

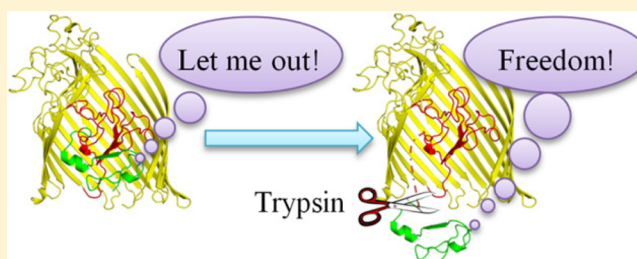
TonB-Dependent Transporter FhuA in Planar Lipid Bilayers: Partial Exit of Its Plug from the Barrel

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S Supporting Information

ABSTRACT: TonB-dependent transporters (TBDTs), which transport iron-chelating siderophores and vitamin B₁₂ across the outer membrane of Gram-negative bacteria, share a conserved architecture of a 22-stranded β -barrel with an amino-terminal plug domain occluding the barrel. We previously reported that we could induce TBDTs to reversibly open in planar lipid bilayers via the use of urea and that these channels were responsive to physiological concentrations of ligands. Here we report that in the presence of urea, trypsin can cleave the amino-terminal 67 residues of the plug of the TonB-dependent transporter FhuA, as assessed by gel shift and mass spectrometry assays. On the bilayer, trypsin treatment in the presence of urea resulted in the induced conductance no longer being reversed upon removal of urea, suggesting that urea opens intact FhuA channels by pulling the plug at least partly out of the barrel and that removal of the urea then allows reinsertion of the plug into the barrel. When expressed separately, the FhuA plug domain was found to be a mostly unfolded structure that was able to occlude isolated FhuA β -barrels inserted into the membrane. Thus, although folded in the barrel, the plug need not be folded upon exiting the barrel. The rate of insertion of the β -barrels into the membrane was tremendously increased in the presence of an osmotic gradient provided by either urea or glycerol. Negative staining electron microscopy showed that FhuA in a detergent solution formed vesicles, thus explaining why an osmotic gradient promoted the insertion of FhuA into membranes.



Gram-negative bacteria use special outer membrane receptors, called TonB-dependent transporters (TBDTs), to acquire vitamin B₁₂ and iron-bound siderophores. These TBDTs, found across many genera including *Escherichia*, *Yersinia*, and *Salmonella*, all possess a conserved architecture consisting of a 22-stranded antiparallel β -barrel with short periplasmic turns and long extracellular loops, along with a roughly 160-residue amino terminus, called the plug domain, which folds into and occludes this very large barrel (for a review, see ref 1).

Genetic experiments have shown that transport of the siderophore across the outer membrane via the TBDTs requires the presence of, and interaction with, the TonB protein of the inner membrane TonB complex (Figure 1, reviewed in ref 2). About five residues (called the TonB box) near the very beginning of the amino terminus of the TBDTs were found to be very well conserved and essential for TonB interaction and TBDT function. The site of interaction with TonB was localized to TonB's carboxy terminus, and the nature of the interaction between TonB and TBDTs was more clearly defined when cocrystal structures of the carboxy terminus of TonB with FhuA³ and BtuB⁴ were solved. These structures showed the carboxy terminus of TonB forming a β -sheet, with the TonB box of the TBDT extending out of the barrel to form another strand of the β -sheet, a so-called strand-exchange interaction.

Co-crystal structures of TBDTs and several known ligands have also been solved. For example, ferric hydroxamate uptake

protein A (FhuA), with its cognate siderophore, ferrichrome, was solved in 1998.⁵ It came as a surprise to many that there were not major conformational changes within the protein, specifically, within the plug domain of the protein, when the siderophore was bound. This left unanswered one of the key questions in the field: how does the siderophore move through an ostensibly occluded barrel to gain access to the periplasm of the cell?

The current hypothesis posits that, upon binding of the siderophore to the TBDT, the TonB box of the TBDT extends into the periplasm to interact with TonB and that this interaction, in an energy-dependent manner, allows the formation of a pathway for the siderophore to move through the lumen of the TBDT. This of course begs the question of what happens to the plug domain during siderophore translocation.

