# Denaturant Unfolding of the Ferric Enterobactin Receptor and Ligand-Induced Stabilization Studied by Site-Directed Spin Labeling†

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*Received July 5, 1995; Revised Manuscript Received August 23, 1995*⊗

ABSTRACT: FepA is an integral outer membrane protein that is the specific receptor for the siderophore, ferric enterobactin, and is thus primarily responsible for iron uptake in many Gram-negative bacteria. A site-specific mutant of FepA, containing a single introduced cysteine in the ligand-binding domain, was spin labeled and used to examine the denaturant-induced unfolding of this receptor with guanidine hydrochloride (Gdn-HCl) and urea. Electron spin resonance (ESR) spectra showed conversion of the spin label from a motionally-restricted, immobilized environment to a freely-accessible, rotationally-mobile state upon denaturation. Unfolding was also followed by nondenaturing polyacrylamide gel electrophoresis (PAGE), which is sensitive to loss of the putative transmembrane $\beta$-structure, and displayed a similar concentration dependence. Unfolding occurred over relatively narrow ranges of denaturant concentration, indicating a high degree of cooperativity. Unfolding was fully reversible under the conditions employed. Rapid, spontaneous refolding occurred in the presence of Triton X-100 and did not require exogenous lipids. Refolding could be induced by either dialysis, dilution to low denaturant concentration, or ethanol precipitation. At ambient temperature the free energy of unfolding extrapolated to zero denaturant concentration ($\Delta G_U°$) was 6.24 ± 0.63 kcal/mol. Values of $\Delta G_U°$ obtained with Gdn-HCl and urea were in good agreement, as were values obtained from linear extrapolation and nonlinear regression fitting to a two-state equilibrium. This is the first report of a quantitative evaluation of the free energy of unfolding for an integral membrane protein.

Studies of solvent-induced denaturation can provide information on the structural stability of a protein and give important insights into the forces involved in protein folding. Numerous studies have utilized guanidine- and/or urea-induced denaturation to determine the free energy of unfolding ($\Delta G_U$).[1] Such studies allow quantitative evaluation of protein stability (Aune & Tanford, 1969; Pace & Vanderberg, 1979; Ahmad & Bigelow, 1982; Ho & DeGrado, 1987; Regan & DeGrado, 1988), destabilization caused by mutations (Kellis *et al.,* 1989; Serrano *et al.,* 1990; Sanz & Fersht, 1993), comparison of secondary structure propensities (Otzen & Fersht, 1995), and insights into the existence of folding intermediates (Sanz & Fersht, 1993; Mücke & Schmid, 1994). These studies typically employ circular dichroism (CD), intrinsic fluorescence, or absorbance in the UV−visible range to monitor loss of protein structure.

The site-directed spin labeling (SDSL) approach (Altenbach *et al.,* 1990; Hubbell & Altenbach, 1994) has recently emerged as an important technique for examining structure−function relationships in proteins. This method involves utilizing site-directed mutagenesis to introduce cysteine residues at desired locations in a protein structure followed by the selective attachment of a sulfhydryl-specific spin label, making it possible to systematically study a variety of sites in a given protein. SDSL provides structural information on a specified location within the protein tertiary structure, as opposed to techniques such as those listed above that monitor global changes in protein conformation. Consequently, with SDSL the potential exists for examining distinct changes in different regions of a protein having multiple structural domains.

We have previously described SDSL of the *Escherichia coli* ferric enterobactin receptor, FepA (Liu *et al.,* 1994). FepA is an 81 kDa outer membrane protein that is responsible for iron uptake *via* binding and translocation of the ferric enterobactin (FeEnt) complex in a wide variety of Gram-negative bacteria (Pollack & Neilands, 1970; Rutz *et al.,* 1991). FepA also serves as the receptor for colicins B and D (Guterman, 1973; Wayne *et al.,* 1976). The FeEnt binding site on FepA has been localized to within a region of approximately 80 surface-exposed amino acid residues by mapping with monoclonal antibodies (Murphy *et al.,* 1991), and we have shown that deletion of this surface domain converts FepA into a nonspecific diffusion channel capable of passing solutes such as rifampin (1500 Da) that normally

† This work was supported by NSF Grant MCB9408737 (P.E.K.) and by NIH Grants RR01008 and GM51339 (J.B.F.).

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⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1995.

[1] Abbreviations: CD, circular dichroism; CROX, potassium tris(oxalato)chromate; ESR, electron spin resonance; FeEnt, ferric enterobactin; $\Delta G_U$, free energy of unfolding; $\Delta G_U°$, free energy of unfolding in the absence of denaturant; Gdn-HCl, guanidine hydrochloride; LDS−PAGE, lithium dodecyl sulfate−polyacrylamide gel electrophoresis; MTSL, methanethiosulfonate spin label; MOPS, 3-(N-morpholino)-propanesulfonic acid; PCR, polymerase chain reaction; SDSL, site-directed spin labeling; SDS, sodium dodecyl sulfate.

