Biochimica et Biophysica Acta 1838 (2014) 364–371

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

FhuA interactions in a detergent-free nanodisc environment

Allan Mills [a,b], Hai-Tuong Le [a,b], James W. Coulton [c], Franck Duong [a,b,*]

[a] Department of Biochemistry & Molecular Biology, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada
[b] Life Sciences Institute, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada
[c] Department of Microbiology and Immunology, Duff Medical Building, McGill University, 3775 University Street, Montreal, QC H3A 2B4, Canada

ARTICLE INFO

Article history:
Received 12 June 2013
Received in revised form 27 September 2013
Accepted 30 September 2013
Available online 16 October 2013

Keywords:
Membrane
Transporters
β-Barrel proteins
Channels
Nanodiscs
Siderophore

ABSTRACT

TonB-dependent membrane receptors from bacteria have been analyzed in detergent-containing solution, an environment that may influence the role of ligand in inducing downstream interactions. We report reconstitution of FhuA into a membrane mimetic: nanodiscs. In contrast to previous results in detergent, we show that binding of TonB to FhuA in nanodiscs depends strongly on ferricrocin. The stoichiometry of interaction is 1:1 and the binding constant K_D is ~200 nM; an equilibrium affinity that is ten-fold lower than reported in detergent. FhuA in nanodiscs also forms a high-affinity binding site for colicin M (K_D ~3.5 nM), while ferricrocin renders FhuA refractory to colicin binding. Together, these results demonstrate the importance of the ligand in regulating receptor interactions and the advantages of nanodiscs to study β-barrel membrane proteins in a membrane-like environment.

1. Introduction

Some metals and vitamins are critical for bacterial cell growth but their availability in the environment is limited. Gram-negative bacteria have evolved a set of outer membrane receptors to efficiently capture and transport these nutrients. FhuA, a prototypical member for the family of TonB-dependent receptor/transporter, binds the siderophores ferricrocin and ferrichrome with nanomolar affinity [1]. FhuA is a 22-stranded β-barrel protein with surface-exposed loops and a large 160-residues N-terminal plug domain buried inside the barrel [2,3]. A conserved sequence at the N-terminus on FhuA, called the TonB box, forms the motif recognized by the inner membrane protein TonB. TonB associates with the proteins ExbB and ExbD to facilitate import of the ligand into the periplasm, in a manner that depends on the proton motive force (for review [4]). FhuA is also a receptor for the antimicrobial protein colicin M [5].

The crystal structure of FhuA, alone or complexed with the C-terminal domain of TonB, provided insights into the mechanism of transport [6]. In the current model, ferricrocin binds to FhuA to trigger the exposure of the TonB box to the periplasm. After recognition by TonB, the plug domain is pulled out of the β-barrel to allow the release of ferricrocin. Alternatively, the plug domain may undergo a conformational change to open a passage sufficient for the transport of the ligand into the periplasm (for review [7]). Certain aspects of the model, such as the mobility of the TonB box, have received genetic and biochemical support [8,9]. Other aspects still require analysis. For example, whether the ligand triggers the recruitment of TonB, or whether TonB is already bound to the transporter is still unclear. Previous analysis indicated that TonB interacts with FhuA with a high affinity (low nM range). However, this high-affinity interaction occurred in the presence or absence of ferricrocin [10]. The same modest effect of the ligand was reported for BtuB and FpvA. These transporters bind TonB with K_D value in the nM and mM range, respectively [11,12]. However, neither ligand vitamin B_{12} and pyoverdin significantly change the equilibrium binding affinity [12,13]. Thus, one would conclude that TonB binds constitutively to the transporters. Alternatively, the systematic use of detergent in these earlier analyses may account for the limited dependence on the ligand.

We report reconstitution of FhuA into nanodiscs, a membrane mimetic that has been successfully employed to analyze the interactions of the SecYEG translocase [14] and the interactions of $MalFGK_2$ permease [15]. Recently, nanodiscs were also developed to conduct structural and functional analyses on β-barrel proteins, including OmpX in *Escherichia coli* and VDAC-1 in mitochondria [16,17]. We show here that FhuA in nanodiscs binds TonB with a much lower affinity (~200 nM) than previously reported in detergent (~20 nM) [10]. Significantly, the binding critically depends on ferricrocin. FhuA in nanodiscs also forms a high-affinity binding site for colicin M and, as earlier observed in intact

Abbreviations: BN, blue-native; CN, clear-native; DDM, dodecyl-β-D-maltoside; LDAO, N-lauryldimethyl amine-N-oxide; Fc, ferricrocin; ITC, isothermal titration calorimetry; MSP, membrane scaffold protein

* Corresponding author at: Department of Biochemistry & Molecular Biology, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada. Tel.: +1 604 822 5975; fax: +1 604 822 5227.
E-mail address: FDuong@mail.ubc.ca (F. Duong).

0005-2736/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.09.022

cells [18], ferricrocin abrogates the binding of the colicin. Together, this quantitative information enhances understanding of the role of ligands in regulating FhuA transporter downstream interactions.

2. Materials and methods

2.1. Protein expression and purification

The DNA fragment encoding TonB (amino acids 32 to 239) was cloned into pET28. This replaced the N-terminal transmembrane domain of TonB with a His_6-tag sequence followed by a short linker and a thrombin cleavage site. A unique cysteine residue was introduced into the N-terminal linker region as previously described [10]. His_6-TonB (32–239) was purified from *E. coli* BL21 (DE3). Briefly, cells were grown in LB media until OD_{600} ~0.6 and induced for 3 h with 1 mM IPTG. Cells were resuspended in buffer A (50 mM Tris HCl (pH = 7.9), 100 mM NaCl, 2% glycerol) and lysed using a microfluidizer (Microfluidics International). The lysate was passed over a 1.5 ml column packed with Ni-NTA agarose and TonB was eluted with a gradient of buffer A containing 600 mM imidazole. TonB was further purified by cation exchange chromatography (HiTrap SP FF column; GE Healthcare) and gel filtration chromatography (Superdex 200 HR 10/30 column) in buffer A.

His_6-FhuA encoded by plasmid pHX405 was expressed in *E. coli* strain AW740 (Δ*ompF* Δ*ompC*) [19]. Cells were grown for 16 h at 37 °C in M9 media plus ampicillin (80 μg/ml). Cells were lysed using a microfluidizer and crude membranes were isolated by high speed centrifugation (200,000 ×*g* for 45 min). Crude membranes (3.0 mg/ml in buffer A) were solubilized with Triton-X-100 (1%, 60 min at room temperature) and centrifuged at 200,000 ×*g* for 45 min. Pellets were resuspended in buffer A (to 3.0 mg/ml) and solubilized overnight at 4 °C with 1% lauryldimethyl amine-N-oxide (LDAO). Solubilized proteins were passed through a 1.5 ml IMAC column equilibrated with buffer B (50 mM Tris HCl (pH = 7.9), 100 mM NaCl, 2% glycerol, and 0.1% LDAO). FhuA was eluted with buffer B plus 600 mM imidazole and was further purified by anion exchange chromatography (HiTrap Q FF column) in Tris–HCl 50 mM (pH = 7.9), 50 mM NaCl, 2% glycerol, 0.1% LDAO.

