Deletion of the proline-rich region of TonB disrupts formation of a 2:1 complex with FhuA, an outer membrane receptor of *Escherichia coli*

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Abstract

TonB protein of *Escherichia coli* couples the electrochemical potential of the cytoplasmic membrane (CM) to active transport of iron-siderophores and vitamin B$_{12}$ across the outer membrane (OM). TonB interacts with OM receptors and transduces conformationally stored energy. Energy for transport is provided by the proton motive force through ExbB and ExbD, which form a ternary complex with TonB in the CM. TonB contains three distinct domains: an N-terminal signal/anchor sequence, a C-terminal domain, and a proline-rich region. The proline-rich region was proposed to extend TonB’s structure across the periplasm, allowing it to contact spatially distant OM receptors. Having previously identified a 2:1 stoichiometry for the complex of full-length (FL) TonB and the OM receptor FhuA, we now demonstrate that deletion of the proline-rich region of TonB (TonB$_{H9004\Delta66-100}$) prevents formation of the 2:1 complex. Sedimentation velocity analytical ultracentrifugation of TonB$_{H9004\Delta66-100}$ with FhuA revealed that a 1:1 TonB–FhuA complex is formed. Interactions between TonB$_{H9004\Delta66-100}$ and FhuA were assessed by surface plasmon resonance, and their affinities were determined to be similar to those of TonB (FL)–FhuA. Presence of the FhuA-specific siderophore ferricrocin altered neither stoichiometry nor affinity of interaction, leading to our conclusion that the proline-rich region in TonB is important in forming a 2:1 high-affinity TonB–FhuA complex in vitro. Furthermore, TonB$_{H9004\Delta66-100}$–FhuA$_{H9004\Delta21-128}$ interactions demonstrated that the cork region of the OM receptor was also important in forming a complex. Together, these results demonstrate a novel function of the proline-rich region of TonB in mediating TonB–TonB interactions within the TonB–FhuA complex.

Keywords: analytical ultracentrifugation; ferricrocin; FhuA; surface plasmon resonance; TonB

TonB-dependent transport systems of Gram-negative bacteria provide high-affinity uptake of essential nutrients such as iron-chelating siderophores and vitamin B$_{12}$. These systems are comprised of an outer membrane (OM) receptor, a periplasmic substrate-binding protein, and an ATP-dependent cytoplasmic membrane (CM) protein complex (Braun et al. 1998). Energy to transport scarce nutrients through OM receptors into the periplasm is provided by the CM-anchored complex TonB–ExbB–ExbD. This complex harnesses the proton motive force (pmf) at the CM to energize TonB, allowing conformationally stored energy to be trans-
duced to OM receptors (Braun et al. 1998; Moeck and Coulton 1998). Transfer of energy requires physical interactions between TonB and the N terminus of OM receptors, a domain referred to as the Ton box. Although other sites of OM receptors have also been suggested to be critical for interactions with TonB (Vakaria and Postle 2002; Ogerman and Braun 2003), none has been fully characterized.

TonB can be divided into three distinct domains that are highly conserved. The N-terminal signal/anchor domain (residues 1–32) spans the CM and participates in energy transduction processes with ExbB/ExbD (Karlsson et al. 1993; Jaskula et al. 1994; Larsen and Postle 2001). Deletion of conserved TonB residues Ser-16 and His-20 disrupts interactions with ExbB, confirming the importance of this domain in transducing pmf (Larsen and Postle 2001). The C-terminal domain of TonB has been the subject of extensive investigation because it contains residues of critical importance for interactions with OM receptors. Residue 160 within this domain is a site of genetic suppressors of Ton box mutations in OM receptors; mutation of this residue resulted in a TonB-uncoupled phenotype (Heller et al. 1988; Bell et al. 1990; Anton and Heller 1993). Residues adjacent to 160 have also been shown to form specific disulphide cross-linkages with the Ton box of BtnB, the OM receptor for cobalamin (Cadieux and Kadner 1999). Two crystal structures of the C-terminal domain were solved independently (Chang et al. 2001; Koedding et al. 2004); they revealed that the C terminus of TonB forms an intertwined dimer. Depending on the size of the C-terminal variant studied, this dimer can interact with the OM receptor FhuA (Khursigara et al. 2004; Koedding et al. 2004), although not with the same affinity as longer monomeric TonB variants (Khursigara et al. 2004). Recently, a high-resolution structure was determined for a third TonB variant (residues 147–239) (Koedding et al. 2004). This TonB fragment crystallized as a dimer, with considerably reduced intermolecular interaction between monomers, and it formed a 1:1 complex with an OM receptor (Koedding et al. 2004).

