ASSEMBLY OF EXBD TRANSMEMBRANE DOMAINS IN THE TONB COMPLEX

A Thesis in
Biochemistry, Microbiology, and Molecular Biology

by
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ABSTRACT

In Gram-negative bacteria, the TonB system is involved in transducing the proton motive force (pmf) of the cytoplasmic membrane to catalyze active transport by high-affinity outer membrane transporters. As essential components of the TonB system, ExbD/ExbB harness the pmf and transmit it to TonB. Subsequently, TonB interacts with outer membrane transporters to provide energy for active transport. However, the detailed function and organization of the ExbD transmembrane domain (TMD) in this system are still unknown.

To analyze the organization of the ExbD TMD, a cysteine scanning study was initiated. Putative ExbD TMD residues (F23 to V43) were systematically replaced with cysteine. Phenotypic analyses showed that all of the ExbD TMD residues cysteine substitutions were sensitive to colicins except D25C, which is consistent with previous research that the conserved aspartate residue was the only functionally important side chain in the ExbD TMD. Disulfide cross-linking analysis showed that six substitutions, ExbD F23C, I24C, L40C, A41C, T42C and V43C, formed homodimers. Especially for the last four mutants, dimer formation was much more prominent, suggesting that these residues were located at the ExbD-ExbD contacting interface, and they were anchoring close enough to interact. Additionally, disulfide bonds formed between ExbD T42C or V43C blocked iron-transport activity of the TonB system, and this deficiency can be rescued by addition of the reducing agent dithiothreitol. Furthermore, the presence of D25N enhanced the efficiency of the disulfide cross-linking of ExbD cysteine substitutions, but aroB only affected the disulfide cross-linking of ExbD T42C. Interestingly, TonB and ExbB have opposite effects on the dimer formation of ExbD: absence of TonB facilitates ExbD dimer formation, while most of ExbD remains monomeric in the absence of ExbB. In this research, we studied the organization of ExbD TMD for the first time and obtained many interesting and controversial results. In order to eliminate these results, some further study should be done.
ABBREVIATIONS

cAMP  cyclic AMP;
CAP    catabolite activator protein;
CAS    Chrome azurol S;
CM     cytoplasmic membrane;
DTT    dithiothreitol;
LPS    lipopolysaccharides;
NEM    N-ethylmaleimide;
NTA    nitrilotriacetate ;
OM     outer membrane;
pmf    proton motive force;
TCA    trichloroacetic acid;
TMD    transmembrane domain.
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ACKNOWLEDGEMENTS

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1.1 Cell envelope of Gram-negative bacteria

The envelope of Gram-negative bacteria, such as *Escherichia coli*, is composed of two concentric membranes, the inner cytoplasmic membrane (CM) and outer membrane (OM), separated by the periplasmic space (Figure 1-1). The CM is composed of phospholipids and proteins that are typically involved in energy conversion, nutrient transport and other essential processes. In Gram-negative bacteria, the outer membrane is very special, and it appears as an asymmetric lipid bilayer composed of phospholipids, lipoproteins, and lipopolysaccharides (LPS). The LPS, located in the outer layer of the outer membrane, consists of a lipid portion extending outward from the bacterial surface (Nikaido 2003). In the inner layer of the outer membrane, phospholipids are the main lipids and lipoproteins serve as the linker between the outer membrane and the peptidoglycan, which is an oligopeptide-crosslinked glycan network in the periplasmic space.

The outer membrane functions as a protective diffusion barrier, but it also hinders the uptake of essential nutrients. Small hydrophilic molecules cross the OM by passive diffusion via aqueous channels formed by porin proteins, but substrates that are either large in mass weight (greater than 600Da) or are present at very low concentrations, such as iron siderophore complexes, vitamin B12, nickel, sucrose, and possibly sulphate, require energized transport for their translocation across the outer membrane through specific OM transporters (Nikaido 2003).
Due to the lack of energy-related proteins, such as ATP-hydrolyzing proteins, and absence of an ion gradient, the OM cannot supply energy for active transport. In order to activate transport across the OM, energy derived from the proton motive force (pmf) at the inner cytoplasmic membrane is required. In addition, a system to transduce the energy from the cytoplasmic membrane to the outer membrane transporter is also required.

The TonB system in Gram-negative bacteria transduces the energy derived from cytoplasmic membrane proton motive force to catalyze active transport across the OM (Postle, K. and Larsen R.A., 2007). While the molecular mechanism of TonB system energy transduction is still unknown, current information suggests that CM proteins ExbB and ExbD harness the energy of the pmf and transmit it to TonB. Subsequently, TonB interacts with OM transporters to provide energy for active transport.

1.2 TonB energy transduction system

The TonB system is a cytoplasmic transmembrane complex composed of the known essential proteins TonB, ExbB and ExbD and potentially some as yet unidentified protein (Postle and Kadner 2003, Skare and Postle 1991). TonB has a similar topological structure to ExbD – both proteins are inserted into the CM by a single transmembrane domain (TMD) and primarily occupy the periplasmic space (Kampfenkel, K. and Braun, V. 1992). In contrast, ExbB is quite different and has three transmembrane domains with the majority of its soluble domains occupying the cytoplasm (Kampfenkel and Braun, 1993) (Figure 1-2). In vivo, ExbB/ExbD/TonB proteins have been confirmed to have physical interaction with each other and the cellular ratio for these three proteins was quantified as 1TonB:7ExbB:2ExbD (Higgs et al 1998; Higgs et al. 2002). The stoichiometry within the energy transduction complex, however, is unknown.
TonB has 239 amino acids and can be divided into three distinct functional domains (Postle and Kadner 2003). At its amino terminus, the predicted TMD (residues 12-32) anchors TonB to the cytoplasmic membrane. This region is required for TonB interaction with ExbB and for conformational changes of the TonB carboxy terminus (Jaskula et al 1994, Ghosh and Postle 2005, Larson et al. 2007). His20 is the only essential residue for TonB activity in its TMD. An all Ala-replacement mutant of the TonB TMD, except Ser16 and His20, still retained 90% of wild-type iron transport activity (Larson et al. 2007). The proline rich domain (66-100aa) is located at the center of the TonB protein. Its role is not clear currently. The carboxy-terminal domain (residues 103-239) occupies the periplasmic space and is required for interaction with outer membrane transporters (Larsen et al 1997). It is known that the carboxy terminus is dynamic. At the energized stage 8–10 aromatic amino acids appears to form a single cluster, and the functional amino-terminal transmembrane domain of TonB and ExbB/ExbD proteins are required for the aromatic cluster formation (Ghosh and Postle, 2005).