0006-2960/95/0434-14230$09.00/0 

cannot cross the outer membrane barrier (Rutz *et al.*, 1992; Liu *et al.*, 1993). Two mutants of FepA were constructed, each of which introduced a cysteine labeling site into the putative extracellular ligand-binding domain. These Cys residues were modified with the small, relatively nonperturbing methanethiosulfonate spin label (MTSL), and the regions occupied by the label were characterized with respect to local packing and accessibility. The mutants, FepA E280C and E310C (replacing a glutamate with a cysteine at residues 280 and 310, respectively), exhibited ligand binding with an affinity similar to that of wild type and were fully functional with regard to colicin B susceptibility and dependence on the inner membrane protein, TonB (Liu *et al.*, 1994). Both were also shown to undergo structural perturbation upon binding of FeEnt (Liu *et al.*, 1994).

In this work we utilize MTSL-labeled E280C to examine unfolding of the ligand-binding domain of FepA upon denaturation with guanidine hydrochloride (Gdn-HCl) and urea. While many studies of denaturant-induced unfolding for globular proteins have been done, there have been few quantitative studies and no prior estimates of $\Delta G_U$ for an intact integral membrane protein. We find that unfolding of the FepA ligand-binding domain is highly cooperative and reversible and that the binding of ferric enterobactin stabilizes the native structure in this region of the receptor.

## MATERIALS AND METHODS

*Protein Purification and Spin Labeling.* Site-specific mutagenesis of FepA by overlap-extension PCR was described previously (Liu *et al.*, 1994). FepAE280C was purified from *E. coli* KDF541 carrying the pITS449E280C plasmid. Bacteria were grown to late exponential phase in iron-deficient MOPS media containing 100 μg/mL streptomycin, 100 μg/mL ampicillin, 0.4% glucose, and 0.2% casamino acids (Neihardt *et al.*, 1974). The receptor was isolated by differential extraction of outer membrane fragments with Triton X-100 followed by ion exchange chromatography on DEAE-cellulose (DE52, Whatman, Hillsboro, OR) as described previously (Fiss *et al.*, 1982). The purified protein was spin labeled at the E280C cysteine by incubation with a 10-fold molar excess of (1-oxy-2,2,5,5-tetramethylpyrrolidin-3-yl)methyl methanethiosulfonate (MTSL, Reanal, Budapest, Hungary) at 4 °C for 5 h. Excess spin label was removed by exhaustive dialysis followed by precipitation with 2 volumes of ice cold ethanol. Protein concentrations were determined by the method of Lowry (Lowry *et al.*, 1951), with 2% SDS present to disrupt Triton X-100 micelles.

*Ferric Enterobactin Preparation.* Purified enterobactin was reconstituted with iron by incubation with $FeCl_3$ at pH 4, purified by chromatography on Sephadex LH-20 (Murphy *et al.*, 1991), and concentrated by rotary evaporation. The concentration of FeEnt was determined by its absorbance at 495 nm using an extinction coefficient of 5600 $M^{-1}$ $cm^{-1}$.

*Polyacrylamide Gel Electrophoresis.* Nondenaturing lithium dodecyl sulfate (LDS)−PAGE was performed as described previously (Liu *et al.*, 1993). Samples contained a final concentration of 3% LDS and were not boiled except where specified. The running buffer contained 0.1% LDS. Electrophoresis on 11% gels (Lugtenberg *et al.*, 1975) was run at 4 °C overnight at a constant current of 4 mA.

*Sample Preparation and ESR Spectroscopy.* Both guanidine hydrochloride and urea were Ultrapure grade (United States Biochemicals, Cleveland, OH) and were used without further purification. MTSL-labeled FepA E280C in 50 mM MOPS and 2% Triton X-100, pH 7.2 (MOPS/Triton), with or without added FeEnt, was further diluted with MOPS/Triton and 8 M Gdn-HCl (or 11 M urea) in MOPS/Triton to give the desired final concentration of denaturant. All samples contained approximately 0.1 mM FepA. Ferric enterobactin, when used, was present at a slight molar excess. ESR spectroscopy was performed on a Varian E-109 Century series spectrometer (Varian Associates Inc., Palo Alto, CA) using 10 mW incident microwave power and 1.0 G magnetic field modulation calibrated with Fremy's salt (potassium nitrosodisulfonate, Aldrich Chemical Co., Milwaukee, WI). Chromium oxalate (potassium tris(oxalato)chromate) was also from Aldrich. Data acquisition was controlled with a PC utilizing the VIKING software package, and spectral subtractions were performed using SUMSPC92 (National Biomedical ESR Center, Milwaukee, WI).

