A Site-Directed Spin-Labeling Study of Ligand-Induced Conformational Change in the Ferric Enterobactin Receptor, FepA†

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ABSTRACT: The ferric enterobactin receptor, FepA, is a TonB-dependent gated porin that transports the siderophore ferric enterobactin across the outer membrane of gram-negative bacteria. We have created two site-directed mutants of *Escherichia coli* FepA, in both cases introducing a cysteine residue into the putative ligand-binding domain. The introduced cysteines were then modified with nitroxide spin labels for structural and dynamic studies using electron spin resonance (ESR) spectroscopy. The mutants were fully functional, as indicated by their ability to grow under iron-limiting conditions, their uptake of [^{59}Fe]enterobactin, and their sensitivity to colicin B. Labeling of the mutant FepA receptors proceeded easily upon incubation with sulfhydryl-specific spin labels, e.g. MTSL, (1-oxy-2,2,5,5-tetramethylpyrrolidin-3-yl)methyl methanethiosulfonate. In contrast, spin labeling of the two native cysteines (Cys486 and Cys493) within wild-type FepA occurred only after treatment with a thiol reducing agent and partial denaturation in urea, suggesting that the native cysteines are disulfide-linked. ESR spectra showed a high degree of motional restriction for all three sites. Continuous wave (CW) saturation studies indicated that one of the mutationally introduced sites (Cys280) was surface-localized as evidenced by its exposure to the aqueous paramagnetic relaxation agent chromium oxalate and its low accessibility to O_2. The other (Cys310) apparently occupies a site near the membrane/aqueous interface. The native cysteines occupy a site tightly packed within the protein structure with low accessibility to both CROX and O_2. A shift in both conventional and saturation-transfer ESR spectra of MTSL-labeled E280C and E310C (but not MTSL-labeled wild type) FepA was observed upon addition of ferric enterobactin. The ESR spectral shift was dependent on ferric enterobactin concentration and did not occur with siderophores not recognized by FepA. Ferric enterobactin binding did not alter the CW saturation properties of MTSL bound to these sites, but did influence their accessibility to O_2. These results provide consistent evidence for a ligand-specific conformational change in the surface peptides of FepA upon the binding of ferric enterobactin.

Under the usual physiological conditions of low iron availability, gram-negative bacteria synthesize and secrete a group of low molecular weight compounds, called siderophores, that bind iron with high affinity (Neilands, 1981). These molecules are too large (M_r = 600−1200) to pass through the porin channels of the bacterial outer membrane (OM)[1] and, therefore, require specific receptors to facilitate their passage across the OM and into the periplasm [reviewed by Nikaido (1992), Postle (1990), Kadner (1990), Braun *et al.* (1991), and Klebba *et al.* (1993)]. The ability to acquire iron through these receptors can be an important factor in the development of pathogenicity, since iron is one factor that limits bacterial growth during infection (Williams, 1979; Lemos *et al.*, 1988). The catecholate ligand enterobactin is the primary siderophore of *Escherichia coli* (Pollack & Neilands, 1970) and many other related enteric bacteria (Rutz *et al.*, 1991). The ferric enterobactin receptor, FepA, is a prototype of ligand-specific siderophore receptors (Murphy *et al.*, 1990; Rutz *et al.*, 1992; Killmann *et al.*, 1993; Liu *et al.*, 1993). FepA is also the receptor of colicins B and D (Guterman & Dann, 1973; Wayne *et al.*, 1976). FepA-mediated uptake of ferric enterobactin through the OM requires the function of another cell envelope protein, TonB (Wang & Newton, 1971), a periplasmic protein, FepB (Pierce & Earhart, 1988), and other cytoplasmic membrane proteins (Shea & McIntosh, 1991). TonB acts in the transport of all ferric siderophores through their OM receptors as well as the uptake of vitamin B_{12} (Postle, 1990; Kadner, 1990; Braun *et al.*, 1991), but the molecular mechanism of its action is not fully understood.

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[1] Abbreviations: CW, continuous wave; CROX, chromium oxalate; DOPC, 1,2-dioleoyl-*sn-glycero*-3-phosphatidylcholine; DTT, dithiothreitol; EPG, egg L-α-phosphatidyl-DL-glycerol; ESR, electron spin resonance; FeEnt, ferric enterobactin; Glu, glutamate; LDS−PAGE, lithium dodecyl sulfate−polyacrylamide gel electrophoresis; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MTSL, (1-oxy-2,2,5,5-tetramethylpyrrolidine-3-yl)methyl methanethiosulfonate; OM, outer membrane; $P_{1/2}$, half-saturation power; PCR, polymerase chain reaction; SDSL, site-directed spin labeling; TE buffer, 50 mM Tris/5 mM EDTA, pH 7.2; TSB, trypticase soy broth; TTE, TE buffer plus 2% (v/v) Triton X-100; wt, wild type.

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Siderophore receptors are TonB-dependent gated porins (Rutz *et al.*, 1992; Killmann *et al.*, 1993; Liu *et al.*, 1993). These receptors bind ligands within a cell surface domain and ostensibly open in response to interaction with TonB to release the ligand into an underlying hydrophilic channel. Deletion of the ligand-binding domain of FepA converted the high-affinity receptor into a nonspecific, passive diffusion channel that allowed uptake of solutes in a TonB-independent fashion (Rutz *et al.*, 1992). The diameter of the channel domain in FepA was defined *in vitro* with liposome-swelling assays as approximately 20 Å, with an estimated exclusion limit of 1200 DA, about twice as large as that of OmpF (Liu *et al.*, 1993). A similar hydrophilic, closed channel was found in another siderophore receptor, the ferrichrome receptor, FhuA (Killmann *et al.*, 1993), and likely exists in the family of TonB-dependent OM receptors.

Since FepA binds ferric enterobactin with high affinity, a subsequent conformational change may be an essential part of the siderophore receptor transport mechanism (Bradbeer, 1991). Such a conformational change may concomitantly release and internalize ferric enterobactin through the OM *via* the underlying channel (Klebba *et al.*, 1993). Furthermore, since there is an apparent physical interaction between TonB and siderophore receptors (Heller *et al.*, 1988; Heller & Kadner, 1985; Hannavy *et al.*, 1990), conformational change may initiate interaction between TonB and FepA. Finally, the bipartite domain character of TonB-dependent receptors also raises the question of whether such putative conformational changes are restricted to specific regions of the protein or are more global in nature. The notion of conformational change is a unifying theme of all models of TonB-dependent transport, yet no experimental evidence exists to substantiate such a mechanism, and systems in which such changes may be examined are not readily available.

