Transport-Defective Mutations Alter the Conformation of the Energy-Coupling Motif of an Outer Membrane Transporter†

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ABSTRACT: The bacterial outer membrane transporter for vitamin B_{12}, BtuB, derives its energy for transport by interacting with the trans-periplasmic membrane protein TonB. This interaction with TonB occurs in part through an N-terminal segment in the BtuB sequence called the Ton box. In the present study, site-directed spin labeling of intact outer membrane preparations was used to investigate the conformation of the Ton box in wild-type BtuB and in two transport-defective mutants, L8P and V10P. In the wild-type protein, the Ton box is folded into the barrel of the transporter. The conformation of this segment is dramatically different in the transport-defective mutants L8P and V10P, where the Ton box is found to be flexible, and undocked from the transporter barrel with a greater exposure to the periplasm. In the wild-type protein, vitamin B_{12} induces an undocking of the Ton box, but its addition to these transport defective mutants produces little or no change in the conformation of the Ton box. Proline substitutions at positions that do not alter transport do not alter the wild-type conformation of the Ton box; thus, the effect of substituting proline at positions 8 and 10 on the docked state of the Ton box appears to be unique. The failure of these mutants to execute the B_{12} transport cycle may be a result of the altered conformation of the Ton box.

Gram-negative bacteria such as *Escherichia coli* contain a family of proteins in their outer membrane that facilitate the binding and active transport of substrates such as iron siderophore complexes and vitamin B_{12} (CNCbl)[1] (*1*−*3*). These outer membrane transporters are β-barrel structures, similar to the outer membrane porins; however, unlike the porins they actively transport substrates. These proteins are termed TonB-dependent transporters because they derive their energy for transport by coupling to the inner membrane protein TonB. TonB functions in a complex with two other inner membrane proteins, ExbB and ExbD, and this complex derives energy from the transmembrane proton potential, $\Delta\mu_{H^+}$, across the inner membrane. TonB-dependent transporters share a number of conserved features, including an N-terminal region called the Ton box that appears to be involved in coupling the transporter to TonB.

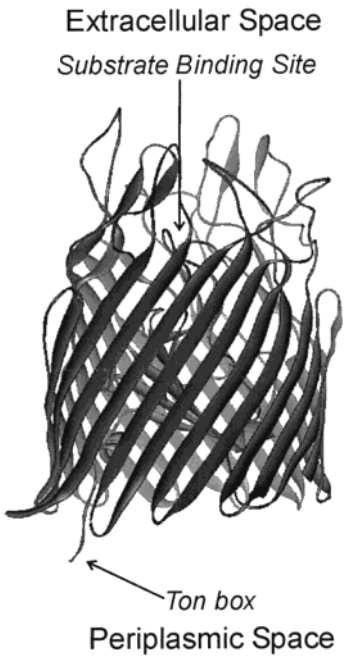

FIGURE 1: Crystal structure of the outer membrane iron transporter FepA (1FEP) (*4*). This structure was obtained with protein both bound and free of substrate. The Ton box is shown as an unstructured strand extending from the β-barrel of the transporter.

High-resolution molecular models are available from X-ray crystallography for two of the iron transporters in this family, FepA (*4*) and FhuA (*5*, *6*). Figure 1 shows the model for FepA, which has a β-barrel composed of 22 antiparallel

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[1] Abbreviations: CNCbl, cyanocobalamin; DPPH, α,α′-diphenyl-β-picrylhydrazyl; DTT, DL-dithiothreitol; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MTSL, (1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate; NiAA, nickel(II) acetylacetonate; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDSL, site-directed spin labeling.

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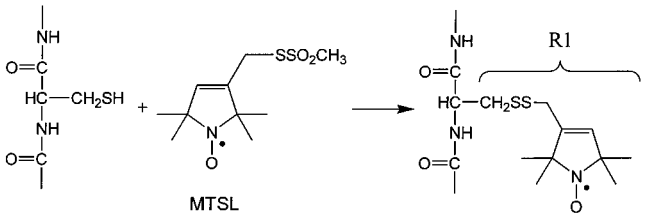
Scheme 1



strands and a core or hatch domain that fills the barrel. Substrate binds to the loop regions on the extracellular face of the barrel and hatch domains of the transporter, and the conserved N-terminal Ton box is localized at the periplasmic face. Crystallography provides limited information on the structure of the Ton box in these transporters. In the case of FepA (Figure 1), the Ton box was assigned to an extended conformation, but the protein crystal used to obtain the diffraction data contained a mixture of substrate-bound and substrate-free transporter. In the case of FhuA, the Ton box was not resolved.

Structural changes in the transporter coincide with the binding of substrate. For example, site-directed spin labeling indicates that there are changes in the extracellular loop regions of FepA upon the binding of substrate (*7*). Crystal structures of FhuA indicate that while much of the structure remains largely unchanged upon the addition of substrate, an N-terminal helix (H1) on the C-terminal side of the Ton box unfolds. Measurements made directly on the Ton box also provide evidence for a structural change in this region upon the addition of substrate. Disulfide cross-linking indicates that the Ton box of the vitamin B_{12} transporter, BtuB, increases its exposure to the periplasmic space in the presence of its substrate, CNCbl (*8*). This finding is consistent with site-directed spin-labeling work indicating that the Ton box is folded into the barrel of BtuB in the absence of substrate but converts to an extended, flexible conformation in the presence of the substrate (*9*).

