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The N-Terminal Domain of a TonB-Dependent Transporter Undergoes a Reversible Stepwise Denaturation

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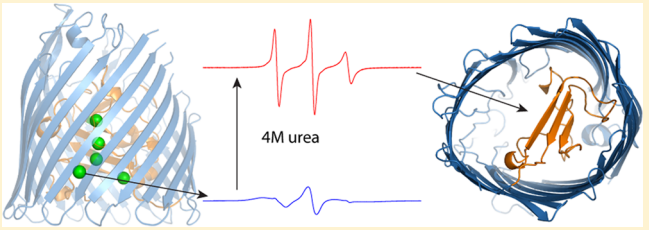
ABSTRACT: Gram-negative bacteria contain a family of outer membrane transport proteins that function in the uptake of rare nutrients, such as iron and vitamin B_{12}. These proteins are termed TonB-dependent because transport requires an interaction with the inner-membrane protein TonB. Using a combination of site-directed spin labeling and chemical denaturation, we examined the site-specific unfolding of regions of the *Escherichia coli* vitamin B_{12} transporter, BtuB. The data indicate that a portion of the N-terminal region of the protein, which occupies the lumen of the BtuB barrel, denatures prior to the unfolding of the barrel and that the free energy of folding for the N-terminus is smaller than that typically seen for globular proteins. Moreover, the data indicate that the N-terminal domain does not unfold in a single event but unfolds in a series of independent steps. The unfolding of the N-terminus is reversible, and removal of denaturant restores the native fold of the protein. These data are consistent with proposed transport mechanisms that involve a transient rearrangement or unfolding of the N-terminus of the protein, and they provide evidence of a specific protein conformation that might be an intermediate accessed during transport.



Gram-negative bacteria acquire rare nutrients by the use of high-affinity transport proteins that are localized within the outer membrane. These transporters are termed TonB-dependent because they interact with the transperiplasmic inner-membrane protein TonB and apparently extract energy for transport from the inner-membrane proton potential.[1−6] Crystal structures for at least 12 unique TonB-dependent transporters have been obtained. They are structurally homologous and consist of two domains: a 22-stranded β-barrel and an N-terminal domain that is folded within the interior of the barrel. At the very N-terminus is an energy coupling segment termed the Ton box, which is required for transport and directly interacts with TonB.[7−9]

Shown in Figure 1 is an X-ray crystal structure of the apo form of BtuB, the *Escherichia coli* TonB-dependent vitamin B_{12} transporter. In addition to the apo form, crystal structures of BtuB have also been obtained in ligand-bound and TonB-bound forms.[9−11] In these and other TonB-dependent transporter structures, there is no obvious passage for substrate through the barrel. Moreover, in the ferrichrome transporter, FhuA, molecular dynamics simulations indicate that there is no water-filled channel through which the substrate might pass.[12] If the barrel of these proteins remains intact during transport, significant conformational rearrangements in the N-terminus would be necessary to facilitate movement of the substrate through the barrel.

Transport mechanisms that involve either the removal of the N-terminal region as an intact domain or a series of unfolding events within the N-terminus have been proposed.[4,13] As a result, the N-terminal domain is sometimes termed a cork, plug, or hatch domain to indicate this proposed role. In the high-resolution crystal structures of BtuB, there are large numbers of waters at the interface between the N-terminal domain and the transporter β-barrel, which suggests that the N-terminal domain may be prone to conformational rearrangements or complete removal.[14] In a steered molecular dynamics simulation, where the Ton box of BtuB is extracted from the periplasmic side of the β-barrel, the N-terminal core is observed to progressively unfold, ultimately exposing a water-filled cavity sufficiently large for passage of the substrate.[15] Denaturation experiments also suggest that the N-terminal domain and the β-barrel behave independently. For example, thermal denaturation indicates that the N-terminal domain and the β-barrel behave as two separate domains, and that the core might undergo structural rearrangements inside a static barrel.[16] Similarly, in a reconstituted planar bilayer system, it has been shown that a pore may be reversibly opened at moderate concentrations of urea in BtuB and related TonB-dependent transporters.[17] Because these transporters retain the ability to bind ligand in the presence of urea, the data suggest that a partial and reversible unfolding of the N-terminal domain will open a channel within the transporter.

In this study, we use site-directed spin labeling (SDSL) to determine whether the N-terminal region of BtuB can be reversibly unfolded while retaining the protein β-barrel structure. This spin labeling approach was used previously to examine the site-specific denaturation of an extracellular loop and transmembrane β-strand in a related TonB-dependent

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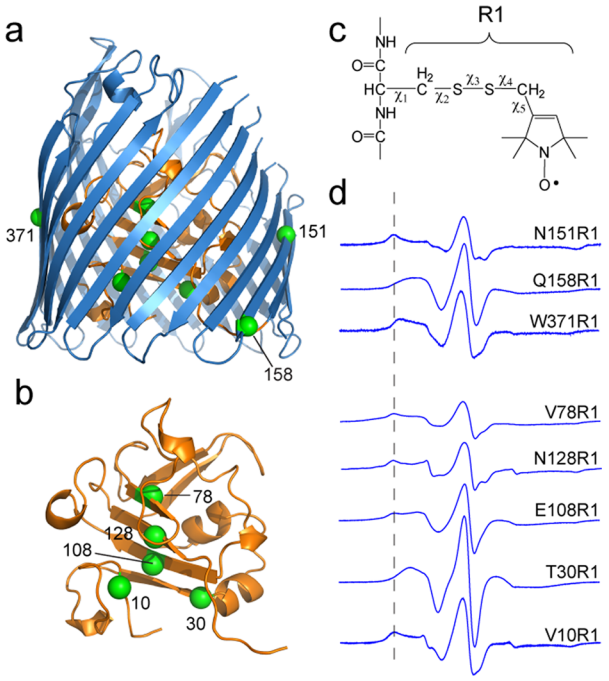