The electron spin resonance (ESR) spin-labeling technique is a sensitive method for examining protein structure and dynamics [Altenbach *et al.*, 1990; Shin *et al.*, 1993; Farahbakhsh *et al.*, 1993; for general reviews, see Berliner (1976, 1978, 1989)]. The recently developed site-directed spin labeling (SDSL) (Altenbach *et al.*, 1989a), in which site-directed mutagenesis or solid-phase synthesis is used to introduce cysteine labeling sites at desired locations, has greatly expanded the applicability of the spin-labeling technique [reviewed by Millhauser (1992)]. Furthermore, each mutation is useful, either as a direct result of inferences that can be made from its effects on receptor function or, if effects on function are minimal, as a relatively benign site for the attachment of a physical probe. In this work, we utilize SDSL to examine structural and dynamic properties of sites within the putative ligand-binding domain of FepA and to evaluate the environment of the native downstream cysteines in FepA primary structure. Our results indicate that FepA residues 280 and 310 (selectively spin labeled with SH-specific nitroxide following site-directed Glu to Cys substitutions) are surface-localized and that the native cysteines of FepA (residues 486 and 493) form a disulfide bond and occupy a tightly packed region of the protein structure. Upon binding of ferric enterobactin to purified FepA, the local region near residues 280 and 310 undergoes a conformational change. This is the first dynamic demonstration of a ligand-induced conformational change in a TonB-dependent OM receptor.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains. *E. Coli* K-12 strain KDF541 (*entA*, *fepA*; Rutz *et al.*, 1992) was used as the host for all plasmids. pITSE280C and pITSE310C are derivatives of pITS449 (*fepA*$^+$; Murphy *et al.*, 1990) carrying the *fepAE280C* and *fepAE310C* alleles, which substitute Cys for Glu at residues 280 and 310, respectively.

Reagents. 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) and egg L-α-phosphatidyl-DL-glycerol (EPG) were obtained from Avanti Polar Lipids (Alabaster, AL). Ferrichrome and ferric enterobactin were purified as described previously (Murphy *et al.*, 1990). The methanethiosulfonate spin label (MTSL) [(1-oxy-2,2,5,5,-tetramethylpyrrolidin-3-yl)methyl methanethiosulfonate] was obtained from Renal (Budapest, Hungary). Dithiothreitol (DTT) and chromium oxalate (CROX) (potassium trioxalatochromate) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Single-stranded oligonucleotides were synthesized at the Protein and Nucleic Acid Core Facility of the Medical College of Wisconsin. Restriction enzymes *Mlu*I and *Cla*I, and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA).

Methods

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by a modification of the polymerase chain reaction (PCR) method of Ho *et al.* (1989). The initial reaction was accomplished with a primer encoding the desired mutation on the sense strand and an external primer that annealed to the nonsense strand and encompassed an upstream restriction site that was subsequently used to insert the mutant fragment into *fepA*. This product was PCR amplified and isolated on a 0.6% acrylamide gel. The purified PCR product was then used as a "megaprimer" in a second reaction with an additional downstream external primer annealing to a second, downstream restriction site (C. K. Murphy, personal communication). PCR amplification resulted in production of the desired full-length mutant product. Use of the megaprimer in this way circumvents the need for purification of the intermediate PCR products of the four-primer method introduced previously (Ho *et al.*, 1989) and in our hands was essential to the success of the four-primer method.

Plasmid pITS449 was utilized as the template for PCR mutagenesis. External primers encompassed the *Mlu*I and *Cla*I sites of the *fepA* gene. The mutant primers were $^{5'}$GACGGTTGGTGCAATCGCCATA$^{3'}$ [for mutation Glu280 → Cys (E280C)] and $^{5'}$ACGGGTGTGGCAGTACTGCAC$^{3'}$ for [Glu310 → Cys (E310C)]. The full-length PCR amplified product described above was digested with *Mlu*I, ethanol precipitated, digested with *Cla*I, ethanol precipitated, and used in the ligation reaction. pITS449 was similarly digested, and the fragment of interest was isolated on a 0.9% agarose gel, purified with a Gene Clean kit (Bio101), and ligated with the PCR mutagenized insert using T4 DNA ligase. The ligation mixture was transformed into KDF541 (Cohen *et al.*, 1972). Plasmid DNA was isolated from ampicillin-resistant clones by alkaline lysis (Kraft *et al.*, 1988) and analyzed on 0.9% agarose gels. Plasmids with the correct mobility were chosen for further analysis by dideoxy chain termination sequencing (Sequenase) using primers that anneal

approximately 20 bases upstream and downstream of the PCR-amplified region.

Protein Purification. For protein purification, bacteria were grown to late exponential phase in MOPS medium (Neidhardt *et al.*, 1974) containing 100 μg/mL ampicillin, 0.4% glucose, and 0.2% casamino acids. FepA and its mutant derivatives E280C and E310C were purified as described previously by differential extraction of OM with Triton X-100 (Fiss *et al.*, 1982). Fractions containing FepA were identified by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on 11% slab gels stained with Coomassie blue. Protein concentrations were determined according to Lowry (1951), in the presence of 2% SDS. FepA, E280C, and E310C were more than 95% pure as estimated by densitometric analysis of Coomassie blue-stained gels.

Spin-Labeling Procedures. Wild-type (wt) FepA did not label with SH-specific spin labels without prior treatment with a thiol reducing agent (e.g., DTT) and partial denaturation by urea. Therefore, FepA (0.1 mM) in 50 mM Tris-HCL, pH = 7.2, 5 mM EDTA, and 2% Triton X-100 (TTE) was first incubated with 6 M urea and 50 mM DDT at 37 °C for 30 min and then at room temperature for 1.5 h. The protein was precipitated with 2 vol of ice-cold 95% ethanol at −20 °C for 2 h, collected by centrifugation, and resuspended in TTE containing 6 M urea and 2 mM MTSL at 4 °C for 8 h. After extensive dialysis (48 h, six buffer changes) to remove urea and unbound spin label, a majority of the protein refolded into its native, compact conformation, as indicated by its relative mobility on non-denaturing lithium dodecyl sulfate (LDS)−polyacrylamide gels (Figure 1; Liu *et al.*, 1993).

Spin labeling of FepA mutant proteins E280C and E310C was accomplished by incubating 0.1 mM protein in TTE buffer with 2 mM MTSL at 4 °C for 8 h. Urea and DTT were not added. Excess free spin label was removed by dialysis, as above.

Liposome Reconstitution. Spin-labeled FepA was reconstituted into liposomes by a method previously established for liposome-swelling experiments (Nikaido *et al.*, 1991; Liu *et al.*, 1993). A thin film of DOPC (2.25 μmol) and EPG (0.25 μmol) was dried onto a glass tube under a soft stream of N_2 and incubated for 10 h *in vacuo* at 25 °C. Approximately 1 mg of spin-labeled protein in 50 mM Tris-HCL, pH = 7.2, and 5 mM EDTA (TE) buffer was added to the lipid film and vortexed to homogeneity. This suspension was dried under vacuum at room temperature. The lipid/protein film was finally hydrated with 100 μL of TE buffer at 37 °C for 30 min and shaken vigorously by hand. In some experiments, CROX (50 mM) was included in the final hydration buffer at the expense of an equimolar amount of NaCl.

Ferric Enterobactin Binding Assay. Two methods were used to determine the binding affinity of FepA and mutant FepA receptors for ferric enterobactin. For *in vivo* determinations (Rutz *et al.*, 1992; Murphy *et al.*, 1990), cells were grown for approximately 8 h in MOPS medium with ampicillin to late exponential phase. To deplete their energy stores, bacteria were collected and suspended at 1×10^9 cells/ml in MOPS medium without glucose, shaken for 2 h at 37 °C, and placed on ice. Bacteria (0.5 mL) were then incubated for 30 min with [^{59}Fe]enterobactin at the desired concentrations (0.1−2 μM), separated by centrifugation, and washed twice with 0.5 mL of MOPS media without glucose. Bound radioactivity was determined by counting the cell pellet on a γ scintillation counter (Packard).