In BtuB and related proteins, the Ton box was identified as a site of mutations that result in an energy-uncoupled transport-defective phenotype. In particular, substitution of proline or glycine at several positions along the Ton box was found to impair transport but not affect substrate binding or transport of TonB-independent substrates (*10*). Disulfide cross-linking studies indicated that these mutations altered the TonB−Ton box interaction. Although the Ton box interacted with TonB, the highly specific interaction that is seen with wild-type BtuB was disrupted in these mutants. Thus, the nature of the transport defect in these mutants might lie in the failure of BtuB to appropriately couple to TonB.

In the present study, site-directed spin labeling was used to examine the structure of the Ton box in wild-type BtuB and in the transport-defective mutants L8P and V10P. Spin labels were incorporated into BtuB by reacting single cysteines, incorporated by site-directed mutagenesis, with the sulfhydryl-reactive methanethiosulfonate spin label (MTSL) shown in Scheme 1. EPR spectroscopy was then used to examine the structure and conformational changes in this energy coupling segment that accompany substrate binding to the transporter. The sulfhydryl-reactive spin label (Scheme 1) is relatively nonperturbing to protein structure (*11*), and this spin label provides information on structure, membrane protein orientation, protein dynamics, and protein conformational changes (*12*, *13*). The results of this study clearly indicate that L8P or V10P substitutions produce dramatic changes in the Ton box structure. In particular, the Ton box in these mutants does not assume its native fold in the absence of substrate but is constitutively undocked from the BtuB barrel. Unlike the wild-type protein, the addition of substrate fails to produce changes in the exposure or tertiary contact of side chains along the length of the Ton box. The implications of these findings for the mechanisms of TonB-dependent transport are discussed.

EXPERIMENTAL PROCEDURES

Materials

The methanethiosulfonate spin label, (1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate (MTSL), was purchased from Toronto Research Chemicals (Ontario, Canada). The paramagnetic relaxation agent nickel(II) acetylacetonate hydrate (NiAA) was obtained from Aldrich (Milwaukee, WI). DL-Dithiothreitol (DTT) was obtained from Sigma (St. Louis, MO), sarkosyl was from Fisher Chemical Co., and phenylmethanesulfonyl fluoride (PMSF) was purchased from Boehringer Mannheim Corp. (Indianapolis, IN).

Methods

Mutagenesis and Isolation of Intact Outer Membranes. A two-step PCR-based site-directed mutagenesis technique was used to produce double mutants in the Ton box of BtuB (*8*). The mutants were expressed in *E. coli* in strain RK5016, which is a derivative of strain MC4100 [Δ(*argF-lac*)*U*169 *araD*139 *rpsL*150 *relA*1 *flbB*5301 *deoC*1 *ptsF*25 *rbsR*22 *non*-9 *gyrA*219] (*14*) having the additional mutations *metE*70 *argH btuB recA*. The cells were grown overnight in minimal medium at 37 °C, harvested by centrifugation at 4000*g* for 10 min, and resuspended in 45 mL of 10 mM HEPES, pH 6.5. The resuspended cells were then lysed using a French press running at a pressure of 18000 psi. To prevent degradation of BtuB, PMSF was added to the suspension to a final concentration of 20 μg/mL. Following lysis, the solution was centrifuged for 10 min at 14500*g* to remove cellular debris. The inner membranes in the supernatant were solubilized by addition of 0.5% sarkosyl, and the outer membranes containing BtuB were pelleted by ultracentrifugation for 1 h at 100000*g*. The outer membrane pellet was resuspended in 40 mL of 10 mM HEPES buffer at pH 6.5, washed by centrifugation for 1 h at 100000*g*, and resuspended in 4 mL of buffer.

Spin Labeling of BtuB Mutants. Approximately 200 μL of the BtuB outer membrane preparation was treated with 0.36 mM DTT in 10 mM HEPES buffer at pH 6.5 for 20 min. The sample was then washed twice in 10 mM HEPES buffer, pH 6.5, to remove the DTT by centrifugation at 100000*g* for 5 min in a Beckman airfuge. The resuspended pellet was labeled with the MTSL by reacting the label at a 3:1 molar ratio (label:BtuB) at room temperature in the dark for approximately 30 min and then washed by centrifugation six times to remove the unreacted spin label. The membrane pellet was suspended in an approximately equal volume of buffer and loaded into a capillary. For the experiments with substrate, cyanocobalamin was added to the spin-labeled protein to a final concentration of 455 μM.

