Published in final edited form as:

Biochemistry. 2018 February 13; 57(6): 1045–1053. doi:10.1021/acs.biochem.7b01223.

A Dynamic Protein-Protein Coupling Between the TonB-dependent Transporter FhuA and TonB

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Abstract

Bacterial outer membrane TonB-dependent transporters function by executing cycles of binding and unbinding to the inner membrane protein TonB. In the vitamin B₁₂ transporter BtuB and the ferric citrate transporter FecA, substrate binding increases the periplasmic exposure of the Ton box, an energy-coupling segment. This increased exposure appears to enhance the affinity of the transporter for TonB. Here, continuous wave and pulse EPR spectroscopy were used to examine the state of the Ton box in the *Escherichia coli* ferrichrome transporter, FhuA. In its apo state, the Ton box of FhuA samples a broad range of positions and multiple conformational substates. When bound to ferrichrome, the Ton box does not extend further into the periplasm, although the structural states sampled by the FhuA Ton box are altered. When bound to a soluble fragment of TonB, the TonB-FhuA complex remains heterogeneous and dynamic, indicating that TonB does not make strong, specific contacts with either the FhuA barrel or the core region of the transporter. This result differs from that seen in the crystal structure of the TonB-FhuA complex. These data indicate that unlike BtuB and FecA, the periplasmic exposure of the Ton box in FhuA does not change significantly in the presence of substrate and that allosteric control of transporter-TonB interactions functions by a different mechanism than that seen in either BtuB or FecA. Moreover, the data indicate that models involving a rotation of TonB relative to the transporter are unlikely to underlie the mechanism that drives TonB-dependent transport.

TOC image

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Supporting Information.

Four figures and one table are provided as supporting information. Figure S1 shows DEER data measuring distances across the FhuA barrel, Figure S2 compares DEER data from reconstituted and outer membrane preparations, and Figure S3 compares EPR spectra with and without sucrose. Figure S4 shows the background corrected DEER data used to determine the distributions shown in Figures 4 and 5 and Table S1 lists the distance restraints used for simulated annealing.

Introduction

In Gram negative bacteria, nutrients such as iron, vitamin B₁₂ and certain carbohydrates are accumulated by a family of specific, high affinity outer membrane transport proteins.^{1, 2} These active transport proteins obtain energy by coupling to the inner membrane protein TonB, and are therefore termed TonB-dependent. Although a number of high-resolution crystal structures have been obtained for members of this family, the molecular steps that mediate the transport process are not presently understood. TonB, which participates in an inner membrane complex with ExbB and ExbD,^{3, 4} is stoichiometrically limited in this system, and transport appears to involve repeated cycles of attachment and dissociation of TonB from the outer membrane transporter. The binding of substrate to TonB-dependent transporters is observed to enhance the affinity of the transporter for TonB⁵ and this enhanced affinity may function to direct TonB to substrate-loaded transporters.

All TonB-dependent transporters consist of a 22-stranded β -barrel, where the N-terminal 130 to 150 residues are folded within the interior of the barrel and form a region referred to as a hatch or core. A highly conserved segment termed the Ton box is located at the N-terminus and is involved in coupling the transporter to TonB.^{5–8} Crystal structures have been obtained for a fragment of TonB interacting with either BtuB, the vitamin B₁₂ transporter from *Escherichia coli*, or FhuA, the *Escherichia coli* ferrichrome transporter.^{9, 10} These structures show that TonB engages in an edge-to-edge β -sheet interaction with the Ton box, while also interacting with the barrel and the hatch regions of the transporter. The mechanism by which TonB drives transport in these TonB-dependent transporters is not resolved. TonB has been proposed to drive conformational changes in the hatch region of the transporter by either a pulling mechanism^{11, 12} or a rotational mechanism,^{13, 14} thereby rearranging or moving the hatch to permit substrate transport.

In BtuB, substrate binding shifts a conformational equilibrium in the Ton box to favor an unfolded state that projects the Ton box into the periplasmic space.^{15–17} As shown in Figure 1a, this substrate-induced disorder transition is clearly observed in EPR spectra from the Ton box as a narrowing of the resonance lines, and it appears to account for the enhanced affinity that is observed between BtuB and TonB in the presence of substrate.^{5, 18} In the *Escherichia coli* ferric citrate transporter, FecA, substrate is also observed to enhance transporter-TonB interactions,¹⁹ and EPR spectra (Figure 1b) indicate that this Ton box also undergoes a transition to a more dynamic state in the presence of substrate.²⁰ However, in the case of FecA, this disordering is not due to an unfolding of the Ton box into the periplasmic space.