Colicin M was expressed from plasmid pMLD189 in the *E. coli* strain BW25113 (ΔFhuA::Cm^R; [20]). Briefly, cells were grown in LB broth to OD_{600} ~0.6 and induced for 2 h with 1 mM IPTG. The cell lysate was passed through a 1.5 ml IMAC column equilibrated with buffer A plus 0.1% β-mercaptoethanol. Colicin M was eluted from the column with a gradient of buffer A plus 600 mM imidazole.

2.2. Reconstitution of FhuA into nanodiscs

The parental plasmid used to construct the membrane scaffold protein MSP-L156 was obtained from the Sligar laboratory [21]. Cells were grown in LB plus kanamycin (25 μg/ml) to OD_{600} ~0.5 and induced with 1 mM IPTG for 3 h. The cell lysate was passed through an IMAC column equilibrated with buffer A and MSP-L156 was eluted with a gradient of buffer A plus 600 mM imidazole. For reconstitution, FhuA and MSP1-L156 were mixed at a molar ratio of 1:5 in buffer A plus 0.1% DDM. Detergent was removed with 1/4 volume BioBeads (Bio-Rad) and gentle shaking overnight at 4 °C. The Nd-FhuA particles were subjected to size exclusion chromatography on a Superdex 200 HR 10/30 column in buffer A.

2.3. Light scattering analysis

Protein samples were applied to a Superdex 200 HR 10/30 column connected in-line to a MiniDAWN TREOS multi-angle light scattering apparatus and an Optilab T-rEX differential refractive index apparatus (Wyatt Technologies). Flow rate was kept at 0.4 ml/min and data were collected using the ASTRA V software (Wyatt Technologies). The molecular mass was calculated using a Debye fit method. Weight averaged molar mass was determined using a dn/dc ratio of 0.185 ml/g.

2.4. Isothermal titration calorimetry

ITC experiments were conducted at 25 °C in a MicroCal ITC-200 apparatus (GE Healthcare). Samples were dialyzed in 50 mM Tris–HCl (pH = 7.9), 100 mM NaCl (2 mM β-mercaptoethanol for ColM) for 16 h using a ~14,000 Da cutoff dialysis membrane (SpectraPor). Where indicated, Nd-FhuA was incubated with a 10-fold molar excess of ferricrocin for one hour at 4 °C prior to dialysis. Proteins were concentrated using an ultracentrifugal concentrator with ~10 kDa cutoff (Millipore). The quality of the material was analyzed by clear-native PAGE before each ITC experiments. TonB (95 μM) was injected (40 injections; 1 μl each) into a cell containing Nd-FhuA or Nd-FhuA-Fc (12 μM). TonB (200 μM) was also injected into a cell containing the complex Nd-FhuA-ColM (16 μM). ColM (500 μM) was injected (20 injections; 1 μl each) into a cell containing Nd-FhuA (25 μM). Colicin M (125 μM) was injected into a cell containing Nd-FhuA-Fc (12 μM). The heat of dilution was obtained by injection of the ligand into a cell containing only buffer, or by injection of the ligand into a cell after saturation of the binding site. Stoichiometry of interaction (N), association constant (K_a; $K_d = 1/K_a$), and enthalpy changes (ΔH) were determined using a single-site binding model. Errors were derived from chi-squared degrees of freedom on Origin 7 software (MicroCal, Inc.;[22]).

2.5. Other methods

Desferrated ferrichrome was purchased from Sigma-Aldrich and incubated with ferric chloride to produce ferrichrome. Protein concentrations were determined using the Bradford assay. Blue-native (BN), clear-native (CN) and electrophoresis conditions were described in Dalal and Duong [23]. Native gels were 4–12% gradient gels and SDS-PAGE gels were 12%, unless otherwise indicated. Molecular weight markers were BSA (67/134 kDa) and ferritin (440 kDa).

3. Results

3.1. Reconstitution of FhuA into nanodiscs

The membrane scaffold protein (MSP) that supports the disc is a repeated amphipathic α-helix (~20 amino acids) interspaced with proline residues. The commonly employed MSP (MSP1E1) contains ten α-helices, producing discs with a diameter of ~10 nm [21]. Since FhuA is relatively small (4.6 × 3.9 nm cross-sectional diameter), we shortened MSP1E1 to seven α-helices (MSP1-L156), giving rise to discs with a calculated diameter of ~5.8 nm. His_6-tagged FhuA was purified in the detergent LDAO and incubated with MSP1-L156 at various ratios to identify the optimal reconstitution conditions (Fig. 1A). The nanodiscs containing FhuA (termed Nd-FhuA) were further purified by gel filtration chromatography to eliminate aggregates, excess of MSPs and traces of detergent (Fig. 1B and Fig. 1C). An analysis by CN and by BN gel electrophoresis showed that Nd-FhuA is stable in aqueous solution (Fig. 1D). The migration behavior indicated that one copy of FhuA is present in the disc; this stoichiometry was also confirmed by dynamic light scattering (see below). The BN-gel indicated that FhuA in detergent can form dimers and perhaps higher order oligomers (Fig. 1D, BN-PAGE, lane 2). In contrast, under the same condition, Nd-FhuA is entirely monodisperse (Fig. 1D, lane 1).

3.2. Siderophore enhances binding of TonB to FhuA

Binding of TonB to Nd-FhuA was analyzed by CN gel electrophoresis (Fig. 2A). Weak interactions can be detected this way because the method increases the protein concentrations during entry into the gel matrix. To perform these experiments without detergent, we employed