EPR Spectroscopy. EPR spectra were obtained on an X-band Varian E-line spectrometer fitted with a conventional two-loop one-gap resonator. Nonsaturated spectra were obtained at a microwave power of 2 mW and 1 G peak-to-peak modulation in either quartz capillaries or gas-permeable TPX capillaries (Medical Advances, Milwaukee, WI). From the line width of the central nitroxide resonance, δ, a parameter termed the scaled mobility, M_s, was determined using the expression:

$$M_s = \frac{(\delta^{-1} - \delta_i^{-1})}{(\delta_m^{-1} - \delta_i^{-1})} \quad (1)$$

where δ_m and δ_i represent the line widths of the most mobile and immobile protein associated line widths observed. In the present case, δ_i and δ_m were taken as 8.5 and 1.8 G, respectively. The parameter M_s provides a relative measure of the rate of spin-label side-chain motion, and it has been shown to reflect secondary and tertiary structure in proteins (*13*).

Continuous-wave power saturation measurements were performed in a manner similar to that previously described (*15*). In these measurements, labeled BtuB samples were placed into TPX capillary tubes having total protein concentrations of 100−400 μM. The peak-to-peak amplitude of the central $m_I = 0$ nitroxide resonance, $A_{pp}(0)$, was then measured as a function of the incident microwave power, P, and the data were fit to the expression:

$$A_{pp}(0) = I\sqrt{P}\left[1 + (2^{1/\epsilon} - 1)\frac{P}{P_{1/2}}\right]^{-\epsilon} \quad (2)$$

where I is a scaling factor, $P_{1/2}$ is the microwave power required to reduce the resonance amplitude to half its unsaturated value, and ϵ is a measure of the homogeneity of the saturation of the resonance (*16*). With I, ϵ, and $P_{1/2}$ as adjustable parameters, a characteristic $P_{1/2}$ is obtained. Values for $P_{1/2}$ were generated for each sample under three different sets of conditions: (1) equilibrated with N_2, (2) equilibrated with air (20% O_2), and (3) equilibrated with N_2 in the presence of 20 mM NiAA. When the change in $P_{1/2}$ produced by O_2 or NiAA [$\Delta P_{1/2}$(oxygen) or $\Delta P_{1/2}$(NiAA)] is normalized against a DPPH standard, a collision parameter is obtained that is related to the collision frequency between the spin label and the second paramagnetic species. For O_2, the collision parameter, Π^{oxy}, is calculated according to

$$\Pi^{oxy} \equiv \frac{\Delta P'_{1/2}(\text{oxygen})}{P'_{1/2}(\text{DPPH})} = \frac{P_{1/2}(\text{oxygen})/\Delta H_{pp} - P_{1/2}(\text{nitrogen})/\Delta H_{pp}}{P_{1/2}(\text{DPPH})/\Delta H_{pp}(\text{DPPH})} \quad (3)$$

where ΔH_{pp} represents the peak-to-peak width of the $m_{I=0}$ resonance (*17*). The collision parameter for NiAA, Π^{NiAA}, is determined in an analogous fashion.

RESULTS

Labeling of Intact Outer Membrane Preparations. Site-directed spin labeling was carried out on intact outer membrane preparations from *E. coli*. Shown in Figure 2 are EPR spectra of two spin-labeled outer membrane preparations. One EPR spectrum was obtained following labeling of an outer membrane preparation containing overexpressed BtuB having a single cysteine substitution at position 6 (D6C). A second EPR spectrum was obtained following labeling of an overexpressed BtuB preparation containing native BtuB, which has no intrinsic cysteine residues. The samples were handled identically in both cases, and similar levels of expression were obtained for each protein. Compared to the labeling of the sample containing D6C, only minor labeling of the outer membranes was observed. For the D6C mutant, this background labeling was approximately 8% of the specific labeling. The difference in signal intensities between background and selectively labeled BtuB seen in Figure 2 was typical of that seen for the other Ton box mutants, and in all of the cases examined here the absolute amplitudes of the background signals were less than 15% of the specifically labeled protein signals. Thus, site-specific labeling of the Ton box in BtuB could be accomplished in these intact membrane preparations without significant interference from the labeling of other sulfhydryls, and purification and reconsitution of the protein sample were not necessary.

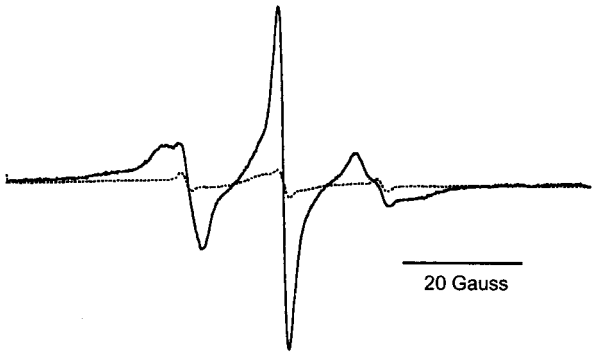


FIGURE 2: EPR spectra of outer membrane preparations containing overexpressed D6C (solid line) or native BtuB (dashed line), which were spin labeled as described in the text. The samples were handled identically, the spectra were taken with identical instrumental settings, and protein expression levels were similar. Native BtuB has no cysteine residues, and the level of background labeling is typical of that seen for some reconstituted membrane protein systems (*23*).

