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Spectroscopic Evidence that Osmolytes Used in Crystallization Buffers Inhibit a Conformation Change in a Membrane Protein†

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ABSTRACT: BtuB is a bacterial outer-membrane protein that transports vitamin B_{12}. Spectroscopic studies using site-directed spin labeling (SDSL) indicate that the N-terminus of BtuB undergoes a dramatic structural change from a docked (folded) to an undocked (unfolded) configuration upon substrate binding. However, this dramatic conformational change is not observed in the crystal structures of BtuB. Here, we make an attempt to resolve this discrepancy and find that the effects of solutes can explain the discrepancy between the results obtained using these two methods. Specifically, if SDSL is performed with the buffers used for the crystallization of BtuB, the substrate-induced order−disorder transition of the N-terminal Ton box observed in intact membranes is blocked. Moreover, poly(ethylene glycol) 3350, which is a component of the crystallization and soaking buffers, is shown to inhibit this structural transition. It is likely that the crystal structure of BtuB in its holo form represents an osmotically trapped conformation. Conformational changes involving relatively modest energy differences and significant hydration changes may be sensitive to solutes used during crystallization, and this example demonstrates the value of combining multiple structural methods in the examination of protein structure and function.

The past decade has witnessed significant progress in X-ray crystallography of membrane proteins, and structures have now been reported for a number of membrane protein classes, including receptors, ion channels, pores, and transporters (*1*−*3*). NMR[1] spectroscopy, as well as lower resolution spectroscopic techniques (*4*−*9*), have also provided useful information about structure and dynamics of membrane proteins. In a few cases, both crystallographic and spectroscopic methods have been used to investigate the structure of a given protein (a few examples include OmpA (*10*), OmpX (*6*), rhodopsin (*11*), and fd coat protein (*12*)). Such comparisons are exceedingly useful because the biophysical methods are complementary and provide, in addition to structure, information about dynamics, conformational changes, and the likely ensemble of protein conformations. However, there are few instances where both crystallographic and spectroscopic approaches have been used to compare conformational transitions in a membrane protein. BtuB is a bacterial transport protein that has been studied both by crystallographic and by spectroscopic methods. In the work presented here, we provide an explanation for differences that are seen in the substrate-bound conformation of BtuB when examined using spectroscopic and crystallographic approaches.

BtuB is a 66 kD outer-membrane TonB-dependent transporter found in *Escherichia coli* that binds vitamin B_{12} (CNCbl) and moves it into the periplasm (*13*). The N-terminus of BtuB contains a highly conserved six to seven amino acid segment near its N-terminus called the Ton box, which is present in all other TonB-dependent transporters (*14*−*17*). The Ton box appears to be responsible for coupling the outer membrane transporter to TonB, a transperiplasmic protein that drives transport by extracting energy from the inner-membrane proton potential in combination with the inner-membrane proteins ExbB and ExbD. The crystal structure of BtuB has been reported (*18*), and a model is shown in Figure 1. Like other TonB-dependent transporters, the structure of BtuB consists of a β-barrel formed from 22 antiparallel strands and an N-terminal core region that occludes the interior of the barrel. In this structure, the Ton box is resolved and is shown folded into the barrel of the transporter. As shown in Figure 1b,c, substrate addition induces a change in the Ton box conformation; however, the fold of most of the Ton box is maintained. The largest change in Ton box structure takes place near residues 6 and 7 and results primarily from a rotation about the ψ angle of residue 7.

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[1] Abbreviations: C_8E_4, *n*-octyl tetraoxyethylene; CNCbl, cyanocobalamin; EGTA, ethyleneglycol-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MTSL, *S*-(1-oxy-2,2,5,6-tetramethylpyrroline-3-methyl) methanethiosulfonate; NMR, nuclear magnetic resonance; OG, octylglucoside; PEG, poly(ethylene glycol); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; R1, cysteine side chain derivatized with MTSL; SDSL, site-directed spin labeling.