ExbD is one of the essential components in the TonB system, and it contains 141 residues. It also can be divided into three domains: a small N-terminal cytoplasmic domain (residues 1–22), a single predicted hydrophobic transmembrane helix (residues 23–43) and a 97-residue C-terminal periplasmic domain (residues 44–141) (Kampfenkel, K., and Braun, V., 1992). ExbD is required for TonB activity and the deletion of the exbD gene rendered the loss of approximately 90% of TonB-dependent activity (Fischer et al., 1989, Brinkman and Larsen, 2008). The exact role of ExbD in the TonB system is still unknown. However, it was recently proposed that ExbD may function as chaperone to regulate the dynamics of TonB conformational changes (Ghosh and Postle, 2005; Larsen et al., 2007).

To understand the role of ExbD in the energy-transducion process, different ExbD mutants have been studied. Currently, only two residues, TMD residue Asp 25 and periplasmic
residue Leu 132, have been identified to be essential for ExbD function, as D25N or L132Q substitutions, rendered ExbD inactive (Braun et al., 1996; Braun and Herrmann, 2004).

In vivo formaldehyde cross-linking analysis has shown that ExbD forms homodimers (Higgs et al., 1998). Recently, Ollis et al. found, ExbD dimer formation was independent of the presence of TonB and ExbB proteins and pmf. Furthermore, ExbD can also formed formaldehyde-linked complexes with TonB and ExbB. The TonB-ExbD complex required the presence of both ExbB and pmf. Additionally, the disulfide-bond formed between two specific cysteine substitutions, ExbD (A92C) and TonB(A150C), was also detected. Interestingly, functional transmembrane domains in both proteins were required for their periplasmic domain interaction since the presence of inactive mutants ExbD(D25N) or TonB(H20A) prevented efficient cross-linking between ExbD and TonB. Two possible models were suggested to explain the pmf-dependent interaction: the TMDs of TonB and ExbD were close enough to interact with each other only in the presence of pmf, or their TMDs configured their respective periplasmic domains as protons are translocated through the ExbB/ExbD/TonB complex. (Ollis A. et al., 2009).

Recently, it was reported that TolR, the homologous protein of ExbD in the TolQRA system, transmembrane residues L22C and V24C formed prominent dimers, with less intense dimers forming along one face of the TMH (Zhang et al, 2009). The disulfide bond formation between L22C or V24C TolR proteins inhibited function of the TolQR complex. Considering that Asp23 was predicted to be part of the proton pathway through the putative TolQR proton channels (Zhou et al., 1998), the authors suggested that two active channels existed in the TolQRA system, and Asp23 oscillated between two positions during proton translocation. The disulfide bond formed between two TolR proteins potentially blocked the oscillation and resulted
These findings with a homologous protein provide an interesting comparison for ExbD TMD.

Although the molecular mechanism of TonB system energy transduction is still unknown, current information suggests a dynamic cyclic model in which TonB protein shuttles energy from the CM to the OM and returns to the CM after transducing this energy to a ligand-loaded outer membrane transporter (Figure 1-3) (Letain and Postle 1997; Larsen et al. 1999, 2003). In this model, TonB exist two distinct states: energized and unenergized states, and these two states can be divided by using the aroB strain, which will be discussed later (Larsen et al., 1999). The energy in this cycle is transduced from pmf that is harnessed by CM proteins ExbB and ExbD (the detailed information are still unclear), and TonB captures the energy through the interaction between its amino terminus and the ExbB/ExbD complexes (Hannavy et al. 1990; Roof et al. 1991; Larsen et al. 1994; Larsen et al. 1999; Larsen and Postle 2001).

1.3 Summary of this study

To understand the mechanism of TonB-dependent energy transduction, it is necessary to understand ExbD TMD organization. In this study, I initiated a cysteine scanning approach to study the assembly of ExbD in vivo. Consistent with previous research (Braun et al., 1996), Asp25 was the only functionally important side chain in the ExbD TMD. A D25C mutation abolished all ExbD functions (based on colicins import and iron transport activities), whereas the other ExbD mutants T42C and V43C only specifically blocked iron transport activity, not colicin import. This study also found that T42C or V43C formed disulfide-linked dimers, suggesting the possibility that the disulfide bonds prevented the iron transport. This was supported by the fact that their iron transport activities were restored by pre-treatment with the reducing agent Dithiothreitol (DTT). We also found that the other mutants L40C and A41C formed disulfide-linked dimer spontaneously in vivo, but these dimers had no effect on ExbD function.
Furthermore, we demonstrated that the absence of ExbB favors monomeric ExbD TMDs, while the absence of TonB facilitates dimer formation of the ExbD TMD.
REFERENCES


Mapping the Interactions between Escherichia coli Tol Subunits – Rotation of the TolR

Function of protonatable residues in the flagellar motor of Escherichia coli: a critical role
**Figure 1-1.** *E.coli* cell envelope and the TonB system. Cell envelope of *E.coli* is composed of outer membrane and plasma membrane, separated by periplasmic space and peptidoglycan. An outer membrane transporter, FepA, binds ferric-enterochelin and transports it into the periplasm. The energy for this active transport is provided by the transmembrane protein complex TonB/ExbB/ExbD system. PBP-periplasmic binding protein, ABC-ATP binding cassette. Figure was referred from Prescott et al. 1996.
Figure 1-2. Three essential components in TonB system. ExbD has a similar topological structure to TonB – both proteins are inserted into the CM by a single transmembrane domain and primarily occupy the periplasmic space (Kampfenkel, K. and Braun, V. 1992). Only charged residue in the TMD of ExbD, D25, was the essential for TonB function.
Figure 1-3. Putative shuttle model for TonB-dependent energy transduction. Membrane proteins ExbB and ExbD harvest the proton motive force from the cytoplasmic membrane (step 1) and then transmit TonB dimer via a conformational change (step 2). TonB shuttles to the outer membrane and transfer to a ligand-bearing TonB-dependent transporter for the active transport (step 3). Subsequently, uncharged TonB return cytoplasmic membrane for next energy cycle (step 4). Figure adapted from Postle K. and Larsen R. A., 2007.
Chapter 2
Experimental Procedures

This chapter describes the experimental procedures and equipment used in this research study.