In the apo state, the Ton box interacts with an N-terminal transcriptional motif and it is sterically blocked from interacting with the TonB. In the presence of substrate the transcriptional motif is displaced from the Ton box thereby allowing TonB to associate with the Ton box.²¹ In FhuA, the affinity of TonB for the transporter also increases in the presence of substrate, and ferrichrome binding is observed to enhance the coupling of TonB to FhuA both in-vivo⁷ and in-vitro.^{18,19,22} However, as seen in Figure 1c, EPR spectra from the FhuA Ton box do not show evidence for a substrate-induced change in local dynamics and the spectra are characteristic of a disordered protein segment in either state.²⁰ Thus, the Ton box of FhuA appears to behave differently than the Ton box in BtuB or FecA, and the allosteric mechanism that regulates the affinity of FhuA for TonB is unclear.

The present work had two objectives: the first was to determine whether substrate produced a change in the position of the FhuA Ton box, perhaps extending it further into the periplasm; the second was to examine the position of the Ton box when bound to TonB to determine whether TonB restrained the position of the Ton box as seen in the FhuA-TonB crystal structure. Both continuous wave and pulse EPR spectroscopy were used to determine the position of the FhuA Ton box and examine its structure in the absence and presence of substrate and when bound to a C-terminal fragment of TonB. In the apo state, the Ton box samples a broad range of conformations as determined by electron-electron double resonance (DEER), consistent with the previously published EPR spectra.²⁰ The distribution remains broad in the presence of substrate and modeling using the major population in these distributions indicates that the Ton box does not extend further into the periplasm. Remarkably, the Ton box samples a wide range of conformations even when bound to a C-terminal fragment of TonB, and the distances measured are much broader and longer than predicted by the FhuA-TonB crystal structure. The result indicates that TonB interacts strongly only with the Ton box, and does not interact strongly with either the FhuA barrel or the N-terminal core of the protein. This dynamic coupling between TonB and FhuA is not consistent with models where a rotation of TonB drives transport. The broad distance distributions sampled by the Ton box in the apo or substrate bound states, indicates that FhuA regulates transporter-TonB interactions through a different mechanism than that in either BtuB or FecA.

Methods

Mutagenesis, expression, purification, and reconstitution of FhuA

The plasmid harboring wild-type FhuA, pHK763, was generously provided by Volkmar Braun (University of Tübingen, Tübingen, Germany). FhuA cysteine mutants were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and subsequently spin labeled. Expression, purification, and reconstitution of FhuA into lipid vesicles followed a procedure described previously, except that 30 column volumes of a linear gradient running from 0 to 1 M LiCl was used for the ion exchange purification.²⁴ For labeling, 30 mL of solubilized protein solution was concentrated down to 10 mL and 1-3 mg of MTSL was added to the mixture (~1 mg for single labeled and 2-3 mg for double labeled samples). The reaction was allowed to proceed overnight at room temperature before purification by FPLC. Each labeled mutant was pure as judged by SDS-PAGE and appeared

to be correctly folded based on the molecular weight. Approximately 1 mg of purified FhuA was reconstituted into DLPC vesicles at a protein:lipid ratio of approximately 1:500. For pulse EPR measurements, the spin-labeled protein was diluted with wild type protein in a 1:3 ratio (mutant protein: wild type) to improve the phase memory time of the samples.¹⁷

Expression and purification of TonB (103-239)

The TonB fragment lacking the N-terminal transmembrane domain and a portion of the periplasmic domain was obtained from Robert Nakamoto (Molecular Physiology, University of Virginia). The plasmid encoding TonB (103-239) was transformed into T7 Express lysY/Iq competent cells (New England Biolabs, Ipswich, MA). The cells were grown in 2xYT media at 37°C until the optical density at 600 nm was 0.7–0.8. The cells were then induced with 0.5 mM IPTG, allowed to grow for an additional 6 hours at 20°C, and then centrifuged at 8275g for 10 minutes at 4°C.