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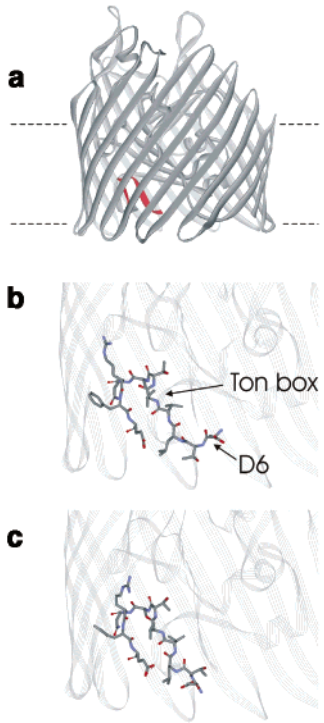

FIGURE 1: Crystal Structures of BtuB. (a) Crystal structure of BtuB in the substrate free (apo) form (*18*). Residues 6−16 on the N-terminus of the protein are highlighted in red and include the Ton box (residues 6−12). Residues 1−5 are not resolved at the N-terminus and are also seen to be unstructured by EPR spectroscopy (*21*). (b) Close-up view of residues 6−16 including the Ton box in the apo form of BtuB. This segment of the protein forms a loop, which buries the Ton box within the protein interior. (c) View of residues 6−16 in the substrate (vitamin B_{12}) bound form of BtuB. The largest change seen in the Ton box is a rotation about ψ of residue 7, which rotates the position of the side chains of residues 6 and 7 by about 180°.

Site-directed spin labeling (SDSL) on native and reconstituted BtuB also indicates that the Ton box is folded or docked into the transporter barrel in the unliganded state. However, in contrast to the crystallographic model, the EPR data indicate that the Ton box undergoes an order−disorder transition upon substrate binding and becomes undocked from the barrel (*19*−*21*). The EPR spectra obtained when substrate is bound contradict the X-ray model in two ways. First, spin labeled side chains (R1) along the Ton box lose tertiary contact with other regions of the protein and show increased solvent accessibility; hence, the Ton box is no longer constrained within the barrel interior. Second, these data also indicate that the N-terminal region of BtuB is highly disordered (not structured) when substrate is present. In fact, a gradient in mobility of the R1 side chain is observed along the Ton box, suggesting that one end of this protein segment is tethered to the core while the other is freely diffusing. These data are dramatically different from and inconsistent with the crystal structure, where the Ton box is resolved and retains its fold in the presence of substrate.

The substrate-induced conformational change in BtuB may be critical to the mechanism of transport. This conformational change is thought to provide a signal to the transperiplasmic protein TonB that the transporter is bound to substrate (*19*, *22*), and this conformational change may allow the Ton box to directly interact with the transperiplasmic protein TonB in an extended conformation (*23*).

The discrepancy between the spectroscopic and the crystallographic results may originate from a variety of factors. For example, the BtuB crystal lacks a membrane environment, and crystal contacts or detergent may prevent the full substrate-induced conformational change. To obtain the holo form of BtuB, vitamin B_{12} and Ca^{2+} were soaked into preformed BtuB crystals rather than being cocrystallized with the protein. On the other hand, in the SDSL experiments, the incorporation of non-native spin labeled side chains could have destabilized the Ton box region and promoted an unfolding that would normally not occur. Indeed, it has been shown that some positions in the Ton box are intolerant to substitution with the spin labeled R1 side chain, large hydrophobic side chains (*21*), or proline mutations (*20*).

In the work presented here, we show that the crystallization buffer, as well as certain solutes, inhibit the substrate-induced structural transition that was observed in previous spectroscopic experiments. There is an extensive literature on the effect of solutes on protein conformations (see, e.g., refs *24*−*26*), and we provide evidence by SDSL that the osmotic stress created by certain solutes in the crystallization buffer is the likely source of the discrepancy between the crystallographic and spectroscopic structural models.

EXPERIMENTAL PROCEDURES

Materials. The sulfhydryl reactive methanethiosulfonate spin label (MTSL), (1-oxy-3-methanesulfonylthiomethyl-2,2,5,6-tetramethyl-2,5-dihydro-1H pyrroline) (*17*) was purchased from Toronto Research Chemicals (Ontario, Canada). Cyanocobalamin (CNCbl), polyethylene glycol (PEG3350), sucrose (gradient grade), Ficoll 400 (20%), lithium chloride, HEPES, and sodium chloride were obtained from Sigma (St. Louis, MO). Octylglucoside (OG) (Anagrade) was purchased from Anatrace (Maumee, OH), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Crystallization buffer solutions were provided by Michael Wiener (Physiology Department, University of Virginia). The reservoir buffer contained 300 mM magnesium acetate, 4−7% PEG 3350, 50 mM cacodylate pH 6.6, and 20 mM C_8E_4 (*n*-octyl tetraoxyethylene). The soaking buffer contained 150 mM calcium chloride, 18% PEG 3350, 25 mM bis-tris pH 6.6, and 10 mM C_8E_4. EPR buffer solutions contained 10 mM HEPES and 130 mM sodium chloride pH 6.5 with a final concentration of 500 μM vitamin B_{12} when added.

