Iron, an essential nutrient for the growth of bacteria, serves as a cofactor for enzymes, as a redox center in electron carriers such as cytochromes and iron-sulfur proteins, and as a global regulator of many cellular biosynthetic and metabolic systems. However, iron is initially inaccessible to bacteria in their natural environments. Within animal fluids and tissues, proteins such as transferrin, lactoferrin, or ferritin sequester iron, while ural environments. Within animal fluids and tissues, proteins such as transferrin, lactoferrin, or ferritin sequester iron, while

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**Binding of Ferric Enterobactin by the *Escherichia coli* Periplasmic Protein FepB**

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The periplasmic protein FepB of *Escherichia coli* is a component of the ferric enterobactin transport system. We overexpressed and purified the binding protein 23-fold from periplasmic extracts by ammonium sulfate precipitation and chromatographic methods, with a yield of 20%, to a final specific activity of 15,500 pmol of ferric enterobactin bound/mg. Periplasmic fluid from cells overexpressing the binding protein adsorbed catecholate ferric siderophores with high affinity: in a gel filtration chromatography assay the $K_d$ of the ferric enterobactin-FepB binding reaction was approximately 135 nM. Intrinsic fluorescence measurements of binding by the purified protein, which were more accurate, showed higher affinity for both ferric enterobactin ($K_d = 30$ nM) and ferric enantiomer enterobactin ($K_d = 15$ nM), the left-handed stereoisomer of the natural *E. coli* siderophore. Purified FepB also adsorbed the apo-siderophore, enterobactin, with comparable affinity ($K_d = 60$ nM) but did not bind ferric agrobactin. Polyclonal rabbit antisera and mouse monoclonal antibodies raised against nearly homogeneous preparations of FepB specifically recognized it in solid-phase immunoassays. These sera enabled the measurement of the FepB concentration in vivo when expressed from the chromosome (4,000 copies/cell) or from multicopy plasmids (>100,000 copies/cell). Overexpression of the binding protein did not enhance the overall affinity or rate of ferric enterobactin transport, supporting the conclusion that the rate-limiting step of ferric siderophore uptake through the cell envelope is passage through the outer membrane.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. E. coli strains BNI071 (49), KDF541 (49), and BL21(DE3) (Novagen) carry the chromosomal fepB* genes, the latter strain carries a chromosomally encoded 17 kDa polymerase under the control of the isopropy-l-β-D-thiogalactoside (IPTG)-inducible lacUV5 promoter. DK24 (35) (provided by C. F. Earhart) is fepB. Plasmid PME13-18 (6) (provided by C. F. Earhart) carries the wild-type E. coli fepB gene under its natural promoter. p72 (provided by M. A. McIntosh) carries fepB* under 17 promoter control, which allowed us to regulate its expression from the plasmid in strain BL21(DE3) by manipulating the concentration of IPTG in the culture medium. pB3 and pB51 are multicopy plasmids that also carry fepB* under the control of its natural promoter. To create them, we PCR amplified fepB and its upstream flanking region from p72 and inserted the product into pUC18 and PHS398 (48), respectively, using BamHI and SalI sites. Both of these clones eliminated the small open reading frame (orf) immediately upstream of fepB and its promoter (33, 34). We sequenced both constructs to verify the integrity of the fepB promoter region and structural gene, using an ALF-Express automated DNA sequenceer (Pharmacia). pTFS449 is a pUC18 derivative that carries wild-type fepA (31).

Bacteria were grown in Luria-Bertani broth (24) and in some experiments were subcultured at 1% to T (22) or morpholinepropanesulfonic acid (MOPS) (27) minimal medium. The cultures were shaken at 37°C with vigorous aeration and induced with 10 (mM IPTG in 900-ml LB aliquots in Fernbach flasks to ensure adequate aeration. All purification procedures were performed at 4°C. The osmotic shock procedure was appropriately scaled to the larger volume of cell suspension, and FepB was precipitated from the periplasmic extracts with a 45 to 80% ammonium sulfate cut. After dialysis against 10 mM Tris (pH 7.4), the protein solution was loaded onto a DE-52 anion-exchange column in the same buffer and eluted with a gradient of 0 to 0.5 M NaCl. FepB eluted at approximate 0.5 M NaCl. After 10 min on ice, the cells were pelleted by centrifugation at 5,000 g for 20 min, and the supernatant, containing the periplasmic fluid, was decanted, not pipetted, into a new tube. The procedure was repeated to maximize the yield.