The Coulton⁶ and later the van der Helm⁷ laboratories, using engineered disulfide bonds to fix the plug to the barrel domain, or engineered intraplug disulfide linkages, concluded that the plug domain remains mostly within the barrel domain during siderophore translocation. However, based on accessibility of fluorescein maleimide to cysteines within the barrel domain of the TBDT FepA, the Klebba laboratory concluded that the plug

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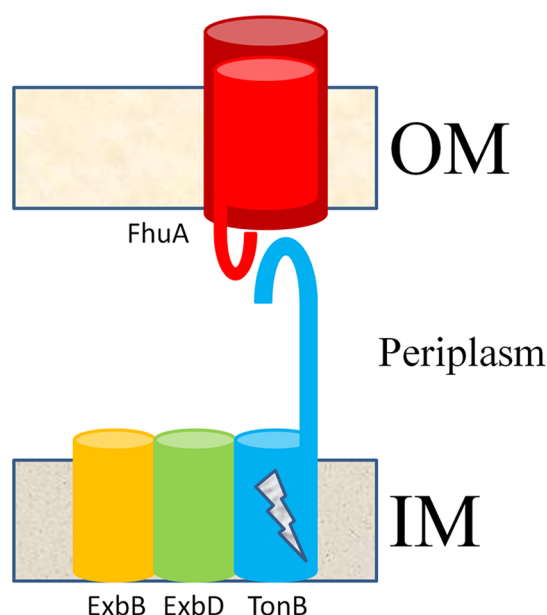


Figure 1. Cartoon of spatial arrangement of the siderophore outer membrane permeation machinery. Siderophore receptors (FhuA is depicted in the figure) are TBDTs that sit in the outer membrane (OM) of Gram-negative bacteria and possess a conserved beta-barrel (dark red) architecture. Upon siderophore binding to the receptor, the plug domain of the receptor (light red) extends its TonB box (red hook), located near the amino terminus of the polypeptide, into the periplasm to interact with the carboxy terminus (blue hook) of the TonB protein of the inner membrane (IM) TonB complex, consisting of ExbB, ExbD, and TonB. In an energy-dependent manner (indicated by a lightning bolt on TonB), TonB's interaction with the receptor's TonB-box somehow allows a pathway to form within the lumen of the receptor's barrel, through which the siderophore diffuses.

domain moves completely out of the barrel.⁸ The Postle laboratory,⁹ using accessibility of BMCC to cysteines on the plug and within the barrel, as well as proteinase K accessibility to the plug in the periplasm, concluded that at least 51 residues of the plug domain exited the barrel in the presence of colicin B, another ligand of FepA. Because of the patterns of the result, however, it was suggested that the plug may come completely out of the barrel but be protected from BMCC and proteinase K by another periplasmic protein.

To this extensive body of *in vivo* evidence, we add data from our *in vitro* studies, in the hope of shedding further light on the issue of the location of the plug domain during siderophore translocation. We find that trypsin can cleave ~40% of the plug domain of FhuA, but only if 4 M urea is present. The barrel domain forms constitutively open channels in planar lipid bilayers, and these open channels can, in fact, be occluded by separately purified plug domain. And finally, the plug domain is mostly unfolded when in solution.

MATERIALS AND METHODS

Protein Expression and Purification. *E. coli* strain AW740¹⁰ has deletions of the genes for the major outer membrane proteins OmpF and OmpC. A *fhuA* mutant derivative of AW740¹¹ bearing the pBR322-based plasmid pHX405, encoding FhuA with a hexahistidine tag inserted after codon 405 of FhuA,¹² was the kind gift of Dr. James Coulton. pHX405 was isolated from this strain and the Stratagene QuikChange Kit (Agilent Technologies, Santa Clara, CA) was

used to delete residues 6–160 of *fhuA* to give FhuA Δ6–160 (FhuA barrel). Strain KJ740 was created by curing the plasmid from the above-described AW740, by extended growth in 5 μg/mL coumermycin A1 (Promega, Fitchburg, WI), as described.¹³ KJ740 is thus Δ*ompF* Δ*ompC* *fhuA*31. DNA sequences encoding FhuA residues 1–160 were amplified from pHX405 by PCR (FhuA plug) and inserted into pET52-b (Novagen, Darmstadt, Germany), which creates an HRV 3C-cleavable Strep II tag at the amino terminus and a thrombin-cleavable His₁₀ tag at the carboxy terminus of the FhuA plug sequence. FhuA barrel was expressed and purified from KJ740 bearing the plasmid FhuA Δ6–160 essentially as described for whole FhuA.¹⁴ FhuA plug was expressed in BL21 (DE3) cells and purified on both Strep-Trap and His-Trap columns (GE Healthcare, Piscataway, NJ) as specified by the manufacturer.