Between TonB’s N-terminal signal/anchor sequence and the C-terminal domain resides the highly conserved proline-rich region (residues 66–102). This domain contains a series of Pro-Glu repeats followed by Pro-Lys repeats, separated by a six-residue linker segment. Nuclear magnetic resonance analysis (NMR) (Evans et al. 1986) of a 33-residue peptide corresponding to the proline-rich region demonstrated that this domain forms a rigid and highly extended structure with an overall length of ~10 nm. The linker segment was proposed to be more relaxed, allowing for swiveling motions. The proline-rich domain of TonB was considered important for linking energy transduction to spatially distant OM receptors (Evans et al. 1986). Additional studies (Brewer et al. 1990; Hannavy et al. 1990) demonstrated that peptides corresponding to the proline-rich region could interact with FhuA. However, in vivo analysis of TonB that was deleted in the proline-rich region (TonBΔ66-100) (Larsen et al. 1993) demonstrated that this motif was not essential for function coupled to OM receptors. Those authors specified that the activity of TonBΔ66-100 was less than that of wild-type TonB in vivo, implying that deletion of the proline-rich domain may have perturbed interactions with accessory proteins, or alternatively, limited TonB’s ability to span the periplasm and thereby contact OM receptors.

Two models describing TonB–OM receptor interaction have been proposed (Postle and Kadner 2003). The propeller model postulated from crystallographic studies of C-terminal TonB (Chang et al. 2001) describes two TonB monomers, intertwined at the C terminus and interacting with OM receptors. This model proposed that dimerized TonB undergoes rotary motion, similar to the mechanism described for the bacterial flagellar motor that is powered by MotA/MotB, homologs of ExbB/ExbD. An alternate model based on in vivo labeling of TonB N terminus with cysteine-specific fluorescent markers described shuttling of TonB between the CM and OM (Larsen et al. 2003). In the shuttle model, TonB in complex with ExbB and ExbD in the CM is present in an unenergized conformation. ExbB/ExbD uses the pmf to energize TonB, allowing its C terminus to interact with the OM. This interaction causes the release of TonB’s N terminus from the CM and transfer of stored potential energy to OM receptors, thereby facilitating ligand import.

Our previous in vitro studies (Khursigara et al. 2004, 2005) of TonB focused on its interactions with FhuA. By combining analytical ultracentrifugation (AUC) and surface plasmon resonance (SPR) we determined stoichiometries and affinities of FhuA with two TonB variants: C-terminal TonB fragment [TonB (CT); residues 155–239] and a full-length TonB minus its signal/anchor sequence [TonB (FL); residues 32–239]. We observed a high-affinity binding region within the N-terminal (residues 32–154) of TonB (FL) that was not present in TonB (CT). Interactions of these TonB variants were also analyzed with FhuAΔ21-128, a FhuA mutant which deleted residues 21–128 in the N-terminal cork domain, eliminating the switch helix (residues 24–29) but not the Ton box. In the work described here, we present AUC and SPR experiments of recombinant TonBΔ66-100 and its interactions with FhuA and FhuAΔ21-128. Our results provide insight into the role of the proline-rich region in forming TonB–FhuA complexes; this region is necessary to form a 2:1 TonB–FhuA complex, and it does not directly interact with FhuA.