Figure 1. Model and EPR spectra from BtuB. (a) High-resolution model of BtuB (Protein Data Bank entry 1NQE) in which the N-terminal region is colored orange. The Cα atoms are colored green for three sites on the β-barrel that were labeled with the MTSL spin-labeled side chain R1 as well as sites in the N-terminus. (b) The N-terminal fold includes the first 136 residues of BtuB and is shown without the barrel. The N-terminal domain was spin labeled in the Ton box at position 10, as well sites on four successive β-strands at positions 30, 108, 128, and 78. The spin-labeled side chain R1 is shown in panel c, and the EPR spectra resulting from these eight sites are shown in panel d. With the exception of sites 78, 108, and 128, the EPR spectra for these sites for BtuB in POPC have been previously reported.[22,39] The dashed line indicates the position of the low-field hyperfine extrema, which is observed for sites in tertiary contact.

transporter, FepA.[18,19] In our case, the data indicate that the urea-induced unfolding of the N-terminus of BtuB does not occur in a single cooperative step but occurs in a series of steps. A region of the N-terminal domain, which includes the first β-strand within the core of BtuB, unfolds prior to the remainder of the core and the β-barrel. In this intermediate state, a channel is likely to be opened within the interior of BtuB that may be sufficiently large to accommodate the substrate. The results demonstrate that the N-terminal domain of BtuB is capable of being reversibly unfolded, and the data suggest a model for an intermediate structural or excited protein conformational state that might be accessed during transport.

MATERIALS AND METHODS

Mutagenesis, Expression, Purification, and Spin Labeling. BtuB mutants were engineered using the Agilent Technologies (Santa Clara, CA) Quick Change Site Directed Mutagenesis Kit. The mutants were overexpressed in *E. coli* strain RK5016 (*metE*).[20] Cells were grown in minimal "A" medium containing 100 μg/mL ampicillin and supplemented with 0.24% (w/v) glucose, 150 μM thiamine, 3 mM $MgSO_4$, 300 μM $CaCl_2$, and 0.01% (w/v) Met and Arg.

Outer membranes were prepared, solubilized, and spin labeled with 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl methanethiosulfonate (MTSL, Toronto Research Chemicals, North York, ON) to introduce the spin-labeled side chain, R1, following a previous protocol.[21] The protein was then purified and reconstituted into POPC vesicles as described previously,[22] and the proteoliposomes were concentrated using a Beckman (Brea, CA) Airfuge.

Sample Preparation and EPR Measurements. Samples were titrated with urea, yielding final urea concentrations ranging from 0 to 12 M. Stock solutions of urea were dried in 0.5 mL tubes overnight using a vacuum concentrator (Savant SpeedVac, Asheville, NC), and accurate final concentrations of the denaturant were obtained by empirically determining the volume occupied by urea in solution. Reconstituted BtuB was added to the dried urea, and the samples were allowed to sit for 1 h at room temperature. The BtuB/urea samples were then subjected to four freeze−thaw cycles using liquid nitrogen and mixed after each cycle to achieve uniform urea accessibility throughout the proteoliposomes. To refold the protein from a urea-denatured state, the sample was diluted 10−20-fold into reconstitution buffer [10 mM HEPES and 130 mM NaCl (pH 6.5)] and concentrated by ultracentrifugation using a Beckman Airfuge.

EPR measurements were performed on 5 μL of sample loaded into glass capillaries with a 0.6 mm inside diameter and a 0.84 mm outside diameter (VitroCom, Mountain Lakes, NJ). These samples had protein concentrations of 100−200 μM. EPR spectra were recorded on a modified Varian E-line 102 Century series X-band spectrometer with a loop gap resonator (Molecular Specialties, Milwaukee, WI). LabVIEW software, provided by C. Altenbach (University of California, Los Angeles, CA), was used for digital collection and analysis of data. All spectra were recorded using a 2 mW incident microwave power and a 1 G peak-to-peak modulation amplitude. The scan range was 100 G, and the spectra were normalized to equivalent spin numbers by double integration of the first-derivative EPR spectra.

Data Analysis. The free energy of unfolding of a protein is known to vary linearly with the concentration of denaturant [23] as given by

$$\Delta G_U = -mC + \Delta G_U^\circ \quad (1)$$

Where ΔG_U° is the standard state free energy for unfolding in the absence of denaturant and C is the concentration of urea. The parameter m is related to the solvent accessible protein surface area exposed to solution upon denaturation. In this case, we use EPR spectroscopy to follow the site-specific denaturation of the protein as described previously.[18,19] The EPR line shapes of the protein-attached nitroxide, R1 (Figure 1c), are defined by the motion of the label on the nanosecond time scale, which is strongly dependent upon the local environment at the labeled site, including the tertiary contact of the label within the protein and the flexibility of the protein backbone to which the label is attached.[24] In the analysis of the data obtained here, we assume that the EPR spectra reflect the protein, or protein domain, in one of two states: a folded form and an unfolded or urea-denatured form. In this case, the EPR spectra at differing concentrations of urea should be a linear combination of the EPR spectra in the two states, and the peak-to-peak amplitude of the normalized first-derivative EPR spectrum, A, will then be given by

$$A = f_F A_f + f_U A_u \quad (2)$$

where f_F represents the fraction of protein in the folded state, f_U represents the fraction of protein in the unfolded state, and

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A_f and A_u are the normalized amplitudes of the EPR spectra in the folded and urea-denatured states, respectively. An inspection of eq 2 indicates that the peak-to-peak amplitude, A, is linearly related to the fraction of unfolded protein as given by $f_U = (A - A_f)/(A_u - A_f)$, and plots of A versus urea concentration should have a shape identical to the shape of those of the fraction of unfolded protein versus urea. Peak-to-peak amplitudes of EPR spectra were measured at each urea concentration for each mutant. The values for the normalized amplitudes as a function of concentration were then plotted and fit as described previously[25] using OriginPro (Origin Lab, Northhampton, MA) to an equation of the form

$$A = \frac{A_F - A_U}{1 + e^{(C-C_0)/dC}} + A_U \quad (3)$$

where C_0 is the value of C, the urea concentration, at which $A = (A_f + A_u)/2$, and dC is a measure of the width in C corresponding to the most significant change in A. The parameter m was calculated from the relationship $m = RT/dC$, where R and T have their usual meanings. Using this method of fitting, the free energy of unfolding in the standard state is given by the equation $\Delta G^\circ_U = mC_0$.