Substrate Addition Undocks the Ton Box. Figure 3A shows the EPR spectra for BtuB labeled with R1 at specific sites along the Ton box in the presence and absence of CNCbl (vitamin B_{12}). These spectra were published previously (*9*) but are shown here in a different form where they are normalized against the total spin concentration. As the label is moved sequentially along the Ton box, the spectra show large variations in amplitude and line width that have a helical periodicity. For these labeled BtuB derivatives, the addition of CNCbl results in a dramatic change in the EPR spectra, with the line shapes becoming narrower and larger in amplitude. The broadest EPR signals, which are lower in amplitude when normalized, are sites at where the label dynamics are slowest.

The EPR line shapes of the side chain R1 are a result of a nanosecond time-scale motional averaging of the anisotropic magnetic interactions of the nitroxide, and both tertiary contact of the labeled side chain and backbone dynamics may contribute to this averaging (*12*, *18*). The broad line shapes seen at positions 7, 8, and 10 along the Ton box are

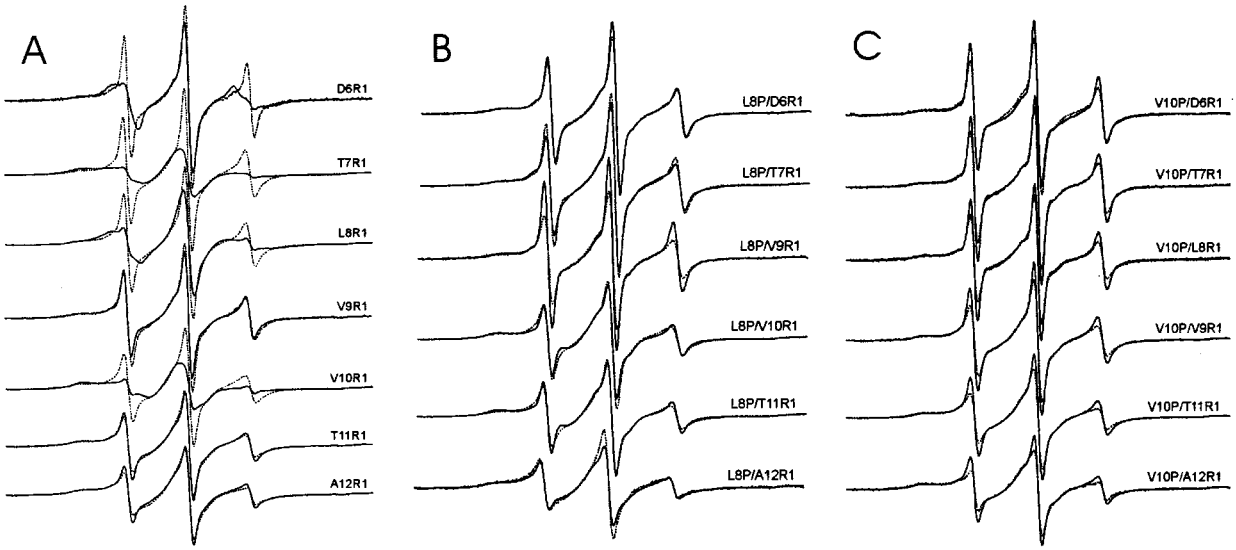


FIGURE 3: EPR spectra of BtuB in the absence (solid line) and presence (dashed line) of substrate. (A) BtuB spin labeled at successive sites along the Ton box of BtuB, (B) BtuB spin labeled at sites along the Ton box in the presence of the L8P mutation, and (C) BtuB spin labeled at sites along the Ton box in the presence of the V10P mutation. The amplitudes of the spectra in this figure have been normalized against the total concentration of spin in the sample. As a result, the amplitudes are directly related to the rates of nitroxide motion.

characteristic of an R1 side chain that is being slowed by strong tertiary contact with other portions of the protein (*19*). The scaled mobility, M_s, may be obtained from the central EPR line width, and it provides an approximate measure of label dynamics, where 0 and 1 correspond to the most restricted and most mobile labeled protein sites found, respectively (*13*). Shown in Figure 4A are values of M_s for the R1 side chain placed along the Ton box. The lowest values of M_s are found for side chains at positions 7 and 10 having values of 0.2−0.3. Following the addition of substrate, the values of M_s are uniformly high and range from 0.65 to almost 0.9. As discussed previously, these changes upon substrate binding are due to an unfolding or undocking of the Ton box of BtuB from a site located within the β-barrel of the transporter (*9*). Henceforth, we will refer to the behavior of the BtuB Ton box shown in Figures 3A and 4A as the wild-type behavior.

It should be noted that virtually all of the spectra shown in Figure 3 contain a broad low-amplitude spectral component, which was previously observed (*9*). Because the M_s values reported in Figure 4 are based on the central line widths, they do not contain information regarding this slower moving component. At the present time, we are not sure of the origin of this component, but it is not seen when these mutant proteins are labeled in a purified reconstituted system (Fanucci and Cafiso, unpublished results).