Concerned about the contribution of the double tags of the plug to plug behavior, we decided to remove these tags to examine the behavior of a “tagless plug” on the system. To this end, we attempted to use thrombin and HRV 3C protease to cleave the C-terminal His₁₀ and N-terminal Strep II tags, respectively, from the plug. Gel shift assays suggested that HRV 3C cleaved the Strep II tag as expected, but exposure of the plug to thrombin resulted in degradation of the plug (data not shown). To overcome this problem, a new plug was engineered with a C-terminal TEV site replacing the thrombin cleavage site (and still retaining the N-terminal HRV 3C cleavage site) and was then purified as described above. Exposure of this plug to HRV 3C (Novagen, Darmstadt, Germany) and AcTEV (Invitrogen, Carlsbad, CA) in a buffer containing 100 mM NaCl, 1 mM DTT, and 20 mM sodium phosphate, pH 7.2, for 3 h at room temperature (~23 °C) yielded the correctly sized fragment as assessed by SDS-PAGE. This reaction was then incubated with Ni-NTA resin (Qiagen, Valencia, CA) to remove both the free His₁₀ and the endoproteases (both possess a poly histidine tag), and the supernatant from this incubation was used directly in bilayer experiments. This tagless plug construct exhibited similar behavior to that of the doubly tagged plug (data not shown).

Reagents. Stock solutions of whole FhuA (0.33 or 2.2 mg/mL in 50 mM ammonium acetate, pH 8.0, 250 mM NaCl, 70 mM imidazole, 0.1% lauryldimethylamine oxide [LDAO]) and FhuA barrel (1.6 mg/mL in 50 mM Tris-Cl, pH 8.0, 500 mM NaCl, 70 mM imidazole, 0.1% LDAO) were stable when kept at 4 °C for over 18 months; FhuA plug (0.3 mg/mL, 100 mM NaCl, 20 mM NaPO₄, pH 7.2) was stored at –80 °C, and the batch used for all experiments reported here was stable when kept at 4 °C for at least 6 months. However, several other batches prepared with identical protocols exhibited degradation with a half-time of several days if kept at 4 °C. Tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical Corp., Lakewood, NJ) was dissolved in 1 mM HCl and frozen at –20 °C until the day of use. For inhibited trypsin, powders of trypsin and soybean trypsin inhibitor (SBTI) (Worthington Biochemical Corp., Lakewood, NJ) were dissolved in 100 mM KCl, 1 mM EDTA, 5 mM CaCl₂, 20 mM HEPES, pH 7.2, at a ratio of 7.5:1 SBTI:trypsin by weight (according to the Worthington documentation, 1 g of SBTI inhibits 2.1 g of trypsin), allowed to incubate 5 min at room temperature, and then frozen at –20 °C until the day of use.

Planar Lipid Bilayer Experiments. Planar lipid bilayers were formed at room temperature by the brush technique¹⁵ across a 0.5 mm hole in a 125 μm thick Teflon partition.

Membranes separated two Lucite compartments, each containing 3 mL of 100 mM KCl, 1 mM EDTA, 5 mM CaCl₂, 20 mM HEPES, pH 7.2, which could be stirred by small magnetic stir bars. The membrane-forming solution was 3% diphtanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in *n*-decane. After the membrane formed, either whole FhuA (1.25 nM) or FhuA barrel (2–20 nM) was added to the cis compartment, which also contained 4 M urea. The final concentrations of FhuA plug, trypsin, and SBTI were 45 nM, 100 μ g/mL, and 750 μ g/mL, respectively, in those experiments in which they were added. Perfusions of each compartment were performed for 8 min via a Polystaltic Pump (Buchler Instruments, Kansas City, MO) at a flow rate of 2 mL/min. All experiments were done under voltage-clamp conditions; voltages are those of the cis solution (to which the whole FhuA or barrel was added) with respect to that of the trans solution, which was held at virtual ground. Current responses were filtered at 10 Hz and recorded concurrently on a Narco physiograph chart recorder (Houston, TX) and an NI6211 Data Acquisition Board (National Instruments, Austin, TX) and stored digitally. Each of the experiments reported here was performed at least three times with similar results.

Proton NMR. A proton 1D NMR spectrum of FhuA plug was obtained on a Varian 600 Instrument (Palo Alto, CA) at 25 °C. The analyzed solution was 0.3 mg/mL FhuA plug in 100 μ M 2,3,5-triisopropylbenzenesulfonyl chloride, 10% D₂O, 100 mM NaCl, 20 mM NaPO₄, pH 7.2.

Circular Dichroism. A circular dichroism spectrum consisting of three averaged scans was acquired from a Jasco J-815 instrument (Easton, MD) analyzing the FhuA plug solution (0.3 mg/mL in 100 mM NaCl, 20 mM NaPO₄, pH 7.2). The circular dichroism spectrum was fed into the K2D2 program¹⁶ for secondary structure estimation.