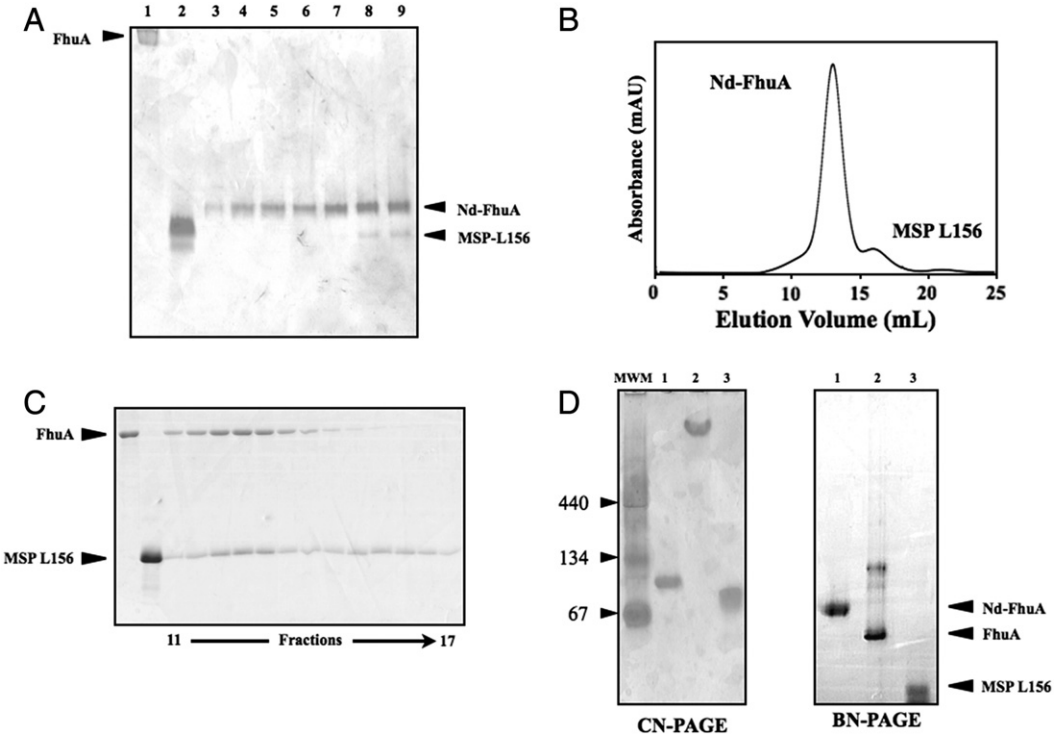

Fig. 1. Reconstitution of FhuA into nanodiscs. (A) FhuA in detergent LDAO (1 nmol) was reconstituted with increasing amounts of MSP-L156 (2 nmol to 8 nmol). Aliquots were analyzed by CN-PAGE and Coomassie staining of the gel. The ratio of 1:6 (*lane 7*) gives the highest yield of reconstituted into nanodiscs and the lowest amount of unincorporated MSP-L156. (B) Nanodisc-reconstituted FhuA (~800 μg) was injected onto a Superdex 200 HR 10/30 column equilibrated in buffer A (50 mM Tris–HCl, pH 7.9, 100 mM NaCl, 2% glycerol). (C) Fractions 11 to 17 were analyzed by 15% SDS-PAGE. For reference, purified FhuA and MSP-L156 are loaded on the same gel. (D) Clear-native and blue-native PAGE analysis of FhuA in nanodisc (*lane* 1), FhuA in LDAO (*lane* 2), and MSP-L156 (*lane* 3). The molecular weight markers are BSA (67/134 kDa) and ferritin (440 kDa).

a version of TonB missing its N-terminal transmembrane domain (called $TonB_{32–239}$). Although this TonB fragment is water-soluble, it did not enter the gel because of its high pI value (~9–10; Fig. 2A lane 2). In contrast, this fragment entered the gel when bound to Nd-FhuA (lane 3). Furthermore, formation of the complex was enhanced in the presence of the siderophores ferricrocin and ferrichrome (Fig. 2A; lane

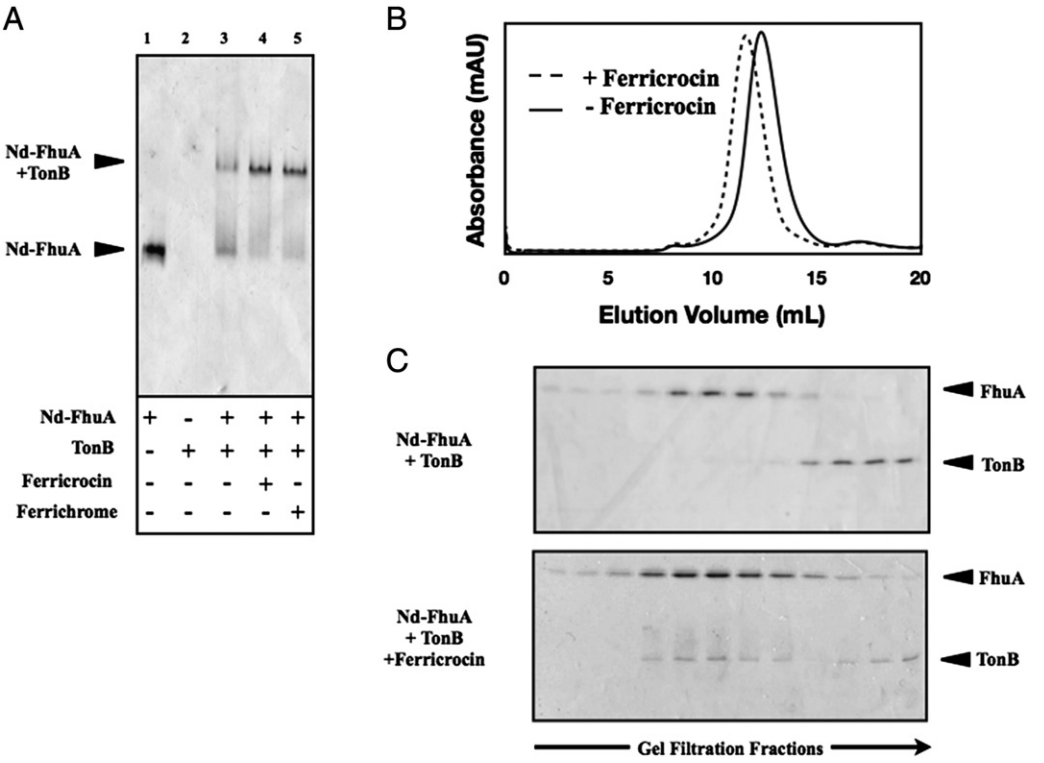

Fig. 2. Formation of a complex Nd-FhuA-$TonB_{32–239}$. (A) Nd-FhuA (5 μg) was incubated with TonB (2 μg) in the absence or presence of ferricrocin or ferrichrome. Proteins were analyzed by clear-native PAGE followed by Coomassie blue staining. (B) Nd-FhuA (300 μg) was mixed with TonB (65 μg), in the absence or presence of ferricrocin, before separation on a Superdex 200 HR 10/30 column. (C) The gel filtration fractions were analyzed by SDS-PAGE followed by Coomassie blue staining.

4 and lane 5). To determine whether the enhancing effect of the siderophore also occurs under different hydrodynamic conditions, TonB and Nd-FhuA were analyzed by size exclusion chromatography (Fig. 2B and C). Without ferricrocin, Nd-FhuA and TonB eluted in separate fractions, around ~12.0 ml and ~14.0 ml, respectively. In the presence of ferricrocin, Nd-FhuA and TonB eluted together in the same fractions (Fig. 2C). A similar result was reported by Moeck and Lettelier [24] who observed that co-elution of TonB and FhuA in detergent solution depends on ferricrocin.