Results and Discussion

The proline-rich region of TonB is essential in forming a 2:1 TonB–FhuA complex

To investigate the role of the proline-rich region of TonB in forming TonB–FhuA complexes in vitro, we expressed a
recombinant TonB lacking the proline-rich region: TonBΔ66-100. Analyses of sedimentation velocity experiments on uncomplexed TonBΔ66-100 were performed using SEDFIT (Schuck 2000) in order to determine sedimentation parameters: sedimentation coefficient, $s$ and buoyant molecular mass, $M_b$. Fits of the data to the Non-Interacting Species model indicated that TonBΔ66-100 sedimented predominantly as a monomer (85.8% of total OD$_{260}$) with an $s$ value of 1.72 and a buoyant molecular mass of 6853 Da. A minor sedimenting species (14.2% of total OD$_{260}$) was also observed with sedimentation parameters consistent with a similar species present in cysteine-substituted TonB (FL) preparations that we reported previously (Khursigara et al. 2004). Analysis of the sedimentation velocity data by the continuous c(S) model of SEDFIT revealed that the fractional ratio of TonBΔ66-100 is 2.27, in contrast to the value (2.39) that we reported for TonB (FL) (Khursigara et al. 2004). The lower fractional ratio of the proline-rich deletion mutant indicates that this protein is less elongated, consistent with previous reports that the proline-rich region forms an extended, rigid structure (Evans et al. 1986; Brewer et al. 1990).

Sedimentation velocity experiments on 2:1 molar mixtures of TonBΔ66-100 and FhuA or TonBΔ66-100 and FhuAΔ21-128, either in the absence or presence of Fc, were analyzed using the Non-Interacting Discrete Species model of SEDFIT (Table 1). Deletion of the proline-rich region prevents formation of a 2:1 TonB–FhuA complex described previously (Khursigara et al. 2004). Both in the absence and presence of Fc, sedimenting species were observed having molecular masses ($M_3$) of 102,923 Da and 101,846 Da, respectively (Table 1, lines 1,2). Given sequence-predicted molecular masses of 80,000 Da (FhuA) and 21,210 Da (TonBΔ66-100), $M_3$ thus corresponds to a 1:1 TonBΔ66-100:FhuA complex. The relative abundance of complex in these mixtures (48% − Fc and 49% + Fc) was similar to the abundance of 1:1 complex formed when TonB (FL) was mixed with FhuAΔ21-128 (Khursigara et al. 2005).

Mixing TonBΔ66-100 with FhuAΔ21-128 also demonstrated a 1:1 complex ($M_3$ = 90,573 − Fc; 90,676 + Fc), but in significantly lower abundance (14% − Fc; 21% + Fc; Table 1, lines 3,4). To form a 1:1 complex, the region of interaction in either TonB (residues 66–100) or FhuA (residues 21–128) is sufficient; the two regions are required to form the 2:1 complex. Deletion of both regions (Table 1, lines 3,4) markedly reduced the abundance of a TonB–FhuA complex. Also, the reduction in sedimentation coefficient upon deletion of both regions ($s$ values of 4.94 vs. 3.13; Table 1, lines 1,3) suggests that the overall shape of this complex differs from the 1:1 TonBΔ66-100–FhuA complex, possibly reflecting loss of an interaction site between residues 66–100 in TonB and the cork domain of the OM receptor.

We previously observed complexes of TonB (FL) with either FhuA or FhuAΔ21-128 that exhibited an increase in $s$ value of −0.2 when the OM receptor was in a ligand-loaded state (Khursigara et al. 2004, 2005). We attributed this change in $s$ to conformational changes of the TonB–FhuA complexes in response to addition of Fc. Neither a complex of TonBΔ66-100–FhuA nor a complex of TonBΔ66-100–FhuAΔ21-128 demonstrated an $s$ value that significantly changed in the presence of Fc (Table 1, lines 1,2 and 3,4). We conclude that the absence of residues 66–100 of TonB prevents Fc-dependent conformational alterations of TonB–FhuA or TonB–FhuAΔ21-128 complexes.