*Analysis of Guanidine Hydrochloride (Gdn-HCl) Denaturation.* The fraction of denatured protein, $f_D$, in any given sample was determined by subtracting an appropriate amount of the ESR spectrum observed in 4 M Gdn-HCl (or 8 M urea) until a lineshape similar to that observed in the absence of denaturant was obtained (see Figure 2), and comparing the integrated spectral intensities before and after subtraction. For a two-state equilibrium between native and denatured protein:

$$K_{dn} = f_D/f_N$$

and the Gibbs free energy of unfolding, $\Delta G_U$, at any denaturant concentration is

$$\Delta G_U = -RT \ln K_{dn}$$

where $f_N$ is the fraction of native protein, $R$ is the gas constant, and $T$ is the absolute temperature. It has been well-established that $\Delta G_U$ varies linearly with [denaturant], such that:

$$\Delta G_U^\circ = \Delta G_U + m[\text{denaturant}]$$

where $\Delta G_U^\circ$ is the Gibbs free energy of unfolding, extrapolated to the absence of denaturant (Schellman, 1978; Pace, 1986; Ahmad & Bigelow, 1986; Sanz & Fersht, 1993; Yao & Bolen, 1995). Gdn-HCl and urea denaturation curves (Figure 4) and linear extrapolations (Figure 5) were fit with nonlinear and linear least-squares regression analyses, respectively, using the program Enzfitter (Elsevier-Biosoft, Cambridge, U.K.). It has been noted that the midpoint of the denaturation curve, $C_m$, may be a more reliable experimental parameter than the extrapolated intercept (Kellis *et al.*, 1989; Serrano *et al.*, 1990). Since at 50% denaturation $K = 1$, $\Delta G_U^\circ = mC_m$. This provides an additional method for determining the free energy of unfolding. In this study, we find $\Delta G_U^\circ$ values calculated by each of these methods to be in good agreement.

## RESULTS

ESR spectra of MTSL-labeled FepAE280C (MTSL-E280C) in various concentrations of Gdn-HCl are shown in Figure 1. As reported previously (Liu *et al.*, 1994), control spectra in 2% Triton X-100 indicated that the nitroxide was strongly immobilized, consistent with tight packing of the

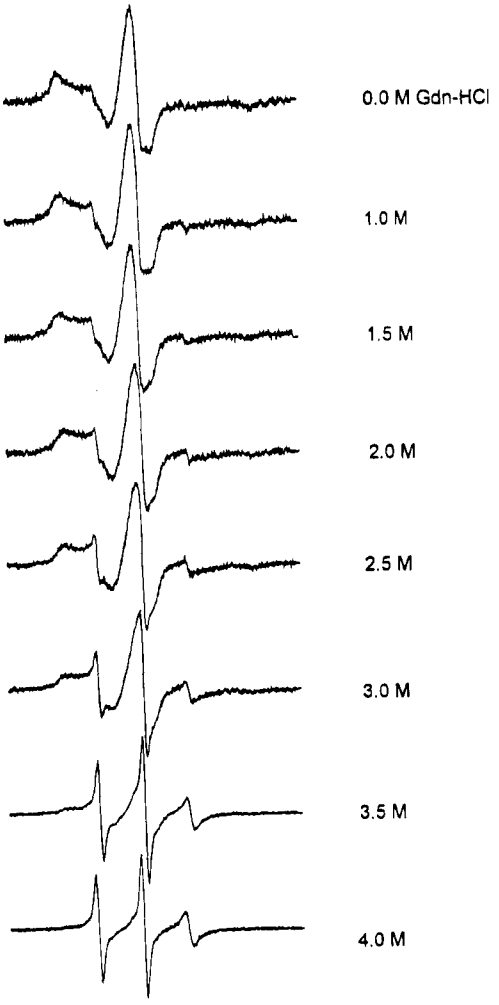


FIGURE 1: Gdn-HCl denaturation of MTSL-E280C. Samples of methanethiosulfonate spin-labeled E280C FepA were mixed with Gdn-HCl and their ESR spectra recorded immediately at ambient temperature (22 °C). Samples contained (top to bottom) 0, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 M Gdn-HCl. Percent denaturation was determined by double integration of the spectra after the rotationally mobile component had been removed by subtraction of the spectrum obtained in 4.0 M Gdn-HCl. All samples contained approximately 0.1 mM (8 mg/mL) FepA, in 2% Triton X-100 and 20 mM MOPS, pH 7.2. Spectra are the average of 8 scans.