2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 2-1 and Table 2-2. All bacteria are derivatives of the *Escherichia coli* K-12 strain W3110. DH5α was used as host strain during plasmid constructions. RA1045 was used as host strain for in vivo cross-linking experiment, and spot titer assay and[^55Fe]-ferrichrome transport assay. KP1509 and RA1017 were host strains for in vivo cross-linking experiments.

All further plasmids are derivatives of pKP999 or pKP1064. Site-directed substitutions were introduced by QuikChange mutagenesis PCR using Phusion Hotstart DNA Polymerase. Complementary pairs of oligonucleotides were designed with the desired base change flanked on both sides by 12 homologous bases. DpnI digestion was used to remove the template plasmid. DNA plasmid was transformed into DH5α by electroporation (1.8kV pulse) and then plated on LB plates with ampicillin for selection. DNA plasmids were isolated from potential strains by QIA®prep Miniprep kit from QIAGEN. Substitutions were verified by DNA sequencing of the relevant amplified gene. All DNA sequencing occurred at The Pennsylvania State University Nucleic Acid Facility, University Park, PA.

Competent target strains were prepared essentially as previously described (Chung et al, 1989). Transformation of target strains was applied by Heat Shock 90 seconds at 42°C.
2.2 Media and culture conditions

Luria–Bertani, tryptone (T)-broth, and M9 minimal salts medium were made as described (Miller J.H., 1972). M9 salts were supplemented with 0.5% glycerol (w/v), 0.2% casamino acids (w/v), 0.4 mg ml⁻¹ thiamine, 1 mM MgSO₄, 0.5 mM CaCl₂, 40 mg ml⁻¹ L-tryptophan and 1.85 mM FeCl₃. Liquid cultures, T-agar plates and T-top agar were supplemented with 100 mg ml⁻¹ ampicillin and mutants-specific levels of glucose or sodium propionate (pH 8), as table 3-1. Liquid cultures were grown with aeration at 37°C.

2.3 Chemicals and reagents

Media components were purchased from Becton, Dickinson and Company. PVDF transfer membrane and ECL immunoblot kits were purchased from Thermo Fisher Scientific. α-ExbD antibodies were prepared as described previously (Larsen et al., 1996) and used at 1:10000. Anti-rabbit IgG-peroxidase antibody produced in goat was purchased from Sigma and used at 1:10000. Oligonucleotides were synthesized by Invitrogen. Restriction Enzymes, DpnI and EcoRI, were purchased from New England Biolabs. Gradient and standard PCR were performed using Phusion Hot-start DNA polymerase from Finnzymes. All other reagents were purchased from Sigma Chemical.

2.4 Colicins preparation

Colicin-producing strain was grown overnight in 5 ml LB and antibiotic if necessary. 2.5 ml of overnight culture were subcultured into 50 ml LB in side arm flasks. Culture was harvested after incubation with 50 μl of 1 mgml⁻¹ mitomycin C for 4 hours at 37 °C with shaking, and then resuspended with 10 ml M9 salts. French Press was applied to lyse the cells. The sample was centrifuged for 5 min at 9000 rpm and supernatant was collected and sterilized by filter.
2.5 Determination of plasmid-encoded ExbD expression level

Saturated overnight cultures were subcultured by 1:100 in T-broth containing 100 μgml⁻¹ ampicillin and different concentrations of sodium propionate (pH 8.0) or glucose. 0.2ODml cultures were harvested at OD₅₅₀ of 0.4 and precipitated with equal volume of 20% trichloroacetic acid (TCA) and then incubated on ice for 15 min. After centrifuge for 5 min at 13000 rpm, cell pellets were resuspended in reducing Laemmli sample buffer (10% glycerol, 2% SDS, 1% β-merceptoethanol, 5μgml⁻¹ Bromophenol Blue, 5mM Tris-HCl pH 7.8) and then boiled at 95°C for 10 min. Samples were resolved on 13% non-reducing SDS-polyacrylamide gels and evaluated by immunoblot analysis. The concentration of sodium propionate or glucose inducing the plasmid-encoded ExbD expression at the chromosomal level exhibited by a wild-type control was selected for the next study.

2.6 Spot titre activity assays

Strains carrying plasmids were grown overnight in 5ml LB with 100 μgml⁻¹ ampicillin. Saturated overnight cultures of strains were subcultured by 1:100 in T-broth containing ampicillin, supplemented with mutants-specific concentrations of sodium propionate (pH 8.0) or glucose. 0.08 ODml cultures per plate were harvested at OD₅₅₀ of 0.4, then suspended in 3 ml of molten T-top agar (55°C) and overlaid onto T-plates. Another 0.2 ODml cultures were harvested for electrophoresis. Both T-top and T-plates were supplemented with ampicillin and sodium propionate as indicated. Serial 5-fold dilutions of colicins and 10-fold dilutions of bacteriophage Φ80 with λ-Ca⁺⁺ buffer (10mM Tris-HCl, pH7.8, 20mM MgSO₄, 5mM CaCl₂) were applied to the plate surface in 10 μl aliquots, then incubated at 37°C for 18 hrs. Results were recorded as the reciprocal of the highest dilution at which clearing of the lawn was evident.

2.7 In vivo disulphide cross-linking assay
Saturated overnight cultures were subcultured by 1:100 in T-broth containing 100 μg/ml ampicillin and mutants-specific glucose and sodium propionate (pH 8.0) as indicated. 0.2ODml cultures were harvested at OD_{A550} of 0.4 and incubate with TCA for 15 min on ice. After centrifuge, cell pellets were resuspended in non-reducing LSB (Laemmli sample buffer) (10% glycerol, 2% SDS, 5μg/ml Bromophenol Blue, 5mM Tris-HCl pH 7.8) containing 50 mM iodoacetamide, and then boiled at 95°C for 10 min. Samples were resolved on 13% non-reducing SDS-polyacrylamide gels and evaluated by immunoblot analysis.