We also determined the parameters for urea denaturation using a complementary approach by finding the fractions of folded and unfolded protein from each experimental spectrum. In this case, we fit the experimental spectrum to a linear combination of the spectra that represent the folded and unfolded protein. The spectrum for the folded protein was taken as that in POPC in the absence of denaturant, and the spectrum of the unfolded protein was taken as that at the highest limiting concentrations of denaturant used. Using a least-squares approach, the best fit yielded the fraction of folded protein, f_F, and the energy for unfolding at each intermediate urea concentration was then calculated from the equation $\Delta G_U = RT \ln(f_F)/(1 - f_F)$. These free energies were plotted as a function of urea concentration, and the data fit with eq 1 to obtain values for m and ΔG°_U.

RESULTS

EPR Spectra of BtuB Indicate That the Core or Hatch Region of the Protein Is Well-Folded. Shown in Figure 1 are locations of eight sites in BtuB into which the spin-labeled side chain, R1, was incorporated as well as their corresponding EPR spectra. Five of these labeled sites are located in the N-terminal core region of BtuB, and three are localized at sites in the β-barrel on strands 2 and 12. The labeled mutant proteins appear to be correctly folded. Each of the labeled mutants exhibited a behavior similar to that of the wild-type protein upon purification and reconstitution into POPC bilayers. In addition, crystal structures were previously obtained for several of these spin-labeled mutants and indicate that there is minimal perturbation of the protein structure upon incorporation of the label.[26,27]

The label at position 10 within the BtuB Ton box, V10R1, has been previously examined in some detail, and the crystal structure for this mutant indicates that the label lies at the base of a pocket on the periplasmic surface where it is in strong tertiary contact within the N-terminal domain of the protein.[26] This is consistent with the EPR spectrum of V10R1, which indicates that the label is restricted in its motion (Figure 1d). Positions 30, 108, 128, and 78 are located on each of four β-strands that form a sheet directed toward the extracellular surface (Figure 1b). The EPR spectrum of T30R1 is the most mobile of the EPR spectra within the N-terminus, and it corresponds to a label having a correlation time of approximately 4−5 ns. This is consistent with its position, which places the label at a site of aqueous exposure. The EPR spectra at positions 108, 128, and 78 indicate that these nitroxides are restricted in their motion because of tertiary contact. Interestingly, when the spectra from these four β-strands are compared, the labels become slightly less mobile, as judged by the normalized amplitudes, when the spin-label is positioned farther into the protein toward the extracellular surface. These spectra indicate that these labels are folded into the protein interior, and that any unfolded population of the protein N-terminal domain (which would have shown up as a mobile component in the EPR spectrum) is small.

Three labels were placed into the β-barrel of BtuB at sites 151 and 158 on strand 2 and site 371 on strand 12. Site 151 faces the interior of the protein, and it yields an EPR spectrum near the rigid motional limit, indicating that the label is present in a sterically restrained environment. The other two sites are located at the hydrocarbon surface of the β-barrel. These spectra are complex but are consistent with R1 at sites facing the hydrocarbon surface of a β-barrel. The origins of line shapes at these hydrocarbon-facing sites of β-barrel proteins have been described in detail elsewhere.[27,28]

EPR Spectra Reveal Residual Protein Structure at the Highest Urea Concentrations Tested. Shown in Figure 2a are normalized EPR spectra recorded at increasing urea concentrations for spin-labels at positions 10, 30, 78, and 108. The changes in these EPR spectra after mixing with urea were complete within 1 h, and spectra that were recorded a day or more after the addition of denaturant exhibited no further change. These spectra are typical of those seen at other sites, and they exhibit a dramatic increase in normalized amplitudes and a narrowing of the EPR line shapes upon urea addition. As indicated previously, the changes in line shape with increasing denaturant concentration are due to a loss of secondary and tertiary structure and an increase in backbone dynamics.[18,19]

Shown in Figure 2b are EPR spectra for a number of sites at the highest urea concentrations tested. In most cases, these are urea concentrations at which the unfolding transition appears to be complete (see Figure 3). The EPR spectra in Figure 2b of sites 10 and 30 result from R1 having a single motional component, corresponding to a label with a correlation time of approximately 0.6 ns. This is consistent with the N-terminal segment assuming a fully unstructured random coil configuration in the presence of urea. However, at all the other sites tested, the spectra are multicomponent and result from at least two motional components of R1: one which is isotropic and rapidly diffusing on the EPR time scale and a second resulting from the label undergoing more restricted motion (arrows in Figure 2b). In these cases, subtraction of the more mobile component and double integration indicate that the population of label undergoing more restricted motion is approximately 40−50% of the total spin signal. The mobile components from labels at sites 78, 108, 121, and 158 are isotropic but exhibit correlation times different from those seen at positions 10 and 30. At high urea concentrations, the label correlation time for V78R1 is approximately 0.3 ns, roughly double the diffusional rate of labels at positions 10 and 30. In contrast, the mobile isotropic signals from sites E108R1, N128R1, and Q158R1 have longer correlation times of approximately 1.0−1.1 ns. These differences in motion appear as a difference in line width

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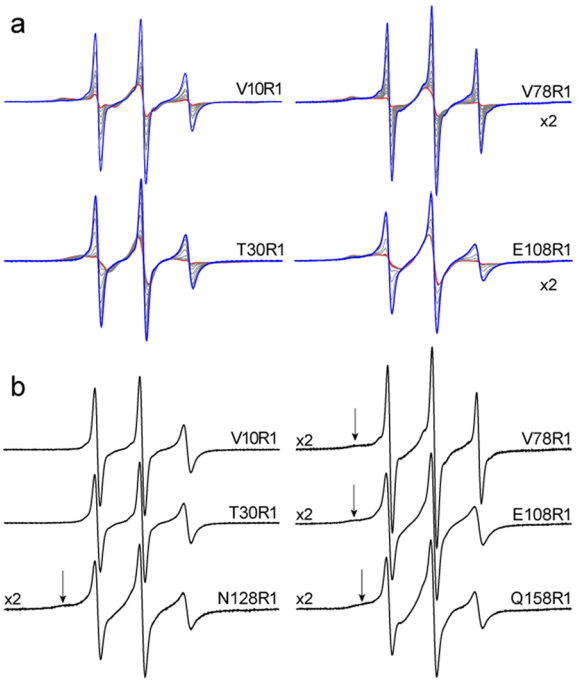

Figure 2. (a) EPR spectra obtained from R1 at four sites in BtuB as a function of added urea concentration. Sequential spectra are shown for V10R1 from 0 to 4 M urea and T30R1 from 0 to 5 M urea as the urea concentration is increased in 0.5 M steps. Sequential spectra are shown for V78R1 from 0 to 9 M in 1 M steps and for E108R1 from 0 to 12 M in 2 M steps. Spectra obtained from BtuB in POPC in the absence of urea are colored red, and EPR spectra obtained at the highest urea concentrations are colored blue. (b) EPR spectra for positions 10, 30, 78, 108, 121, and 158, at the highest urea concentrations used. These concentrations were 6, 5, and 11 M urea for sites 10, 30, and 78, respectively, and 12 M urea for the remaining sites. The arrows indicate the position of a second motional component due to a more compact conformation in the urea-denatured state.

and a difference in the ratio of amplitudes between the high-field and central nitroxide resonances.