protein in the region occupied by the spin label. Both wild type FepA and E280C bind FeEnt with high affinity ($K_d$ approximately 0.2−0.5 $\mu$M for wild type; Hollifield & Neilands, 1978) in 2% Triton X-100, suggesting that the native structure of FepA is largely retained in this nonionic detergent (Hollifield & Neilands, 1978; Liu *et al.*, 1994). The ESR spectra of MTSL-E280C remained essentially unchanged up to 1.5 M Gdn-HCl, following which there was a concentration-dependent increase in a narrow spectral component characteristic of much more rapidly tumbling spin labels. This spectral component had a nitrogen hyperfine coupling constant ($a_N$) of 16.1 G, the same as observed for free MTSL dissolved in 4 M Gdn-HCl, and was completely broadened by hydrophilic relaxation agents such as chromium oxalate (CROX) and $Ni^{2+}$. The motional characteristics, coupling constant, and accessibility to CROX and $Ni^{2+}$ all indicated that this mobile spectral component corresponds to an unfolded, denatured state of FepA in which the spin label is exposed to the aqueous phase. At 4 M Gdn-HCl, denaturation was complete (Figure 1). Similar results were

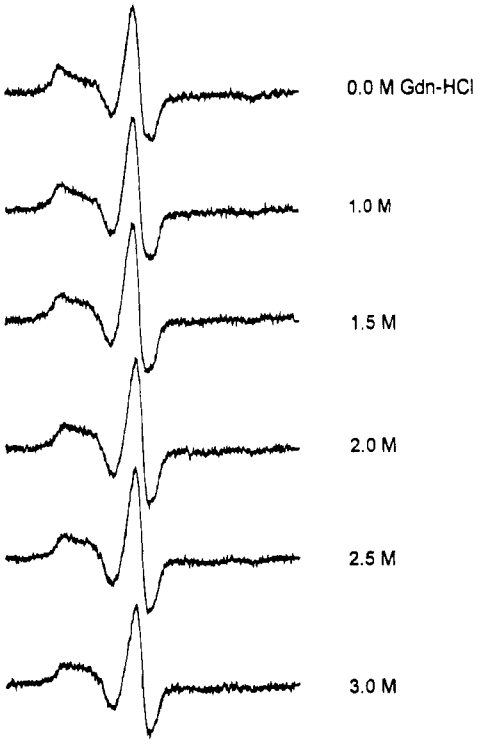


FIGURE 2: Difference spectra obtained by subtracting the ESR spectrum of MTSL-E280C in 4.0 M Gdn-HCl from the experimental spectra obtained immediately following addition of Gdn-HCl. Spectra correspond to those in Figure 1 and contain (top to bottom) 0, 1.0, 1.5, 2.0, 2.5, and 3.0 M Gdn-HCl.

obtained using urea, although somewhat higher concentrations were required, consistent with Gdn-HCl being the stronger denaturant (Greene & Pace, 1974; Pace & Vanderberg, 1979; Santoro & Bolen, 1988).

Difference spectra, with the denatured component removed by subtraction of an appropriate amount of the spectrum obtained in 4 M Gdn-HCl, were largely unchanged from the control spectrum observed in the absence of Gdn-HCl (Figure 2). This indicates that for the fraction of protein remaining in the nondenatured state the spin label binding site was not significantly perturbed and is consistent with a concerted unfolding process lacking stable intermediates (Creighton, 1990). When Gdn-HCl was removed by dialysis from samples that had been fully denatured in 4 M Gdn-HCl, the ESR spectrum indicated that the spin label had returned to its initial, immobilized state, thus demonstrating that unfolding of the ligand-binding domain was reversible. Refolding could also be observed by ESR when samples denatured in 4 M guanidine were diluted to ≤1 M Gdn-HCl. Under these conditions, refolding was rapid, occurring in less than the 1−2 min needed for placing the sample in the ESR cavity. Urea unfolding of FepA was also reversible, as indicated both by ESR and by polyacrylamide gel electrophoresis (discussed below). These results are consistent with a two-state equilibrium between folded (native) and unfolded (denatured) conformations of the ligand-binding domain.

Denaturation and refolding were also demonstrated by LDS−PAGE. FepA, like the porins, migrates with an anomalously low apparent molecular mass (ca. 56 kDa) on polyacrylamide gels, due to the high content of compact $\beta$-sheet (Schweizer *et al.*, 1978; Mizuno & Kageyama, 1979; Dornmair *et al.*, 1990). Denaturation, for example, by boiling in 2% SDS, restores migration to an apparent