To prohibit proteolysis, the purification of TonB (103-239) was performed on ice. The cell pellet was resuspended in 25 mL of resuspension buffer (25 mM Tris, pH 7.5) to which protease inhibitors AEBSF, aprotinin, and leupeptin were added as well as dithiothreitol (DTT). The sample was homogenized by hand and the cells were disrupted by passing the sample three times through a French Press. Following 15 minute centrifugation at 17,000 × g, the supernatant was retained and loaded onto a nickel column with equilibration buffer (25mM Tris, pH 7.4, 300 mM NaCl, 20 mM imidazole). The column was then washed with 3 column volumes of equilibration buffer and twice with 5 mL of a 100 mM imidazole buffer (25 mM Tris, pH 7.4, 300 mM NaCl, 100mM imidazole) to remove nonspecifically bound proteins. The bound protein was eluted with 25 mL of elution buffer (25 mM Tris, pH 7.4, 300mM NaCl, 250 mM imidazole), the purity of the fractions checked by SDS-PAGE, and the pure fractions pooled. The sample was then concentrated by ultrafiltration using a 3 kDa molecular mass cut-off membrane to yield a 1.5 mL solution of approximately 370 μM. To bind TonB (103-239) to membrane reconstituted FhuA, the membrane preparation was taken through 6 or 7 freeze-thaw cycles to insure that the TonB fragment had access to both membrane vesicle surfaces.

Electron paramagnetic resonance (EPR) measurements

Continuous wave EPR spectra were collected at room temperature using an X-band EMX spectrometer (Bruker Biospin, Billerica, MA) equipped with an ER 4123D dielectric resonator. All EPR spectra were recorded with 150 G magnetic field sweep, 1 G modulation, and 2.0 mW incident microwave power. Samples of 3-5 μL were loaded into glass capillary tubes (0.6 mm ID × 0.84 mm OD round capillary, Vitrocom, Mountain Lakes, NJ). Scaled mobilities provide a relative measure of nitroxide motion within proteins and are defined as:

$M_s = (\delta^{-1} - \delta_i^{-1}) / (\delta_m^{-1} - \delta_i^{-1})$, where δ is the central resonance linewidth and δ_i and δ_m are the least and most mobile R1 side chains found in proteins.²⁵ Values for δ_i and δ_m were taken as 8.5 and 1.8 Gauss, respectively. Fits to the EPR spectra and estimates of the label correlation time were made using the LabVIEW program Multicomponent (Christain Altenbach, UCLA), which implements the Microscopic Order Macroscopic Disorder (MOMD) model developed by Freed and co-workers.²⁶ Magnetic parameters used were those found previously for solvent exposed sites.²⁷

Double electron-electron resonance (DEER) experiments were performed on a Bruker Elexsys E500 EPR spectrometer running at Q-band using an EN5107D2 dielectric resonator. Measurements were made on 10-15 μL of sample at a protein concentration of approximately 100 μM that was loaded into quartz capillaries (2.0 mm ID $\times$ 2.4 mm OD) and flash frozen in a dry ice/isopropanol bath. DEER data were acquired using a 4-pulse sequence²⁸ with 16 and 32 ns $\pi/2$ and π observe pulses, respectively, separated by a 36 ns π pump pulse. The pump frequency was set to the maximum of the nitroxide spectrum and the observer frequency was set at a 75 MHz lower frequency offset from the pump frequency. The dipolar evolution data were processed and distance distributions determined using Tikhonov regularization incorporated in the DeerAnalysis2016 software package,²⁹ where the regularization parameter was taken near the inflection point in the L-curve. Confidence limits in the distances and distance distributions are determined by the refocusing echo time and were set as described previously.³⁰ Expected distance distributions from crystal structures were obtained using the program MMM³¹ and a spin label rotamer library based upon a density functional theory analysis of the energetics of the R1 side chain.³²

Simulated annealing—All runs were carried out using Xplor-NIH version 2.40.³³

Structure Generation and *in silico* labeling—Three crystal structures of FhuA were used as a starting points for structure generation in the apo (PDB ID: 1BY3), ferrichrome (PDB ID: 1BY5) and TonB (PDB ID: 2GRX) conditions. For the apo and ferrichrome conditions, structures were labeled *in silico* with the R1A label at the sites used in the DEER experiments using the program MMM. The label CHI 1 and 2 angles were then manually set to the m, m (−60, −60) rotamer using Pymol. Finally, the label atom names were manually adjusted to the CYSP convention used for R1 labels in Xplor-NIH.