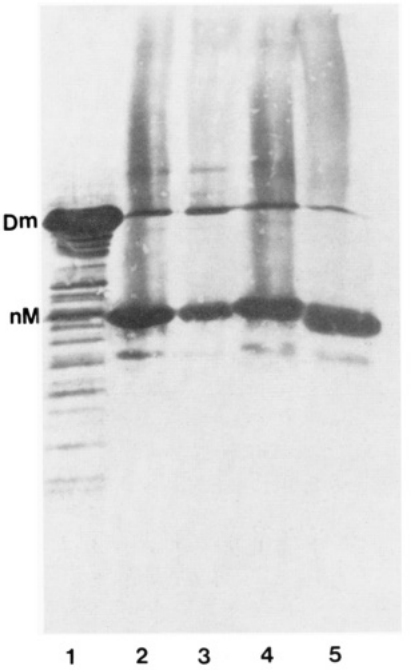

FIGURE 1: LDS−PAGE analysis of protein vesicles. Purified wt FepA (30 μg) was subjected to LDS−PAGE at 2 °C (Liu *et al.*, 1993), electrophoretically transfered to nitrocellulose, and immunoblotted with anti-FepA MAb 29 (Murphy *et al.*, 1989). Lane 1, heat-denatured FepA in 2% Triton X-100; lane 2, non-denatured FepA in Triton; lane 3, protein that was first partially denatured by urea (4 M) and then ethanol precepitated; lane 4, non-denatured, spin-labeled FepA in Triton; lane 5, non-denatured, spin-labeled FepA after reconstitution into phospholipid liposomes. The positions of native FepA monomer (nM) and denatured FepA monomer (Dm) are indicated. Note that the percentage of unfolded FepA in liposome (lane 5) is less than that in detergent (lane 4). FepA degradation products are seen in lane 1.

For purified proteins, ferric enterobactin binding was assessed by equilibrium dialysis (Cantor *et al.*, 1980). Typically, 0.1 mg of protein was dialyzed against 100 mL of TTE buffer containing [^{59}Fe]enterobactin at concentrations ranging from 0.1 to 5 μM for 48 h at 4 °C. Radioactivity retained within the dialysis bag and in the buffer were measured, and K_d was determined from Scatchard plots.

Ferric Enterobactin Nutrition Assay. Bacteria were grown overnight in LB broth, and 10^8 cells from this culture were diluted into 2 mL of TSB top agar containing 25 μg/mL ampicillin and 100 μM deferriferrichrome A to induce iron starvation (Wayne *et al.*, 1976). Filter discs (5 mm diameter) containing 2 μL of 10 μM freshly prepared ferric enterobactin or ferrichrome were placed on top of the agar and were scored for growth as determined by a halo of cell growth around the disc after 8−16-h incubations at 37 °C.

Electron Spin Resonance (ESR) Spectroscopy. ESR measurements were made on a Varian E-109 spectrometer (Varian Associates Inc., Palo Alto, CA) operating at X-band, fitted with a two-loop−one-gap resonator (Froncisz & Hyde, 1982) and a Varian field-frequency lock. Data acquisition was controlled by a PC interfaced to the spectrometer utilizing the Viking software package (C. C. Felix, National Biomedical ESR Center, Milwaukee, WI). Samples were contained in a gas-permeable TPX plastic sample capillary (Popp & Hyde, 1981) with an active volume of ap-

Table 1: Functional Activity of the FepA Transport System[a]

strain/plasmid	growth on Fe(enterobactin)	growth on Fe(ferrichrome)	colicin B susceptibility
RWB18-60 (*fepA*)	−	+	−
KDF541/pITS449	+	−	+
KDF541/E280C	+	−	+
KDF541/E310C	+	−	+
KDF571/E280C (*tonB*)	−	−	−
KDF571/E310C (*tonB*)	−	−	−

[a] Strains RWB18-60 (Rutz *et al.*, 1992), KDF541 with plasmids, and KDF571 with plasmids (Rutz *et al.*, 1992) were assayed for growth with ferric enterobactin or ferrichrome as the sole source of iron and for susceptibility to killing by colicin B as described in the text. All strains exhibited similar concentration dependencies.

proximately 2 μL. Spin label concentration was determined by double integration of spectra and comparison to a standard solution, using the SUMSPC92 software package (National Biomedical ESR Center, Milwaukee, WI).

Conventional ESR spectra were recorded at 0.5 mW incident microwave power, with a 100-kHz magnetic field modulation of 1.5 gauss. When performing continuous wave power saturation experiments were performed, the sample in TPX was continuously purged either with nitrogen gas or with a N_2/O_2 mixture containing the desired concentration of O_2. The $M_I = 0$ resonance line was scanned over 10−20 G as a function of microwave power in the range of 0.1−40 mW. Peak-to-peak amplitudes of the first-derivative line were measured and plotted against the square root of microwave power, and $P_{1/2}$ values were determined as the intersection of the experimental curve with a straight line having one-half the initial slope of the saturation curve (Subczynski and Hyde, 1981; Altenbach *et al.* 1989; e.g., see Figure 4).

Saturation-transfer ESR (STESR) spectra were recorded in the second-harmonic, 90° out of phase, absorption mode (V_2' display) at a modulation frequency of 50 kHz and a modulation amplitude of 5 G. The phase was set by the self-null method at a subsaturating microwave power level of 0.5 mW (Thomas *et al.*, 1976).

RESULTS

Functional Analysis of FepA Mutants. To determine the effects of the E280C and E310C mutations on FepA physiology, we examined the ability of the altered receptors to function in siderophore nutrition assays, colicin B sensitivity tests (Murphy *et al.*, 1990), [^{59}Fe]enterobactin uptake measurements *in vivo*, and [^{59}Fe] enterobactin binding determinations *in vivo* and *in vitro*. Nutrition assays with *E. coli* strain KDF541 (*fepA, tonA, cir*) containing pITS449 (*fepA*$^+$), pITSE280C, or pITSE310C indicated that the mutant receptors utilized ferric enterobactin for growth under conditions of low iron availability. The same bacteria displayed wild-type sensitivity to colicin B (Table 1). Ferrichrome as the sole iron source did not support growth of KDF541 (*tonA*) either with or without the plasmids of interest, indicating that the mutant receptors were not in a permanently "open" channel state. These data confirm the specificity of the transport through FepA. Similarly, ferric enterobactin uptake was TonB-dependent (Table 1), demonstrating the requirement for functional interaction between TonB and the mutant FepA receptors. The ability of the mutant receptors to assimilate ferric enterobactin and interact correctly with TonB indicated that the site-directed changes have not caused global alterations in FepA structure. This was further substantiated cytofluorimetrically using MAbs that bind the surface epitopes of wt FepA (Murphy *et al.*, 1990). These antibodies also recognized the two site-directed FepA mutants (Table 2).