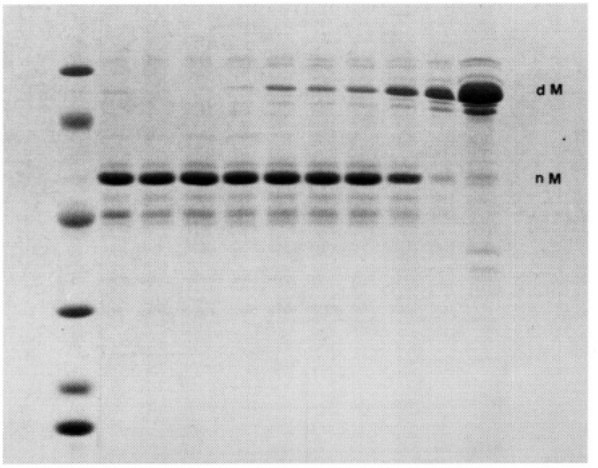


FIGURE 3: Nondenaturing LDS−PAGE of MTSL-E280C FepA demonstrating conversion of the 58 kDa apparent molecular mass native structure to the 81 kDa apparent molecular mass denatured structure following incubation for 24 h with urea. (1) molecular mass standards; (2−10) of 20 $\mu$g FepA and urea concentrations of (2) no urea, (3) 1 M, (4) 2 M, (5) 4 M, (6) 4.5 M, (7) 5 M, (8) 5.5 M, (9) 6 M, or (10) 8 M; (11) FepA boiled in 2% SDS.

molecular mass of 81 kDa in agreement with the primary sequence. Titration with urea caused progressive conversion from the compact native state to the denatured state (Figure 3). Urea was used exclusively for these studies since Gdn-HCl is incompatible with LDS−PAGE. Again, no intermediate species was observed, and samples could be induced to refold by either dialysis, dilution to [urea] $\leq$2 M, or ethanol precipitation. Importantly, the concentration range over which transition from the native to denatured state occurred matched closely with that observed by ESR (Figure 4B).

The rate of unfolding for MTSL-E280C FepA was dependent on both the concentration of denaturant and temperature. For example, for [Gdn-HCl] between 1.5 and 2.5 M at room temperature (22 °C), the fraction of unfolded protein increased gradually to an equilibrium value reached after 16−24 h. Transition to the unfolded state at ambient temperature was complete in 3 h or less for Gdn-HCl concentrations $\geq$2.5 M (immediate in 4 M Gdn-HCl or 8 M urea). The ESR spectra of samples containing less than 1 M guanidine (2 M urea) remained unchanged (i.e., in the native state) for several days. Consequently, equilibrium denaturation curves were determined on samples incubated 24 h at room temperature.

Gdn-HCl and urea denaturation curves, obtained from ESR data with the fraction of highly mobile spin labels (e.g., in the presence of 4.0 M Gdn-HCl or 8 M urea) plotted against concentration of the denaturant, are shown in panels A and B, respectively, of Figure 4. Curves for both early (immediately following addition of denaturant) and equilibrium (after 24 h at room temperature) conditions are shown for Gdn-HCl (Figure 4A). All of the curves indicate a highly cooperative unfolding of FepA, occurring over relatively narrow concentration ranges. Equilibrium transition midpoints ($C_m$) occurred at 1.95 ($\pm$0.06) M Gdn-HCl and 5.48 ($\pm$0.03) M urea.

Figure 5 shows the dependence of the free energy of denaturation ($-RT \ln K_{dn}$) on denaturant concentration. This

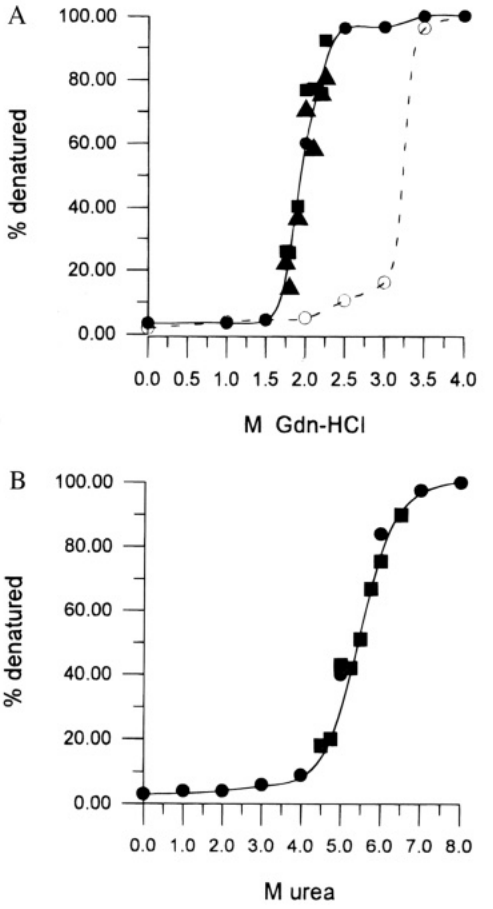


FIGURE 4: (A) Gdn-HCl denaturation curves for MTSL-E280C FepA derived from ESR data obtained (open symbols) immediately after Gdn-HCl addition, and (closed symbols) after 24 h at room temperature. Percent denaturation was determined by spectral subtraction with 4 M Gdn-HCl as the fully-denatured reference. The solid lines are best fits of the data to a two-state equilibrium model. The different symbols represent independent experiments. (B) Urea denaturation curve for MTSL-E280C FepA obtained after 24 h at room temperature. Percent denaturation was determined by spectral subtraction with 8 M urea as the fully-denatured reference. The solid line is the best fit of the data to a two-state equilibrium model. The different symbols represent independent experiments.