Expression, Labeling with MTSL, Purification, and Reconstitution of BtuB. Expression, labeling with MTSL, purification, and reconstitution of BtuB mutants were performed as previously described (*27*). Reconstituted BtuB samples were prepared in 10mM HEPES, 130 mM NaCl, pH 6.5.

Measurement of Solution Osmolalities. The solution osmolality was measured with a Wescor 5500 vapor pressure osmometer. Sample sizes were 10 μL. Measurements were made in duplicate for each solution, and most solutions were prepared in triplicate to ensure reproducibility. The average values (±5%) were used in all calculations and figures. Because EPR sample volumes are typically less than 5 μL, separate solutions for osmolality measurements were prepared. These solutions mimicked those used for EPR except

that there was no protein present in the samples used for osmometry measurements.

EPR Measurements. EPR spectroscopy was performed on a Varian E-line 102 X-band spectrometer equipped with a loop−gap resonator (Medical Advances, Milwaukee, WI). Labview software, which was generously provided by Drs. Christian Altenbach and Wayne Hubbell (UCLA), was used for digital collection and analysis of data. All spectra for line shape analyses were recorded at 2.0 mW incident power with a modulation amplitude of 1.0 G, and these samples were prepared in glass capillary tubes with a 0.8 mm i.d. (VitroCom, Mountain Lakes, NJ). Titration experiments were performed by preparation of individual protein samples where the appropriate amount of a stock solution of osmolyte was added. Osmolyte solutions were either added directly to BtuB reconstituted into POPC vesicles (with three to five freeze thaw cycles in liquid N_2; with or without B_{12}) or to samples of BtuB in POPC that were solubilized with OG (POPC/OG 1:17). For incorporation into crystallization solutions, protein samples that were reconstituted into POPC were added to an equivalent volume of the crystallization solution that had been taken to dryness. In these samples containing crystallization buffers, the C_4E_8 detergent concentrations were sufficient to clarify the reconstituted membrane preparation.

RESULTS AND DISCUSSION

Substrate-Induced Structural Transition in BtuB Is Inhibited in Solutions Used for Crystallization. The solutions used in the crystallization of BtuB differ from those used in the SDSL experiments. The crystallization solutions have higher concentrations of solutes, including divalent salts and poly(ethylene glycol) 3350 (*28*). We measured the osmolality of the reservoir buffer used during crystallization as well as the soaking solution used to incorporate CNCbl into the BtuB crystal and find both values to be in excess of 1000 mmol/kg. These osmolalities are considerably higher than those of the reconstitution buffer used in the EPR spectroscopic measurements (320 mmol/kg). To determine whether solutions with high osmolality can inhibit the conformational change observed by SDSL, we produced two reconstituted spin labeled BtuB samples with the R1 nitroxide side chain (Figure 2) placed either at position 7 or 10 within the Ton box. These positions were chosen because their line shapes exhibit dramatic changes upon substrate binding and because label incorporation at these sites does not appear to interfere with the normal fold of the Ton box (*21*).

Shown in Figure 2b are EPR spectra for labels at positions 7 and 10 in BtuB when the protein is reconstituted into POPC (with the reconstitution buffer) in the presence and absence of substrate. In the absence of substrate, each spectrum is dominated by a relatively broad signal that is characteristic of an R1 side chain that is in tertiary contact with some other part of the protein. When substrate is added, there is a dramatic narrowing and increase in amplitude of the EPR spectra for both T7R1 and V10R1. These line shape changes result from enhanced motional averaging of the magnetic interactions of the R1 side chain and indicate that the Ton box segment is unfolded and that these sites have lost tertiary contact with other regions of the protein (*8*, *29*, *30*). Shown in Figure 2c are EPR spectra of the same two samples in the presence and absence of substrate, except that the samples are prepared in the solutions that were used for crystallization