To determine the ratio of ExbD dimer to monomer, the samples are diluted as (1:1, 1:2, 1:3, 1:4, 1:5 and 1:10) with non-reducing LSB, and then evaluated by western blot. The ratios of dimer to monomer are equal to the quotient of two dilution factors that the dimer and monomer bands appear to have similar grey intensity.

In order to enhance the intensity of disulfide cross-linking, oxidative cross-linking was applied. When necessary, the cells were treated with 0.3mM copper-1, 10-phenanthroline for 10 min at 37°C with shaking, and then the reaction was blocked with N-ethylmaleimide (NEM) solution (20mM Tris-HCl pH7.8, 8mM NaH$_2$PO$_4$, 12.5mM EDTA, 5mM NEM, 1.25% SDS, 25% Sucrose, 2.5 mg/ml BPB) after washing in sodium phosphate buffer (pH 7.4).

2.8 [$^{55}$Fe]ferrichrome transport

The strain carrying plasmids encoding either wild-type ExbD (pKP999) or ExbD cysteine substitutions was grown to OD_{A550} of 0.45 in supplemented M9 salts with 100 μg/ml ampicillin and mutants-specific glucose and sodium propionate (pH 8). 1.4 ODml cultures were harvested and then resuspended in 1.4 ml M9 assay culture containing 0.1 mM nitrilotriacetate (NTA), followed by incubation with shaking for 5 min at 30°C. 0.2 ml samples were precipitated with TCA for electrophoresis. Before the transport assay, [$^{55}$Fe]ferrichrome was prepared by pre-incubation of ferrichrome with [$^{55}$Fe]Cl$_3$ at a 6.7:1 molar ratio in 10 mM HCl at 37°C for 15 min.
and then placed on ice. The samples was incubated at 30 °C with shaking after addition of 5μl of 
$[^{55}\text{Fe}]$ferrichrome. 50 μl samples were harvested at the indicated time points (1, 3, 5, 7, 9 min.) by 
filtration onto filter disc, which were then washed three times with 3 ml of 0.1 m LiCl and dried 
by vacuum pump. Incorporated $[^{55}\text{Fe}]$ was determined by liquid scintillation counting.
Table 2-1 Strains used in this study

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Table 2-2 Plasmids used in this study

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<tr>
<td>pKP1393</td>
<td>ExbD(V43C)</td>
<td>Present study</td>
</tr>
<tr>
<td>pKP1412</td>
<td>ExbD(D25NL40C)</td>
<td>Present study</td>
</tr>
<tr>
<td>pKP1413</td>
<td>ExbD(D25NA41C)</td>
<td>Present study</td>
</tr>
<tr>
<td>pKP1425</td>
<td>ExbD(D25NT42C)</td>
<td>Present study</td>
</tr>
<tr>
<td>pKP1406</td>
<td>ExbD(D25NV43C)</td>
<td>Present study</td>
</tr>
</tbody>
</table>
Chapter 3

Results

3.1 Cysteine Scanning Mutagenesis and Expression Levels of Cys Substitutions

Previous, it has been shown that ExbD can form homodimer in vivo (Higgs et al., 1998, Ollis A. et al., 2009). In order to obtain the detailed information about the organization of ExbD transmembrane domain in the homodimer, cysteine scanning study was initiated. Cysteine is average in bulk and relatively hydrophobic, and its specific modification is very favorable for the study of membrane proteins, so Cys-scanning mutagenesis is a very popular approach to be used to study structure–function relationships in membrane proteins (Frillingos, et al 1998; Zhang et al 2009).

By β-lactamase fusion assay (Kampfenkel K, Braun V. 1992) and MPEx (http://blanco.biomol.uci.edu/mpex/), predicted ExbD TMD was determined from residue F23 to V43. So these 21 residues were individually replaced with cysteine (Figure 3-1). Mutants were introduced into pKP999 plasmid, which is derivative of plasmid pBAD24 and expresses wild-type ExbD, as described in Vakharia-Rao H. et al., 2007. The \textit{exbD} gene in pKP999 plasmid is regulated by \textit{prpBCDE} promoter expression system (prpB-PprpR) (Figure 3-2). At the presence of 2-methylcitrate that is generated from propionate by chromosomal encoded enzyme, the transcriptional activator, prpR, activates the expression of cloned genes (\textit{exbD}) under the control of \textit{PrprpB} (Lee and Keasling, 2005).

RA1045 (W3110, Δ\textit{exbD}, Δ\textit{tolQRA}) was used as host strain to express cysteine substituted ExbD proteins. In order to make sure the every mutant protein is actually present and keep the cellular ratio of ExbD:TonB:ExbB as 2:1:7, overall expression levels of mutated ExbD
proteins were determined by western blot. At the present of mutants-specific concentration of sodium propionate (table 3-1), all of the substituted ExbD proteins were expressed at the chromosomal level compared to the native ExbD except D25C. As shown in Figure 3-3 A, mutation of the D25C was highly overexpressed in the absence of inducer. In order to determine whether the ExbD D25C is more stable, its half-life was determined in vivo. The results showed that the half-life of ExbD D25C was approximately 60 minutes which was very close to wild-type ExbD (Figure 3-3 B), suggesting ExbD D25C overexpression probably is due to its high transcription, but not the stability. Since the prpBCDE promoter has CAP-dependent activation, the prpR-PprpB system can be suppressed by addition of glucose. So in order to maintain its expression at chromosomal level, 0.05% (v/v) glucose was used to repress ExbD D25C expression (Figure 3-3 A).