The existence of broader components in these EPR spectra at high denaturant concentrations has been observed previously for FepA,[18] and these features provide a strong indication that at the highest urea concentrations reached regions of the N-terminal core of BtuB, beyond the first β-strand, are in an equilibrium between populations of highly unstructured (largely random coil) and more compact protein conformations. The fact that the motionally mobile components vary in line shape and exhibit significantly different correlation times indicates that the unstructured state is not uniform, and that the protein backbone dynamics in the most unfolded state varies with position on the nanosecond time scale. These observations are consistent with the finding that nativelike protein structure can persist even in the presence of high concentrations of denaturants,[29] and with the view that denatured states of proteins are more complex and more compact than a truly random coil.[30]

Sites in the N-Terminal Domain of BtuB Unfold at Different Urea Concentrations and Have Differing Solvent Exposures. Shown in Figure 3 are plots of the normalized EPR amplitudes (obtained from spectra such as those shown in Figure 2a) as a function of urea concentration for a number of sites in BtuB. Also shown are fits to these data using eq 3. A comparison of the denaturation curves for labels in the N-terminus, particularly sites 10 and 30, indicates that they undergo an unfolding transition at lower urea concentrations than do sites deeper in the N-terminal core or in the BtuB barrel. The data were also fit as described in Materials and Methods by directly calculating the fraction of unfolded protein and performing a linear extrapolation to the data for the free energy versus urea concentration according to eq 1. Several of these plots are shown in Figure 4, and a summary of the data obtained for the eight mutants is given in Table 1. It should be noted that each experimental spectrum could be fit with a linear combination of the spectra that represent the folded and unfolded protein, consistent with the assumption that the spectra report two structural states.

The data listed in Table 1 indicate that the thermodynamic stabilities measured at sites within the N-terminal domain are similar, with the values of ΔG_U° varying from ~2.2 to ~3.2 kcal/mol. These values are smaller than those typically obtained for the denaturation of globular proteins,[23,31,32] and they are smaller than those previously seen using SDSL for the barrel of a homologous TonB-dependent transporter, FepA.[18] The values of ΔG_U° obtained by SDSL should be viewed with some caution, because the spin-labeled side chain, R1, is known to modulate ΔG_U° particularly when placed at deeply buried sites or at sites in strong tertiary contact.[24] Nonetheless, if these values are perturbed by the spin-labels, the values are remarkably similar, and this includes the value of ΔG_U° obtained from site 30, which should not be highly perturbed because of the aqueous exposure at this site.

Urea Denaturation Indicates That the N-Terminal Domain Does Not Unfold in a Single Step. Unlike the values of ΔG_U°, which are remarkably consistent, the values of m vary substantially. As shown in Table 1, there is an approximately 3-fold variation in the values of m for sites in the N-terminus, with sites 10 and 30 yielding the highest values of m and sites 78 and 128 yielding the lowest. The values of C_0 (the urea concentration at the midpoint of unfolding) is also substantially different for sites 10 and 30, which is expected because the product of m and C_0 yields the free energy of unfolding. Interestingly, the values of m become progressively smaller as the spin-label is moved farther toward the extracelluar surface of BtuB within the N-terminal domain. The parameter m strongly correlates with the amount of protein surface area that becomes exposed to solution upon the unfolding of the protein with urea.[33] Because these numbers vary substantially within the N-terminus, this region must not unfold in a single concerted step but must unfold in a number of independent steps. Sites 10 and 30 unfold at the lowest concentrations of urea with similar values of m, suggesting that the Ton box and the first β-strand C-terminal to the Ton box unfold in a single step with the largest exposure of protein surface area. Strands farther into the protein interior unfold at higher urea concentrations and involve progressively smaller exposures of surface area.

The three labels within the β-barrel region unfold at urea concentrations that are much higher than those at sites 10 and 30, and they yield similar values for protein stability compared to those elsewhere in BtuB. These values are smaller than those observed previously for the stability of a β-strand in FepA, but the measurements taken here were acquired in a lipid mixture different from that used previously. It should be noted that bilayer integrity seems to be maintained in the presence of urea, even at relatively high denaturant concentrations.[17,34,35]