FepB purification. BL21(DE3) p72 (13.5 liters) was grown to mid-log phase and induced with 10 mM IPTG in 900-ml LB aliquots in Fernbach flasks to ensure adequate aeration. After 10 min on ice, the cells were pelleted by centrifugation at 5,000 g for 20 min, and the supernatant, containing the periplasmic fluid, was decanted, not pipetted, into a new tube. The procedure was repeated to maximize the yield.

Gel filtration of FepB on Sephacryl S-100 HR, with appropriate protein standards, showed a molecular mass of 33.8 kDa. Rf measurements for purified FepB from SDS-PAGE (Fig. 1) also estimated its molecular mass as 33.8 kDa, suggesting that its native form is monomeric. SDS-PAGE also revealed a second major periplasmic protein in BL21/p72, of 31.5 kDa, that reacted with anti-FepB sera in immunoblots. The N-terminal 15 amino acids of the 33.5- and 31.5-kDa protein bands were identical to each other and to those of the deduced primary structure of the mature protein (33, 34). The first amino acid of mature FepB, which corresponds to A27 in the deduced primary structure of the mature protein (33, 34).

RESULTS

Purification of FepB. The binding protein in periplasmic fluid from BL21(DE3)/p72 precipitated over a broad range of ammonium sulfate concentrations, from 45 to 80%. After dialysis of the precipitate in 10 mM Tris (pH 7.4), the protein solution was loaded onto a 80-ml DE-52 anion-exchange column (5.7 by 22 cm). The column was washed with 5 volumes of 10 mM Tris (pH 7.4) and eluted with a linear gradient of NaCl. Fractions containing FeEnt binding activity were pooled and concentrated by ammonium sulfate precipitation, and samples with the highest specific activity were fractionated on a column of Sephacryl S-100 HR (1.3 by 117 cm) in TBS (pH 7.4). The purest fractions were pooled and stored (Fig. 1). This procedure resulted in 23-fold purification of FepB: the 9 mg we obtained from 75 g of wet cell paste had a specific activity of 15,500 pmol of 59FeEnt bound/mg (Table 1), a stoichiometry of approximately 0.5.

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not derive from the presence of FepA, because similar FepA contamination occurred in extracts from the \textit{fepB} strain DK214, which did not measurably bind FeEnt in the assay.

**Affinity of purified FepB for catecholate ferric siderophores.** Aliquots of periplasmic fluid containing overexpressed FepB were mixed with different concentrations of $^{59}$FeEnt and chromatographed on Sephadex LH20. The counts for the first peak were summed and denoted as bound ligand. The summed counts for the second peak, which contained unbound $^{59}$FeEnt, were denoted as free ligand. From these data, we determined the ratio of bound ligand to free ligand at equilibrium (Fig. 3a). Analysis of binding data from crude FepB, in periplasmic extracts, produced an apparent $K_d$ of 124 $\pm$ 17 nM; analogous experiments with purified FepB yielded a $K_d$ of 145 $\pm$ 29 nM.

Intrinsic fluorescence spectroscopy of the four tryptophans in FepB (residues 29, 89, 152, and 209) provided another measure of the affinity of the interaction with FeEnt. Binding of the ferric siderophore did not shift the excitation or emission maxima of purified FepB (Fig. 1, pooled fractions 15 to 21; 90% 33.8-kDa band), suggesting that the tryptophans did not experience any significant change in environment. However, saturation with FeEnt reduced the fluorescence intensity of FepB by approximately 70%. The concentration dependence of this decrease showed a midpoint ($K_d$) at 29 $\pm$ 1.4 nM (Fig. 3b), roughly fivefold lower than that observed in the column assay. The discrepancy between the two measurements probably derives from the much shorter time frame of the spectroscopic method, which increases the accuracy of the bound/free ratio determinations (see Discussion).