Trypsin Digest for SDS-PAGE. A reaction buffer of 0.05% LDAO, 100 mM KCl, 5 mM CaCl₂, 1 mM EDTA, 20 mM HEPES, pH 7.2 containing 100 μ g/mL whole FhuA (and, where indicated, 4 M urea), 100 μ g/mL trypsin, or preincubated trypsin (100 μ g/mL) with SBTI (750 μ g/mL) was allowed to incubate at room temperature (~23 °C) for 20 min. The reaction was then treated with reducing sample buffer, boiled for 1 min, and resolved on a 4–15% acrylamide gradient SDS gel, followed by visualization with Coomassie blue staining.

Trypsin Digest for Mass Spectrum Analysis. The sample for mass spectrum analysis was prepared as follows: a reaction buffer of 4 M urea, 100 mM KCl, 5 mM CaCl₂, 1 mM EDTA, 0.05% LDAO, 20 mM HEPES, pH 7.2 containing 800 μ g/mL whole FhuA and 400 μ g/mL trypsin was incubated at 37 °C for 120 min, and then at room temperature for an additional 60 min, before being quenched with 0.1% trifluoroacetic acid. The deconvoluted masses, along with enzyme identity and FhuA sequence (from PDB file 1QKC) were then entered into ExPASy's FindPept, ProtParam, and PeptideCutter programs to infer possible trypsin cleavage sites.

Electron Microscopy. Solutions of whole FhuA were diluted 10-fold in 100 mM KCl, 5 mM CaCl₂, 1 mM EDTA, 20 mM HEPES, pH 7.2, and used to perform negative staining.

RESULTS

With FhuA in planar lipid bilayers, we normally find a reversible conductance increase and decrease corresponding to introduction and removal of 4 M urea in the cis solution (a urea cycle).¹⁷ However, if the FhuA-containing membrane was

exposed to both 4 M urea and trypsin at the same time, we found not only an increase in conductance beyond that seen with 4 M urea alone but also little conductance loss upon removal of urea (Figure 2A). (It is known that even in 10 mM

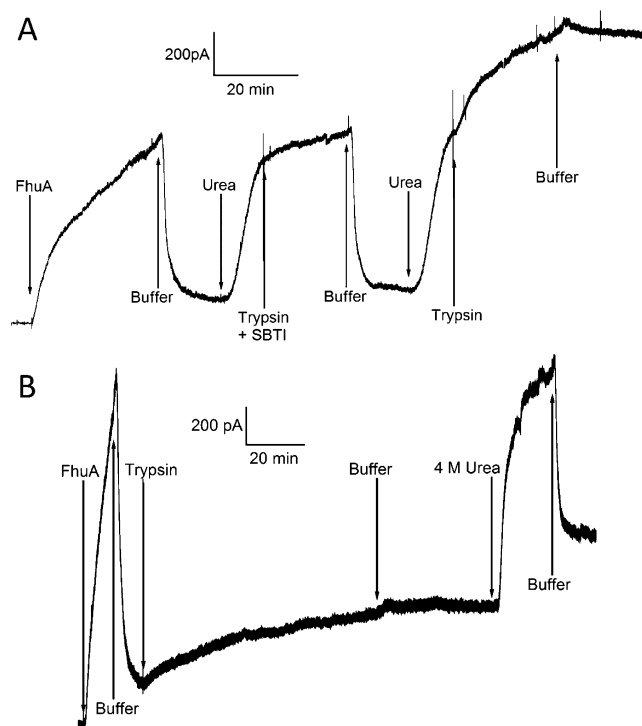


Figure 2. Trypsin in the presence of urea, but not in its absence, resulted in loss of reversibility of conductance. Both current traces begin with 4 M urea in the cis compartment and the voltage held at -20 mV throughout the experiment. (A) Addition of FhuA (1.25 nM) to the cis compartment and removal of urea via buffer perfusion (labeled "Buffer") resulted in the standard rise and fall, respectively, of conductance. Subsequent exposure of the membrane to cis trypsin (100 μ g/mL) with bound soybean trypsin inhibitor (750 μ g/mL) (labeled "Trypsin + SBTI") in the presence of urea did not alter the conductance reversal level when the urea (plus trypsin and SBTI) were perfused out. However, addition of cis trypsin (100 μ g/mL), without the trypsin inhibitor, in the presence of urea resulted in a conductance increase beyond that seen without trypsin, and more importantly, there was little fall in conductance upon buffer perfusion of the compartment. (B) Addition of FhuA (1.25 nM) to the cis compartment and removal of urea via buffer perfusion resulted in the standard rise and fall, respectively, of conductance. When trypsin was added to the cis compartment (in the absence of urea), there was a small, slow rise of conductance that terminated after the trypsin was removed via perfusion with buffer. More significant, however, was the fact that now when urea was cycled in and out of the cis compartment, the response was similar to that seen when no trypsin was added. Contrast the final buffer perfusion in (B) with that in (A).