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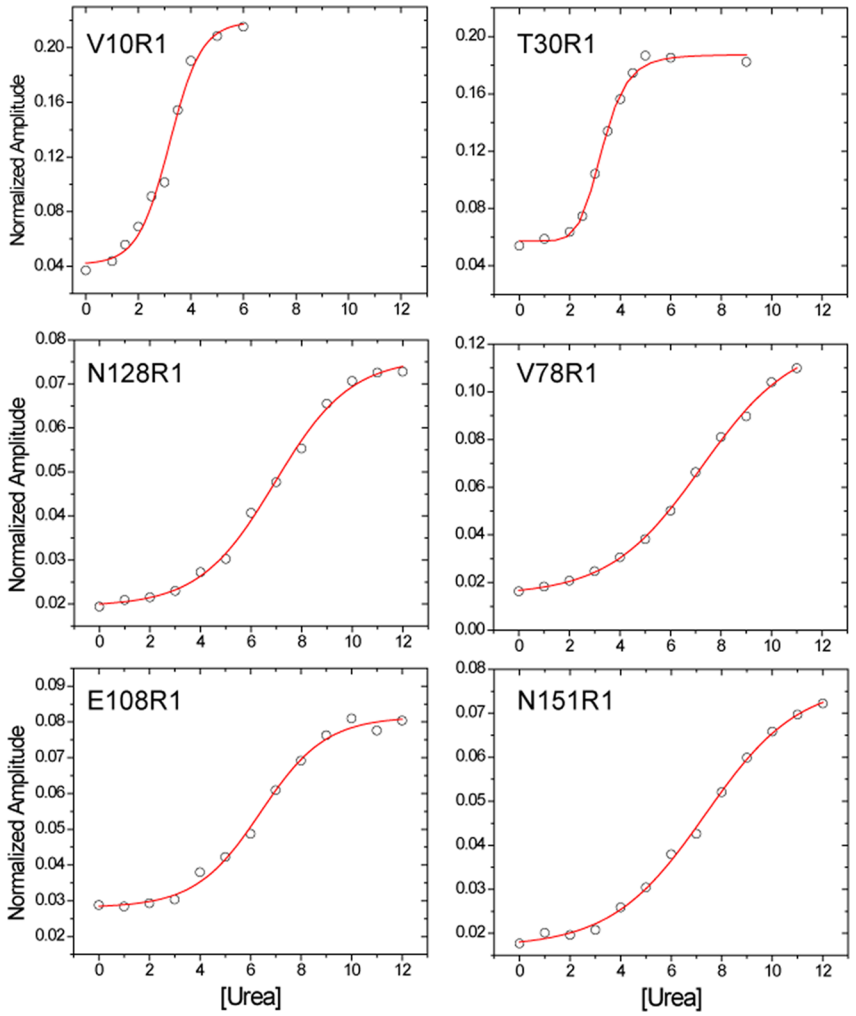

Figure 3. Plots of the normalized amplitudes of the EPR spectra from five core mutants and one of the barrel mutants as a function of urea concentration. As indicated in Materials and Methods, the normalized amplitude is linearly related to the fraction of unfolded protein. The lines represent the best fits to the data using eq 3 (see Materials and Methods). This fit assumes a two-state model in which the protein exists in a folded or unfolded conformation.

Unfolding of the N-Terminal Core Region Is Reversible. Once denatured, the N-terminal domain of BtuB can be refolded by the removal of urea. For the data shown here, urea was removed by dilution of the lipid/denaturant mixture (see Materials and Methods) and centrifugation to concentrate the sample. Shown in Figure 5 are EPR spectra of sites T30R1, E108R1, V78R1, and N128R1 at the highest concentration of urea indicated in Figure 3 and following the removal of the urea by dilution. The EPR spectra recorded after dilution of urea are identical to those taken after reconstitution into POPC and indicate that the protein refolds under these conditions to restore local structure around the labeled site.

DISCUSSION

The N-terminal domain that occludes the β-barrel of TonB-dependent transport proteins is generally thought to undergo a significant conformational rearrangement during transport. In one proposed transport mechanism, the N-terminal domain reversibly unfolds, where the unfolding is driven by an interaction between the transporter's Ton box and TonB.[4,13] In this study, site-directed spin labeling was used to determine the stability of the N-terminal domain and to characterize intermediates that could be accessed using reversible urea denaturation. The fact that the unfolding is reversible at every site examined suggests that the intermediates observed may be intermediates that are sampled during the folding of the protein.[36] Moreover, these intermediates might be sampled during transport that would be driven by a TonB-dependent unfolding.

The denaturation data obtained here provide strong evidence that the N-terminal domain of BtuB unfolds in a stepwise and localized fashion. The denaturation data shown in Figures 3 and 4 can be fit by a simple two-state mechanism; however, the apparent surface areas that are exposed during denaturation (as judged by the m values in Table 1) vary significantly, as do the concentrations of urea required to produce denaturation. As a result, the urea-induced unfolding of the N-terminal domain must not take place in a single cooperative step. The behavior of sites 10 and 30 with respect to urea denaturation is different than at other sites in BtuB. First, this region of the protein unfolds at significantly lower urea concentrations compared to other sites in BtuB. Second, the EPR spectra in the denatured state are similar, and they reflect a single isotropic motional component of the R1 side chain. Unlike other sites in BtuB, there is no evidence of residual compact structure in this segment of the protein at the completion of the urea titration.

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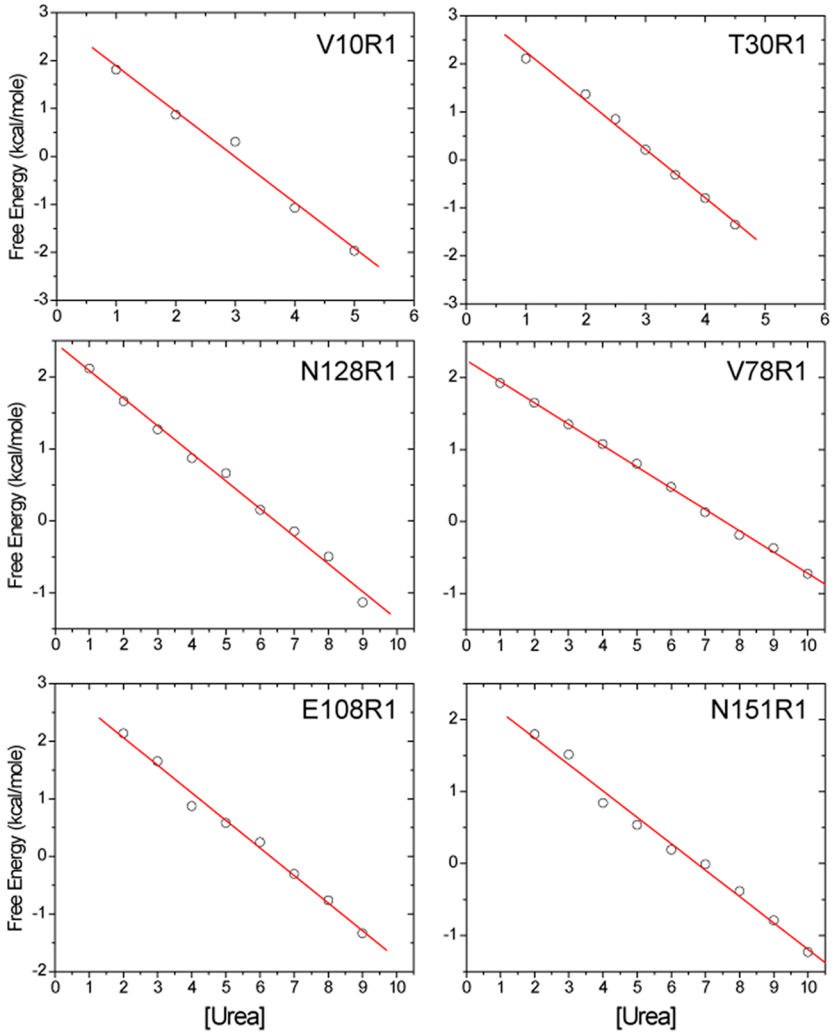