analysis considers the unfolding process to be a two-state equilibrium between native and denatured states. Consistent with this are the lack of change in the native ESR spectrum at denaturant concentrations below the transition region, the reversibility of the ESR spectral changes, and the lack of change in difference spectra at denaturant concentrations below the transition region (e.g., Figure 2 at 1.0 M Gdn-HCl). However, the increase in signal intensity in the region between the low-field turning point maxima and the center line that is observed at higher denaturant concentrations (e.g., Figure 2 at 2.5−3.0 M Gdn-HCl) indicates that folding intermediates may exist. Similar spectral observations have recently been reported for spin-labeled carbonic anhydrase (Lindgren *et al.*, 1995; Svensson *et al.*, 1995). However, both Gdn-HCl and urea denaturation curves show only a single transition, and there are no identifiable intermediates observed on nondenaturing polyacrylamide gels, suggesting that these may be transient states. In the present case, the two-state model serves as a useful initial approximation. Extrapolation to zero guanidine concentration provides an estimate of the free energy of folding in the absence of denaturant, $\Delta G_U°$ (see Materials and Methods). $\Delta G_U°$ values were also determined by fitting the denaturation curves

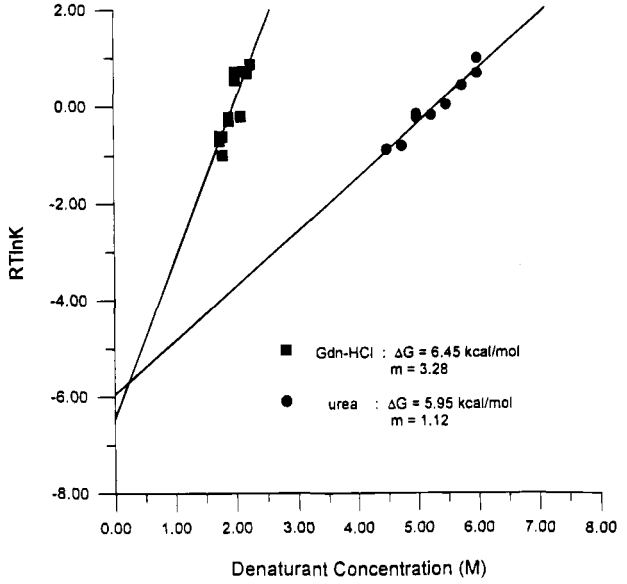


FIGURE 5: Free energy of denaturation for the ligand-binding domain of FepA. $RT$ ln $K$ values were calculated for $K$ = [(% denatured)/(% native)] from the transition region of the Gdn-HCl (squares) and urea (circles) denaturation curves after 24 h at ambient temperature. Extrapolation by linear regression analysis to zero denaturant concentration gives an estimate of $\Delta G$ for unfolding in the absence of denaturant, $\Delta G_U°$, and the slope, $m$, is a measure of cooperativity (see Table 1).

Table 1: Thermodynamic Parameters for Denaturant Unfolding of FepA[a]

| denaturant | $\Delta G_U°$ (kcal mol$^{-1}$) | $m$ (kcal mol$^{-1}$ M$^{-1}$) | $C_m$ (M) |
|---|---|---|---|
| Gdn-HCl | 6.46 ± 1.08[b] | 3.28 ± 0.05 | 1.95 ± 0.06 |
| | 6.40 ± 0.64[c] | | |
| urea | 5.96 ± 0.56[b] | 1.12 ± 0.11 | 5.48 ± 0.03 |
| | 6.14 ± 0.23[c] | | |

[a] $\Delta G_U°$ is the free energy of unfolding in the absence of denaturant calculated [b] from linear extrapolation (Figure 5) and [c] from $mC_m$. $m$ is the dependence of the unfolding free energy on denaturant concentration, obtained from the slope of the linear extrapolation. $C_m$ is the midpoint concentration of the unfolding transition at equilibrium, calculated by fitting the experimental data to reversible two-state equilibria (Figure 4).