Quantitative assessment of receptor function, by binding and uptake of [^{59}Fe]enterobactin, indicated that, for both the E310C and the E280C mutant, binding affinity relative to that of wild-type FepA was virtually unchanged (Figure 2). Energy-dependent uptake into bacteria expressing either E280C or E310C was also similar to wild type (data not shown). We also measured binding of [^{59}Fe]enterobactin to the purified receptors before and after spin labeling by equilibrium dialysis. For all samples (wild type and both mutants, before and after spin labeling), a K_d in the range of 0.3−0.6 μM was obtained, in good agreement with published values (Hollifield & Neilands, 1978). These results confirmed that the Glu → Cys site-directed mutations at 280 and 310 had little impact on the structure or function of the receptor, and that covalent attachment of MTSL either to the native cysteines of wild type FepA or to the inserted cysteines of the mutants had little effect on ligand binding.

Spin Labeling of Purified Receptors. Wt FepA has two cysteines located at residues 486 and 493. No labeling of wt FepA with MTSL was observed without prior treatment with a disulfide reducing agent (DTT or β-mercaptoethanol) and partial unfolding in urea (≥4 M). Similar results were obtained with MAL-6 [*N*-(2,2,6,6-tetramethylpiperidinin-4-yl-1-oxyl)maleimide], a more hydrophobic label that readily penetrates membranes. These results indicate that Cys486 and Cys493 are disulfide-linked and occupy a tightly foded region of the protein. Concentrations of urea less than 4 M led to incomplete labeling. Under optimal conditions, we observed binding of approximately two labels per FepA monomer. In contrast, the E280C and E310C mutants were readily labeled without either reduction or denaturation, and they consistently gave labeling stoichiometries (spin label: FepA) of approximately 1:1. Labeling without DTT and urea thus allowed selective spin labeling of mutationally inserted Cys residues under circumstances in which the native cysteines were not modified.

Conventional ESR. The conventional (first harmonic, in phase, absorption) ESR spectra of MTSL-labeled wt FepA and the two mutants in phospholipid liposomes are shown in Figure 3. In each case, the spectra consist of two motionally distinct components. The large majority of labels, identified as component A (Figure 3), arise from spin labels that are strongly immobilized in the conventional ESR time scale and give rise to a characteristic powder pattern. Component B (Figure 3) arises from labels with greater motional freedom. Similar two-component spectra have been observed in a variety of systems including spin-labeled bacteriorhodopsin (Altenbach *et al.*, 1989), rhodopsin (Farahbakhsh *et al.*, 1993), and hemoglobin (Ogawa & McConnell, 1967). Alternations in the relative intensities of the various components can provide a sensitive means by which to detect changes in protein conformation [e.g., Farahbakhsh *et al.* (1993)]. For wild type protein, a fraction (ca. 5%) of relatively mobile spins (identified as *C*, Figure 3) correlates well with the amount of receptor that does not refold after denaturation as assessed by gel electrophoresis (Figure 1).

Table 2: Cytofluorimetric Localization of the Surface and Buried Epitopes of FepA and FepA Mutants[a]

	mean fluorescence intensity																	
	FepA residues, buried epitopes									FepA residues, surface epitopes								
	100–142a, MAb					100–142b, MAb		444–475,	495–566,	200–227, MAb		258–290, MAb		314–399, MAb				382–400, MAb	
	5	7	11	27	38	2	3	MAb 57	MAb 64	33	34	16	44	31	35	37	45	23	24
strain/plasmid																			
KDF669																			
KDF669/pITS449										153	149	135	186	180	185	178	205	195	175
KDF669/*fepA*E280C										200	198	135	203	207	217	200	214	212	199
KDF669/*fepA*E310C										163	168	133	192	193	194	197	214	208	197

[a] The deep rough *E. coli* strain KDF669 (Rutz *et al.*, 1992) either without plasmid or carrying pITS449 (*fepA*$^+$) or its derivatives *fepA*E280C and *fepA*E310C was grown to late log phase, stained with MAb to FepA and fluorescein isothiocyanate-labeled antibodies, and analyzed on an EPICS Profile II flow cytometer. Values are mean fluorescence intensities obtained as described previously (Rutz *et al.*, 1992). No data is shown for negative samples.

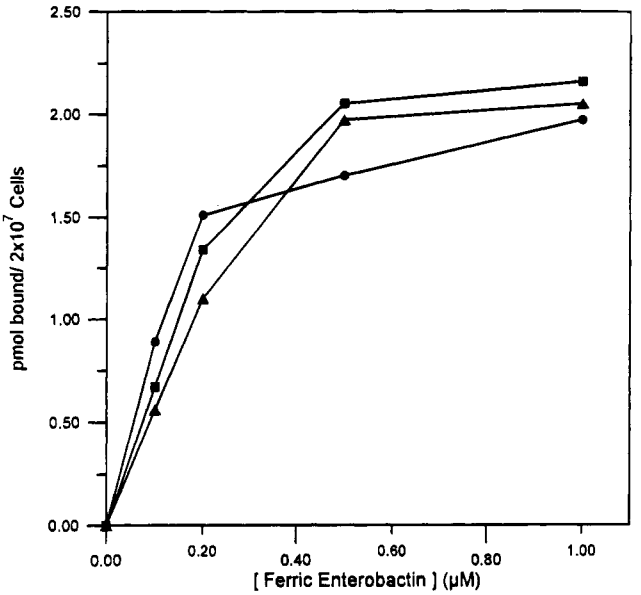

FIGURE 2: Ferric enterobactin binding assay. KDF541/pITS 449 (■), KDF541/E280C (▲), and KDF541/E310C (●) were grown for approximately 8 h in MOPS medium to the late exponential phase, collected by centrifugation, and washed. Bacteria were shaken for 2 h at 37 °C in MOPS without glucose. Cells were incubated for 30 min with [^{59}Fe]enterobactin and washed, and their radioactivity was determined.

Localization of Cys280, Cys310, Cys486, and Cys493. Determination of whether a particular spin-labeled site on a protein is buried or surface-exposed, and its localization with respect to the membrane bilayer, can be accomplished by examining accessibility of the bound nitroxide to lipid- or water-soluble paramagnetic probes (Altenbach *et al.*, 1989, 1990; Farabakhsh *et al.*, 1992; Resek *et al.*, 1993). Molecular oxygen and CROX have been successfully used as lipid- and water-soluble exchange reagents, respectively (Altenbach *et al.*, 1989, 1990; Yin & Hyde, 1987). Spin exchange with fast relaxing agents like O_2 and CROX may be measured *via* continuous wave (CW) power saturation (Subczynski & Hyde, 1981). CW saturation measurements provide an experimental parameter, $P_{1/2}$ (see Figure 4), and the change ($\Delta P_{1/2}$) is proportional to the bimolecular collision rate between the spin label and the probe (Subczynski & Hyde, 1981; Altenbach *et al.*, 1989). The effect of 20% O_2 on the CW saturation curve for wild-type FepA labeled at its native cysteines is shown in Figure 4. Interaction of the protein-bound spin label with O_2 makes saturation more difficult to achieve, shifting the curve (and $P_{1/2}$) to higher powers.