Figure 4. Linear extrapolation of free energies as a function of urea concentration to obtain the free energy of unfolding (eq 1). The free energies were determined from the populations of folded and unfolded protein. These populations were estimated by determining the linear combination of folded and unfolded spectra that provide the best fit for each spectrum in the presence of urea (see Materials and Methods).

Table 1. Thermodynamic Data from Urea Denaturation of BtuB

mutant in POPC	ΔG_U° (kcal/mol)[a]	ΔG_U° (kcal/mol)[b]	m (kcal mol^{-1} M^{-1})	C_0 (M)
V10R1	2.77 ± 0.21	3.01 ± 0.24	0.93 ± 0.06	3.25 ± 0.13
T30R1	3.23 ± 0.11	3.33 ± 0.12	1.00 ± 0.04	3.32 ± 0.04
V78R1	2.22 ± 0.02	2.12 ± 0.05	0.29 ± 0.00	7.23 ± 0.13
E108R1	2.99 ± 0.11	3.04 ± 0.13	0.47 ± 0.02	6.40 ± 0.14
N128R1	2.39 ± 0.05	2.53 ± 0.08	0.36 ± 0.01	6.95 ± 0.12
N151R1	2.42 ± 0.09	2.63 ± 0.11	0.36 ± 0.01	7.34 ± 0.14
Q158R1	2.89 ± 0.18	2.69 ± 0.42	0.39 ± 0.06	6.91 ± 0.22
W371R1	2.90 ± 0.07	3.88 ± 0.50	0.55 ± 0.07	7.05 ± 0.08

[a]Free energy of unfolding calculated by linear extrapolation (y-intercept). [b]Free energy of unfolding calculated as mC_0, where C_0 is the midpoint of the unfolding transition calculated by fitting a sigmoidal curve to urea concentration vs peak-to-peak amplitude (see Materials and Methods).

Third, the value of m, which reflects the protein surface area exposed to solvent during denaturation, is identical within experimental error at sites 10 and 30 and larger than at other sites examined. Taken together, the data suggest that at modest urea concentrations (4 M) a significant segment of the N-terminus is unfolded. This includes sites 10 and 30 and may include regions farther toward the C-terminus, but not extending to site 78.

What do these data tell us about a model for transport involving a transient unfolding of the N-terminus? First, the data suggest that regions of the N-terminus are substantially less stable than typical globular proteins (perhaps by a factor of 2 or 3) and that the N-terminus might be relatively easy to unfold. This finding is consistent with previous NMR measurements, which indicate that the N-terminal domain of FepA is not well-folded when expressed as an isolated domain.[37] Second, the data indicate that the N-terminus of

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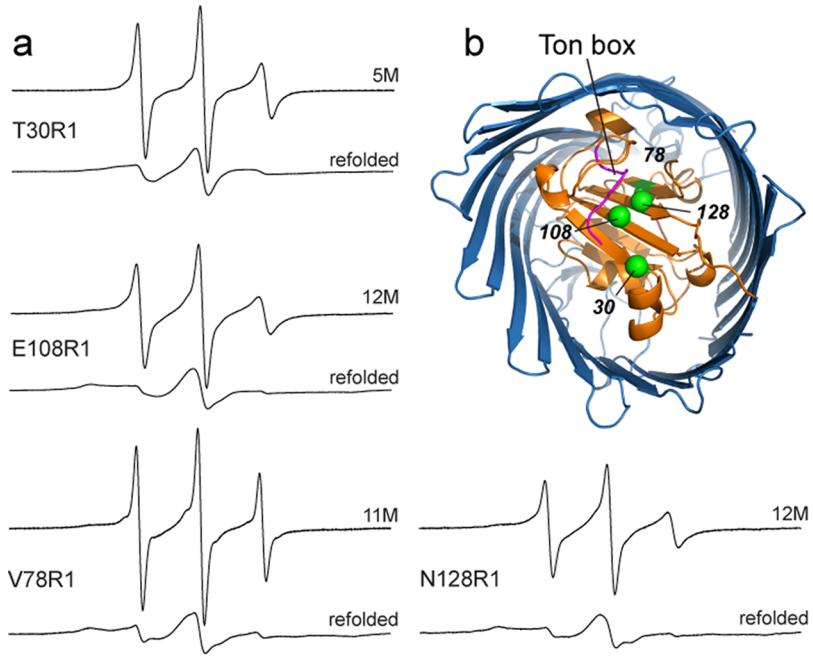

Figure 5. (a) EPR spectra of four R1 mutants within the core of BtuB following the removal of urea by dilution. Spectra for each site in the presence of high urea concentrations and following the removal of urea are shown. At each site, the EPR spectrum following removal of urea closely resembles the spectrum before denaturation, indicating that the local protein fold is restored at each of these sites. (b) Positions of these four sites within the core region of BtuB. In this model, the N-terminal domain is colored orange and the Ton box magenta.