(Figure 4) to a two-state equilibrium, and from the product ($mC_m$). These parameters, along with the midpoint concentrations of unfolding, $C_m$, are given in Table 1. The $\Delta G_U°$ values calculated by these different methods are in good agreement. The free energy change determined from urea and Gdn-HCl agreed within experimental error, indicating that it is characteristic of the protein and not dependent on specific interactions with a given denaturant. The slope in Figure 5 also provides a measure of folding cooperativity. A comparison of these values with those for several water-soluble proteins (discussed below) confirms the apparent high cooperativity of the observed transition.

It is noteworthy that the EPR spectrum of the native state (e.g., top spectra in Figures 1 and 2) is not a pure powder pattern, having some spectral intensity in the region between the low-field parallel and perpendicular turning points. Signal intensity in that region of the spectrum could arise from dipolar interaction between labels (Lihktenstein, 1976; Anthony-Cahill *et al.*, 1992), or the presence of a small population of spin labels in structural states that allow greater rotational freedom than the fully folded conformation (discussed above). Since there is a spin label:protein

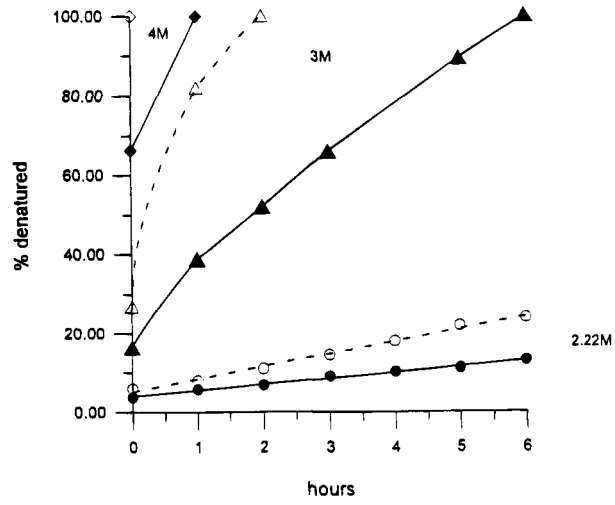


FIGURE 6: Effect of ferric enterobactin on denaturation rate. Samples containing MTSL-E280C (0.1 mM) in the presence (closed symbols) or absence (open symbols) of a slight molar excess of ferric enterobactin were mixed with Gdn-HCl, their ESR spectra were followed as a function of time at ambient temperature, and the percent denaturation was determined by spectral subtraction. Final concentrations of Gdn-HCl were (◇, ◆) 4.0 M, (△, ▲) 3.0 M, and (○, ●) 2.22 M.

stoichiometry of 1:1 (Liu *et al.*, 1994), the possibility of dipolar interaction is unlikely. Given the localization of the spin label site in a surface loop that is part of the ligand-binding domain of FepA, the existence of some flexibility in this region with rapid interconversion between states due to protein breathing would not be surprising.

*Effect of Ferric Enterobactin.* To assess the effect of ligand binding on the structural stability of FepA, we followed the rate of denaturation at various Gdn-HCl concentrations in the presence and absence of ferric enterobactin (Figure 6). As discussed above, the rate of denaturation was dependent on Gdn-HCl concentration. In the absence of FeEnt, denaturation was complete within an hour (at 22 °C) for samples containing 3 M Gdn-HCl and within minutes at 4 M guanidine, whereas in the presence of FeEnt unfolding took considerably longer. At 1.75−2.5 M Gdn-HCl, samples both with and without FeEnt unfolded more gradually, and binding of FeEnt to MTSL-E280C prior to Gdn-HCl addition again dramatically decreased the rate of unfolding (Figure 6).

## DISCUSSION

These studies demonstrate the use of SDSL to follow denaturant-induced unfolding of an integral membrane protein. Such studies are proving increasingly useful for providing insights into the factors that influence the stabilization of protein structure. Use of the ESR spin labeling technique to examine changes in protein conformation is well-established (e.g., Ogawa & McConnell, 1967; for reviews see Berliner, 1976, 1979), with several successful applications in the past few years (reviewed by Hubbell & Altenbach, 1994). Spin labeling is particularly well-adapted to the study of membrane, vesicular, or micellar systems since ESR is not influenced by the optical or light-scattering properties of the sample. These factors can often limit the application of CD and other optical techniques.

The free energies of unfolding obtained from SDSL studies will be distinctly characteristic of the region of the protein

occupied by the spin label and could well be different for labels at different sites. Given that most membrane receptors, indeed most proteins, are thought to be composed of multiple domains, this method provides an opportunity to examine differences in the structural integrity in distinct regions of a protein. A recent study of the extracellular region of CD4 examined interaction among domains by characterizing their denaturation when expressed separately and in combination (Tendian *et al.,* 1995). The ability to selectively introduce single conformationally-sensitive probes at desired locations would allow such studies to be done on the intact protein, providing a powerful approach to examining the forces stabilizing multipartate receptors.