3.3. Thermodynamics of the TonB-FhuA association

We employed isothermal titration calorimetry (ITC) to determine the binding affinity of TonB to Nd-FhuA, in addition to other thermodynamic parameters that characterize the interaction. The titration of Nd-FhuA with TonB was done in the presence of ferricrocin (Fig. 3A). The thermogram indicated an equilibrium binding affinity (K_D) around ~200 nM (±29 nM) and a binding stoichiometry (N) of ~0.98 (Table 1). The reaction was exothermic (free energy $\Delta G = -9.1$ kcal $\cdot mol^{-1}$) due to a highly favorable enthalpy ($\Delta H = -9.2$ kcal $\cdot mol^{-1}$) and a modest unfavorable entropy ($T\Delta S = -0.05$ kcal $\cdot mol^{-1}$). When the titration was repeated without siderophore (Fig. 3B), no heat was generated. Thus, in agreement with the gel filtration analysis (Fig. 2), the binding of TonB to FhuA is strongly enhanced by the cognate ligand of the receptor.

3.4. FhuA binds TonB in a 1:1 molar ratio

The ITC result above suggests that FhuA and TonB are interacting in 1:1 molar ratio. To confirm this, TonB was stabilized as a dimer after introducing a unique cysteine at the N-terminus of the protein [10]. Purification of this dimeric TonB was achieved by size exclusion chromatography without reducing agent (Fig. 4A, lane 2). This covalently bound TonB dimer could bind to Nd-FhuA (Fig. 4B, lane 4), but the resulting complex migrated at a higher position compared to the wild type (lane 2). After addition of DTT to disrupt the TonB dimer, the complex migrated to a position similar to the wild-type (lane 5). We also determined the molecular mass of the complexes using multi-angle light scattering (Fig. 4C). The mass of TonB was ~25 kDa (±5%) and that of Nd-FhuA was ~130 kDa (±0.4%), in good agreement with the prediction (25 kDa for TonB and 122 kDa for Nd-FhuA, respectively). When TonB and Nd-FhuA were incubated together with ferricrocin, the mass of the complex increased to ~160 kDa (±3%) (Fig. 4C). Together, these results show that Nd-FhuA and TonB interact in a 1:1 molar ratio under the conditions tested.

Table 1
Binding affinities and thermodynamics between Nd-FhuA, TonB and ColM.

Titrant	Cell	N	K_D (nM)	ΔH (cal/mol)	ΔS (cal/mol/deg)
TonB	Nd-FhuA apo	–	–	–	–
TonB	Nd-FhuA-Fc	0.977 ± 0.0100	200.4 ± 29.3	−9186 ± 133.4	−0.164
Colicin M	Nd-FhuA apo	0.906 ± 0.0016	3.48 ± 1.09	7969 ± 33.3	65.4
Colicin M	Nd-FhuA-Fc	–	–	–	–
TonB	Nd-FhuA-ColM	–	–	–	–

The estimated errors are based on a $\chi 2$ minimized fit of the experimental data to a single-site binding model using Origin 7.0 software (OriginLab).

3.5. Binding of colicin M to FhuA is inhibited by siderophore

The antimicrobial colicin M (ColM) binds the receptor FhuA in order to enter the periplasmic compartment of the cell [25,26]. Colicin M was purified and incubated with Nd-FhuA to evaluate the biochemical basis of the interaction. On CN gel, although ColM was not detected on its own, the complex between Nd-FhuA and ColM was apparent (Fig. 5A, lane 4). Significantly, formation of this complex was strongly inhibited by ferricrocin (lane 5). The thermodynamics of the interaction were determined by ITC (Fig. 5B and Table 1). In the absence of ferricrocin, the affinity of ColM to Nd-FhuA was high, with a K_D value of ~3.5 nM

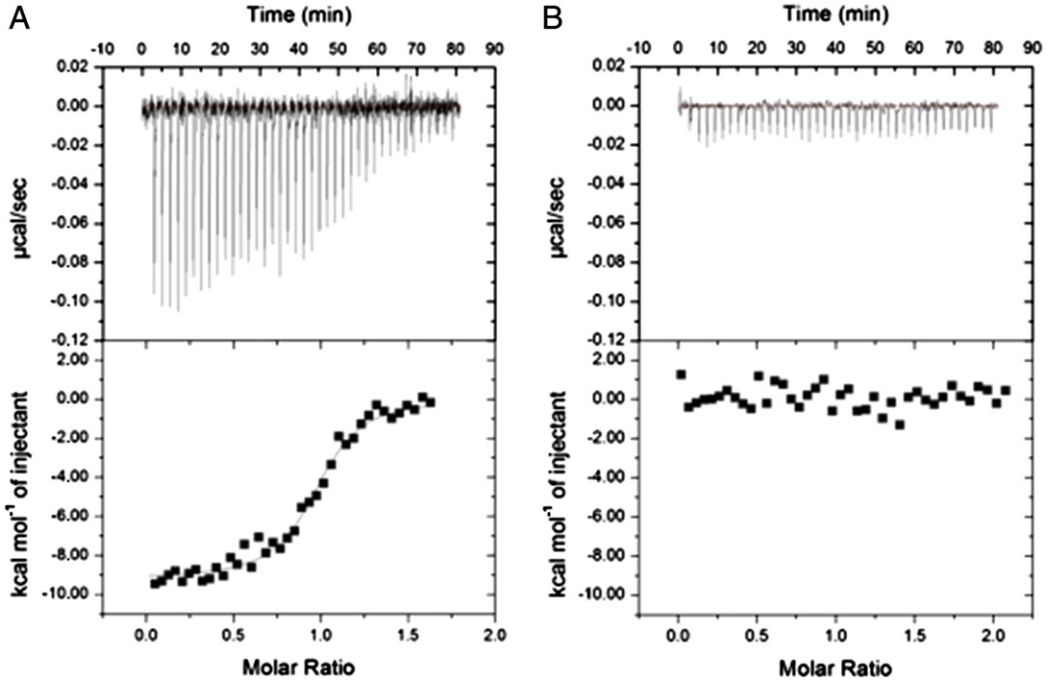

Fig. 3. Binding affinity between Nd-FhuA and $TonB_{32-239}$. The ITC thermograms show the interaction of Nd-FhuA with TonB in the presence (A) or absence of ferricrocin (B). Raw ITC traces and integrated heats of interactions are presented. Each titration was performed by injecting 1 µl of TonB (95 µM) into a cell contained Nd-FhuA (12 µM) or Nd-FhuA-Fc (12 µM), 39 times with 2 min between injections. Each titration was initiated with a single 0.5 µl injection. The thermodynamic parameters are reported in Table 1.