BtuB can be refolded from this denatured state and that refolding from a TonB-driven unfolding event should be spontaneous. Finally, if a segment including residues 10 and 30 and extending up to residue 60 or 65 is unfolded from the interior of BtuB, a cavity is opened within the interior of BtuB, as shown in Figure 6. This unfolded segment includes several

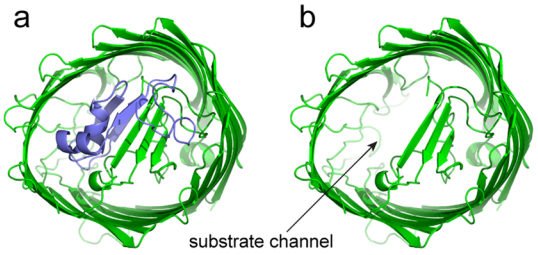

Figure 6. Unfolding a section of the N-terminus including the first β-strand within BtuB should open a pore that allows the passage of the substrate. (a) View from the periplasmic surface of BtuB in its folded state where residues 6−65 are highlighted in blue. (b) View of the periplasmic surface with the first 65 residues removed from the core of the protein.

substrate contact sites that are conserved in TonB-dependent transporters.[14] A cavity similar to that shown in Figure 6 was observed in steered molecular dynamics simulations where extraction of the Ton box of BtuB eventually opened a channel sufficiently large for substrate passage.[15] Moreover, a cavity such as that shown in Figure 6 may be the source of the ion conduction observed for reconstituted BtuB under similar denaturing conditions.[17] As a result, the opening of this cavity, for example, by an interaction of the Ton box with TonB, might release bound vitamin B_{12} from its extracellular binding pocket and allow passage of the substrate into the periplasm. It should be noted that although these denaturation experiments indicate the types of conformational transition states that might be sampled during a directed unfolding of the N-terminus of BtuB, there are no data to indicate that such a unfolding takes place during transport. Further work under conditions where active transport is taking place or where an intermediate state is trapped would be required to verify such a model.

The data obtained here by SDSL for the denatured state of BtuB provide a unique view of membrane protein structure under highly denaturing conditions. It is generally believed that the urea-denatured states of proteins are not random coils and that a significant level of compact structure exists even at relatively high urea concentrations.[29,30] The EPR data obtained here support this view. Two distinct motional components are observed for most sites in BtuB, suggesting that significant populations of an extended (random coil) and a more compact protein form are present under denaturing conditions. At X-band, conversion between these states must be slower than a few tens of nanoseconds for these states to be resolved, and additional experiments examining the relaxation rates of these labels could put a time scale on the rate of conversion between these forms.[38] In addition to compact and more extended forms, the EPR spectra indicate that the backbone dynamics of the extended form varies as a function of position along the sequence. Thus, even the more open form seen for the denatured protein is not a true random coil. This mixture of states generally applies to both the N-terminal domain and the membrane-imbedded β-barrel of BtuB. As a result, the ability of the protein to refold at every site examined is perhaps not surprising, because significant residual structure is likely maintained even under strongly denaturing conditions.

In summary, active transport requires the input of energy, and in the case of TonB-dependent transport, this energy is believed to be derived from the inner-membrane proton potential through an interaction with TonB. The results presented here indicate that a region of the N-terminus of

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BtuB can be unfolded under moderately denaturing conditions and that the N-terminus is denatured in a series of steps. The data are consistent with a model for transport that involves a partial unfolding of the N-terminal domain of BtuB, promoted by an interaction with energized TonB. This partial unfolding would lead to a release of the substrate from its binding site and could facilitate diffusion of the substrate through the BtuB barrel.

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Notes

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ABBREVIATIONS

EPR, electron paramagnetic resonance; MTSL, methanethiosulfonate spin-label; POPC, palmitoyloleoylphosphatidylcholine; R1, spin-labeled side chain produced by derivatization of a cysteine with the MTSL; SDSL, site-directed spin labeling.

REFERENCES

(1) Ferguson, A. D., and Deisenhofer, J. (2002) TonB-dependent receptors: Structural perspectives. *Biochim. Biophys. Acta 1565*, 318–332.

(2) Faraldo-Gomez, J. D., and Sansom, M. S. (2003) Acquisition of siderophores in Gram-negative bacteria. *Nat. Rev. Mol. Cell Biol. 4*, 105–116.

(3) Postle, K., and Kadner, R. (2003) Touch and go: Tying TonB to transport. *Mol. Microbiol. 49*, 869–882.

(4) Wiener, M. C. (2005) TonB-dependent outer membrane transport: Going for Baroque? *Curr. Opin. Struct. Biol. 15*, 394–400.

(5) Braun, V., and Endriss, F. (2007) Energy-coupled outer membrane transport proteins and regulatory proteins. *BioMetals 20*, 219–231.

(6) Noinaj, N., Guillier, M., Barnard, T. J., and Buchanan, S. K. (2010) TonB-dependent transporters: Regulation, structure, and function. *Annu. Rev. Microbiol. 64*, 43–60.

(7) Cadieux, N., and Kadner, R. J. (1999) Site-directed disulfide bonding reveals an interaction site between energy coupling protein TonB and BtuB, the outer membrane cobalamin transporter. *Proc. Natl. Acad. Sci. U.S.A. 96*, 10673–10678.

(8) Pawelek, P. D., Croteau, N., Ng-Thow-Hing, C., Khursigara, C. M., Moiseeva, N., Allaire, M., and Coulton, J. W. (2006) Structure of TonB in complex with FhuA, *E. coli* outer membrane receptor. *Science 312*, 1399–1402.

(9) Shultis, D. D., Purdy, M. D., Banchs, C. N., and Wiener, M. C. (2006) Outer membrane active transport: Structure of the BtuB:TonB complex. *Science 312*, 1396–1399.

(10) Chimento, D. P., Mohanty, A. K., Kadner, R. J., and Wiener, M. C. (2003) Substrate-induced transmembrane signaling in the cobalamin transporter BtuB. *Nat. Struct. Biol. 10*, 394–401.

(11) Cherezov, V., Yamashita, E., Liu, W., Zhalnina, M., Cramer, W. A., and Caffrey, M. (2006) In meso structure of the cobalamin transporter, BtuB, at 1.95 Å resolution. *J. Mol. Biol. 364*, 716–734.

(12) Faraldo-Gomez, J. D., Smith, G. R., and Sansom, M. S. (2003) Molecular dynamics simulations of the bacterial outer membrane protein FhuA: A comparative study of the ferrichrome-free and bound states. *Biophys. J. 85*, 1406–1420.

(13) Buchanan, S. K., Smith, B. S., Venkatramani, L., Xia, D., Esser, L., Palnitkar, M., Chakraborty, R., van der Helm, D., and Deisenhofer, J. (1999) Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. *Nat. Struct. Biol. 6*, 56–63.

(14) Chimento, D. P., Kadner, R. J., and Wiener, M. C. (2005) Comparative structural analysis of TonB-dependent outer membrane transporters: Implications for the transport cycle. *Proteins 59*, 240–251.