3.2 Phenotypic characterization of cysteine substituted ExbD Mutants

The activities of these ExbD cysteine substitutions were assayed through spot titer, as discussed in chapter 2. Four agents were used in this assay: colicin B, colicin Ia, colicin M and Φ80. Colicins are proteins produced by some strains of Escherichia coli that are lethal for related strains of E. coli. They take use of different OM transporters to cross the membrane and kill the target cell (Cascales et al, 2007). So spot titer provides an indirect measure of TonB system activity, and the highest dilution at which clearing of the lawn was evident is used to reflect the activity of ExbD mutants. This assay was applied after transformation of mutant plasmids into RA1045 (W3110, ΔexbD, ΔtolQRA) and all of the ExbD mutants were expressed at the chromosomal level in the presence of proper concentration of sodium propionate. As shown in table 3-2, the ExbD transmembrane segment was shown to be remarkably tolerant to cysteine substitution because all of the mutants but ExbD D25C displayed wild-type phenotype, sensitive to colicins.
3.3 Disulfide Bond Formation between ExbD TMD Residues

In vivo studies have previously shown that ExbD appears to form a homodimers via the periplasmic region (Ollis et al., 2009). The role that the TMDs of ExbD dimer played in the membrane is still unknown. To map regions of interaction between two ExbD TMDs, the spontaneous disulfide cross-linking was monitored on non-reducing SDS-polyacrylamide gels. The ExbD TMD is predicted to be a single $\alpha$-helix, and whether the cysteines in adjacent transmembrane helices are close enough to form a disulfide linkage depends on the relative rotations of the helices around their long axes. Only if the cysteines in both ExbD TMDs were positioned close to the interacting face could they form a disulfide bond spontaneously.

As shown on Figure 3-4 A, four substitutions, ExbD L40C, A41C, T42C and V43C, formed high levels of homodimers, with less intense dimers formed by ExbD F23C or I24C, suggesting the TMDs of ExbD dimer were anchoring close enough to interact, and that these six residues were located at the ExbD-ExbD contacting interface. As seen on a helical wheel projection summarizing extent of dimer formation (Figure 3-1), these 6 residues forming homodimers are located on the four different faces, which suggests there may be at least two ExbDs in one complex, which is consistent with the cellular ratios, and their relative position is highly dynamic.

In order to enhance the intensity of dimer formation, I applied the oxidative cross-linking with copper-1,10-phenanthroline (Zhang et al., 2009). The major factor of copper-1,10-phenanthroline is probably to trap the intermediates of ExbD rotation, which would allow stabilizing of more-distorted conformations. With copper-1,10-phenanthroline treatment, another six Cys-substituted residues, V26C, F34C, M35C, V36C, A37C and P39C, were detected to form disulfide cross-linking, which scattered around the helical wheel projection, with no apparent pattern (Figure 3-5).
3.4 Disulfide bond formed by ExbD T42C or ExbD V43C prevented the $[^{55}\text{Fe}]$ferrichrome transport

$[^{55}\text{Fe}]$ferrichrome transport is another assay to evaluate the activity of ExbD mutants since it take place over a very short time frame and be able to discriminate different levels of TonB system activity which spot titer assays cannot offer. This assay is applied in M9 medium as described previously. As expected, the strain producing ExbD D25C was inactive for the iron transport. Similar phenotypes were observed in the strains expressing ExbD T42C and ExbD V43C. Their $[^{55}\text{Fe}]$ferrichrome transport activities were significantly less efficient, providing for the transport at only 18.96% and 7.61% of the rate conferred by wild-type ExbD, respectively (table 3-3). This reduced rate of transport did not reflect lower levels of expression, as immunoblots verified that each of these mutants was present at levels similar to that of chromosomally encoded wild-type ExbD expressed from the native promoter (lower bands in Figure 3-6A). Because of disulfide bond formed by ExbD T42C or V43C, we suspected the deficiency of these two mutants on iron transport activity is likely due to prevention of ExbD conformational change by covalently bound.

To test whether the decreased levels of Fe transport observed in these two mutants were due to disulfide bond formation, we performed $[^{55}\text{Fe}]$ferrichrome transport in presence and absence of the reducing agent DTT. All of the ExbD cysteine substitutions were keeping at the monomer form verified by western blots (Figure 3-6 A). The $[^{55}\text{Fe}]$ferrichrome transport rate of ExbD T42C and ExbD V43C were respectively restored to 54% and 62% of the rate conferred by chromosomally encoded wild-type ExbD, close to the plasmid-encoded wild-type ExbD (Figure 3-6 B). These results suggested that the phenotypes of ExbD T42C and ExbD V43C are mainly caused by the disulfide bonds and not by the cysteine residue.
However, as shown in the figure 3-6 A, ExbD V43C monomer was degraded on the non-reducing gel with DTT treatment. The degradation of ExbD monomer was also found in the absence of ExbB, even after TCA precipitation and boiled at 95°C (Figure 3-7). In order to minimize the effect of ExbD degradation, the protein sample should be stored on ice during the experiment, and prepared fresh daily.

### 3.5 The disulfide cross-linking is more efficient in the absence of TonB, but not ExbB.

Previously, formaldehyde cross-linking study was shown that ExbD dimer formation occurred in the absence of TonB or ExbB, and ExbD also formed formaldehyde-linked complexes with TonB and ExbB that required the presence of pmf (Ollis et al, 2009). In order to determine whether and how TonB and ExbB affect the disulfide bond formation of ExbD dimer TMDs, immuo-detection of disulfide cross-linking was applied after transformation of ExbD cysteine-substituted plasmids into *tonB* or *exbB* background strains.

KP1509 (W3110, ΔexbD, ΔtolQRA, ΔtonB::kan) was used in this study. As shown on figure 3-4B, the pattern of disulfide cross-linking of ExbD TMD did not change obviously, except A38C. However, compared with the dimer formation in RA1045 expressing chromosomally encoded TonB, all of the four mutants ExbD L40C, A41C, T42C and V43C formed significant higher level of homodimer in the strains without TonB protein (Figure 3-9), and the yields of dimer increased about 1-fold (Figure 3-10). Additionally, ExbD A38C, a new residue forming the disulfide bond, appeared in the *tonB* background strain. These results suggested that the absence of TonB protein favored the dimer formation of ExbD. Results are summarized in Table 3-4.