Through the use of in vivo cross-linking, Larsen et al. (1993) showed that TonBΔ66-100 interacted with FepA in the E. coli cell envelope. Although TonBΔ66-100 was demonstrated to adsorb bacteriofage Φ80 at wild-type levels, they reported that the mutant was overall less effective in siderophore transport and manifested elevated resistance to colicins B and D. Our in vitro results confirm the ability of TonBΔ66-100 to interact with an OM receptor. However, the stoichiometry of the resulting complex is altered compared to that of TonB (FL). Furthermore, interactions are

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### Table 1. Sedimentation velocity parameters of two complexes: TonBΔ66-100–FhuA and TonBΔ66-100–FhuAΔ21-128

<table>
<thead>
<tr>
<th>TonB Δ66-100</th>
<th>FhuA</th>
<th>FhuA Δ21-128</th>
<th>Fc</th>
<th>$s_1$</th>
<th>$s_2$</th>
<th>$s_3$</th>
<th>$M_3$ (Da)</th>
<th>$\Phi_{avg}$ (mL/g)</th>
<th>$M_3$ (Da)</th>
<th>$c_{S1}/c_{S2}/c_{S3}$ (%)</th>
<th>r.m.s.d. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>−</td>
<td>(3.50)</td>
<td>(1.72)</td>
<td>4.94</td>
<td>25,319</td>
<td>0.754</td>
<td>102,923</td>
<td>45/03/48</td>
<td>0.0054</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>+</td>
<td>(3.60)</td>
<td>(1.72)</td>
<td>4.97</td>
<td>25,054</td>
<td>0.754</td>
<td>101,846</td>
<td>39/03/49</td>
<td>0.0055</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1</td>
<td>−</td>
<td>(3.04)</td>
<td>(1.72)</td>
<td>3.13</td>
<td>22,915</td>
<td>0.747</td>
<td>90,573</td>
<td>71/15/14</td>
<td>0.0065</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1</td>
<td>+</td>
<td>(3.04)</td>
<td>(1.72)</td>
<td>3.10</td>
<td>22,941</td>
<td>0.747</td>
<td>90,676</td>
<td>67/12/21</td>
<td>0.0078</td>
</tr>
</tbody>
</table>

[a] Values in columns 1 to 3 represent molar ratios.

[b] An apparent $vbar$ [$\Phi = (1-M_3/M_{avg})p$] was used to calculate molecular mass [$M_r = M_3/(1-\Phi)$] values of TonB–FhuA complexes ($M_3$) as described previously (Khursigara et al. 2004).

[c] Relative abundances of $s_1$, $s_2$, and $s_3$ expressed as percentages of total absorbance. Values determined from SEDFIT’s Non-Interacting Discrete Species Model.

[d] Best fits were obtained by modeling different stoichiometric combinations of $s_1$, $s_2$, and $s_3$ and repeating the constrained analyses until fully random distributions of residuals and minimal r.m.s.d. errors were obtained.

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independent of the ligand-loaded state of the receptor. We therefore propose that the previously observed inefficiency of TonBΔ66-100 to transport iron-bound siderophores (Larsen et al. 1993; Skare et al. 1993) is due to an inability to form the 2:1 complex in vivo. Clearly, the proline-rich region is essential in assembly of an optimal TonB–OM receptor complex and does not simply function to extend the C-terminal region of TonB towards the OM (Larsen et al. 1993).