For the TonB bound state, the crystal structure was missing residues 403-407. To avoid problems with residue numbering, the atoms for these residues were built into the crystal structure using the addatoms.py script that is included with Xplor-NIH. For this structure, the labels were also attached using the same addatoms.py script, and the CHI 1 and 2 angles were again manually set to the m, m rotamer.

Creation of restraints from distance measurements—Main distance restraints were placed between the ring nitrogen atom (NS1) in the R1 side chain of sites on the barrel, and the beta carbon of the label at site 13 in the Ton box. Values for each restraint were obtained by taking the value of the distance distribution which showed the maximum probability, and subtracting 6 angstroms (approximately the expectation length of the MTSL side chain) to reflect the use of a beta carbon restraint at position 13. This change prevents the resulting modeled structures from showing fully extended, strained label conformations that were observed for restraints solely between both ring nitrogens. Restraints were modeled in Xplor-NIH as square well potentials using the NOE potential term. The uncertainty in these ring nitrogen to beta carbon restraints was set to 2 Angstroms. A secondary restraint between the two ring nitrogens was also employed, using the same square well form. These restraints had the same values as the previous set but used an uncertainty of 8 Angstroms.

Annealing Protocol

Apo and Ferrichrome—Backbone atoms were fixed for the majority of the protein (residues 30-714) for the duration of dynamics and annealing. Residues 1-30 were left free, and the spin label CHI 1 and CHI 2 angles were fixed to the previously noted m, m rotamer. The protein was first subjected to 3000 steps of dynamics at 2000 K to disorder the ton box region using the BOND, ANGL, IMPR, and CDIH potential terms. No NOE restraints were used during the dynamics. The simulated annealing then stepped down from 2000 K to 200 K with 20 K stepping, using the aforementioned potential terms, and adding the NOE potential term restraints. Runs were conducted on the University of Virginia's Rivanna computing cluster, and a total of 400 structures were generated per condition. Structures were scored based on the energy of the NOE distance restraint potential, and the top 40-60 structures having restraint violations < 0.5 Å were collated together for visualization using Pymol.(Schrodinger, LLC (2015) The PyMOL Molecular Graphics System (Schrodinger, LLC, New York), Version 1.8.).

TonB—These simulations proceeded as above, with the following exceptions. Since the starting structure contained TonB, these atoms (residues 158-235) were grouped together in a separate selection. Residues 1-13 of the FhuA structure were also included in the TonB grouped selection, in order to preserve the beta sheet contact observed between these entities in the crystal structure.

The order of the simulations was the same, but the initial dynamics section used 8000 steps and a higher temperature of 5000 K to ensure that the larger mobile segment with TonB was sufficiently displaced prior to annealing, which stepped down from 5000 K to 200 K with 20 K stepping.

Results

FhuA binds substrate and is correctly folded into proteoliposomes

Many of the experiments described here were carried out for FhuA that was purified and reconstituted into DLPC liposomes. As found previously for other TonB-dependent transporters, the shorter chain lipid²¹ as well as magnetic dilution of the labeled protein¹⁷ improved the phase memory times and the range of distances that could be measured for FhuA using DEER. FhuA appears to be correctly folded in these preparations. The membrane reconstituted FhuA binds ferrichrome²⁰ as shown previously using a fluorescence based assay,³⁴ and distance measurements made using DEER between pairs of labels placed across FhuA on the extracellular and periplasmic sides of the barrel (Figure S1) are consistent with those expected based upon the FhuA crystal structure. DEER data from the reconstituted system were found to be consistent with data obtained from FhuA in native outer membrane preparations. In these native preparations, FhuA was not taken through detergent solubilization and membrane reconstituted (Figure S2).