There is considerable interest in the mechanisms by which membrane proteins undergo folding and insertion into the lipid bilayer. The outer membrane protein OmpA can be completely unfolded in 8 M urea and will spontaneously refold upon dilution in the presence of small unilamellar vesicles (Surrey & Jähning, 1992). OmpA will also refold following thermal denaturation in the presence of octyl glucoside (Dornmair *et al.,* 1990) or lipopolysaccharide (Schweizer *et al.,* 1978). Similarly, *E. coli* porin will refold into its native structure following complete denaturation in 6 M Gdn-HCl (Eisele & Rosenbusch, 1990). Bacteriorhodopsin, after acid denaturation, refolds upon addition of SDS followed by mixing with phospholipids, cholate, and retinal (Huang *et al.,* 1981). FepA, after denaturation in Gdn-HCl or urea, was induced to refold by ethanol precipitation followed by solubilization in MOPS/Triton, dialysis (against MOPS/Triton) to remove the denaturant, or in some cases simple dilution. Refolding was observed by ESR, where the spectrum returned to that of the native state; or on polyacrylamide gels, where FepA returned to its compact, rapidly migrating structure. Renaturation occurred in the presence of Triton X-100 without addition of exogenous lipids. This is in contrast to porin, which requires the presence of an anionic amphiphile (e.g., SDS) and/or lipids to regain structure from a random coil state (Eisele & Rosenbusch, 1990), but similar to OmpA, which requires only the hydrophobic environment provided by a nonionic detergent (Dornmair *et al.,* 1990). Although our ESR studies probe only the local environment of the spin label and may not reflect overall structure, the compact structure responsible for anomalous migration on gels (presumably the $\beta$-structure of the transmembrane domain) was also regained upon removal of denaturant. Previously, we showed that, following a procedure that involved denaturation in 6 M urea and reduction and labeling of the native cysteine residues, FepA retained its ability to bind ferric enterobactin after dialysis to remove the urea (Liu *et al.,* 1994). Further studies on the conditions required for folding of the various regions of FepA are in progress. With regard to characterizing the thermodynamics of unfolding, refolding indicates that denaturation of FepA by either Gdn-HCl or urea is a reversible process.

The free energy change associated with unfolding of FepA, approximately 6 kcal/mol, is similar to that observed for several water-soluble globular proteins. Myoglobin, lysozyme, ribonuclease, and barnase all have $\Delta G_U$ values reported in the range of 5−10 kcal/mol (e.g., Pace & Vanderberg, 1979; Ahmad & Bigelow, 1982; Serrano *et al.,* 1990; Yao & Bolen, 1995). Surface domains of T-cell CD4 have somewhat lower $\Delta G_U$ values, in the range of 2−4 kcal/mol (Tendian *et al.,* 1995). The cooperativity of FepA unfolding, as estimated from the slope of the $RT \ln K_{dn}$ *vs* [Gdn-HCl] extrapolation (Figure 5), is relatively high compared to that of the above proteins. We are not aware of a quantitative evaluation of $\Delta G_U°$ for any other integral membrane protein.

Because ferric enterobactin has limited stability at room temperature, it was not possible to obtain an equilibrium denaturation curve for ligand-saturated FepA, which would have provided an estimate of the free energy of stabilization imparted by binding of the ligand. Initial rates of unfolding were considerably slower in the presence of FeEnt than in its absence, clearly demonstrating receptor stabilization. The increased resistance of FepA to Gdn-HCl denaturation upon binding of FeEnt is consistent with our previous results on the effects of FeEnt binding (Liu *et al.,* 1994), where we observed a ligand-induced conformational change that resulted in a more compact, structured environment in the region around E280C as indicated by a decrease in the rotational mobility of the spin label. Studies aimed at determining the free energy of unfolding in the presence of FeEnt by characterizing the initial rates of both the forward (unfolding) and reverse (refolding) reactions are in progress.

In summary, we have shown that a spin-labeled mutant of the ferric enterobactin receptor, FepA, undergoes reversible denaturation in the presence of Gdn-HCl or urea. Unfolding was highly cooperative and was strongly inhibited by the bound ligand. The unfolding transitions for the ligand-binding domain, as monitored by ESR spin labeling, and the proposed transmembrane $\beta$-barrel, as monitored by nondenaturing gel electrophoresis, occurred over a similar range of denaturant concentrations. Further SDSL studies with additional FepA mutants will help determine if localized domains exist in this receptor that undergo independent folding−unfolding transitions.

## ACKNOWLEDGMENT

The authors wish to thank Dr. M. Claire Kennedy for a careful reading of the manuscript and several helpful discussions.

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BI951496D