Although the pattern of contact shown in Figure 3A is approximately that of an α-helix, several of the EPR spectra, for example, spectra for V9R1, T11R1, and A12R1, are not characteristic of an R1 side chain that is placed into a well-formed helix. These line shapes are relatively isotropic, having correlation times on the order of 1 ns or less, and they are much narrower than spectra for the R1 side chain on exposed, flexible helices (*18*). Thus, if this segment is helical, it is likely a disordered irregular helix.

Transport-Defective Mutations Alter the Ton Box Structure. Panels B and C of Figures 3 show a series of spectra obtained for labels placed along the Ton box in the presence of the transport-defective mutations L8P and V10P, respectively. Compared to the protein lacking these mutations (Figure 3A), labels placed along the Ton box in these transport-defective mutants (Figure 3B,C) yield significantly different line shapes in the absence of substrate. Remarkably, the spectra are similar along the length of the Ton box for either proline substitution, and the line shapes indicate that there is relatively rapid motion of these nitroxides with correlation times on the order of 1 ns or less. These line shapes resemble those for labels that have been placed into highly flexible protein segments (*19*). Panels B and C of Figure 3 also show the EPR spectra for these labeled mutants following the addition of substrate, CNCbl. Unlike the protein lacking these proline substitutions (Figure 3A), the addition of substrate produces little or no change in the EPR line shapes.

From the EPR spectra shown in Figure 3B,C, the scaled mobilities for the R1 side chain on the transport-defective BtuB mutants were determined and are shown in Figure 4B,C. These data illustrate in a more quantitative fashion that the sequence-dependent mobilities seen in Figure 4A are eliminated by either of these two proline mutations. Furthermore, the addition of substrate does not produce dramatic changes in side chain mobility which were seen for the wild-type BtuB.

It is immediately evident from these spectra that proline substitutions at positions 8 and 10 prevent the Ton box from folding into a conformation that resembles the wild-type conformation. The Ton box appears to be in a conformation that is highly dynamic, and unlike the wild-type protein there are no labeled sites in strong tertiary contact. Furthermore, while there are small changes in the motion of these side chains in the presence of substrate, the dramatic changes in tertiary contact that are characteristic of the wild-type protein are not seen. The simplest interpretation of these data is that the Ton box in these transport-defective mutants is no longer docked within the BtuB barrel in the absence of substrate.

Collision Accessibilities Indicate That the Ton Box Is More Exposed to the Periplasm in the Transport-Defective Mutant V10P. In addition to measurements of mobility, the EPR

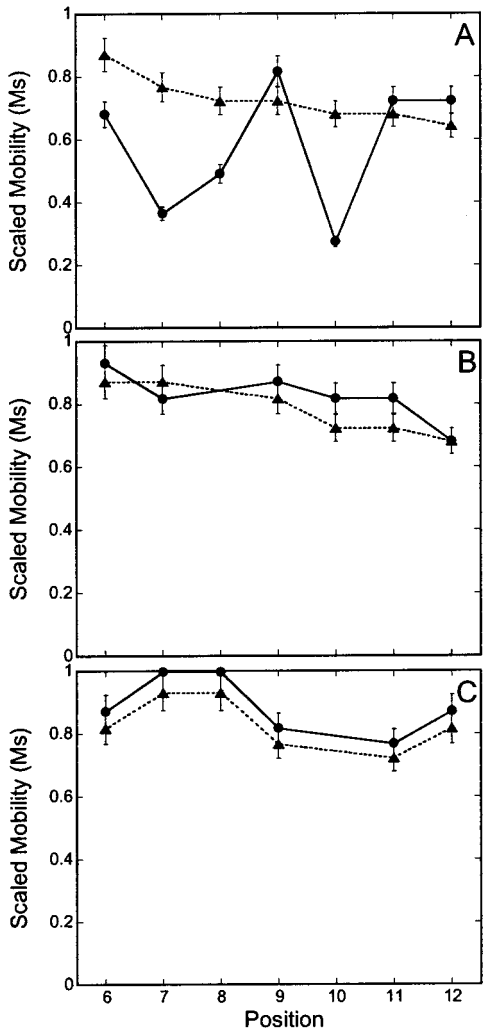


FIGURE 4: Scaled mobilities, M_s, determined from the central EPR line width according to eq 1 for the spectra shown in Figure 3 in the absence (●) and presence (▲) of substrate. Values of M_s for the R1 side chain (A) substituted along the Ton box in the wild-type protein, (B) in the presence of the L8P mutation, and (C) in the presence of the V10P mutation. For the calculation of M_s, δ_i and δ_m are taken as 8.5 and 1.8 G, respectively. The error bars shown are based on a ±0.1 G uncertainty in the line width measurement.