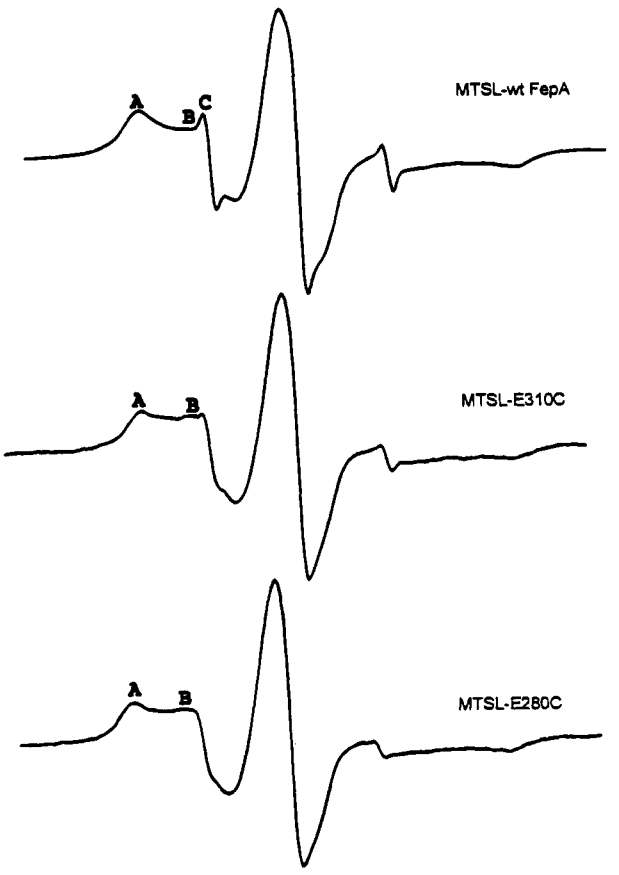

FIGURE 3: Conventional ESR spectra of MTSL-labeled wild-type FepA and E280C and E310C mutants in DOPC/EPG (9:1) liposomes. The protein concentration is approximately 0.1 mM, and the lipid:protein ratio is 200:1. Spectra were recorded at room temperature with a total scan range of 100 G. Signals A and B represent the immobile and mobile components, respectively. Signal C is due to small fraction of protein not refolded.

Results for the interactions of both O_2 and CROX with all three spin-labeled receptor preparations are summarized in Table 3. The nitroxide bound at E280C was much more strongly affected by CROX than O_2, clearly indicating that it occupies a site in an extracellular domain that is relatively accessible to the aqueous phase. Compared to the E280C labeling site, MTSL bound at E310C was less affected by CROX and interacted more strongly with O_2 (Table 3). The $\Delta P_{1/2}$ values for MTSL-E310C were in an intermediate range,

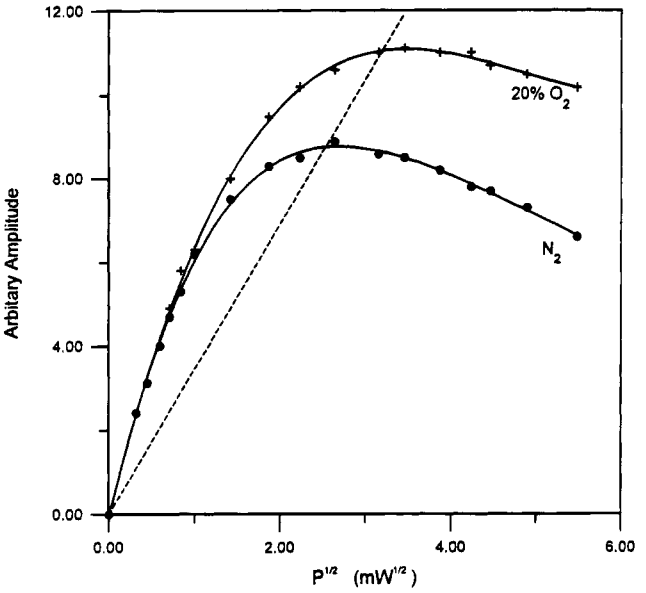

FIGURE 4: Continuous wave power saturation curves. The peak-to-peak amplitude of the first-derivative ESR signal of the center line in arbitrary units is plotted against the square root of the incident microwave power. The dashed straight line has one-half the initial slope of the experimental curves and intersects each at a value corresponding to the square root of $P_{1/2}$. Data shown is for MTSL-labeled wt FepA.

Table 3: $\Delta P_{1/2}$ Parameters for Purified, MTSL-Labeled FepA and FepA Mutants in Vesicle[a]

	$\Delta P_{1/2}$ (20% O_2) (mW)	$\Delta P_{1/2}$ (50 mM CROX) (mW)
wt FepA in liposomes	3.78	1.53
E310C in liposomes	2.64	3.05
E280C in liposomes	1.48	8.77

[a] $\Delta P_{1/2} = P_{1/2} - P_{1/2}^0$, where $P_{1/2}$ and $P_{1/2}^0$ are half-saturation values in the presence or absence of relaxing agent, respectively.

making that site difficult to categorize with regard to its structural locale. The results with MTSL-E310C may reflect a location near the membrane surface where interaction with CROX is reduced by electrostatic repulsion between the probe and the negatively charged bilayer surface (Altenbach *et al.*, 1989). Steric hindrance of the paramagnetic probe molecules by protein structure may also diminish the interaction between spin label and spin probe. For our studies the wild-type Cys residues must be considered as a single site, since they are not resolved either motionally or by their relaxation properties. MTSL-wt FepA interacts only weakly with CROX and somewhat more strongly with O_2 (Table 3). The low accessibility to CROX and the moderate accessibility to O_2 suggested that the wild-type Cys residues are buried. The extreme conditions required for labeling these native Cys residues (≥4 M urea, in addition to thiol reduction) further indicate that they occupy a tightly packed site within the protein structure.

Ligand-Induced Conformational Change. The effects of ferric enterobactin binding on the conventional ESR spectra of MTSL-labeled E280C and E310C FepA in liposomes (Figure 5) show that addition of ferric enterobactin to either MTSL-E310C or MTSL-E280C caused substantial alterations in their respective ESR spectra, in which the relative populations of spin label conformations were changed. The spectral changes that we described in FepA were similar to those observed for light-induced conformational change in