Saturation of FepB with ferric enantioenterobactin (FeEnEnt) decreased the fluorescence intensity of the binding protein 75%, slightly more than that observed during the binding of FeEnt. The midpoint of this transition reflected a $K_d$ of 14.5 $\pm$ 1 nM. Again, the excitation and emission maxima did not change. Comparable experiments with the apo-siderophore enterobactin showed that it also bound to FepA with high affinity ($K_d = 60$ nM). However, ferric agrobactin did not specifically adsorb to FepB. Whereas enterobactin, FeEnt, and FeEnEnt engendered a $>$70% decrease in the intrinsic fluorescence of FepB at saturation (0.2 $\mu$M), ferric agrobactin did not manifest saturation binding (Fig. 3b). Its addition to solutions of FepB only marginally changed the fluorescence of the binding protein (<15% at concentrations up to 6 $\mu$M), presumably as a result

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total activity (U) $^a$</th>
<th>Amt of protein (mg/ml)</th>
<th>Sp act (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periplasmic fluid</td>
<td>500.0</td>
<td>687,000</td>
<td>2.0</td>
<td>687</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>20</td>
<td>321,000</td>
<td>7.1</td>
<td>2,260</td>
<td>3.3</td>
<td>47</td>
</tr>
<tr>
<td>DE-52 chromatography</td>
<td>20.5</td>
<td>149,000</td>
<td>0.9</td>
<td>8,075</td>
<td>11.8</td>
<td>22</td>
</tr>
<tr>
<td>Sephacryl S-100 chromatography</td>
<td>11.2</td>
<td>139,000</td>
<td>0.8</td>
<td>15,513</td>
<td>22.6</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$ Activity units are arbitrary, based on the binding of $^{59}$FeEnt measured by the column chromatography assay.

**TABLE 1. Purification of FepB**
of collisional quenching. Thus, FepB did not recognize ferric agroactin.

**Anti-FepB sera.** We raised polyclonal antisera against purified FepB in rabbits and monoclonal antibodies to it in mice. Western blots with the rabbit anti-FepB confirmed the absence of the binding protein in the fepB strain DK214 and its presence in the fepB⁻ (chromosomal or plasmid) strains (Fig. 4). FepB expressed from the chromosome or from low-copy-number plasmids was difficult to visualize in Coomassie blue-stained SDS-PAGE gels.

Monoclonal antibodies from the 37 anti-FepB hybridomas that we raised, purified, and subeluted reacted with FepB in an enzyme-linked immunosorbent assay and with denatured FepB in Western blots. Protein A did not react with any of the MAbs; we used the polyclonal rabbit antisera for quantitation of FepB expression.

**Effect of FepB overexpression on ⁵⁹FeEnt uptake.** Using the variety of fepB-containing plasmids, we expressed the binding protein at various levels (Fig. 4) and measured the effects on ⁵⁹FeEnt uptake (Table 2). Although FepB was necessary for transport of the ferric siderophore, variations in its concentration did not affect either the overall affinity or the rate of ⁵⁹FeEnt uptake. This was best seen in ⁵⁹FeEnt uptake experiments by strains expressing FepB from multicopy plasmids (pIB3, pIB51, and p72). These constructions produced FepB at 9- to 35-fold-higher levels than those resulting from chromosomal expression by the natural promoter, with little effect on the rate of ⁵⁹FeEnt transport (Table 2, Fig. 4). Furthermore, we compared the concentrations of chromosomal fepB in three different strains (KDF541/pITS449, BN1071, and BL21) and plasmid-mediated fepB in two different backgrounds (DK214 and BL21). In Table 2 the ⁵⁹FeEnt binding data and also the colicin susceptibility measured the affinity and amount of FepA in the outer membrane (from Kₒ and capacity, and percent killing, respectively), and we observed some variation of this parameter in the different strains. The transport data characterized the overall affinity and rate of the uptake process into the cytoplasm (from Kₒ and V_max, respectively). The comparison of FepB expression levels and ⁵⁹FeEnt uptake rates in the different strains demonstrated that variations in the periplasmic expression of FepB in Western blots. Protein A did not react with any of the MAbs; we used the polyclonal rabbit antisera for quantitation of FepB expression.