The N-terminal segment of FhuA is tethered near residue 20

Shown in Figure 2 are EPR spectra taken from single spin labels placed along the N-terminal end of FhuA from sites 14 through site 23 in the presence and absence of the

substrate, ferrichrome. Spin labels at sites 14 and 15 yield highly averaged EPR lineshapes typical of unstructured protein segments, where the label correlation time is roughly 1.5 ns. Addition of substrate has no effect upon these lineshapes, which is consistent with a result seen previously for labeled sites within the Ton box (residues 7-13).²⁰ Spectra from sites 16 to 19 undergo a progressive increase in correlation time as one moves away from the N-terminus, and the relative scaled mobilities (Ms) of the sites scanned are shown in Figure 3a (open circles). A value of Ms near 1 indicates a label has a mobility that is among the most mobile seen for the R1 side chain in proteins, whereas a value near 0 indicates that the label is among the least mobile or most restricted. Sites 20 and 21 are the first sites where evidence of tertiary contact between the label and protein are observed, indicating that this segment is in contact with other regions of the protein. Substrate addition has differential effects on the EPR lineshapes along this segment. Substrate slows the label motion at sites 16 and 17, but increases it at site 18. As seen in the inset for sites 20 and 21 (Figure 2), many of these spectra are actually a composite of at least two modes of nitroxide motion, one which is more mobile and one which is less mobile and in tertiary contact (for example sites 19 to 23). The components in these spectra are modulated by sucrose addition (Figure S3) where sucrose decreases the more mobile component and increases the immobile component. The sensitivity to sucrose indicates that these components arise from structural substates that are in equilibrium.^{35, 36} There are clearly substrate-induced structural changes in this region of FhuA and as seen for S20R1 and A21R1 (Figure 2), substrate shifts the equilibrium between protein substates. For example, at site S20R1, there is one major component of intermediate motion in the apo state. With substrate addition, this component splits into more mobile (m) and less mobile components (i) representing 13 and 87% of the spins. However, unlike residues that are C-terminal to the Ton box in BtuB,¹⁵ there is no evidence for unfolding of this segment upon substrate binding.

In addition to recording these spectra following the addition of substrate, a C-terminal fragment of TonB (residues 103 to 239) was bound to FhuA either with or without substrate. As seen in Figure 3a, the mobilities of the nitroxides decrease slightly towards the Ton box upon TonB binding, but are not dramatically altered elsewhere on this segment. Spectra for R1 placed within the Ton box (Figure 3b, positions 8, 11 and 13) undergo a dramatic restriction in R1 motion upon TonB binding. The spectrum for V11R1 shows evidence for strong tertiary contact upon TonB binding, and inspection of the crystal structure for the FhuA/TonB complex¹⁰ indicates that this may be because R1 at this position is interacting strongly within a pocket at the Ton box/TonB interface. The large change in the spectra from labels at sites 8, 11 and within the Ton box allows us to estimate the fraction of FhuA that is bound to TonB under these conditions. This is found to be on the order of 95%. Thus, the N-terminal segment that includes the Ton box in FhuA is dynamic and appears to be unfolded until residue 20; however, neither the addition of substrate nor TonB significantly unfolds this segment of the protein from the N-terminal hatch domain.

The FhuA Ton box samples a broad conformational space under any condition

As indicated above, substrate enhances the binding of TonB, but as shown in Figures 2 and 3, substrate does not unfold the Ton box or any of the ten sites immediately C-terminal to the Ton box. To determine whether substrate shifts the position of the Ton box, five nitroxide

pairs were constructed where one label was placed at the end of the Ton box (position 13) and a second label was placed on the periplasmic edge of the FhuA barrel. The β -barrel of FhuA is expected to be rigid and DEER measurements between the barrel and position 13 should reflect changes in the position of the Ton box. Shown in Figure 4 are distance distributions measured for five sites on the barrel to the end of the Ton box in the apo and substrate bound states. Samples of the primary data for these distributions are shown in Figure S4. These generally have good signal-to-noise with echo times in the 2 to 3 μ s range, except for the substrate bound cases where the echo times are shorter due to reduced phase memory times. As indicated in Figure 4 the distance distributions are reliable over most of the distribution. The distance distributions observed are broad, where there is often more than one resolved distance within the distribution. It should be noted that FhuA is diluted with wild-type protein, minimizing intramolecular contributions to the dipole interaction. As discussed below, these distributions are much broader than can be explained based upon the likely label rotameric states of the spin labels and they are consistent with the EPR spectra from the Ton box and regions C-terminal to the Ton box (Figures 1c and 2), which indicate that this segment is unfolded and samples multiple conformational states. Substrate addition produces little change in the distributions or peak distance for 13R1/161R1 and 13R1/663R1; however, it lengthens the primary distance for 13R1/583R1, and it shortens the distances measured to 228R1 and 373R1. One interpretation of this result is that substrate moves the Ton box away from the side of the barrel where 583 is located and towards the opposite surface. We do not detect longer distances appearing with substrate addition, which is consistent with the EPR spectra in Figure 2 and suggests that the Ton box and the region C-terminal to the Ton box are not unfolding further into the periplasm with substrate addition.