CaCl₂ and 5 M urea, trypsin retains ~75% of its activity after 1 h at 37 °C.¹⁸) In contrast, a FhuA-containing membrane exposed to trypsin in the absence of urea subsequently displayed a urea cycle response comparable to that seen without trypsin exposure (Figure 2B) (The exposure to trypsin did produce a small conductance increase.) Thus, in the absence of urea, the sites of trypsin action were not readily accessible to trypsin.

In an effort to identify the sites of trypsin action, we incubated FhuA (in its LDAO-containing solution) with trypsin

in the presence and in the absence of urea (see Materials and Methods) and resolved the products on SDS-PAGE. The Coomassie-visualized proteins shown in Figure 3 reveal that in

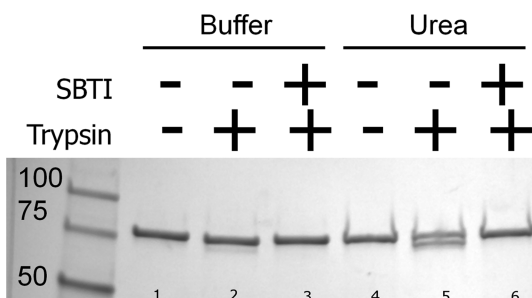


Figure 3. Trypsin treatment of whole FhuA in the presence of 4 M urea resulted in a faster-migrating band on SDS-PAGE (see Materials and Methods for details of the trypsin treatment). Whole FhuA protein migrates at ~ 80 kDa when boiled in either buffer or urea, as shown in lanes 1 and 4 (numbers at bottom of figure). In the presence of trypsin alone (lane 2), there may be a very faint band representing a faster migrating species, whereas trypsin and urea together (lane 5) converted about half of the 80 kDa band to the faster migrating species. A preincubation of trypsin with soybean trypsin inhibitor resulted in no faster migrating species (lanes 3 and 6).

the absence of urea, trypsin had a very slight effect on the FhuA band, whereas in the presence of urea, two protein bands appeared: one migrating at the normal position and another faster-migrating band, seemingly several kilodaltons less in size. We then performed the same reaction to completion (see Materials and Methods), quenched with trifluoroacetic acid, and did a mass spectrum analysis. Using the deconvoluted masses with tools from ExPASy's Web site (see Materials and Methods), we found several logical sites for trypsin action on FhuA. Entities corresponding to 1–31, 32–64, and 39–64 were found. The mass spectrometer also found entities of mass that corresponded to the carboxy terminus of the FhuA polypeptide after trypsin cleavage at sites R31, K64, and K67. Together, these results suggest a total of four trypsin cleavage sites (Figure 4), with K67 being the furthest from the amino terminus in the primary sequence.

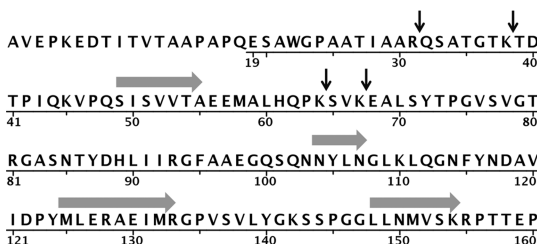


Figure 4. Results of mass spectrum analysis of whole FhuA incubated with trypsin in 4 M urea (see Materials and Methods). Three small amino-terminal peptides from whole FhuA were identified, corresponding to trypsin cleavage sites R31, K38, and K64. Carboxy-terminal polypeptides were also identified corresponding to trypsin cleavage sites at R31, K64, and K67. The black vertical arrows indicate the trypsin cleavage sites, while the gray horizontal arrows indicate the residues that form the beta strands composing the central β -sheet of the plug (PDB file 1QKC; residues 49–55, 104–107, 125–133, and 148–154). The first 18 residues of FhuA in this PDB structure were disordered, denoted by the lack of an underline for those residues.

Because it seems that in 4 M urea the plug of whole FhuA is somewhat exposed to solution, we decided to separately express the barrel and plug domains of FhuA and look for interactions on the bilayer. We found that if the FhuA barrel was exposed to the membrane in the presence of either 3 M glycerol or 4 M urea, we could induce robust channel conductance across the membrane (Figure 5). This is due to the formation of ~ 100 nm