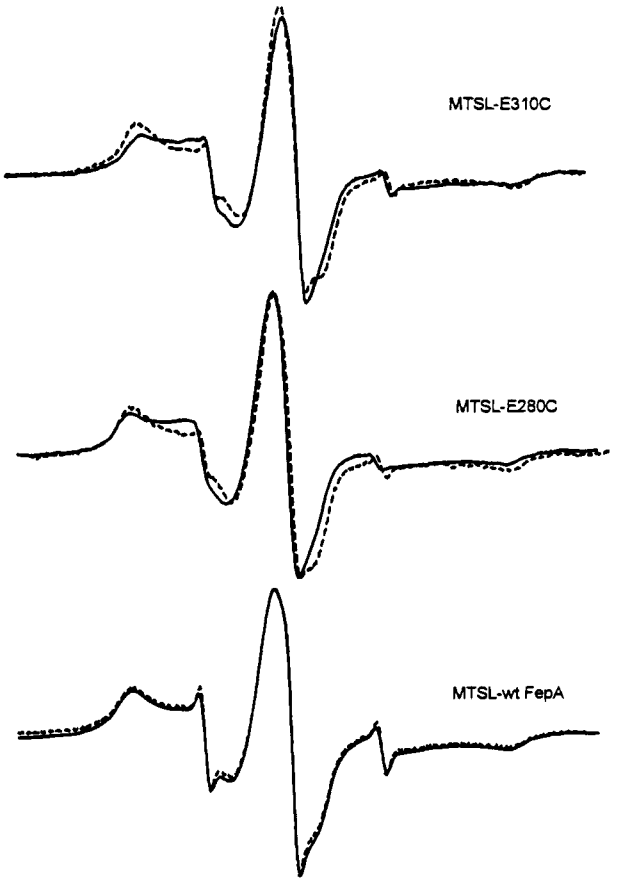

FIGURE 5: Effects of ferric enterobactin on the conventional ESR spectra of MTSL-labeled FepA mutants. Multilamellar vesicles containing 0.1 mM FepA and 20 mM lipid (DOPC/EPG, 9:1) were hydrated with phosphate buffer either with (dashed lines) or without (solid lines) 100 μM ferric enterobactin, and the ESR spectra were recorded. Spectra shown are the sum of eight averages.

MTSL-labeled rhodopsin (Farahbakhsh *et al.*, 1993). In contrast, the ESR spectrum of the MTSL-labeled downstream cysteines of wt FepA does not change significantly upon addition of ferric enterobactin (Figure 5).

Because the spin labels in all three receptors are close to the slow motional limit of conventional ESR measurements, we used STESR to further examine the rotational mobility of the spin-labeled receptors. The effects of ferric enterobactin on the STESR spectra of MTSL-labeled E280C and E310C in phospholipid liposomes are shown in Figure 6. Spectral parameters L''/L and C'/C (Thomas *et al.*, 1976) are given in Table 4. Intensities in the high-field region of the spectra were too weak for resolution of the H''/H parameter. Due to the complexity of these systems, it was not possible to accurately determine rotational correlation times. However, effective correlation times may be estimated by comparing the defined spectral parameters to a model system. The C'/C parameter gave values of approximately 0.8×10^{-7} s for wt and E310C and 3×10^{-7} s for E280C, based on the standard curves of Thomas *et al.* (1976). The L''/L values give correlation times of approximately 0.8×10^{-5} s for wt and E280C and 0.3×10^{-5} s for E310C. These large differences in estimates from different spectral positions may reflect anisotropic motion (Delmelle *et al*, 1980; Fajer & Marsh, 1983), as would be expected for an integral transmembrane protein.

Although precise rotational correlation times could not be determined, changes in the STESR parameters do accurately

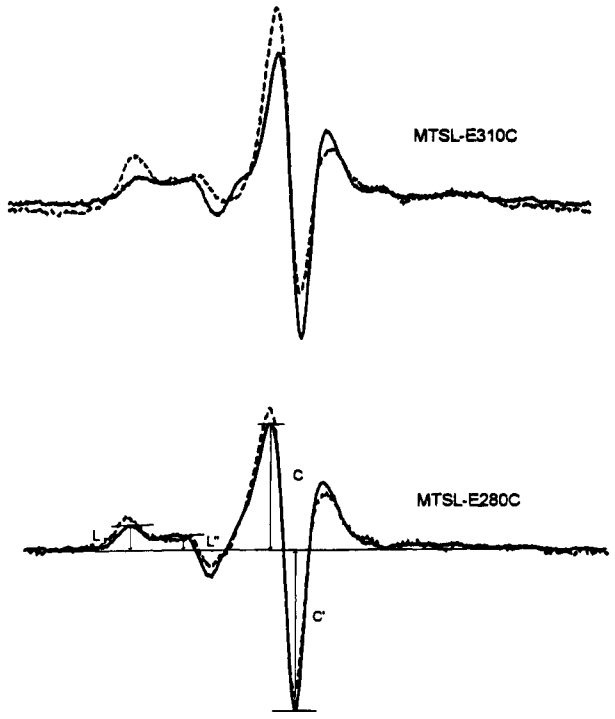

FIGURE 6: Effects of ferric enterobactin on the STESR of MTSL-labeled FepA mutants. Samples were the same as described in Figure 5, with (dashed lines) or without (solid lines) 100 μM ferric enterobactin. Spectra were signal averaged 16 times each. The spectral parameters C, C', L, and L'' are indicated in the lower spectrum.

Table 4: Spectral Parameters C'/C and L''/L from STESR Measurements in Liposomes[a]

	C'/C	L''/L
wt FepA in liposomes	−0.90	0.64
wt FepA + FeEnt	−0.86	0.65
E280C	−1.30	0.60
E280C + FeEnt	−1.05	0.38
E310C	−0.92	0.83
E310C + FeEnt	−0.42	0.66

[a] The parameters, C, C', L, and L'' are depicted in Figure 6, as defined by Thomas *et al.* (1976).

reflect trends in the motion upon ligand binding. Addition of ferric enterobactin caused an apparent decrease in the rotational mobility of MTSL bound to residues 310 and 280 in the corresponding mutant receptors that was reflected in both L''/L and C'/C parameters (Table 4). MTSL bound to residues 486 and 493 of wt FepA was not significantly perturbed by ferric enterobactin binding (Figure 5; Table 4). These observations verify the conventional ESR results using a display that is more sensitive in the slow motional regime. The observed changes of rotational mobility were dependent on the concentration of ferric enterobactin, as shown for MTSL-E310C in Figure 7. Addition of ferrichrome (a siderophore not recognized by FepA) produced no change in either the conventional or STESR spectra of any of the spin-labeled receptors (data not shown).

The spectral changes observed in the conventional ESR spectra of MTSL-E280C and MTSL-E310C upon addition of ferric enterobactin could conceivably arise from either a conformational change in the protein or physical perturbation of spin labels by the ligand. These mechanisms, as well as alterations in spin label relaxation properties caused by the

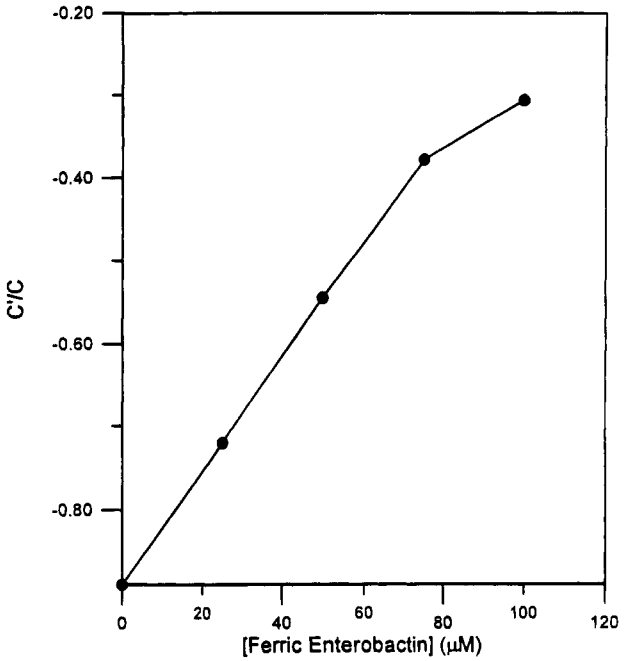

FIGURE 7: Dependence of the C'/C value on the concentration of ferric enterobactin. MTSL-E310C in liposomes (0.1 mM) was added with different concentrations of FeEnt; the C'/C values (depicted in Figure 6) from STESR spectra were plotted against the concentration of FeEnt. Note that the curve begins to saturate at 100 μM ferric enterobactin, approximately equimolar with the receptor.

paramagnetic iron center of ferric enterobactin, could also produce the observed ligand-induced changes in the STESR spectra.