To generate models for the average or most populated position of the FhuA Ton box without and with substrate, we took the major distance from each of these distributions and used XPLOR-NIH (see Methods) to generate structural models consistent with the DEER data. The shaded regions shown in the distributions in Figure 4 were taken as distance restraints and Figures 4b and c show the 50 lowest energy models for the position of site 13 (the C-terminal end of the Ton box) relative to the FhuA barrel that is consistent with these distances. Only the C β atom of position 13 is shown for clarity. It should be noted that we could not satisfy these restraints using the apo and substrate bound crystal structures of FhuA, but needed to allow movement in the segment encompassing residues 1 to 29 in order to satisfy the restraints. As seen in Figure 4, substrate produces a slight shift in position 13, but the changes are not large and there is no significant extension of the Ton box towards the periplasm with substrate. In either state, the C-terminal end of the Ton box is positioned 15 to 20 Angstroms from the periplasmic turns of the transporter.

Distance distributions to the Ton box are broad in the presence of TonB and not consistent with the FhuA-TonB crystal structure

DEER measurements between each of the five positions in the FhuA barrel to position 13 in the Ton box were made in the presence of a C-terminal fragment of TonB (103-239). As indicated above, EPR spectra from sites 8, 11 and 13 indicate that TonB is fully bound (>90%) to the Ton box under the conditions used to incorporate TonB. The resulting

distance distributions in the presence of TonB are shown in Figure 5a. Although slightly different than the distributions in the apo or substrate bound states, these distributions are very broad and indicate that the Ton box (when bound to TonB) retains a high degree of conformational heterogeneity.

The distance distributions in the presence of TonB were compared with distance predictions based upon the FhuA-TonB crystal structure using a standard rotamer library for the R1 side chain (see Methods). The resulting predictions are shown as the magenta histograms in Figure 5a. In every case, the distributions predicted by the crystal structure are relatively narrow and are represented in the shorter end of these distributions. In all cases, the experimental DEER distributions are 15 to 25 Angstroms broader and much longer than those expected. Since TonB is anchored to the Ton box, the result indicates that TonB is not anchored to either the FhuA β -barrel or to the hatch region of FhuA at a defined position. Instead, TonB, like the Ton box to which it is attached, samples multiple conformational states when it is bound to FhuA.

We also took distance restraints from the distributions in Figure 5a and used this to generate a family of structures where the TonB fragment was bound to the Ton box in an edge-to-edge manner as seen in the FhuA-TonB crystal structure. It was not possible to satisfy the distance restraints between the barrel edge and site 13 using the crystal structure, and we did not restrain TonB. The resulting structures are shown in Figure 5b, and indicate that TonB must sample a broad conformational space when bound to FhuA. In reality, TonB likely samples a broader distribution than that seen in Figure 5b, since only a portion of the measured distributions are taken in the modeling.

Discussion

The binding of TonB to FhuA is known to be regulated by substrate,^{7, 18, 19, 22} and in the present work we tested the idea that interactions with TonB might be regulated by an extension of the FhuA Ton box further into the periplasm. For FhuA, substrate-dependent changes in the Ton box were relatively minor and not as obvious as those seen for the Ton box in BtuB (the vitamin B₁₂ transporter) or FecA (the ferrichrome transporter). The distance distributions seen in Figure 4 changed with substrate addition, indicating a shift in the conformational states sampled by the Ton box. However, the major conformation in each state was similar and placed the Ton box 15 to 20 Angstroms below the periplasmic interface. The EPR spectra from the N-terminal region of FhuA indicated that the Ton box and several segments C-terminal to the Ton box are disordered under all conditions, and a gradient of R1 motion along this segment demonstrates that it is tethered near residue 20. There is no indication in the continuous wave EPR spectra recorded here or previously²⁰ of unfolding near or within the hatch domain that might extend the Ton box. Thus, both the pulse and continuous wave EPR are consistent, but the mechanism regulating the enhanced TonB binding is unclear.