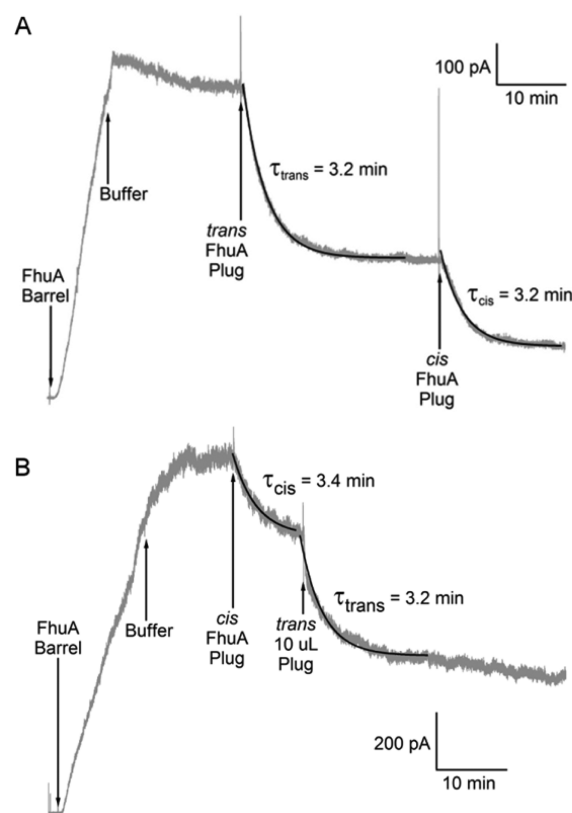


Figure 5. FhuA plug occluded FhuA barrel. Current traces begin with either 4 M urea (A) or 3 M glycerol (B) in the cis compartment, and the voltage was held at -20 mV throughout the experiment. Conductance was induced by the addition of FhuA barrel (9 nM) to the cis solution, and unlike whole FhuA, this conductance persisted even when urea was removed. (Also unlike whole FhuA, conductance was induced by 3 M glycerol.) Furthermore, the addition of FhuA plug (45 nM) to either side of the membrane resulted in some loss of conductance. (Addition of cis plug resulted in a 25% and 22% loss of conductance in panels A and B, respectively; addition of trans plug resulted in a 50% and 40% loss of conductance in panels A and B, respectively.)

vesicles in the FhuA–detergent solution (Figure 6). As has been shown,¹⁹ an osmotic gradient across the membrane greatly facilitates the fusion of vesicles to that membrane. It should be noted that the vesicles only formed in detergent solution with FhuA present and not in detergent alone.

In contrast to the results with whole FhuA, the conductance induced by the barrel was not reversed upon removing the urea. (Also unlike whole FhuA, significant conductance was induced in the presence of 3 M glycerol.) This was to be expected, if the other effect of urea (besides its effect on the fusion of the vesicles with the membrane) is disruption of the plug domain. The addition of the FhuA plug to either the cis or trans compartment resulted in a loss of conductance ranging from 55 to 90% of total conductance (Figure 5), with 15–30% conductance loss due to plug action from the cis compartment

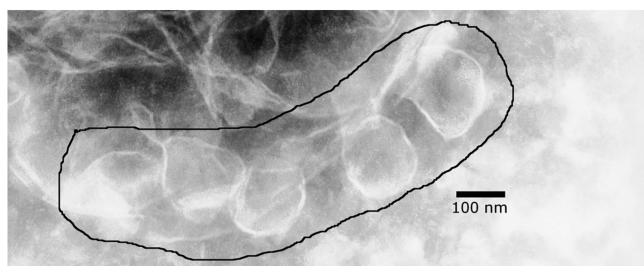


Figure 6. FhuA forms vesicles in detergent solution. Shown here is a negative stained electron microscopy image at 50 000 magnification of whole FhuA after it had been diluted 10-fold into the buffer used in the bilayer experiments. (This resulted in a 0.03 mg/mL FhuA protein concentration and a 0.01% LDAO detergent concentration.) The black line encloses several ~100 nm vesicles. No vesicles were observed in solutions with detergent alone (not shown).

and 40–60% loss due to plug action from the trans compartment. The conductance block by the plug followed exponential kinetics with a time constant from the cis and trans sides of 4.69 ± 1.05 min ($n = 8$) and 4.45 ± 1.45 min ($n = 13$), respectively. Unexpectedly, the subsequent addition of urea did not cause the channels to reopen (data not shown) (see Discussion).

Since the plug seems able to enter and occlude the barrel, we sought to determine the secondary structure characteristics of the plug in solution. The NMR spectrum (Figure 7) of the plug