However, the results were quite different in the *exbB* strains. ExbD disulfide-linked dimers were suppressed partially after the introduction of ExbD cysteine substituted plasmid into RA1017 (W3110, ΔexbBD, ΔtolQR) (figure 3-4 C, figure 3-9, table 3-4), suggesting the absence
of ExbB facilitated the maintenance of ExbD monomer. Consistent with previous study (Fischer et al., 1989), we found that ExbD was very unstable in the absence of ExbB, and more sodium propionate were required to express them in the chromosomal level (table 3-1).

3.6 *aroB* has no effect on the disulfide cross-linking of ExbD TMDs except ExbD (T42C)

In the *aroB* strains, the endogenous synthesis of enterochelin is prevented, resulting in the prevention of the progression of TonB from an energized conformation to a discharged conformation (Larsen et al., 1999). Therefore, by using the *aroB* strains, the TonB energy transduction cycle can be divided into two stages. To investigate the organization of ExbD TMD in these two stages, disulfide bond formation of cysteine substituted ExbD protein was detected in an *aroB* mutant strain KP1526 (RA1045 *aroB*).

Chrome azurol S (CAS) plate is used to detect the *aroB* mutant strains. The Fe(III) gives the agar a rich blue color, while siderophores excreted by wild-type strains result in a color change to orange (Alexander and Zuberer, 1991). As shown on the figure 3-8A, after 18-hour incubation at 37°C, compared with the orange region growing wild-type strain W3110, the areas growing the strains expressing ExbD mutants were still blue, suggesting all of these strains were *aroB* mutants.

The immunoblot results showed that ExbD mutants, ExbD L40C, A41C and V43C, still formed high level of disulfide-linked dimers in the *aroB* mutant strains and ratios of dimer to monomer was no significant different from the *aroB* strains (Figure 3-8 B), whereas the disulfide cross-linking of ExbD (T42C) was ~1:3 dimer: monomer in the *aroB* mutant strains, which is less than in the *aroB* strains. This suggests that the ExbD TMD organization can be slightly affected by the presence or absence of enterochelin.
3.7 Inactive ExbD mutant D25N boosts its TMD disulfide bonds formation

It is proved that Asp25 was the functionally important side chain in the ExbD TMD (Braun et al., 1996). The ExbD mutant, D25N rendered ExbD inactive due to steric hindrance. Meanwhile, the D25N substitutions in the ExbD TMD prevented formaldehyde cross-linking to TonB, but not to ExbB (Ollis et al, 2009). Although ExbD(D25N) did not affect the homodimer formation, the molecular mass of ExbD (D25N) dimer was slightly less than wild-type (Ollis et al, 2009). Therefore, we hypothesized that this inactive mutant might alter the conformational structure of ExbD dimer.

In order to examine this hypothesis, four double mutants, a single cysteine substitution in the ExbD D25N template, ExbD (D25N, L40C), ExbD (D25N, A41C), ExbD (D25N, T42C) and ExbD (D25N, V43C) were constructed. These double substitutions mutants were tested for their ability to form disulfide cross-linking in vivo. As shown on figure 3-10, all of these four double mutants formed high levels of homodimers, even higher than the single cysteine substitutions. These results suggested that the functional ExbD TMD is not required for the ExbD dimer formation, and to some extent, the disulfide cross-linking is more efficient in the presence of D25N. Results are summarized in Table 3-4.
Table 3-1 The concentrations of sodium propionate are used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>RA1045 (W3110,ΔexbD, ΔtolQR)</th>
<th>RA1017 (W3110,ΔexbBD, ΔtolQR)</th>
<th>KP1509 (W3110, ΔexbD, ΔtolQRA, ΔtonB::kan)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pExbD</td>
<td>0.5 mM</td>
<td>3 mM</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>ExbD(F23C)</td>
<td>0.25 mM</td>
<td>5 mM</td>
<td>0 mM</td>
</tr>
<tr>
<td>ExbD(I24C)</td>
<td>0.5 mM</td>
<td>5 mM</td>
<td>0.25</td>
</tr>
<tr>
<td>ExbD(D25C)</td>
<td>0.05%Glucose</td>
<td>2 mM</td>
<td>0.1% Glucose</td>
</tr>
<tr>
<td>ExbD(V26C)</td>
<td>1 mM</td>
<td>5 mM</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>ExbD(M27C)</td>
<td>0.25 mM</td>
<td>5 mM</td>
<td>0 mM</td>
</tr>
<tr>
<td>ExbD(L28C)</td>
<td>1 mM</td>
<td>10 mM</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>ExbD(V29C)</td>
<td>0.005%Glucose</td>
<td>3 mM</td>
<td>0 mM</td>
</tr>
<tr>
<td>ExbD(L30C)</td>
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<td>10 mM</td>
<td>0 mM</td>
</tr>
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<td>0.5 mM</td>
</tr>
<tr>
<td>ExbD(I32C)</td>
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<td>10 mM</td>
<td>0.5 mM</td>
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<tr>
<td>ExbD(I33C)</td>
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<td>10 mM</td>
<td>0 mM</td>
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<td>10 mM</td>
<td>0 mM</td>
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<td>ExbD(M35C)</td>
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<td>0.25 mM</td>
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<tr>
<td>ExbD(V36C)</td>
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<td>0 mM</td>
</tr>
<tr>
<td>ExbD(A37C)</td>
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<td>0 mM</td>
</tr>
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<td>ExbD(A38C)</td>
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<td>0 mM</td>
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<td>0 mM</td>
</tr>
<tr>
<td>ExbD(T42C)</td>
<td>0 mM</td>
<td>2 mM</td>
<td>0 mM</td>
</tr>
<tr>
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<td>2 mM</td>
<td>0 mM</td>
</tr>
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<td>0.005% Glucose</td>
<td></td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>ExbD(D25NT42C)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ExbD(D25NV43C)</td>
<td>0.1 mM</td>
<td></td>
<td></td>
</tr>
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</table>

Data indicate the required concentration of sodium propionate or glucose needed to express protein at chromosomal levels in T-broth. pExbD indicates plasmid-encoded wild-type ExbD.
Table 3-2 Phenotypes of strains producing Cys-substituted ExbD proteins