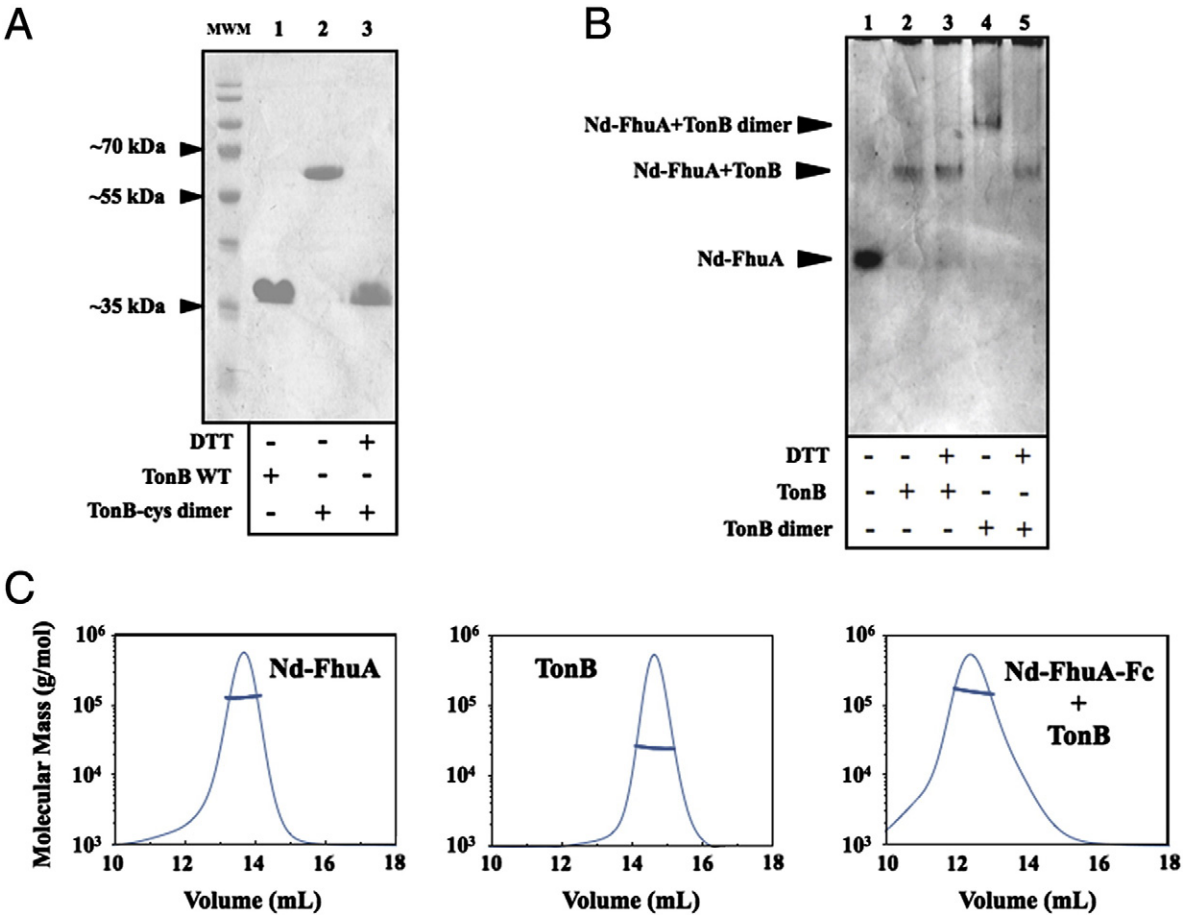

Fig. 4. Binding stoichiometry between Nd-FhuA and $TonB_{32-239}$. (A) TonB WT (6 μg) and disulfide-linked TonB dimer (6 μg) were incubated for 2 min at 37 °C in the absence (*lane 2*) and presence (*lane 3*) of DTT (1 mM). Samples were analyzed by SDS-PAGE and Coomassie blue staining. (B) Nd-FhuA (5 μg) was incubated with wild-type TonB or disulfide-linked TonB (4 μg each) in buffer A. The indicated samples were incubated with DTT (1 mM) for 2 min at 37 °C prior to analysis by clear-native PAGE. (C) Multi-angle light scattering (MALS) analysis of Nd-FhuA, TonB and Nd-FhuA-TonB in the presence of ferricrocin. Proteins (400 μg) were loaded on a Superdex 200 HR 10/30 column equilibrated in buffer A. The measured molecular masses are the following: Nd-FhuA ~130 kDa (±0.4%); TonB ~25 kDa (±5%); Nd-FhuA-Fc-TonB ~160 kDa (±3%).

(±1 nM). This interaction was strongly endothermic (free energy $\Delta G = -11.5$ kcal·mol^{-1}), due to an unfavorable enthalpy ($\Delta H = 7.9$ kcal·mol^{-1}) and a favorable entropy ($T\Delta S = 19.5$ kcal·mol^{-1}). When the titration was performed using Nd-FhuA with bound ferricrocin, binding of ColM was no longer detected, showing that ferricrocin renders FhuA refractory to the colicin (Fig. 5C). This later result is consistent with the native gel analysis above, and also with observation that siderophores protect cells against the killing action of colicin M [18]. The binding of siderophore may change the conformation of the FhuA external loops, so that ColM can no longer recognize them. The stoichiometry of interaction of ColM to FhuA was determined by light scattering (Fig. 5D). The determined molecular mass of ColM was ~27 kDa (±3%) and that of Nd-FhuA bound to ColM was ~162 kDa (±2%), showing a 1:1 stoichiometry of interaction.

3.6. Colicin M does not increase the binding of TonB to Nd-FhuA

Colicin M possesses at its N-terminus a sequence resembling a TonB-box. This TonB box-like sequence may be recognized by TonB to help translocation of the colicin across FhuA and the outer membrane [27]. Accordingly, mutations in the TonB box of FhuA diminish the transport of colicin M [8]. We therefore tested if the binding characteristics between FhuA and TonB are modified in the presence of colicin M. Nd-FhuA, ColM and TonB were incubated together and analyzed by CN-PAGE (Fig. 6A). A band corresponding to the ternary complex ColM-FhuA-TonB was readily detected (Fig. 6A, lane 4). This ternary complex was not formed in the presence of ferricrocin (lane 5), since ferricrocin abrogates the binding of ColM to FhuA (Fig. 5A). We then determined the stability of the ternary complex by size exclusion chromatography (Fig. 6B). Nd-FhuA and ColM eluted together as a stable complex, but TonB eluted alone in the later fractions. We also employed ITC to detect the heat variations during the interaction (Fig. 6C). The complex Nd-FhuA-ColM was purified by gel filtration before incubation with increasing amounts of TonB. In these conditions, the thermogram did not reveal any significant change in heat production (Fig. 6C). Thus, in the conditions of our experimental setup, colicin M does not induce an interaction of TonB with FhuA.