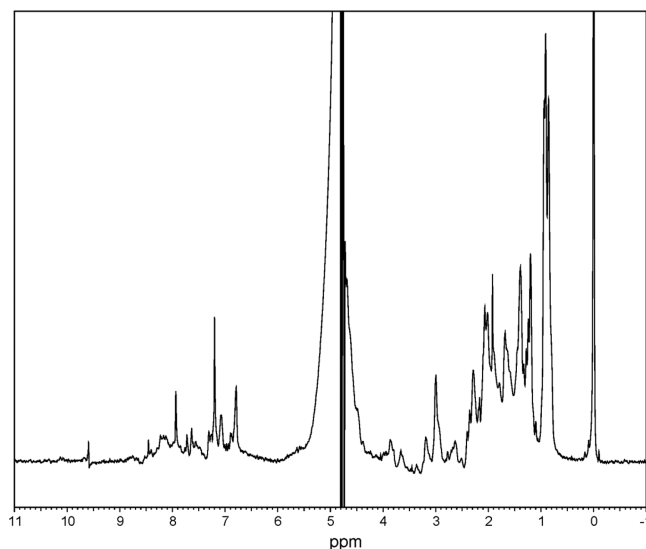


Figure 7. FhuA plug has no protein core. This NMR spectrum shows most of the peaks in the range of 0.5–3 and 7–8.5 ppm, suggesting that the methylene groups and amine groups of the protein, respectively, are not in a hydrophobic core.

showed no peaks below 0.5 ppm or above 8.5 ppm, suggesting there is no hydrophobic protein core, but not ruling out secondary structure completely. To check for secondary structure, we performed circular dichroism on the plug (Figure 8), and analysis of the spectrum found a mostly unfolded polypeptide with some β -sheet structure.

DISCUSSION

We have shown for FhuA reconstituted in planar lipid bilayers that trypsin can, in the presence of 4 M urea, prevent the loss of

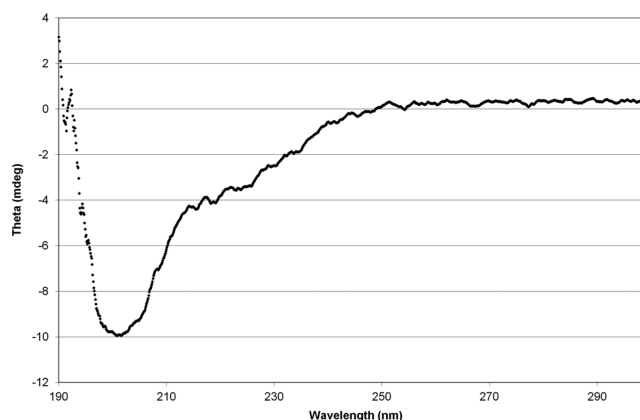


Figure 8. FhuA plug has little secondary structure. This CD spectrum shows no obvious minima at the canonical secondary structure locations, but analysis by the K2D2 program suggests a ~26% β -sheet structure and ~8% α -helix structure, with the rest being unstructured.

conductance normally induced by the removal of urea (Figure 2A). Combined with the gel shift assay (Figure 3), we interpret these data to mean that in the presence of urea, at least some part of the plug comes out of the barrel and is exposed to the action of trypsin. The fact that this response was much less in the absence of urea (Figure 2B) suggests that trypsin was acting on something that it has more access to in the presence of urea, i.e., the plug, and not the loops of the protein. Mass spectrum analysis indicates that the first 67 residues of FhuA are accessible to trypsin (Figure 4), in agreement with data previously obtained by the Coulton laboratory.¹¹ It should be noted, however, that while in that case complete conversion of FhuA to the faster-migrating species on SDS-PAGE occurred in the absence of urea, that conversion took 20 h of incubation at 37 °C. This is not inconsistent with our result of very little whole FhuA conversion (in the absence of urea) to a faster migrating band, since our exposure was only 20 min at room temperature. A mutant FhuA lacking residues 21–128 was also exposed to trypsin,²⁰ and K154 was found to be a trypsin-accessible site. However, we believe that the different results (trypsin action at K154 versus K67) observed are due to the differing constructs used in the trypsin digest experiments; hence, the data obtained with this FhuA Δ 21–128 are not necessarily comparable to our studies.

The fact, however, that trypsin action on whole FhuA was constrained to the initial 67 residues of the polypeptide, either in the presence (this work) or absence (this work and ref 11) of urea, suggests that the plug domain of FhuA may normally be transiently exposed to solution, and urea may be accelerating the rate of plug movement into the solution and/or decelerating re-entry of the plug domain back into the lumen of the barrel. In fact, molecular dynamics simulations of the action of TonB on BtuB²¹ indicated that unwinding of the plug domain of BtuB by TonB was “a relatively low-force event”. This unwinding included the “unzipping” of the first β -strand from the rest of the four-stranded β -sheet of the plug domain of BtuB. In FhuA, the first 67 residues of the plug domain also encompass this first β -strand of the β -sheet present in the plug domain (Figure 4), lending support to the notion that the action of urea on FhuA mimics to some extent the *in vivo* behavior of FhuA (that is, the action of TonB upon FhuA).