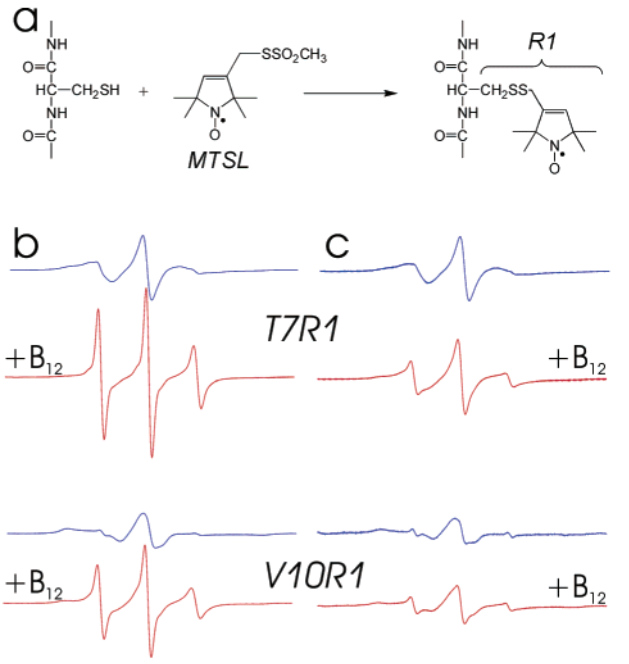

FIGURE 2: EPR spectra from the Ton box region of BtuB. (a) Single cysteine substitutions in the Ton box of BtuB are labeled with the MTSL to produce the spin labeled side chain R1. (b) EPR spectra of T7R1 and V10R1 in POPC in the absence and presence of substrate (these spectral changes occur when excess Ca^{2+}/B_{12} or $EGTA/B_{12}$ are added and are therefore not dependent upon the presence of calcium). (c) EPR spectra of T7R1 and V10R1 in POPC in the absence and presence of substrate when the sample is suspended in the reservoir buffer and soaking buffer, respectively, used for crystallization. Protein concentrations are approximately 0.20 μM, and vitamin B_{12} is added to concentrations of about 0.5 mM.

(*28*). In this case, the EPR spectra do not change significantly upon the addition of vitamin B_{12}, and the spectra resemble those in the unliganded state. Hence, the EPR spectra indicate that the dramatic substrate-induced order−disorder transition previously seen in the Ton box (Figure 2b) does not take place when samples are prepared in the crystallization solution. Interestingly, the EPR spectrum of T7R1 does change slightly from the substrate-free form. This is not unexpected because the conformation at this position does change in the BtuB crystal structure in the Ca^{2+} and B_{12} liganded state. In the presence of substrate and soaking solution, the EPR spectra of T7R1 and V10R1 are now consistent with the crystal structure, and the result indicates that different solutes in the sample solutions can account for the discrepancy observed between SDSL and crystallography for BtuB.

It should be noted that the crystallization soaking solution contains Ca^{2+}, which is known to play a role in the binding of vitamin B_{12} to BtuB (*31*). Although Ca^{2+} dramatically increases the affinity of BtuB for its substrate and coordinates within the external binding loops of BtuB (*18*), the substrate-induced unfolding of the Ton box, as observed by EPR, takes place either in the presence or absence of Ca^{2+} (data not shown). The EPR experiments described here are carried out using high concentrations of protein and substrate, and BtuB is fully bound to substrate even if substrate affinity is diminished.

PEG 3350 Blocks the Substrate-Induced Transition by Imposing an Osmotic Stress on the Protein. It is well-known that conformational transitions in proteins may be modulated

by the addition of certain solutes, such as poly(ethylene glycol)s and sugars. Solutes have been shown to modulate conformational changes in ion channels, enzymes, and hemoglobin (*32*−*35*). In general, the addition of these solutes reduces the chemical potential of water, which will modify the free energy of the protein (*25*, *36*). For example, if a conformational change involves the creation of a water-filled cavity to which the solute is inaccessible, the resulting decrease in the chemical potential of water will generate an osmotic stress on the protein (*25*). The conformational transition may be blocked if the free energy difference between conformers is close to the work required for opening the cavity against the imposed osmotic pressure. In general, any conformational change that involves the exposure of the protein surface to the solution may be sensitive to solutes because protein surfaces are likely to be differentially solvated by solvent and solute (*37*).