spectra for one of these mutants were power saturated to determine the collision frequencies between R1 and either O_2 or NiAA. Shown in panels A and C of Figure 5 are collision accessibilities Π^{oxy} for the spin-labeled side chain R1 along the Ton box for the native protein and for the mutant V10P in either the absence or the presence of substrate. As was shown previously, the collision accessibilities to O_2 or paramagnetic metals increase for the native protein in the presence of substrate (*9*); however, for the transport-defective mutation V10P, the average accessibility to oxygen along the Ton box upon substrate addition remains roughly constant (although there are positions, for example, position 8, where substrate does produce a significant change in the collision parameter). In addition, the values of Π^{oxy} are on average higher than that seen for native BtuB in the absence of substrate. As expected, these results are consistent with the information obtained from EPR line widths and indicate that the Ton box in the mutant V10P in the absence of substrate has a solvent exposure similar to that of the Ton box in the wild-type protein when it is bound to substrate. The same general trends are seen for the wild-type protein and the V10P mutant (Figure 5B,D) using NiAA as a paramagnetic reagent; however, there are differences at specific sites when the two reagents are compared that likely reflect the size and polarity differences between NiAA and O_2.

Proline Mutations That Do Not Alter Transport Do Not Alter the Ton Box Conformation. The proline substitutions L8P and V10P appear to be unique in their ability to interfere with vitamin B_{12} uptake, and most other proline mutations along the Ton box are not detrimental to transport (*10*). We tested the ability of two proline mutations that do not affect transport, D6P and A12P, to alter the conformation of the Ton box. This was accomplished by investigating the line widths for several labeled sites along the Ton box in the presence of these mutations. Shown in Figure 6 are EPR spectra for R1 at position 7 in the presence of either the D6P or A12P mutation. Comparison of these spectra with those in Figure 3A indicates that these side chains are in strong tertiary contact as they are in the Ton box lacking any proline substitution. In the presence of these proline mutations the Ton box appears to have a wild-type behavior, and the addition of CNCbl results in a dramatic increase in signal intensity that reflects the undocking of the Ton box from the BtuB barrel. Thus, for two proline mutations that do not affect transport, the Ton box assumes conformations that resemble those of the wild-type Ton box.

DISCUSSION

Several recent studies provide evidence that the Ton box in BtuB undergoes a substrate-dependent conformational change. Site-directed spin-labeling studies indicate that the Ton box is bound within the β-barrel of BtuB and that the addition of substrate converts the Ton box into an extended conformation that has greater exposure to the periplasmic space (*9*). Evidence for a conformational change upon CNCbl binding has also been obtained from site-directed disulfide cross-linking studies. Cysteines placed within the Ton box of BtuB undergo cross-linking to cysteines placed at specific sites on TonB (*8*), and this cross-linking and the dimerization between BtuB monomers are increased in the presence of the substrate. This is consistent with the idea that the Ton box increases its periplasmic exposure upon substrate binding. In addition, for the related TonB-dependent proteins FhuA and FepA, the interactions between TonB and the outer membrane iron transporters are enhanced in the presence of substrate (*1*, *20*). Taken together, these results suggest that substrate binding promotes an interaction between TonB and the outer membrane transporter and that a key feature of the transport is a cycle of docking and undocking of the Ton box to the transporter.

In the absence of substrate, the pattern of side chain contact that is seen using site-directed spin labeling follows a roughly helical pattern (*9*). However, this is a relatively short segment, and several types of structural arrangements of the Ton box could account for the pattern of contact. In fact, the short correlation times seen at several positions indicate that this segment does not assume a regular well-ordered helical structure when docked to BtuB. Experiments are currently underway to measure the dipolar interactions