A surprising finding made here was that the binding of a C-terminal fragment of TonB did not limit the distribution of the Ton box. Even when bound to TonB, the position of the Ton box remained widely distributed relative to the FhuA barrel. This presents a different picture

of the FhuA-TonB interaction than that presented by either the FhuA-TonB crystal structure¹⁰ or by the BtuB-TonB structure.⁹ In these structures, TonB is seen to interact with the Ton box, but it also makes interactions with the hatch region and the β -barrel of the transporter. The crystal structure of the FhuA-TonB complex is shown in Figure 6, and in this structure, residue R166 in TonB interacts closely with A26, E56, A591 and N594 on FhuA to place helix1 on TonB in contact with both the barrel and hatch. The data in Figure 5 clearly shows that this interaction cannot dominate the TonB-FhuA interaction since interspin distances that are consistent with this structure represent only a minor portion of the broad distance distributions that are observed.

There are several reasons why models from crystallography may present a different or limited set of protein conformations compared to those observed with spectroscopic techniques. In addition to interference due to incorporation of a label¹⁵ or distortion due to contact in the unit cell, the precipitants used for crystal growth are stabilizing osmolytes³⁷ that will bias conformations that are in equilibrium towards the least hydrated state. For example, when membrane reconstituted BtuB and BtuB in the protein crystal were compared using EPR, a conformational equilibrium in the Ton box of BtuB was seen to be shifted by 3 kcal/mol between the two environments. This made it impossible to observe a substrate-induced extension of the Ton box by crystallography.³⁸ Similarly, we think it is likely that the FhuA-TonB interaction seen in Figure 6, represents one trapped substate among many. Both the DEER data and the EPR spectra generated here from segments near the Ton box demonstrate that the coupling between the two proteins is structurally heterogeneous and dynamic. This result is also consistent with recent cross-linking data obtained for the interaction of FepA (the *Escherichia coli* iron enterobactin transporter) with TonB, which indicates that there are multiple sites of interaction for TonB on the periplasmic interface of the transporter.³⁹

The results presented here show that interactions between the transporter and TonB are mediated by interactions with the Ton box, and that this interaction alone is responsible for the high-affinity binding between these outer and inner membrane proteins. At the present time, the precise mechanism by which TonB-transporter interactions mediate substrate transport is not clear, and there are two general models that have been proposed for this system. One mechanism involves pulling, where the movement of TonB away from the outer membrane and towards the inner membrane drives an unfolding or rearrangement of the transporter hatch domain.^{11, 12} A second mechanism involves the rotation of TonB, which subsequently rearranges the hatch domain.^{13, 14} The observations made here are not inconsistent with a pulling mechanism, but the fact that TonB is flexibly linked to the hatch domain is not consistent with rotational mechanisms. The presence of 4 or 5 unstructured residues between the Ton box and the hatch when TonB is bound indicates that the transfer of a rotational torque from TonB to the hatch would be inefficient. There are difficulties with the pulling models as well, which suggest that large extensions of the Ton box in BtuB are necessary to open a channel within the hatch domain to facilitate substrate movement. In one simulation 85 to 95 Angstroms of extension is proposed to occur,¹¹ and in another up to 200 Å of pulling is proposed.¹² How such large extensions of the Ton box can take place over dimensions that are comparable to or larger than the separation between outer and inner membranes is unclear. Previous work using EPR demonstrated that binding of the C-

terminal fragment of TonB to BtuB lowered substrate affinity and could partially release bound substrate.⁴⁰ These observation suggests that the substrate binding site is allosterically coupled to the Ton box, and that TonB binding alone might lower the energy of intermediate conformational states that facilitate transport.

In summary, EPR based measurements in reconstituted membrane systems demonstrate that the Ton box of FhuA remains disordered upon the addition of substrate, and that in apo and substrate bound states it is positioned 15 to 20 Angstroms into the periplasmic space. Upon the addition of TonB, the position of the Ton box is heterogeneous with respect to the barrel and residues immediately C-terminal to the Ton box remain disordered. The result suggests that TonB is tethered to FhuA through the Ton box, and that interactions between TonB and the hatch or barrel that are seen in the crystal structure are not energetically favored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding information

This work was supported by a grant from the National Institutes of Health, NIGMS, GM035215 to DSC.