In the case of BtuB, we have shown that the Ton box exists in equilibrium between a docked and an undocked form depending upon the presence of substrate (*38*). We find that the undocking of the Ton box alters the hydration of the protein, exposing the N-terminal region of the protein to solution and opening a water-filled cavity. The newly exposed protein surface is not equally accessible to both solvent and solutes, such as PEG 3350 in the crystallization solution, and we hypothesize that the resulting lowering of the chemical potential of water drives the protein to its most dehydrated state (in this case, the docked conformation). In other words, an osmotic stress is imposed on the protein that shifts the equilibrium of the Ton box toward the docked state. The dependence of this equilibrium on the osmotic pressure is expected to have the following behavior:

$$\frac{\partial \ln K}{\partial \pi} = -\frac{\Delta \nu_i}{kT}$$

where K is the equilibrium constant between docked and undocked conformations, π is the osmotic pressure, and $\Delta\nu_i$ is the effective volume exposed during the transition from which solute is excluded (*32*).

Here, SDSL is used to show that the free energy difference between docked and undocked Ton box states has a linear dependence on solution osmolality (Figure 3). A BtuB sample in the substrate bound form (Ton box undocked) is titrated with increasing amounts of PEG 3350 in the buffer, and the EPR spectra are recorded as a function of increasing PEG concentration (Figure 3A). As the PEG 3350 concentration is increased, the spectrum undergoes a progressive change, from one dominated by a relatively sharp line shape superimposed upon a broad component to one that is dominated by the broad component. This change reflects a shift in the equilibrium from an undocked to a docked form. The fraction of docked and undocked populations can be determined directly from the EPR spectra by spectral subtraction (*38*), and the equilibrium constant, K, can be determined. For each sample, the osmolality of the buffer solution was also measured. Shown in Figure 3b is a plot of the dependence of ln(K) upon solution osmolality. The data are well-fit to a linear dependence, and the slope of this curve indicates that an effective volume change of approximately 10 000 Å^3 (or about 300 water molecules) takes place during this transition. As indicated previously, this change represents the increased hydrated volume from which solute is excluded,

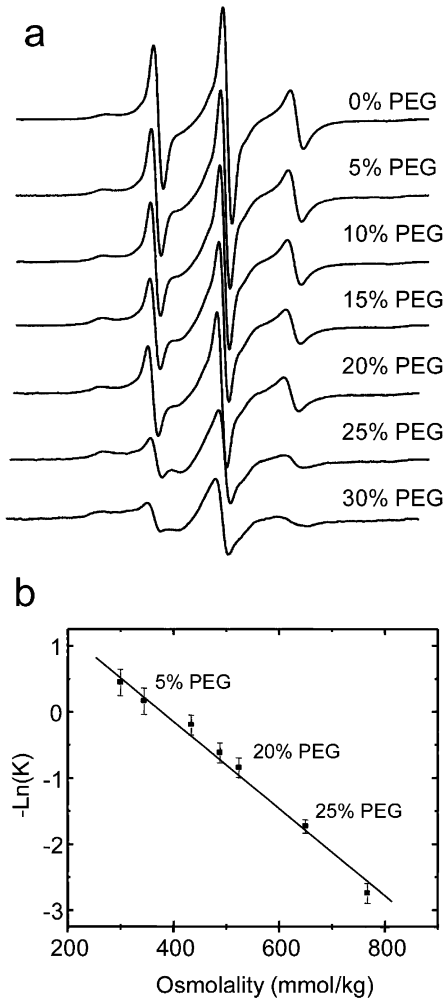

FIGURE 3: Effect of PEG 3350 on the equilibrium of the Ton box. (a) EPR spectra of the BtuB mutant V10R1 in POPC in the presence of the BtuB substrate (vitamin B_{12}), titrated with increasing concentrations (% by weight) of PEG 3350. The EPR spectra result from V10R1 in both docked and undocked populations of the Ton box. Addition of PEG 3350 converts the Ton box from an undocked to a docked form. (b) Natural log of the equilibrium constant (−ln-(K)) obtained from the EPR spectra as a function of solution osmolality. Here, K is defined as [docked]/[undocked]. The slope of the line would be produced by the formation of a volume of approximately 10 000 Å^3 that was inaccessible to solute.

and it could result either from the formation of a water-filled cavity and/or the hydration of additional protein surface (such as the exposed N-terminus).