TonBΔ66-100 undergoes a rearrangement upon initial interactions with FhuA

Previous experiments by us (Khursigara et al. 2005) and others (Muller et al. 1998; Hancock et al. 2004; Svensson et al. 2004) demonstrated that time-lapse SPR analysis is a reliable method of determining whether biomolecular interactions are comprised of multiple steps. By varying injection times of analyte at fixed concentrations, the resulting dissociation profiles identify interactions that follow a simple 1:1 Langmuirian model as described by the equation A + B ⇌ AB, or that are more complex. For example, if interactions between FhuA and TonBΔ66-100 follow a simple Langmuirian model, the observed dissociation profile should not change with respect to the duration of FhuA injections. However, if the mechanism of interaction were comprised of sequential steps, the observed dissociation profiles may vary as a function of changes in duration of injection. Figure 1 presents normalized responses of FhuA–Fc (1000 nM) injected over TonBΔ66-100 (67 RUs); injection times of FhuA varied between 20 sec and 240 sec. Sensorgrams were labeled to indicate injection and dissociation phases and are aligned in order that dissociation of each injection began at 0 sec. We observed that the dissociation of each injection was comprised of distinct steps: a fast dissociation step and a slow dissociation step. As injection times increased, the apparent rate for the fast dissociation component varied (Fig. 1, blue lines). However, the slow dissociation component for all injection times was constant (Fig. 1, red lines); the rate did not change after 200 sec of dissociation. The variable dissociation profiles signify sequential steps for interactions between TonBΔ66-100 and FhuA, thus deviating from a simple Langmuirian 1:1 interaction model.

These observations are consistent with our previous analyses of two TonB variants, TonB (FL) and TonB (CT) (Khursigara et al. 2005). For TonB (FL), deviations from a simple model were attributed to an intramolecular rearrangement that occurs when monomeric TonB (FL) interacts with FhuA. Koedding et al. (2004) advanced their hypothesis that the C terminus of TonB folds back to make interactions with a yet unidentified portion of the same TonB molecule. They concluded that the dimeric C-terminal portion of TonB was a crystallographic anomaly, formed as a result of the absence of residues 1–153 of TonB. In the case of TonB (CT), we (Khursigara et al. 2005) attributed deviations from a simple model to the interaction of FhuA with a preformed dimer of TonB (CT). We proposed that FhuA initially interacts with one chain of the TonB (CT) dimer, and then rearranges to a more stable and higher-affinity complex in which the second TonB (CT) chain, within the dimer, also contacts FhuA. To determine whether similar mechanisms were involved in initial TonBΔ66-100–FhuA interactions, we performed kinetic assays as follows.

For kinetic SPR experiments, refinements commonly used to reduce or eliminate experimental artifacts were closely followed (Rich and Myszka 2000, 2001, 2002). These include use of low-loaded TonBΔ66-100 surfaces, increased flow rates, and control-correcting sensorgrams by employing the double referencing method (Rich and Myszka 2000). Interactions between thiol-coupled TonBΔ66-100 and FhuA (~FC + Fc) were examined using CM4 sensor chips. Concentrations of FhuA (~FC + Fc) from 0 nM to 3000 nM were injected (Fig. 2A,B). No differences were observed in the level of response between FhuA injections in the absence or presence of Fc. This result differs from our previous kinetic SPR analysis of initial TonB (FL) interactions with FhuA (Khursigara et al. 2005), in which TonB (FL) demonstrated ability to promote 2:1 TonB–FhuA interactions in the presence of Fc. Such observations are supported by our AUC analyses of TonBΔ66-100–FhuA interactions: Fc did not enhance formation of this complex (Table 1).
Given the intricate mode of interaction observed in the time-lapse experiments, TonBΔ66-100–FhuA interactions were initially analyzed using a simple kinetic model. Non-random residuals and standard deviations demonstrated poor fits (Fig. 2C,D) to a simple model. Because sources of artifact that might contribute to deviations from a simple model were addressed by our experimental design, the observed deviations are consistent with a more complex mechanism of interaction between TonBΔ66-100 and FhuA. Analyses of the data using the rearrangement model yielded excellent fits and standard deviations (Fig. 2E,F). We conclude that during its initial interaction with FhuA, TonBΔ66-100 behaves more like TonB (FL) than TonB (CT) (Khursigara et al. 2005). This conclusion is supported by the apparent dissociation constants (Table 2) for TonBΔ66-100–FhuA interactions (Kd app; 55.0 nM – Fc and 65.3 nM + Fc) which are similar to those reported for TonB (FL) (25.7 nM – Fc and 11.5 nM + Fc) (Khursigara et al. 2005). However, the presence of Fc did not enhance interactions between TonBΔ66-100 and FhuA as it did for TonB (FL). In the context of our previously proposed model (Khursigara et al. 2005), conformational changes within FhuA that are caused by Fc are required to recruit a second TonB. It is now evident from our kinetic SPR experiments that the absence of the proline-rich region negatively effects recruitment of a second TonB in an Fc-independent manner.