Since we were led to believe that the plug of whole FhuA came at least partly out of the barrel in the presence of urea and

then reinserted into the barrel with the removal of urea, we thought to separately express and purify the plug and barrel of FhuA and attempt to observe interactions on planar lipid bilayers. It should be noted that this has been previously observed *in vivo* for the proteins FhuA,²² FepA,⁸ and FpvA.²³ Likewise, we also observed interactions of the plug and barrel on the planar lipid bilayer. As seen in Figure 5, the addition of the plug to either the *cis* or the *trans* compartment was able to cause a loss of conductance, suggesting that (i) the plug is plugging the barrel of FhuA and (ii) like their full-length brethren, the FhuA barrels also insert bidirectionally into the membrane.¹⁷ Experiments with colicin M, however, suggest an alternative possibility. Colicin M, when added to the *trans*, but not *cis*, compartment after FhuA barrels had been inserted with a urea cycle, yielded an increase in conductance (Figure S2), suggesting that the barrel channels may all be oriented extracellular side *trans*, and two plugs may be occluding the same barrel from different sides of the membrane.

We could not unplug the barrels that had been plugged by the exogenous plug, using 4 M urea (not shown). This may be attributed to a non-native fold of the plug into the barrel of FhuA. It is also possible that the lack of a linker between the plug and barrel is what is responsible for this inability to pull the plug out of the barrel with 4 M urea, since the whole polypeptide (barrel plus plug) had been essentially reconstituted save for the one covalent linkage between the plug and barrel.

We were able to reproduce previously published data showing conductance induced across the membrane with FhuA barrel (and no osmotic gradient) in the presence of symmetric 1 M salt solutions,²⁴ a result also demonstrated for other FhuA mutants^{25–27} and even wild-type FhuA.²⁸ However, we obtained much smaller conductances than we obtained in 0.1 M KCl with an osmotic gradient (Figure S1). For this reason, we used 0.1 M KCl and an osmotic gradient in all our experiments with the FhuA barrel construct. The greater rate of barrel insertion into the membrane induced by 3 M glycerol (or 4 M urea) is explained by the negative staining images of the detergent solutions, showing the formation of vesicles in the presence of FhuA (Figure 6). As demonstrated by Niles et al.,¹⁹ an osmotic gradient across the membrane greatly facilitates fusion of vesicles into the membrane. The phenomenon of glycerol inducing robust conductance across the membrane in conjunction with the FhuA barrel, as shown in Figure 4B (glycerol with whole FhuA does not have this effect), suggests that the barrels are open channels and, once inserted into the membrane, allow ions to flow freely through them.

The Braun²⁴ and Movileanu²⁸ laboratories demonstrated frequent flickering of the conductance of the FhuA barrel channels in symmetric 1 M KCl solutions, a result we cannot directly confirm due to the lack of temporal resolution in our system. They also demonstrated that the deletions of both the plug and loop 4 converts FhuA into a large open pore, different from the pores formed by the single deletions of plug and loop 4. It is therefore possible that the extracellular loops and plug can each modify the other's behavior. Our results and their interpretation, however, are independent of possible changes in the local orientation and flexibility of the large extracellular loops that may accompany changes in plug conformation. The presence of channel currents across the membrane with wild-type FhuA observed by Mohammad et al.²⁸ is something that we nor others (e.g., refs 17, 24, and 29) have observed. However, given the amount of protein in the system, and our

hypothesis that the plug may transiently exit the barrel, it is not unreasonable that one may observe stochastically opened channels with the wild-type protein.

Isolated FepA plug was reported to be a mostly unstructured polypeptide,²⁰ and because we can get the FhuA plug to occlude the FhuA barrel, we examined the secondary structure characteristics of the isolated FhuA plug, to see if the plug entered the barrel as a folded or unfolded structure. A one-dimensional proton NMR spectrum suggested a lack of a hydrophobic core within the polypeptide but did not rule out isolated secondary structure without a tertiary fold (Figure 7). Further analysis by circular dichroism (Figure 8) revealed that ~25% of the polypeptide had assumed a β -strand configuration. It is possible that these β -strands are free β -strands that are supposed to form the four-stranded β -sheet of the plug domain observed in the many crystal structures of TonB-dependent transporters solved to date.

■ ASSOCIATED CONTENT

📄 Supporting Information

Description of our own experiences in using the barrel-only construct of FhuA in 1 M KCl and our results with colicin M. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ ABBREVIATIONS

EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDAO, lauryldimethylamine oxide; TBDT, TonB-dependent transporter; BMCC, 1-biotinamido-4-(4'-(maleimidoethylcyclohexane)carboxamido)-butane; SBTI, soybean trypsin inhibitor; HRV 3C, human rhino virus 3C protease; TEV, tobacco etch virus protease; DTT, dithiothreitol.

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