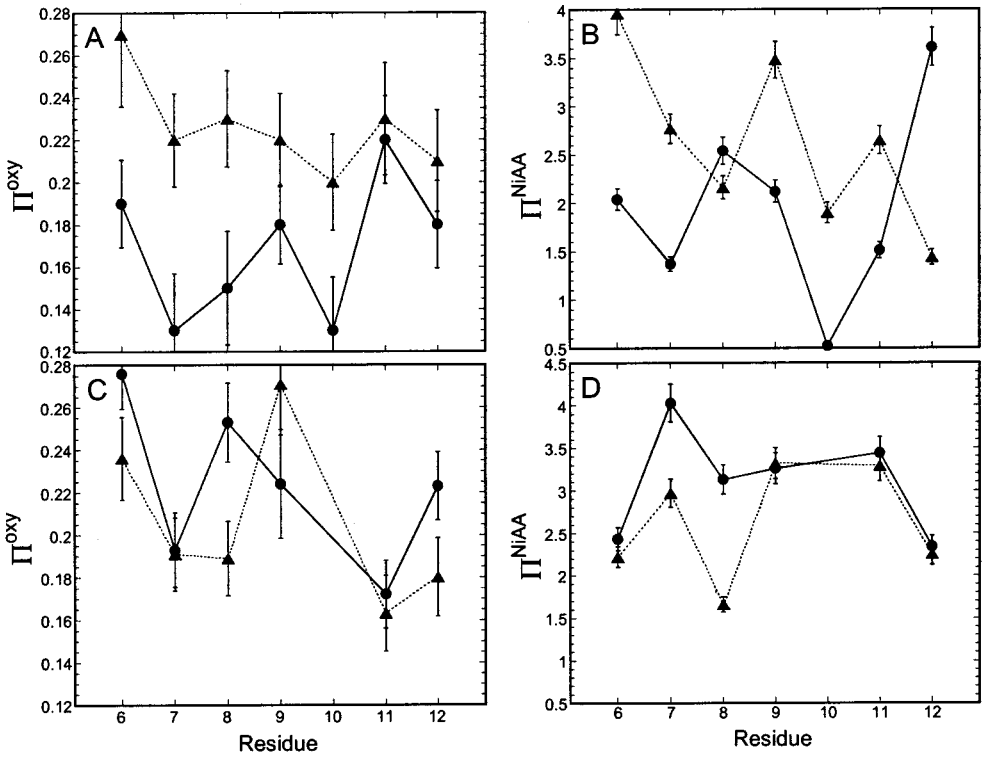


FIGURE 5: Collisional parameters for oxygen, Π^{oxy}, in the absence (●) and presence (▲) of substrate (A) for the Ton box in wild-type BtuB and (C) for the Ton box having the V10P mutation. Collisional parameters for NiAA, Π^{NiAA}, in the absence (●) and presence (▲) of substrate (B) for the wild-type Ton box and (D) for the Ton box having the V10P mutation. The error bars shown represent experimental uncertainty due to the propagated error in the measurement of $P_{1/2}$.

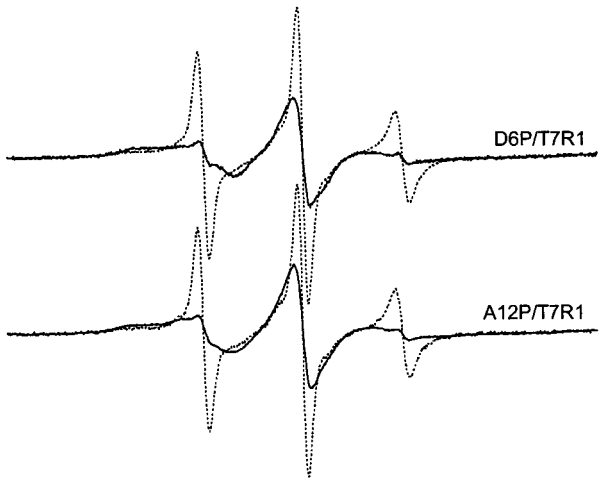


FIGURE 6: EPR spectra of D6P/T7R1 and A12P/T7R1 in the absence (solid line) and presence (dashed line) of CNCbl. These proline substitutions do not alter the transport efficiency of BtuB and yield EPR spectra similar to those for the native protein (see Figure 3).

between a number of pairs of spin-labeled sites within the Ton box of BtuB to better define the structure of this energy-coupling segment.

In the work presented here, the conformation of the Ton box was examined in the presence of specific proline mutations, which result in a transport-defective phenotype. The data demonstrate that the Ton box in the transport-defective mutants V10P or L8P assumes a dramatically different conformation than it does in the wild-type BtuB protein. In particular, the Ton box appears to be in an extended, highly flexible conformation that is similar to the conformation seen for the wild-type protein in the presence of substrate. The uncoupled phenotype that results from these mutations appears to be unique to proline substitution at the 8 and 10 positions. For two positions where proline substitution does not alter transport, A12P and D6P, the Ton box appears to assume a configuration that is characteristic of wild-type BtuB. Thus, there is a correlation between the transport-defective phenotype and the normal docking of the Ton box.

Recent work using site-specific disulfide cross-linking indicates that the interaction between the Ton box and TonB is altered by these mutations (*10*). In these studies, the ability of single cysteine substitutions in TonB to cross-link with cysteine substitutions in the Ton box of BtuB was examined. In the native protein there is a high degree of residue selectivity in the cross-linking, indicating that the Ton box interacts with TonB in a well-defined manner. In the uncoupling mutants, L8P and V10P, the two proteins also cross-link; however, the pattern of cross-linking is dramatically altered, and there no longer appears to be high residue specificity in the interaction between the Ton box and TonB. Thus, in addition to not correctly docking within the BtuB barrel, the cross-linking data suggest that the transport-defective proline mutants no longer correctly interact with TonB.

Several other observations regarding these cross-linking data are noteworthy. First, cross-linking between the Ton box and the BtuB mutants V10P or L8P is stronger than that seen with native BtuB (*10*). This observation is consistent with the EPR data presented here, showing that the Ton box in the BtuB mutants is more mobile and more exposed than the wild-type Ton box. Second, the defect in CNCbl