Attempts to assess interactions between TonBΔ66-100 and FhuAΔ21-128 by SPR used low amounts of thiol-coupled TonBΔ66-100 coupled to CM4 sensor chips. These experiments did not demonstrate any significant binding response (data not shown). To increase responses, higher amounts of TonBΔ66-100 were coupled: 425 RUs of FhuA that are caused by Fc are required to recruit a second TonB. It is now evident from our kinetic SPR experiments that the absence of the proline-rich region negatively effects recruitment of a second TonB in an Fc-independent manner.

Table 2. Apparent kinetic and thermodynamic constants of FhuA (−/+ Fc) interacting with TonBΔ66-100 fit to the rearrangement model

<table>
<thead>
<tr>
<th>Parameters</th>
<th>FhuA (−Fc)</th>
<th>FhuA (+Fc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_m (M⁻¹s⁻¹)</td>
<td>(31.9 ± 0.7) × 10³</td>
<td>(29.9 ± 0.8) × 10³</td>
</tr>
<tr>
<td>k_off app (s⁻¹)</td>
<td>(1.8 ± 0.2) × 10⁻³</td>
<td>(1.9 ± 0.3) × 10⁻³</td>
</tr>
<tr>
<td>K_app (nM)</td>
<td>550 ± 5</td>
<td>65.3 ± 7</td>
</tr>
</tbody>
</table>

Values correspond to average value ± the standard deviation of three independent experiments.
TonBΔ66-100, rather than 72 RU as in Figure 1. Concentrations of FhuAΔ21-128 (–/+ Fc) from 0 nM to 3000 nM were injected over this surface (Fig. 3). The amounts of FhuAΔ21-128 (~ Fc, gray curves; + Fc, black curves) that bound to the highly loaded TonBΔ66-100 surface were still markedly below the response detected for TonBΔ66-100–FhuA interactions (Fig. 1). Our interpretation is that deletion of residues 21–128 of the FhuA cork significantly reduced FhuA’s ability to interact with TonBΔ66-100. These observations are supported by AUC experiments (Table 1): The total percentage of TonBΔ66-100–FhuAΔ21-128 complex formed is significantly less (14% – Fc and 21% + Fc) than TonBΔ66-100–FhuA complexes (48% – Fc and 49% + Fc). Our previous AUC experiments (Khursigara et al. 2004, 2005) demonstrated that TonB (FL) and FhuAΔ21-128 could form significant amounts of complex, similar to that of TonBΔ66-100–FhuA, implying that the presence of both the proline-rich region of TonB and cork residues 21–128 of FhuA are individually necessary and jointly sufficient for formation of the high-affinity 2:1 TonB–FhuA complex.