Abbreviations

AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
DTT	dithiothreitol, DEER, double electron-electron resonance
DLPC	1,2-dilauroyl-sn-glycero-3-phosphocholine
EPR	electron paramagnetic resonance
MTSL	(<i>S</i> -(1-oxyl-2,2,5,5-tetramethyl- 2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonylthioate
NOE	nuclear Overhauser effect
R1	spin-labeled side chain produced by derivatization of a cysteine with the MTSL
SDSL	site-directed spin labeling
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris	tris(hydroxymethyl)aminomethane

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Figure 1.

Structures and structural changes in the N-terminal region of TonB-dependent transporters.

a) In BtuB, the binding of substrate promotes the unfolding of the energy coupling segment (Ton box) into the periplasm. Selected EPR spectra along the Ton box reflect the unfolding transition;¹⁵ **b)** In FecA, substrate binding alters the position of the N-terminal extension or transcriptional domain so that the Ton box is no longer sterically blocked.²¹ Selected EPR spectra reflect the increased disorder in the Ton box resulting from this change.²⁰ **c)** In FhuA, the Ton box is not resolved in crystal structures for the apo (PDB ID: 1BY3) and substrate-bound states (PDB ID: 1BY5).²⁵ EPR spectra along the Ton box of FhuA indicate that it is disordered in either state.²⁰

Figure 2. EPR spectra from the N-terminal segment of FhuA. **a)** Model of FhuA docked to the C-terminal fragment of TonB showing positions 14, 19 and 23 on the N-terminal segment (PDB ID: 2GRX). **b)** Spin labeled side chain R1 resulting from the reaction of the MTSL spin label with a site-directed cysteine. **c)** X-band EPR spectra from sites 14 to 23 on the C-terminal side of the Ton box (residues 7 to 13) in the apo (black trace) and substrate loaded states (red trace). Many of the spectra contain both a mobile (*m*) and immobile (*i*) motional component, as see in the expanded spectra for sites 20 and 21. All spectra are normalized to spin count, except the expanded spectra for sites 20 and 21.

Figure 3.

EPR spectra in the presence of TonB (103–239). **a**) Scaled mobility²⁵ determined from the EPR spectra obtained from sites 14 to 23 in the apo state (open symbols) and presence (closed symbols) of the C-terminal TonB fragment. **b**) EPR spectra in the apo (black trace) and TonB bound states (red trace) for selected sites in the N-terminus. Significant lineshape changes in this N-terminal region are only detected within the Ton box (residues T8R1 and V11R1).

Figure 4.

a) Distance distributions obtained using DEER between five pairs of labels on FhuA where one label is at the C-terminal end of the Ton box at position 13 and a second label is placed on the FhuA barrel near the periplasmic interface. Distributions are shown for both the apo (blue trace) and substrate bound (red trace) states. For the apo state, the area between the first and second dashed lines indicates the region where distances but not distributions are reliable. Beyond the second (red) dashed line, the distances are not reliable. For the substrate bound distributions, this region is moved by about 5 Angstroms towards shorter distances. The distance restraints used in the annealing are indicated as the shaded regions in the distributions (blue for apo, red for substrate bound) using the most populated distance from the distributions. **b, c)** Model showing the position of the C β carbon from site 13 on the Ton box (the C-terminal position) in the apo (blue) and substrate-bound states (red) for the 50 lowest energy structures obtained by simulated annealing (see Methods).

Figure 5.

a) Distance distributions obtained by DEER for the five pairs of spin labels shown in Figure 4 when FhuA is bound to TonB. The histograms (magenta) represent the predicted distributions for each pair based upon the crystal structure of the FhuA-TonB complex (PDB ID: 2GRX)¹⁰ (see Methods). The area between the first and second dashed lines indicates the region where distances but not distributions are reliable. Beyond the second (red) dashed line, the distances are not reliable. The blue shaded regions indicate the distance restraints that were used in simulated annealing to generate a position for TonB. **b)** Position of the TonB fragment (red) when bound to FhuA that satisfy the distance restraints shown in a). Fifty structures for TonB are shown. The orientation of TonB is not restrained, but it was not possible to satisfy the restraints to site 13 (green spheres) if TonB was restrained to the orientation seen in the crystal structure.

Figure 6.

Crystal structure of the TonB-FhuA complex (PDB ID: 2GRX),¹⁰ showing contact of residue R166 in TonB (green ribbon) with residues in the hatch region (A26 and E56) and barrel (A591 and N594) (shown in magenta). The placement of the Ton box in this structure is not consistent with the majority of the distance populations sampled by the FhuA-TonB complex in bilayers.