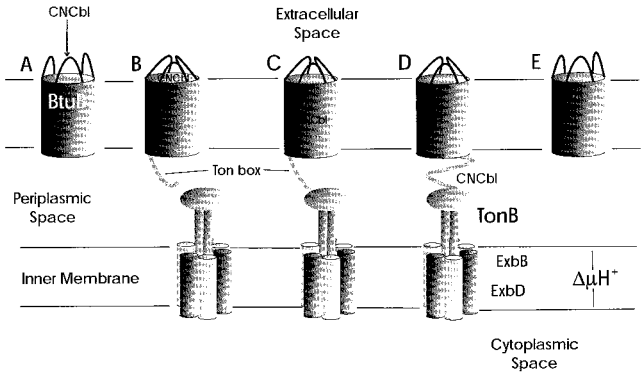


FIGURE 7: Model for the mechanism of CNCbl transport by BtuB. (A) The Ton box of BtuB is docked into the barrel of the transporter, and BtuB is dissociated from the inner membrane complex TonB/ExbB/ExbD. (B) The substrate, CNCbl, binds to BtuB, producing a conformation change that releases the Ton box into the periplasmic space. (C) The exposure of the Ton box into periplasmic space provides the recognition signal for the binding of BtuB by TonB. (D) TonB induces a conformational change within the interior hatch region of BtuB that permits CNCbl to diffuse across the outer membrane. (E) TonB dissociates from BtuB and resets the outer membrane transporter to its native state.

utilization by L8P and V10P could be substantially suppressed by certain mutations in TonB, in particular, Q160C. Although these mutations restored cell growth, CNCbl uptake corresponded to no more than 1 molecule per BtuB, suggesting that L8P and V10P in the presence of Q160C can only undergo one round of transport.

It has been proposed that this transport system can exist in at least three distinct states (*10*). In its normal functional state, BtuB can undergo repeated transport cycles accumulating CNCbl within the periplasm. In a second state, CNCbl will bind to BtuB, but it cannot be released into the periplasmic space. This state occurs both for wild-type BtuB in the absence of TonB and for the mutants L8P or V10P. In a third state, bound CNCbl can be released into the periplasm, but the transporter fails to undergo further rounds of transport, and the stoichiometry of CNCbl:BtuB never exceeds 1:1.

We speculate that the transport process for BtuB involves the series of steps depicted in Figure 7. Upon the binding of substrate to the extracellular surface, the Ton box undocks from the BtuB barrel and becomes exposed to the periplasmic space. The Ton box then associates with TonB. Once bound to BtuB, TonB catalyzes a second conformational change in the BtuB, which may involve a partial unfolding of the protein hatch. This second conformational step permits diffusion of the substrate across the outer membrane. Following transport, the Ton box dissociates from TonB, and the hatch of BtuB refolds into its native state.

As indicated above, the data obtained from BtuB−TonB cross-linking, combined with the EPR data presented here, indicate that the L8P and V10P mutations have at least two consequences for BtuB transport. First, the Ton box no longer correctly interacts with TonB, a defect that may partially be restored by mutations such as Q160C. Second, the Ton box no longer assumes its wild-type fold. This later conformational defect may prohibit these mutants from undergoing more than one round of transport. As indicated in Figure 7, the last step in the transport cycle involves refolding of the core of BtuB so that the protein assumes its native state, and a nativelike interaction between the Ton box with the BtuB barrel may be necessary to reset the system to its resting state. However, it should be noted that there are other reasonable possibilities that could explain these results. For example, since the Ton box is always extended in these mutants, the Ton box might never be able to disengage from TonB once bound. Such a constitutive interaction between TonB and BtuB could clearly prevent the hatch of BtuB from refolding into its native state and could block the normal transport cycle.

It is interesting to note that the spin label is highly mobile at positions 9, 11, and 12 but is highly restricted at several adjacent positions (see Figure 3A). Such a pattern presumably reflects the side chain contact that is present for the Ton box in its docked state. However, it is possible that the R1 side chain when present at positions 9, 11, or 12 undocks the Ton box, an event that is not hard to imagine especially if the docked and undocked configurations are close in energy. Although we are presently carrying out experiments to evaluate the effect of the spin label, several observations argue against this possibility. First, BtuB transport activity appears to be remarkably tolerant of single amino acid substitutions along the Ton box. In fact, all the single cysteine BtuB mutants of the Ton box exhibit normal transport. Second, the R1 side chain assumes a compact conformation along the backbone (*21*), and it appears to be no more disrupting than any other single amino acid substitution (*11*).

It is not surprising that the inclusion of proline at specific positions prevents the appropriate docking of the Ton box in the BtuB barrel. Prolines tend to disrupt secondary structure (*22*): they have a hindered ψ angle, they place steric constraints on adjacent residues, and the lack of an amide proton interrupts hydrogen bonding. It is interesting to note that R1 at positions 8 and 10 shows particularly strong contact in the native protein, indicating that side chain or backbone interactions at these positions may be particularly important. Proline substitutions at these positions may alter the geometry required to dock the Ton box to BtuB, or they may eliminate the hydrogen bonding that is important for docking of the Ton box.

In summary, the data presented here indicate that proline mutations within the Ton box of BtuB that lead to a transport-defective phenotype alter the normal structure of the Ton box. Rather than being docked into the barrel of BtuB in the absence of substrate, the Ton box in these mutants is in an extended conformation similar to that seen for wild-type BtuB in the presence of substrate. Unlike the functional wild-type protein, the addition of substrate produces little or no change in the configuration of the Ton box.

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