Biological role of the proline-rich region in nutrient transport

The NMR-derived structure (Evans et al. 1986) of the proline-rich region of TonB supports the hypothesis that TonB extends from the CM to the OM, thereby delivering energy. An ~10-nm distance and a rigidity mediated by the X-Pro linkages provide the necessary length and potential conformation. The postulated function of the proline-rich region was tested by a series of in vivo experiments by Larsen et al. (1993), demonstrating that TonBΔ66-100 is capable of supporting most TonB-dependent processes, although at a reduced efficiency compared to wild-type TonB. Their study also confirmed that TonBΔ66-100 can interact with the OM receptor FepA. By our SPR experiments, we extend their observations, substantiate their conclusions, and now demonstrate that affinities of TonBΔ66-100–FhuA are similar to those of TonB (FL)–FhuA. However, our results suggest that TonB residues 66–100 mediate TonB–TonB interactions as opposed to TonB–FhuA interactions as previously reported (Brewer et al. 1990).

In light of TonB’s apparent shuttling between the CM and the OM (Larsen et al. 2003), what might be the significance of an extended proline-rich motif? Our previous studies (Khursigara et al. 2004, 2005) and present work implicate the proline-rich motif in modulating intermolecular interactions between two TonB proteins within the 2:1 TonB–FhuA complex. This modulation occurs only after an initial TonB–OM receptor interaction. Our hypothesis is also supported by in vivo studies (Sauter et al. 2003) where a bacterial two-hybrid system demonstrated that full-length TonB formed dimers. Although those authors suggested that this dimerization is not necessarily attributed to residues 33–164 of TonB, it is not evident from their study whether TonB interacts with OM receptors prior to dimerization, a step that is deemed critical by our in vitro studies.

In summary, our results demonstrate that the proline-rich motif of TonB is required to form 2:1 TonB–FhuA complexes, and that upon its initial interaction with FhuA, TonBΔ66-100 undergoes a rearrangement similar to TonB (FL). We also demonstrate that a significant portion of the FhuA cork domain is required to interact effectively with TonBΔ66-100. Many aspects of TonB-dependent transport systems remain elusive. To further elucidate the role of TonB in energy transduction between the CM and OM, specific sites of TonB–TonB and TonB–OM receptor interactions within the high-affinity complex need to be identified.

Materials and methods

Strains and cloning

A plasmid expressing TonBΔ66-100 was constructed from E. coli strain K1096 (provided by K. Postle, Washington State University). Briefly, tonB Δ66-100 was PCR amplified from K1096 genomic DNA and doubly digested with NdeI and XhoI. The resulting fragment encoding the internally deleted tonB gene was then ligated into pET28 vector previously digested with the same enzymes. This construct corresponded to TonBΔ66-100 with the addition of a 32-residue linker containing a hexahistidine tag at the N terminus. The plasmid was then transformed into E. coli host strain ER2566 for expression. Additional of a unique cysteine residue in the N terminus linker was accomplished using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, no. 200519) as described (Khursigara et al. 2005). FhuA and FhuAΔ21-128 were previously described (Carmel and Coulton 1991; Ferguson et al. 1998; Khursigara et al. 2005).

Protein expression and purification

FhuA and FhuAΔ21-128 were purified (Carmel and Coulton 1991; Ferguson et al. 1998; Khursigara et al. 2005) in N-lauryldimeth-
yalmine oxide (LDAO) (Fluka) and detergent-exchanged using Q-Sepharose anion exchange media (Amersham Pharmacia) into 100 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Tween 20 (Calbiochem); this solution is designated Biacore running buffer. TonBΔ66-100 was purified using Ni2+-NTA Superflow resin (QIAGEN) followed by cation exchange on SP-Sepharose (Amersham Pharmacia). Prior to SPR experiments, TonBΔ66-100 was diazylized into Biacore running buffer.