(15) Gumbart, J., Wiener, M. C., and Tajkhorshid, E. (2007) Mechanics of force propagation in TonB-dependent outer membrane transport. *Biophys. J. 93*, 496–504.

(16) Bonhivers, M., Desmadril, M., Moeck, G. S., Boulanger, P., Colomer-Pallas, A., and Letellier, L. (2001) Stability studies of FhuA, a two-domain outer membrane protein from *Escherichia coli*. *Biochemistry 40*, 2606–2613.

(17) Udho, E., Jakes, K. S., Buchanan, S. K., James, K. J., Jiang, X., Klebba, P. E., and Finkelstein, A. (2009) Reconstitution of bacterial outer membrane TonB-dependent transporters in planar lipid bilayer membranes. *Proc. Natl. Acad. Sci. U.S.A. 106*, 21990–21995.

(18) Klug, C. S., and Feix, J. B. (1998) Guanidine hydrochloride unfolding of a transmembrane β-strand in FepA using site-directed spin labeling. *Protein Sci. 7*, 1469–1476.

(19) Klug, C. S., Su, W., Liu, J., Klebba, P. E., and Feix, J. B. (1995) Denaturant unfolding of the ferric enterobactin receptor and ligand-induced stabilization studied by site-directed spin labeling. *Biochemistry 34*, 14230–14236.

(20) Heller, K., and Kadner, R. J. (1985) Nucleotide sequence of the gene for the vitamin B_{12} receptor protein in the outer membrane of *Escherichia coli*. *J. Bacteriol. 161*, 904–908.

(21) Coggshall, K. A., Cadieux, N., Piedmont, C., Kadner, R., and Cafiso, D. S. (2001) Transport-defective mutations alter the conformation of the energy-coupling motif of an outer membrane transporter. *Biochemistry 40*, 13946–13971.

(22) Fanucci, G. E., Cadieux, N., Piedmont, C. A., Kadner, R. J., and Cafiso, D. S. (2002) Structure and dynamics of the β-barrel of the membrane transporter BtuB by site-directed spin labeling. *Biochemistry 41*, 11543–11551.

(23) Yao, M., and Bolen, D. W. (1995) How valid are denaturant-induced unfolding free energy measurements? Level of conformance to common assumptions over an extended range of ribonuclease A stability. *Biochemistry 34*, 3771–3781.

(24) McHaourab, H. S., Lietzow, M. A., Hideg, K., and Hubbell, W. L. (1996) Motion of spin-labeled side chains in T4 lysozyme. Correlation with protein structure and dynamics. *Biochemistry 35*, 7692–7704.

(25) Hong, H., Park, S., Jimenez, R. H., Rinehart, D., and Tamm, L. K. (2007) Role of aromatic side chains in the folding and thermodynamic stability of integral membrane proteins. *J. Am. Chem. Soc. 129*, 8320–8327.

(26) Freed, D. M., Horanyi, P. S., Wiener, M. C., and Cafiso, D. S. (2010) Conformational Exchange in a Membrane Transport Protein Is Altered in Protein Crystals. *Biophys. J. 99*, 1604–1610.

(27) Freed, D. M., Khan, A. K., Horanyi, P. S., and Cafiso, D. S. (2011) Molecular origin of electron paramagnetic resonance line shapes on β-barrel membrane proteins: The local solvation environment modulates spin-label configuration. *Biochemistry 50*, 8792–8803.

(28) Flores Jimenez, R. H., Freed, D. M., and Cafiso, D. S. (2011) Lipid and Membrane Mimetic Environments Modulate Spin Label Side Chain Configuration in the Outer Membrane Protein A. *J. Phys. Chem. B 115*, 14822–14830.

(29) Shortle, D., and Ackerman, M. S. (2001) Persistence of native-like topology in a denatured protein in 8 M urea. *Science 293*, 487–489.
(30) Dill, K. A. (1990) Dominant forces in protein folding. *Biochemistry 29*, 7133–7155.
(31) Pace, C. N., and Vanderburg, K. E. (1979) Determining globular protein stability: Guanidine hydrochloride denaturation of myoglobin. *Biochemistry 18*, 288–292.
(32) Ahmad, F., and Bigelow, C. C. (1982) Estimation of the free energy of stabilization of ribonuclease A, lysozyme, α-lactalbumin, and myoglobin. *J. Biol. Chem. 257*, 12935–12938.
(33) Myers, J. K., Pace, C. N., and Scholtz, J. M. (1995) Denaturant m values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding. *Protein Sci. 4*, 2138–2148.
(34) Pastoriza-Gallego, M., Oukhaled, G., Mathe, J., Thiebot, B., Betton, J. M., Auvray, L., and Pelta, J. (2007) Urea denaturation of α-hemolysin pore inserted in planar lipid bilayer detected by single nanopore recording: Loss of structural asymmetry. *FEBS Lett. 581*, 3371–3376.
(35) Feng, Y., Yu, Z. W., and Quinn, P. J. (2002) Effect of urea, dimethylurea, and tetramethylurea on the phase behavior of dioleoylphosphatidylethanolamine. *Chem. Phys. Lipids 114*, 149–157.
(36) Religa, T. L., Markson, J. S., Mayor, U., Freund, S. M., and Fersht, A. R. (2005) Solution structure of a protein denatured state and folding intermediate. *Nature 437*, 1053–1056.
(37) Usher, K. C., Ozkan, E., Gardner, K. H., and Deisenhofer, J. (2001) The plug domain of FepA, a TonB-dependent transport protein from *Escherichia coli*, binds its siderophore in the absence of the transmembrane barrel domain. *Proc. Natl. Acad. Sci. U.S.A. 98*, 10676–10681.
(38) Bridges, M. D., Hideg, K., and Hubbell, W. L. (2010) Resolving Conformational and Rotameric Exchange in Spin-Labeled Proteins Using Saturation Recovery EPR. *Appl. Magn. Reson. 37*, 363.
(39) Fanucci, G. E., Coggshall, K. A., Cadieux, N., Kim, M., Kadner, R. J., and Cafiso, D. S. (2003) Substrate-induced conformational changes of the periplasmic N-terminus of an outer-membrane transporter by site-directed spin labeling. *Biochemistry 42*, 1391–1400.