ferric enterobactin is not the best ligand for FecA. On the other hand, it was demonstrated in our earlier report that the binding of FecA with ferric enterobactin can be enhanced by ferric citrate. This indicates that ferric citrate does not compete with the binding site of FecA with ferric enterobactin. It will be interesting to further characterize the binding and transport characteristics of FecA by physical methods.

Acknowledgments

This work was supported by NIH grant GM21822. The authors wish to thank Dr P. Klebba for advice and donation of the ascite solutions of monoclonal antibodies and to Marjorie Montague for excellent technical assistance.

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