To further explore the magnetic interactions between bound ligand and spin label, we performed CW saturation experiments with and without the addition of ferric enterobactin. The results, summarized in Figure 8, indicate that ferric enterobactin alone (under N_2) did not change the $P_{1/2}$ values of MTSL bound at either the 280 or the 310 site. These data demonstrate a lack of significant dipolar interaction between these spin label sites and ferric enterobactin. Furthermore, direct spin−lattice relaxation time measurements by saturation recovery ESR between 5.3 K and 85.3 K showed that T_1 values of the spin labels at these sites were not changed by addition of ferric enterobactin (data not shown). These results suggest that the observed changes in STESR spectra do not result from changes in the relaxation properties of the spin labels. This result also makes it unlikely that the altered motion of spin labels upon binding of ferric enterobactin is due to steric hindrance or perturbation by the ligand, because it is doubtful that the ligand could be close enough to the nitroxide spin label for direct contact and still not affect its T_1. Thus the observed spectral changes indicate a structural alteration in FepA in the extracellular peptide domain encompassing residues 280 and 310. These effects apparently do not alter the local protein structure in the region occupied by the native cysteine residues 486 and 493.

Further evidence of ligand-induced conformational change in FepA came from the effects of ferric enterobactin binding on oxygen accessibility. If the local environment of a spin label changes, then it is likely that its accessibility to a given paramagnetic probe will also change (Farahbakhsh *et al.*, 1993). CW saturation data showed that oxygen accessibili-

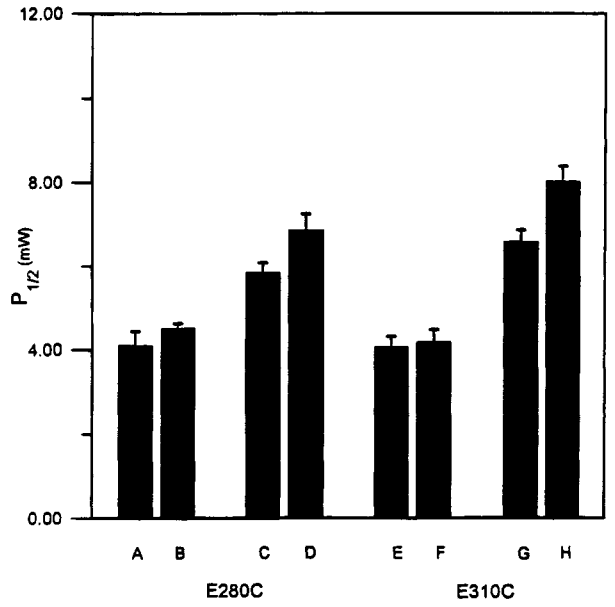

FIGURE 8: Effects of ferric enterobactin on the accessibility of spin labels to O_2: $P_{1/2}$ values of MTSL-labeled E280C and E310C under different conditions. (A) MTSL-E280C under N_2; (B) MTSL-E280C with 100 μM ferric enterobactin under N_2; (C) MTSL-E280C under 20% O_2; (D) MTSL-E280C with ferric enterobactin under 20% O_2; (E–H) are the same as A–D, respectively, except the data are from MTSL-labeled E310C. Values are averages of five experiments ± SD. The differences between C and D and between G and H are significant with $p < 0.05$ (Student's t test). There is no significant difference between A and B or between E and F.

ties increased for both MTSL-E280C and MTSL-E310C on the addition of ferric enterobactin (Figure 8). This result provides direct evidence for a conformational change in the ligand-binding domain of FepA. In summary, these data show that, upon the binding of ferric enterobactin to FepA, a conformational change takes place in the local region around residues 280 and 310, as reflected by both the motion and the accessibility of the attached nitroxide spin labels at these sites.

DISCUSSION

Site-directed spin labeling (SDSL) has emerged as a powerful method for examining structure–function relationships of proteins. The ability to introduce labeling sites at desired locations, rather than relying on endogenous cysteines, makes this a generally applicable approach. It is particularly suitable for large membrane proteins that are not amenable to structural analysis by current NMR methods. FepA, an 81–kDa receptor responsible for ferric enterobactin uptake across the bacterial OM, clearly fits this category. Elucidation of structure-function relationships within FepA may provide general insights into the physiological mechanisms of TonB-dependent receptors.

The spin label probe we chose for this study, MTSL, has certain advantages. It reacts exclusively with free sulfhydryl groups and has a relatively small molecular volume, similar to that of a phenylalanine residue (Altenbach *et al.*, 1989). Thus it introduces a minimal structural perturbation in the protein. This nitroxide spin label has been used extensively in recent SDSL studies of bacteriorhodopsin (Altenbach *et al.*, 1989), rhodopsin (Farahbakhsh *et al.*, 1993), colicin E1 (Shin *et al.*, 1993), and peptides (Mchaourab *et al.*, 1993, 1994; Fiori *et al.*, 1993).

For the initial SDSL studies of FepA, we modified glutamate residues 280 and 310. These sites are located within the putative extracellular peptide domain that, on the basis of mapping with a panel of monoclonal antibodies (Murphy *et al.*, 1990) and [^{59}Fe]enterobactin binding studies with FepA deletion mutants (Rutz *et al.*, 1992), contains the ligand-binding site. Furthermore, the negative charge of these residues makes it unlikely that they interact directly with the anionic ligand ferric enterobactin (net charge 3–). Thus, we expected them to provide relatively safe sites for mutation and attachment of a molecular probe. Siderophore nutrition assays, colicin susceptibility, and [^{59}Fe]enterobactin binding and uptake studies indicate that this is indeed the case. Flow cytometry and non-denaturing LDS–PAGE of the reconstituted receptors also suggest that FepA mutants E280C and E310C retain their native, functional conformation.