4. Discussion

In the current model of transport, FhuA exposes its TonB box to the periplasm when the siderophore binds the receptor. The exposed TonB box is then captured by TonB to trigger opening of the channel [7]. This signaling mechanism is thought to be important to the cell because the number of TonB copies is less than the total number of receptors [28]. It is therefore predicted that TonB interacts transiently and only when the transporter is bound with the ligand. Yet, the current experimental data is not entirely consistent with this model. Surface plasmon resonance experiments showed that FhuA binds TonB with a high affinity (K_D ~ 25 nM), but in a manner quasi-independent from

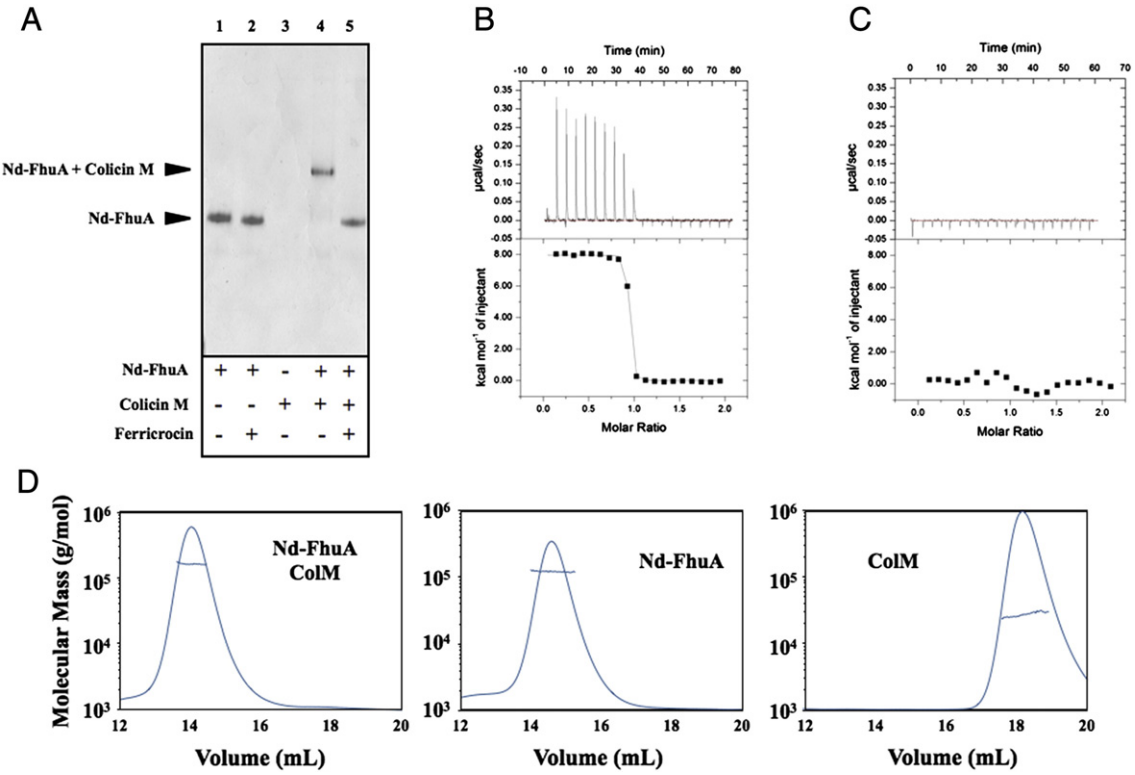

Fig. 5. Ferricrocin prevents binding of colicin M to Nd-FhuA. (A) Nd-FhuA (5 μg) was incubated with ColM (5 μg) with or without ferricrocin. The resulting complexes were separated on clear-native PAGE and visualized by Coomassie blue staining. (B, C) ColM (500 μM) was injected (20 injections; 1 μl each) into a cell containing apo-FhuA or ligand-loaded FhuA (25 μM). Raw ITC traces and integrated heat of interactions are presented. The thermodynamic parameters are reported in Table 1. (D) MALS analysis of Nd-FhuA, Colicin M, and Nd-FhuA-ColM. Proteins (~300 μg) were loaded on a Superdex 200 HR 10/30 column equilibrated in buffer A. The measured molecular masses are the following: Nd-FhuA ~123 kDa (±6%); Colicin M ~27 kDa (±3%); Nd-FhuA-ColM ~162 kDa (±2%).

ferricrocin [10]. Vitamin B_{12} had also a limited effect on the high-affinity interaction of BtuB with TonB [13]. In the case of FpvA, TonB was bound to the receptor with a modest affinity (μM range), but an enhancing effect of pyoverdine was still not seen [12]. Remarkably, these earlier biochemical studies were all performed in detergents, which perhaps modify protein interacting surfaces. For example, FptA does not bind

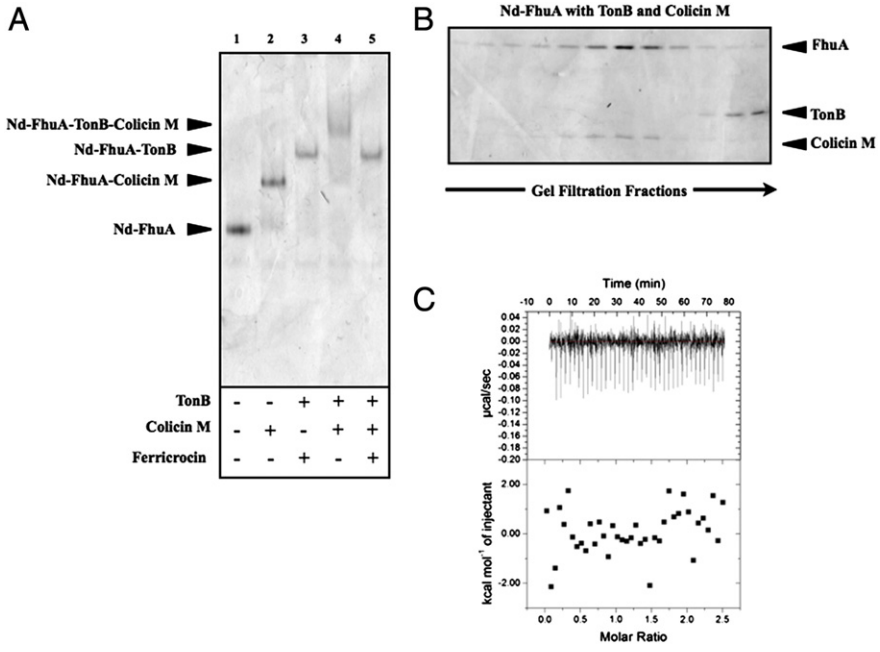

Fig. 6. Colicin M does not trigger the binding of TonB to Nd-FhuA. (A) Nd-FhuA (6 μg), TonB (4 μg), and colicin M (4 μg) were incubated together in buffer A. The mixture was analyzed by clear-native PAGE. Where indicated, Nd-FhuA was pre-incubated with ferricrocin. The gel was stained with Coomassie blue. (B) The mixture in (A) was applied on a Superdex 200 HR 10/30 column equilibrated in buffer A. The gel filtration fractions were analyzed by SDS-PAGE. (C) TonB (200 μM) was injected into a cell containing the complex Nd-FhuA-ColM (16 μM) isolated by gel filtration on Superdex 200 HR 10/30 column. The raw ITC traces and integrated heats of the interaction are presented. The thermodynamic parameters are reported in Table 1.