**Table 2.** ⁵⁹FeEnt uptake by strains with chromosome- or plasmid-encoded FepB.

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>KDF541/pITS449</td>
<td>0.09</td>
<td>111</td>
<td>0.3</td>
<td>181</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>BN1071</td>
<td>0.103</td>
<td>27.6</td>
<td>0.829</td>
<td>120</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>DK214</td>
<td>0.938</td>
<td>11.4</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td></td>
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<tr>
<td>DK214/pIB3</td>
<td>0.584</td>
<td>11.6</td>
<td>1.45</td>
<td>83</td>
<td>2</td>
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<tr>
<td>DK214/pIB51</td>
<td>0.896</td>
<td>27.3</td>
<td>1.5</td>
<td>179</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>BL21</td>
<td>1.2</td>
<td>77.6</td>
<td>0.817</td>
<td>127</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>BL21/p72</td>
<td>0.924</td>
<td>67.3</td>
<td>0.957</td>
<td>140</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>BL21/p72 + IPTG</td>
<td>0.538</td>
<td>83.2</td>
<td>0.711</td>
<td>116</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Kₒ (nanomolar) was determined by measurement of ⁵⁹FeEnt binding to bacteria at 0°C. All kinetic and thermodynamic constants were obtained by analysis of the data with Grafit 4 (Erithacus Software Ltd., London, United Kingdom).
* ⁵⁹FeEnt binding capacity (picomoles/10⁹ cells).
* K_max (nanomolar) was determined for the ⁵⁹FeEnt uptake reaction into live bacteria at 37°C.
* V_max (picomoles/10⁹ cells/minute) was determined from ⁵⁹FeEnt uptake measurements.
* Colicin B or D killing is expressed relative to that of KDF541/pITS449 (24), which contains chromosomal FepB and multicopy plasmid-encoded FepA (pUC18 derivative).
* ND, not determined.
mic concentration of FepB did not significantly change the velocity of $^{59}$FeEnt transport. For example, in the isogenic series of BL21, an approximately 10-fold increase in FepB concentration did not alter the $V_{\text{max}}$ of $^{59}$FeEnt uptake. Instead, the transport rate depended on the amount of FepA in the outer membrane, as established by the isogenic pair KDF541/pITS449 and BN1071, which have the same amount of FepB (chromosomal level) but different amounts of FepA. These results support the idea that passage through the outer membrane is the rate-limiting step of $^{59}$FeEnt transit through the cell envelope. Furthermore, from the chromosomal expression results, we calculated that each bacterial cell contains approximately 3,800 FepB proteins. The multicopy plasmids produced as many as 135,000 copies per cell (p72 plus IPTG).

**DISCUSSION**

The native structure of the FepB protein may resemble that of other gram-negative bacterial periplasmic binding proteins, including those of the sugar (MalE [43], MgtB [50], RbsB [25], and AraF [9]), amino acid (HisJ [32], LivJ [40], LivK [38], and GltP [12]), and iron (3) transport systems: a double-lobed kidney bean that closes around the ligand during binding. However, the FeEnt-FepB system differs from other binding protein-dependent transport systems in several ways. First, FepB and another iron binding periplasmic protein of E. coli, FhuD (4, 17) are synthesized at low levels relative to sugar (MalE [16]) and amino acid (HisJ [45]) binding proteins and the iron binding protein Fbp from *Haemophilus influenzae* (10). Even when derepressed by iron starvation, chromosomally encoded FepB was not detected in Coomassie blue-stained gels of periplasmic extracts. We needed overexpression to see FepB in SDS-PAGE, and similar findings were reported for FhuD (17, 18).