<table>
<thead>
<tr>
<th>ExbD</th>
<th>ø80</th>
<th>colicin B</th>
<th>colicin Ia</th>
<th>colicin M</th>
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<td>3, 3, 3</td>
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<td>V43C</td>
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<td>5, 5, 5</td>
<td>5, 4, 5</td>
<td>3, 3, 3</td>
</tr>
</tbody>
</table>

Phenotype of ExbD mutants were evaluated using spot titer assays. Cultures with the strains expressing ExbD mutated proteins at the chromosomal level were plated on T-plates and spotted with fivefold serial dilutions of colicins and 10-fold serial dilutions of bacteriophage ø80. Values were recorded as the reciprocal of the highest dilution at which clearing of the lawn was evident after 18 h of incubation at 37°C. ‘T’ indicates tolerance.
Table 3-3 Iron transport activity of ExbD cysteine substitutions

<table>
<thead>
<tr>
<th></th>
<th>%rate relative to pExbD without DTT</th>
<th>%rate relative to pExbD with DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA1045</td>
<td>0.15%</td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>121%</td>
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</tr>
<tr>
<td>pExbD</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>L40C</td>
<td>101.9%</td>
<td></td>
</tr>
<tr>
<td>A41C</td>
<td>103.6%</td>
<td></td>
</tr>
<tr>
<td>T42C</td>
<td>22.9%</td>
<td>77%</td>
</tr>
<tr>
<td>V43C</td>
<td>9.2%</td>
<td>89%</td>
</tr>
</tbody>
</table>

Percentiles of transport rates calculated by slopes derived from linear regression analysis of the individual data points, as described in Ghosh J., and Postle, K. (2005).
Table 3-4 Ratio of dimer to monomer in three different backgrounds

<table>
<thead>
<tr>
<th>ExbD</th>
<th>RA1045 (W3110, ΔexbD, ΔtolQR)</th>
<th>RA1017 (W3110, ΔexbBD, ΔtolQR)</th>
<th>KP1509 (W3110, ΔexbD, ΔtolQRA, ΔtonB::kan)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F23C</td>
<td>Medium (&lt;1:1)(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I24C</td>
<td>Medium (&lt;1:1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L40C</td>
<td>High (5:4)</td>
<td>Low (&lt;1:10)</td>
<td>High (3:2)</td>
</tr>
<tr>
<td>A41C</td>
<td>High (2:1)</td>
<td>Medium (3:4)</td>
<td>High (5:1)</td>
</tr>
<tr>
<td>T42C</td>
<td>High (2:1)</td>
<td>Low (&lt;1:4)</td>
<td>High (5:1)</td>
</tr>
<tr>
<td>V43C</td>
<td>High (10:1)</td>
<td>High (2:1)</td>
<td>High (&gt;10:1)</td>
</tr>
<tr>
<td>D25NL40C</td>
<td>High (5:1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D25NA41C</td>
<td>High (5:1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D25NT42C</td>
<td>High (5:1)</td>
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<td></td>
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<tr>
<td>D25NV43C</td>
<td>High (10:1)</td>
<td></td>
<td></td>
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\(^a\) The ratios of dimer to monomer are calculated by the dilution factors that the dimer and monomer bands appear to have similar grey intensity in the figure 3-10.
Figure 3-1 Putative ExbD Transmembrane Domain residues (F23-V43). The ExbD TMD is represented on a helical wheel projection. The hydrophilic residues as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles. Hydrophobicity is color coded as green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Average yields of ExbD homodimer are represented by bars.
Figure 3-2 Map of pKP999 plasmid. The pKP999 plasmid is derivative of pPro expression vector. This vector contains the prpBCDE promoter (prpB promoter) responsible for expression of the propionate catabolic genes (exbD in pKP999 plasmid) and prpR, the positive regulator of this promoter. It also carries a pBR322 origin of replication, an M13 intragenic region and an Ampicillin resistance gene for selection. Since the prpBCDE promoter has CAP-dependent activation, the prpR-PprpB system can be suppressed by addition of glucose.
Overexpression of ExbD D25C and its Half-life. A, ExbD D25C protein was overexpressed in vivo. In order to down-regulate its expression to the chromosomal level, 0.05% Glucose was added to repress its transcription. B, ExbD D25C mutant protein has the same half-life as the chromosome or plasmid encoded wild-type ExbD, about 60 min. Synthesis of protein was blocked by addition of chloramphenicol at the indicated time points (0, 15, 30, 60, 90, and 120 min). Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted using polyclonal anti-ExbD antibody (Higgs et al., 2002). RA1045 (W3110, ΔexbD, ΔtolQR) was used as the host strain in this study.

* 0.5 mM sodium propionate was used to induce the plasmid-encoded wild-type ExbD expressing at the chromosomal level.
Figure 3-4. Profile of ExbD dimer formation in three different strains. To map regions of interaction between two ExbD TMDs, the spontaneous formation of disulfide-linked homodimers was monitored on non-reducing SDS-polyacrylamide gels. *Escherichia coli K-12* strain W3110 and its derivatives, RA1045 (ΔexbD, ΔtolQR), RA1017 (ΔexbBD, ΔtolQR) and KP1509 (ΔexbD, ΔtolQRA, ΔtonB::kan), were used in this study. Four substitutions, ExbD L40C, A41C, T42C and V43C, formed high levels of homodimers in 3 strains, with less intense dimers forming by F23C, I24C and A38C, suggesting that these residues were located at the ExbD-ExbD contacting interface and they were anchoring close enough to interact.

“NC” indicates negative control which corresponds to the target strains in each experiment. pExbD indicates plasmid-encoded wild-type ExbD. The positions of ExbD monomers and ExbD dimers are indicated on the right. *, low levels of dimerization; **, high levels of dimerization.
Figure 3-5 Profile of ExbD dimer formation after the addition of copper-1, 10- phenothaline.