TonB until the detergent octyl-POE is replaced by N-decyl-β-D-maltoside [29]. It was also reported that the structure of TonB box of BtuB is affected by detergents [30].

An increasing number of studies show that nanodiscs provide an advantageous environment to study membrane proteins, including β-barrels ones [16,17]. Here, we shortened the scaffold protein (MSP-L156) to adapt the diameter of the disc to the diameter of FhuA. With these particles, the effect of the ligand on the TonB-FhuA interaction was evident: no high-affinity interaction was detected until ferricrocin is bound to the receptor. Once liganded, the equilibrium binding affinity TonB-FhuA was ~200 nM and the complex was readily isolated by gel filtration (Fig. 2C). These results do not exclude a low affinity association in the absence of ferricrocin, as we detect on native gel (Fig. 2A), but the binding affinity of TonB to FhuA in aqueous solution is obviously much weaker (~10-fold) than in detergent solution (200 nM versus 20 nM; 10,13). This weak affinity seems more consistent with the notion that TonB binds FhuA in a transient manner during ligand transport [28]. Detergents therefore seem to increase the exposure to the TonB box, which explain the observed limited effect of the ligand on TonB-FhuA interaction.

Based on analytical ultracentrifugation data, it was proposed that FhuA binds two TonB [31]. More recently, it was observed that TonB dissociates into monomers upon binding to BtuB [13]. In both cases, the possible effect of the detergent on the oligomeric state of the transporter and the TonB dimer equilibrium is not known. From our native gels and light scattering analysis, we report that FhuA and TonB interact in 1:1 molar ratio, unless TonB is stabilized as a dimer. Significantly, an increase in enthalpy was observed during the interaction. This phenomenon was also reported for the receptor HasR binding to HasB, a protein homologous to TonB [32]. Since the TonB box of FhuA becomes more structured after binding TonB [6,11], it was proposed that an increased entropy accounts for the increased enthalpy [33].

The FhuA monomer in nanodisc is also a receptor for colicin M, and this interaction is abrogated by ferricrocin. The binding constant is very low, around 3.5 nM. A similar high-affinity association was reported for a fragment of colicin E3 with BtuB [34] and for colicin Ia with Cir [35]. Strong affinity perhaps explains the endothermic character of the interaction and the increased entropy. This thermodynamic behavior usually indicates displacement of water molecules from hydrophobic surfaces [36]. In this context, it is noteworthy that helix α1 of colicin M is rather apolar; deletion of this helix reduces the ability of the colicin to interact with FhuA [37], and the mouth of FhuA where ferricrocin binds is a region rich in aromatic amino acids [3]. Such an overlapping of the ColM and ferricrocin binding sites is consistent with the observation that ferrichrome protects the cell from the killing action of the bacteriocin [18]. Finally, we tested whether the TonB-like box of colicin M becomes exposed to TonB after the colicin binds to FhuA. This mechanism explains why transport of colicin M depends on TonB [27]. We could detect the ternary complex ColM, FhuA and TonB in our native gel (Fig. 6A). However, the gel filtration chromatography and ITC experiments showed that the ternary complex did not gain stability, showing that colicin M does not trigger the exposure of the TonB box at least as much as ferricrocin. Nevertheless, the possibility to reproduce the initial transport steps in the nanodisc environment, such as binding of ferricrocin and recruitment of TonB, or binding of colicin M and displacement by ferricrocin, facilitates further analysis of this important class of outer membrane transporters.

Acknowledgments

We thank Dominique Mengin-Lecreulx for providing plasmid-encoding for colicin M and Filip van Petegem and Kelvin Lau for helping with the ITC experiments. We also acknowledge Jean-François Montariol for the nanodisc preparation. This research was supported by a grant from Natural Sciences and Engineering Research Council (NSERC) of Canada to FD. AM is recipient of a Fellowship from NSERC's CREATE program: *Cellular Dynamics of Macromolecular Complexes*.