The ESR spectra of all three MTSL-labeled receptors exhibited a remarkable degree of rotational immobilization. This was unexpected for the mutationally inserted sites at residues 280 and 310, which are presumably located in a cell-surface domain (Murphy *et al.*, 1990). MTSL labeling of a surface loop of colicin E1 gives rise to an ESR spectrum characteristic of rapid rotational motion (Todd *et al.*, 1987). However, as with our studies, immobilization was observed for several MTSL-labeled sites located in aqueous-exposed, cell-surface domains in SDSL studies of bacteriorhodopsin (Altenbach *et al.*, 1989), apparently due to contacts between the spin label and the protein. Our SDSL studies of FepA suggest a high degree of structural organization in the ligand-binding domain, consistent with the high affinity and specificity of FepA for its ligand. This perception of FepA surface loops is also consistent with the conformation of surface loops in other OM proteins, as in the *Rhodobacter* porin and the *E. coli* OmpF-type porins, for example, which have been characterized by X-ray crystallography as tightly packed and folded (Weiss *et al.*, 1991; Cowan *et al.*, 1992).

Oxygen and CROX accessibility studies indicate that residues 280 and 310 are localized in or near an extracellular surface domain. These results are compatible with a model for the membrane topology of FepA by Murphy *et al.* (1990) proposed on the basis of sequence analysis and binding of monoclonal antibodies. Further mapping of solvent exposure for residues in this region should allow the development of a consensus structure for this extracellular domain. The somewhat diminished collision rate between MTSL-E310C and CROX (relative to MTSL-E280C) may indicate that Cys310 lies near the negatively charged membrane surface. The low accessibility to CROX and O_2 of nitroxide bound to native cysteines at 486 and 493 and the extreme conditions required for labeling the native cysteines suggest that they are buried residues, which agrees with the prior finding (Rutz *et al.*, 1992) that certain surface loops of the receptor overlay others. The native cysteines of FepA, which are postulated as being in a small exterior loop (Murphy *et al.*, 1990), are apparently rendered inaccessible and immobile by other elements of FepA tertiary structure. In this sense, they are analogous to the native cysteines of *E. coli* LamB (Luckey *et al.*, 1991).

In this report we demonstrate, for the first time, that a TonB-dependent receptor protein undergoes conformational change when it binds its natural ligand, ferric enterobactin. Changes in receptor conformation are an integral part of

transmembrane signaling or transport mechanisms, yet they are not easily demonstrated. ESR spin labeling has been widely used in protein conformation studies due to its high sensitivity and large dynamic range. In our studies and others (Altenbach *et al.*, 1989; Farahbakhsh *et al.*, 1993), the spin label spectra cannot in general be fitted with a single motional model. The relationship between the motional restriction of the spin label and the local protein structure has been investigated recently by molecular dynamics techniques (Altenbach *et al.*, 1994). ESR spectra computed from trajectories of the nitroxide reorientational motion for different model peptide structures show that the ESR spectrum of a spin-labeled side chain reflects tertiary interactions of the nitroxide, and therefore is a sensitive indicator of small conformational movements in the protein. Site-directed fluorescence labeling, a technique closely analogous to SDSL, was recently used to demonstrate ligand-induced changes in the tertiary structure of lactose permease (Jung *et al.*, 1994). In the present work we have utilized SDSL to demonstrate a conformational change in the extracellular domain of FepA upon ligand binding. Addition of ferric enterobactin to reconstituted MTSL-E280C and MTSL-E310C resulted in substantial changes in their respective conventional ESR and STESR spectra. The absence of an effect caused by ferric enterobactin on the relaxation properties of the spin label at these sites excluded the possibility that the observed spectral alterations are due to dipolar-induced changes in line shape or relaxation properties. This could be due to ferric enterobactin being a poor relaxing agent. The effect of a spin probe on relaxation of a nearby spin label has been discussed by Hyde *et al.* (1979). When T_1 of the spin probe is so short that $(\omega_{pm} - \omega)T_{1k} \ll 1$, $(\omega_{pm} + \omega)T_{1k} \ll 1$, and $\omega T_{1k} \ll 1$ (where ω is the resonance frequency of the spin label, ω_{pm} is the resonance frequency of the paramagnetic metal in the magnetic field H_0 used to observe resonance of the spin label, and T_{1k} is the spin−lattice relaxation time of the metal ion), it may not provide an effective relaxation pathway for the spin label even when the spin label and metal ion are within the limit of dipolar interactions. We find T_1 of iron in ferric enterobactin to be too fast to measure even at 8 K by saturation recovery ESR ($T_1 < 2$ μs on our instrument, limited by the ring time of the cavity). This is consistent with the iron center of ferric enterobactin being in the high-spin state (Neilands, 1981). Thus, ferric enterobactin could be within the distance limit for dipolar interaction (typically ≤25 Å; Dalton *et al.*, 1987; Gettins *et al.*, 1988) and yet not act as a relaxing reagent. Fortunately, another sensitive indicator of protein conformation, the accessibility of the spin label to O_2, did provide definitive evidence for a ligand-induced conformational change in FepA. Addition of ferric enterobactin to MTSL-E280C and MTSL-E310C resulted in a significant increase of the accessibilities of these spin labels to O_2, indicative of a structural change in the protein. Since the sample conditions were the same in this measurement as in the conventional ESR and STESR experiments, this result also suggested that the observed spectral changes in conventional and saturation-transfer ESR are due to a conformational change in FepA, and not to dipolar effects or physical perturbation by the paramagnetic ligand. Ferric enterobactin did not induce changes in O_2 accessibility of MTSL bound to the native cysteine residues at 486 and 493. The absence of significant perturbation in the region occupied by native cysteines 486 and 493 suggests that the conformational change is not global in nature, but several additional sites must be analyzed before final conclusions can be made.

Conformational changes are of particular interest in the FepA-mediated siderophore transport system because of the number of accessory proteins involved in ferric enterobactin uptake, the unusual coupling of proton-motive force energy between inner and outer membranes, and the importance of TonB in this and several other transport processes. TonB is likely anchored to the cytoplasmic membrane *via* a hydrophobic helix near its N-terminus. It also contains a structurally rigid X-Pro region that may span the periplasm to allow interaction with TonB-dependent OM receptors (Hannavy *et al.*, 1990; Kadner, 1990). The location of the TonB C-terminus is unknown, and various models of TonB function have been proposed on the basis of different localizations of the C-terminus within the cell envelope [reviewed by Postle (1993) and Klebba *et al.* (1993)]. A common theme among all of these functional models is that energy transduction from the inner membrane is transmitted to TonB, which further interacts directly or indirectly with an OM receptor, causing a conformational change that releases bound ligand into the periplasmic space. An additional aspect of TonB function is the recognition of receptors complexed with their respective ligands by TonB. This would seem essential since, first, the ratio of the OM siderophore receptors to TonB in an iron-deficient environment is at least 10:1 (Klebba *et al.*, 1993), and second, TonB-dependent OM receptors are ligand-specific gated porins that exist in a "closed" configuration prior to the binding and internalization of ligands. This closed conformation maintains the restricted permeability of the OM. A conformational change in the receptor upon siderophore binding may thus provide a mechanism to trigger the action of Ton or provide the receptor sampling mechanism for TonB (Postle, 1993). The demonstration of a ligand-induced conformational change in FepA in our present study may support this idea, although it is not apparent how this structural change can be recognized by TonB. Further work utilizing the methodological foundation of this study may provide the structural and dynamic information necessary for understanding the transport mechanism of TonB-dependent OM receptors.

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