Although we have not exhaustively studied the effects of different size osmolytes, we have examined the effect of Ficoll 400 and sucrose on the redocking of the Ton box. A 10% Ficoll 400 solution produces a viscous sample but has no significant effect on the equilibrium observed with EPR for these labeled Ton box sites. Ficoll 400 is a 400 000 MWT sucrose polymer, and the osmolality of the Ficoll solution used in this EPR experiment was less than 100 mmol/kg. In contrast, the low molecular weight uncharged solute sucrose also blocks the transition, but much higher molar concentrations corresponding to much higher osmolalities are required when compared to PEG 3350. This difference may result because sucrose is not completely excluded from the protein surface or cavity that forms during the conformational change. These findings and the linearity of the curve shown in Figure 3 suggest that the origin of the inhibitory effect of

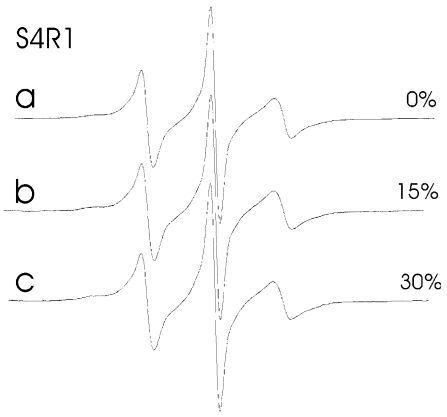

FIGURE 4: Effect of PEG 3350 on a disordered protein segment. Spin labeled BtuB, S4R1, reconstituted into POPC (a) without addition of PEG 3350, (b) in the presence of 15% w/v PEG 3350, and (c) in the presence of 30% w/v PEG 3350.

PEG 3350 on the Ton box equilibrium is the solution osmolality, or the change in the chemical potential of water due to solute.

Solutes Will Not Promote the Folding of Natively Disordered Sites in BtuB. The data discussed previously indicate that the Ton box favors an unfolded state when substrate is bound but adopts a folded state upon the addition of PEG 3350. Unlike the Ton box, residues 1−5 are not resolved in the crystal structure and are also observed by SDSL to be disordered either in the presence or in the absence of substrate (*21*). Because they are not resolved in the crystal structure, EPR spectra taken from this segment of the protein would be expected to remain unfolded in the presence of PEG 3350. We tested this expectation by examining the spectrum of a mutant having the spin labeled side chain R1 placed at position 4. Shown in Figure 4 are EPR spectra taken of the spin labeled mutant S4R1 (unliganded state) in the absence and presence of 15 and 30% PEG 3350. In the absence of PEG 3350, the spectrum is narrow, consistent with a protein segment that is unfolded. Addition of either 15 or 30% PEG 3350 produces no significant change in the EPR spectrum and indicates that PEG fails to fold this segment of the protein.

The result shown in Figure 4 indicates that the effects of PEG 3350 seen for sites in the Ton box are not generally seen at natively disordered sites in the protein. Unlike the Ton box, a folded conformation for the extreme N-terminus must not be energetically accessible. The fact that there are no changes in the EPR spectrum at this flexible protein site also indicates that changes in the EPR spectra obtained from the Ton box in the presence of PEG 3350 are not due to solution viscosity.

Sensitivity to Osmolytes May Be a General Feature of Conformational Transitions that Involve Small Energy Differences and Significant Changes in Hydration. From EPR spectroscopy, the energy difference between the docked and the undocked forms of the Ton box is estimated to be approximately 2 kcal/mol (*38*). Addition of substrate shifts this energy difference, so that the equilibrium slightly favors the undocked form of the Ton box. The data obtained here demonstrate that this equilibrium can be reversed by high solution osmolality, a result that can account for the difference between the spectroscopic and the crystallographic results. At present, it is not known how general this phenomena is or whether it might play a role in other protein crystal structures. Protein crystallography is clearly capable of probing conformational changes, and there are numerous examples of protein structures determined from crystals precipitated with osmolytes that do show conformational changes. Nonetheless, when protein conformational changes involve both modest energy differences and significant increases in protein hydration, conformational transitions maybe be sensitive to the presence of high concentrations of certain solutes.