**Sedimentation velocity analytical ultracentrifugation and data analysis**

TonBΔ66-100 and FhuA or FhuAΔ21-128 were mixed in a 2:1 molar ratio, in the absence (−) or the presence (+) of ferriocin (Fc). Sedimentation velocity experiments were conducted with a Beckman XL-I Analytical Ultracentrifuge, with sample and reference sectors having a 1.2-cm path length. Sample and reference cells were filled with 400 μL of protein + AUC buffer (100 mM HEPES [pH 8.0], 150 mM NaCl, 0.4% n-octylbeta-D-thiogalactopyranoside [C8E4; Bachem]) or AUC buffer alone. All sedimentation velocity runs were performed at 40,000 rpm, with absorbance scans monitored at 280 nm in 10-min intervals over a total spin time of 4 h at 24.6°C.

Sedimentation velocity data were analyzed using the computer program SEDFIT (Version 8.5) (Schuck 2000). Absorbance data were fit to the Continuous c(S) Model, allowing for initial estimates of sedimentation coefficients. Buoyant molecular mass (Mb) values and refined s values for uncomplexed species (s1 = FhuA or FhuAΔ21-128; s2 = TonBΔ66-100) were obtained by fits to SEDFIT’s Non-Interacting Discrete Species model. Initial Mb values were estimated from the sequence-predicted molecular mass of individual protein species. Sedimentation parameters determined for uncomplexed species were input as constrained parameters (parentheses) for analysis of sedimentation velocity data of heterogeneous protein mixtures. Unconstrained parameters of TonB–FhuA complexes (s3, Mb,3) were refined using the nonlinear regression algorithms of SEDFIT.

**Surface plasmon resonance experiments and data analysis**

SPR measurements were performed using Biacore 2000 and 3000 optical biosensors. The data collection rate and temperature were set to 10 Hz and 25°C, respectively. TonBΔ66-100 was immobilized on CM4 sensor chips. Activation of chip surfaces was achieved with N-hydroxy succinimide/N-ethyl-N-(3-dimethylaminopropyl) carbodiimide-hydrochloride (NHS/EDC, 25 μL) and 2-(2-pyridylihydrothio) ethanolamine (PDEA) (dissolved in borate buffer pH 8.5, 30 μL). TonBΔ66-100 was diluted in 10 mM acetic buffer (pH 4.5) and was coupled to surfaces by manual injection at 5 μL/min (final concentration; 125 nM) until the desired amount of TonBΔ66-100 was immobilized. Deactivation of remaining activated groups was accomplished by injection of L-cysteine solution (35 μL) dissolved in 0.1 M formate buffer, pH 4.5. Control surfaces were prepared using the same procedure by replacing TonBΔ66-100 injections with Biacore running buffer. Before conducting experiments, TonBΔ66-100 surfaces were conditioned by buffer injections followed by two 50-μL pulses of 5 mM NaOH, 0.1% Tween 20, and an EXTRACLEAN procedure (flow rate set at 100 μL/min). Time-lapse experiments were conducted by injecting FhuA–Fc (1 μM) at 50 μL/min for 20, 30, 60, and 240 sec. The sensorgrams were control-corrected using the double referencing method (Myszka 1997) and normalized to an arbitrary value at the end of each injection phase. The beginning of the dissociation phase for each curve was aligned on the X-coordinate, X=0 sec. All kinetic experiments were carried out with the flow rate set to 100 μL/min on CM4 sensor chips (Biacore). Different concentrations of FhuA (in the absence of Fc or presence of 1 μM Fc) were injected for 60 sec over multiple TonBΔ66-100 surfaces; FhuA injections were randomly performed in triplicate. Analyte injections were followed by buffer injection for 180 sec. Between each FhuA injection, regeneration of sensor chip surfaces was achieved by two 50-μL pulses of 5 mM NaOH, 0.1% Tween 20, followed by an EXTRACLEAN procedure. Data were prepared using the double referencing method (Rich and Myszka 2000), also described by Khursigara et al. (2004). All curves were reduced to 700 evenly spaced sampling points. For each individual curve corresponding to various concentrations of injections FhuA over TonBΔ66-100 surface, kinetic analysis of the data set was carried out using different models (simple and rearrangement models) available in the SPR Evolution software package (De Crescenzo et al. 2000, 2001).

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