Second, the affinity of FepB for its ligand is apparently greater than that of other characterized periplasmic binding proteins. The $K_a$ of the FeEnt-FepB interaction lies in the nanomolar range, while those of FhuD (37) and sugar or amino acid binding proteins (MalE [47] and HisJ [51]) are micromolar. It is most relevant to address this difference in light of the similar fluorescence measurements that were recorded for MalE, FhuD, and FepB and in relationship to the nature of the outer membrane components of the three transport systems. The comparison suggests that in specific porin-mediated transport, the affinity of the outer membrane protein (LamB: $K_a = 2 \mu M$ [15, 46, 47]) for the sugar maltose is comparable to that of the periplasmic protein (MalE: $K_a = 1 \mu M$ [47]), whereas in the ligand-gated porin systems, the affinity of the outer membrane protein (FepA: $K_a = 0.1 \mu M$ [31]; FhuA: $K_a = 50$ to $100 \mu M$ [19]) for the siderophore considerably exceeds that of the periplasmic protein (FepB: $K_a = 30 \mu M$ [this study]; FhuD: $K_a = 1 \mu M$ [37]). Thus, specific porin and ligand-gated porin transport occur with fundamentally different parameters. The low-affinity nature of maltose uptake means that the carbon source must reach high external concentrations to achieve uptake into the periplasm. In this case, in which solute efflux may occur through the open maltoporin channel, equilibration across the outer membrane creates high periplasmic concentrations of the sugar, which are appropriately matched by the micromolar $K_a$ of the MalE binding reaction. In ferric siderophore transport systems, on the other hand, efflux of the solute apparently does not occur, because of the gating of transport through the TonB- and energy-dependent outer membrane receptor protein. However, ferric siderophore uptake systems accumulate iron from very low external concentrations, and under such conditions the periplasmic concentration of the solute may not reach micromolar levels, hence the need for a binding protein with higher affinity. In both transport systems, the binding proteins function with sufficient affinity to create a periplasmic pool of bound solute; this complex presumably is the substrate for the inner membrane permease system.

Our results also show differences between the ferric catecholate binding protein, FepB, and the ferric hydroxamate binding protein, FhuD. The latter functions in the transport of ferrichrome, aerobactin, and coprogen. All three siderophores protect FhuD from degradation by proteinase K (17) and have measurable affinity for His-tagged FhuD (37). The broad specificity and lower affinity of FhuD may stem from the lack of a deep cleft in its tertiary structure (5). Conversely, FepB exclusively bound ferric enterobactin (natural or enantiol); it did not accept the relatively similar catecholate ferric agrobactin. Other experiments suggest that FepB does not recognize ferric vibriobactin, a catecholate siderophore with structural similarity to ferric agrobactin (52).

The existence of multiple electrophoretic forms of FepB represents a third difference from sugar and amino acid binding proteins. Like others (6), we observed three FepB isoforms, of 36.5, 33.8, and 31.5 kDa. However, they were only apparent when FepB was heavily overexpressed (Fig. 4): normal production from the chromosome produced a homogeneous band of 33.8 kDa. The 31.5- and 33.8-kDa polypeptides had identical N termini, refuting the possibility of different signal peptidase cleavage sites. Thus, the 33.8-kDa band is not pro-FepB (predicted from the sequence as 34.3 kDa). Rather, the 36.5-kDa band (6) is probably unprocessed pro-FepB. The 33.8-kDa protein which was the major FepB isoform both in vivo and after purification, may contain a posttranslational modification with lipid (for a review, see reference 39), as is found for other FeEnt binding proteins, CueE of *Campylobacter jejuni* and ViuP of *Vibrio cholerae* (52). FepB does not contain the most common lipid attachment site (Cys in the motif LLAAC [11, 48]); in fact, the protein does not contain cysteine, and so if lipidation does occur, it takes place at a novel site. The 31.5-kDa band probably represents mature, nonposttranslationally modified FepB (predicted to be 31.6 kDa), which appears under overexpression conditions because such high concentrations of the binding protein overload the lipidation system. Unlike FepB, FhuD has only a proform and a mature form (17, 18), and so lipidation is apparently not a requisite feature of binding proteins associated with TonB-dependent transport systems.

The Sephadex LH20 chromatography assay independently demonstrated binding between FeEnt and FepB, but it was of limited value for quantitative affinity determinations, because of the time required to perform the experiment. Assuming a simple equilibrium with adsorption of FeEnt to FepB at the diffusion limit, a $K_a$ of 30 nM predicts a dissociation half-life for FeEnt-FepB of <1 s. Therefore, as the complex traverses the resin, some of the FeEnt will release from it and fractionate away from the bound peak, causing an underestimation of the equilibrium concentration of FeEnt-FepB. This explains the lower estimate of affinity: the $K_a$ of 130 nM that we obtained from the chromatographic method suggests that about 75% of the FeEnt that initially adsorbed to FepB dissociated and separated from the binding protein during chromatography.

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