In the case of BtuB, a conformational change that was detected by SDSL is no longer observed when this lipid−protein sample is placed in the solution that was used for crystallization. In addition, the EPR data show that PEG 3350 has the capacity to shift the conformational equilibrium of the Ton box to the docked state even in the presence of Ca^{2+} and vitamin B_{12}. Other components in the crystallization solutions may contribute to the osmotic stress on the protein. For example, there are salts in these buffers, and high salt concentrations (0.5−1 M $LiCl_2$, data not shown) also inhibit the conformational transition as detected using EPR, presumably because ions are unable to completely solvate the protein surface that becomes hydrated. As mentioned previously, sucrose solutions (>1 M) also block the transition, but much higher molar concentrations of this solute are required when compared to PEG 3350. The higher concentrations needed of the smaller molecular weight solute may indicate that sucrose has some access to a hydrated cavity that is formed. Previous work has shown that the dependence on polymer size can be used to estimate the radius of ion channel pores (*32*).

X-ray crystallography has produced some exciting structures of membrane proteins in recent years. Although SDSL can be used to generate low-resolution protein structures (*39*, *40*), its strongest applications involve studies of conformational changes, dynamics, and orientation of peptides or proteins in the membrane or at the membrane surface (*41*−*44*). Membrane proteins are particularly amenable to study by SDSL because these investigations can be performed in a variety of membrane or membrane-like environments, where the protein is reconstituted into micelles, bicelles, or lipid bilayers. Significantly, SDSL can be carried out on some membrane proteins even within intact biological membranes and cells (*19*, *45*). Additionally, unlike crystallography, SDSL is often performed on nonoriented powder-like samples, thus negating the need for agents to precipitate the protein. However, SDSL is not without its own potential drawbacks. For example, when conformational changes involve small energy differences, the results of label incorporation should be viewed cautiously. The R1 side chain substitution is generally well-tolerated in proteins, and it has been found to have little effect on the folding energy of proteins particularly when incorporated into solvent exposed sites (*29*). However, the effect of R1 is more significant when it is incorporated into the hydrophobic core of a protein. For example, in the T4 lysozyme, the R1 side chain has been observed to alter the protein-folding energy by 3−4 kcal/mol when placed into the hydrophobic core (*29*). In the case of the T4 lysozyme, this energy change is not sufficient to alter the protein fold, but this may not be true of structures that are less stable. Previously, we provided evidence that R1 at certain positions in the Ton box of BtuB unfolded this region of the protein in the unliganded state (*21*). Indeed,

the EPR spectra of R1 at these positions are not consistent with those predicted from the BtuB crystal structure in its apo form.

The data presented here can account for the different models of the Ton box provided by SDSL and crystallography. However, which view correctly depicts the substrate-bound form of the Ton box? One possibility suggested by the data presented here is that the crystal structure represents an osmotically trapped conformation. Another possibility is that the incorporation of spin labels along the N-terminal region of the protein destabilizes the Ton box producing an artifactual substrate-induced change. There are a number of arguments that can be made against such a probe-induced artifact. First, 10 labeled sites along the N-terminus of BtuB yield changes in EPR spectra that are consistent with an unfolding of the Ton box (*21*), an unfolding that is also observed by EPR in intact outer membranes (*19*). Furthermore, seven of these sites are surface exposed, including sites 7 and 8 in the Ton box, as well as sites 1−5 (which are observed to be disordered both by EPR and by crystallography). As shown previously, surface substitutions with R1 are not highly perturbing (*29*), and it is very unlikely that a large number of sites, some of which are exposed and some of which are buried, should all generate the same artifact. In addition to SDSL, chemical derivatization of cysteine residues along the Ton box with a suflhydryl specific reagent of BtuB in intact *E. coli* indicates that access to the reagent dramatically increases along the Ton box upon substrate binding (*46*). The change in conformation indicated by SDSL is consistent with this enhanced labeling. As a result, we believe that the spectroscopic method provides the correct view of the substrate-bound form of the Ton box and that the crystal structure model is an osmotically trapped conformation. However, further work is underway to test this conclusion. This includes EPR spectroscopy and X-ray diffraction of BtuB crystals with selected spin labeled Ton box mutants.

In summary, the addition of substrate to the outer-membrane transport protein BtuB is observed by SDSL to induce a significant structural change in the Ton box. This structural change is not depicted in the protein crystal structure. The conformation of the Ton box in BtuB exists in equilibrium between a docked and an undocked state (*38*). This report shows that solution osmolality shifts the equilibrium toward the docked (less hydrated) state. The osmotic stress created by solutes used during the crystallization process may account for the discrepancy between the models reported by crystallography and EPR spectroscopy. The sensitivity of conformational changes to solutes may be a feature of many protein conformational changes, and the comparison made here illustrates the value of an approach that uses multiple experimental techniques.

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