References

[1] K.P. Locher, J.P. Rosenbusch, Oligomeric states and siderophore binding of the ligand-gated FhuA protein that forms channels across Escherichia coli outer membranes, Eur. J. Biochem. 247 (1997) 770–775.
[2] A.D. Ferguson, E. Hofmann, J.W. Coulton, K. Diederichs, W. Welte, Siderophore-mediation iron transport: crystal structure of FhuA with bound lipopolysaccharides, Science 282 (1998) 2215–2220.
[3] K.P. Locher, B. Rees, R. Koebnik, A. Mitschler, L. Moulinier, J.P. Rosenbusch, D. Moras, Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and ferricrhome bound states reveal allosteric changes, Cell 95 (1998) 771–778.
[4] K.D. Krewulak, H.J. Vogel, TonB or not TonB: is that the question? Biochem. Cell Biol. 89 (2011) 87–97.
[5] V. Braun, K. Schaller, H. Wolf, A common receptor protein for phage T5 and colicin M in the outer membrane of Escherichia coli B, Biochim. Biophys. Acta 323 (1973) 87–97.
[6] P.D. Pawelek, N. Croteau, C. Ng-Thow-Hing, C.M. Khursigara, N. Moiseeva, M. Allaire, J.W. Coulton, Structure of TonB in complex with FhuA, *E. coli* outer membrane receptor, Science 312 (2006) 1399–1402.
[7] N. Noinaj, M. Guillier, T.J. Barnard, S.K. Buchanan, TonB-dependent transporters: regulation, structure, and function, Annu. Rev. Microbiol. 64 (2010) 43–60.
[8] H. Schöffler, V. Braun, Transport across the outer membrane of *Escherichia coli* K12 via the FhuA receptor is regulated by the TonB protein of the cytoplasmic membrane, Mol. Gen. Genet. 217 (1989) 378–383.
[9] F. Endriß, M. Braun, H. Killmann, V. Braun, Mutant analysis of the *Escherichia coli* FhuA protein reveals sites of FhuA activity, J. Bacteriol. 185 (2003) 4683–4692.
[10] C.M. Khursigara, G. De Crescenzo, P.D. Pawelek, J.W. Coulton, Kinetic analyses reveal multiple steps in forming TonB-FhuA complexes from *Escherichia coli*, Biochemistry 44 (2005) 3441–3453.
[11] D.D. Shultis, M.D. Purdy, C.N. Banchs, M.C. Wiener, Outer membrane active transport: structure of the BtuB:TonB complex, Science 312 (2006) 1396–1399.
[12] H. Adams, G. Zeder-Lutz, I. Schalk, F. Pattus, H. Celia, Interaction of TonB with the outer membrane receptor FpvA of *Pseudomonas aeruginosa*, J. Bacteriol. 188 (2006) 5752–5761.
[13] D.M. Freed, S.M. Lukasik, A. Sikora, A. Mokdad, D.S. Cafiso, Monomeric TonB and the TonB box are required for the formation of a high-affinity Transporter-TonB complex, Biochemistry 52 (2013) 2638–2648.
[14] M. Alami, K. Dalal, B. Lelj-Garolla, S.G. Sligar, F. Duong, Nanodiscs unravel the interaction between the SecYEG channel and its cytosolic partner SecA, EMBO J. 26 (2007) 1995–2004.
[15] H. Bao, F. Duong, Discovery of an auto-regulation mechanism for the maltose ABC transporter $MalFGK_2$, PLoS One 7 (2012) e34836.
[16] F. Hagn, M. Etzkorn, T. Raschle, G. Wagner, Optimized phospholipid bilayer nanodiscs facilitate high-resolution structure determination of membrane proteins, J. Am. Chem. Soc. 135 (2012) 1919–1925.
[17] T. Raschle, S. Hiller, T.-Y. Yu, A.J. Rice, T. Walz, G. Wagner, Structural and functional characterization of the integral membrane protein VDAC-1 in lipid bilayer nanodiscs, J. Am. Chem. Soc. 131 (2009) 17777–17779.
[18] R. Wayne, K. Frick, J.B. Neilands, Siderophore protection against colicins M, B, V, and Ia in *Escherichia coli*, J. Bacteriol. 126 (1) (1976) 7–12.
[19] G.S. Moeck, P. Tawa, H. Xiang, A.A. Ismail, J.L. Turnbull, J.W. Coulton, Ligand-induced conformational change in the ferrichrome-iron receptor of *Escherichia coli* K-12, Mol. Microbiol. 22 (1996) 459–471.
[20] M.E. Ghachi, A. Bouhss, H. Barreteau, T. Touzé, G. Auger, D. Blanot, D. Mengin-Lecreulx, Colicin M exerts its bacteriolytic effect via enzymatic degradation of undecaprenyl phosphate-linked peptidoglycan precursors, J. Biol. Chem. 281 (2006) 22761–22772.
[21] I.G. Denisov, Y.V. Grinkova, A.A. Lazarides, S.G. Sligar, Directed self-assembly of monodisperse phospholipid bilayer nanodiscs with controlled size, J. Am. Chem. Soc. 126 (2004) 3477–3487.
[22] M.W. Freyer, E.A. Lewis, Isothermal titration calorimetry: experimental design, data analysis, and probing macromolecule/ligand binding and kinetic interactions, in: J.J. Correia, H.W. Detrich III (Eds.), Methods in Cell Biology, vol. 84, Academic Press, San Diego, CA, 2008.
[23] K. Dalal, F. Duong, Reconstitution of the SecY translocon in nanodiscs, Methods Mol. Biol. 619 (2010) 145–156.
[24] G.S. Moeck, L. Letellier, Characterization of in vitro interactions between a truncated TonB protein from *Escherichia coli* and the outer membrane receptors FhuA and FepA, J. Bacteriol. 183 (9) (2001) 2755–2764.
[25] H. Killmann, G. Videnov, G. Jung, H. Schwarz, V. Braun, Identification of receptor binding sites by competitive peptide mapping: phages T1, T5, and Φ80 and colicin M bind to the gating loop of FhuA, J. Bacteriol. 177 (3) (1995) 694–698.
[26] V. Braun, S. Helbig, S.I. Patzer, Import of periplasmic bacteriocins targeting the murein, Biochem. Soc. Trans. 40 (2012) 1449–1455.
[27] E. Cascales, S.K. Buchanan, D. Duche, C. Kleanthous, R. Lloubes, K. Postle, M. Riley, S. Slatin, D. Cavard, Colicin biology, Microbiol. Mol. Biol. Rev. 71 (2007) 158–229.
[28] R.J. Kadner, K.J. Heller, Mutual inhibition of cobalamin and siderophore uptake systems suggests their competition for TonB function, J. Bacteriol. 177 (1995) 4829–4835.

[29] S. Choul-Li, H. Adams, F. Pattus, H. Celia, Visualization of interactions between siderophore transporters and the energizing protein TonB by native PAGE, Electrophoresis 29 (2008) 1333–1338.
[30] G.E. Fanucci, J.Y. Lee, D.S. Cafiso, Membrane mimetic environments alter the conformation of the outer membrane protein BtuB, J. Am. Chem. Soc. 125 (2003) 12933–13932.
[31] C.M. Khursigara, G. De Crescenzo, P.D. Pawelek, J.W. Coulton, Enhanced binding of TonB to a ligand-loaded outer membrane receptor: role of the oligomeric state of TonB in formation of a functional FhuA–TonB complex, J. Biol. Chem. 279 (2004) 7405–7412.
[32] J. Lefèvre, P. Delepelaire, M. Delepierre, N. Izadi-Pruneyre, Modulation by substrates of the interaction between the HasR outer membrane receptor and its specific TonB-like protein, HasB, J. Mol. Biol. 378 (2008) 840–851.
[33] K. Brillet, L. Journet, H. Célia, L. Paulus, A. Stahl, F. Pattus, D. Cobessi, A β-strand lock exchange for signal transduction in TonB-dependent transducers on the basis of a common structural motif, Structure 15 (2007) 1383–1391.
[34] G. Kurisu, S.D. Zakharov, M.V. Zhalnina, S. Bano, V.Y. Eroukova, T.I. Rokitskaya, Y.N. Antonenko, M.C. Wiener, W.A. Cramer, The structure of BtuB with bound colicin E3 R-domain implies a translocon, Nat. Struct. Biol. 10 (2003) 948–954.
[35] J. Konisky, B.S. Cowell, Interaction of colicin Ia with bacterial cells. Direct measurement of Ia–receptor interaction, J. Biol. Chem. 247 (1972) 6524–6529.
[36] P.D. Ross, S. Subramanian, Thermodynamics of protein association interactions: forces contributing to stability, Biochemistry 20 (1981) 3096–3102.
[37] S. Helbig, V. Braun, Mapping functional domains of colicin M, J. Bacteriol. 193 (2011) 815–821.