Oxidative cross-linking assay was applied as described in chapter 2. With the treatment of copper-1, 10- phenothaline, another six Cys-substituted residues, V26C, F34C, M35C, V36C, A37C and P39C, were determined to form disulfide cross-linking. RA1045 (W3110, ΔexbD, ΔtolQR) was used as the host cell in this study. The positions of ExbD monomers and ExbD dimers are indicated on the right.
Figure 3-6. $^{55}$Fe-ferrichrome transport of ExbD T42C and ExbD V43 in the absence or presence of DTT. A, Disulfide cross-linking of ExbD was applied in W3110 or RA1045 carrying plasmid-encoded ExbD as described in Experimental procedures. Samples were resolved on a 13% SDS-polyacrylamide gel and immunoblotted. ‘+’ or ‘−’ indicates the presence or absence of DTT, respectively. Positions of ExbD monomer or dimer are indicated on the right. The lower bands indicated the ExbD monomer level, monitored on reducing SDS-polyacrylamide gels. B. The slope represents the iron transport rate. The iron transport rate of ExbD(T42C) was 19.0% relative to W3110, while it increase to 54% after addition of DTT. The iron transport rate of ExbD(V43C) was restored to 62% from 7.6% relative to W3110 after addition of DTT. Adding DTT had no effect on iron transport in W3110.
Figure 3-7 ExbD is unstable in the absence of ExbB. Strains expressing plasmid-encoded Cys-substituted ExbD in ExbD⁻ (RA1045), ExbB⁻ (RA1017) or TonB⁻ (KP1509) backgrounds were incubated at the presence of proper concentration sodium propionate as indicated. Overall expression level of ExbD was determined on a 13% reducing SDS-polyacrylamide gel and immunoblotted (lower figure). The disulfide cross-linking was detected on a 13% non-reducing SDS-polyacrylamide gel (upper figure). ExbD was visualized with ExbD-specific polyclonal antibodies. Positions of ExbD dimer and monomer are indicated on the right.
**Figure 3-8.** *aroB* has no effect on the disulfide cross-linking of ExbD mutants except ExbD T42C. 

A, Chrome azurol S (CAS) plate is used to detect the *aroB* mutant strains. The area growing *aroB* strain develops a blue color, while wild-type strains extract iron from the place, resulting in a color change to orange, after incubation at 37°C for 18 hrs. W3110 is used as a positive control, and KP1270 and KP1526 are *aroB* strain. The KP1526/ExbD(T42C) 1# is contaminated. B, western blot was applied as described in Experiment procedure. The positions of ExbD monomers and dimers are indicated on the right.
Figure 3-9. Comparison of dimer formation in three different backgrounds. Four ExbD cysteine substitutions, L40C (A), A41C (B), T42C (C) and V43C (D), were transduced into RA1045 (ΔexbD, ΔtolQR), RA1017 (ΔexbBD, ΔtolQR) and KP1509 (ΔexbD, ΔtolQRA, ΔtonB::kan). The dimer formation was monitored on 13% non-reducing SDS-polyacrylamide gels. Positions of ExbD monomer or dimer are indicated on the right. The upper bands indicate the ExbD monomer level, monitored on reducing SDS-polyacrylamide gels.
Figure 3-10. Comparison of ExbD dimer formation in different conditions. To determine the ratio of ExbD dimer to monomer, the protein samples were diluted as (1:1, 1:2, 1:3, 1:4, 1:5 and 1:10) with non-reducing LSB respectively, and then resolved on a 13% SDS-polyacrylamide gel and immunoblotted. RA1045 (ΔexbD, ΔtolQR) and KP1509 (ΔexbD, ΔtolQRA, ΔtonB::kan) were used in this study to determine the effect of TonB on the ExbD dimer formation. Four single cysteine substitutions, ExbD L40C, A41C, T42C and V43C, and four double mutations, ExbD D25NL40C, D25NA41C, D25NT42C and D25NV43C, were expressed in RA1045 strains to verify the impact of D25N on ExbD disulfide cross-linking. The ratios of dimer to monomer are calculated by the dilution factors that the dimer and monomer bands appear to have similar grey intensity. They are indicated on the top of each figure. The positions of ExbD monomers and dimers are indicated on the right.
Chapter 4

Discussion

The TonB/ExbB/ExbD proteins are involved in transducing the proton motive force to catalyze active transport of iron siderophore and vitamin B12 through high-affinity outer membrane transporters (Postle, K. and Larsen R.A., 2007). In this energy transduction cycle, ExbB and ExbD appear to harness the pmf and transmit it to TonB, and then energized TonB protein interacts with OM transporters to provide energy for active transport, after that, TonB becomes uncharged states and returns to CM (Fischer et al., 1989; Larsen et al., 1999; Larsen et al., 2007). As one of essential components, ExbD is required for TonB to conformationally respond to pmf and associate with the CM (Held, K. and Postle K., 2002). However, its exact role in this process is still unclear.

Dimer Model of ExbD TMD

In vivo, ExbD forms homodimer via its periplasmic region (Ollis A.et al., 2009). The disulfide cross-linking data provide further evidence to show the ExbD TMD organization within the dimer. Six ExbD cycteine substitutions formed the disulfide cross-linking spontaneously, and the more efficient cross-linking of Cys-substituted residues, L40, A41, T42 and V43, are located on four different interfacial positions based on the helical wheel projection (Figure 3-1), which suggested that at least two ExbD TMDs were in close contact in the TonB complex and rotated around each other, since these four permanent cross-linkings cannot be formed by their corresponding residues at the same time.

However, after treatment of copper- 1, 10-phenanthroline, another six Cys-substituted residues, V26C, F34C, M35C, V36C, A37C and P39C, were determined to form disulfide cross-linking, scattering around the helical wheel projection. Copper-1, 10-phenanthroline serves to trap
the intermediates of ExbD rotation, so the cross-linking results suggested that there residues were close enough transiently during the rotation of ExbD dimer. However, due to the lack of apparent location pattern of these residues, it is difficult to visualize the possible model of ExbD dimer organization at present.

These results can be compared to a similar study of the paralogous TolR protein. TolR is anchored in the cytoplasmic membrane via residues 21 to 41 which has been proved by computational and experimental evidences (Kampfenkel K, Braun V. 1993), while the transmembrane segment of ExbD was predicted as the residues from 23 to 43 (Kampfenkel K, Braun V. 1992). Alignment of ExbD and TolR TMD showed that they have highly conserved transmembrane domains (Figure 4-1). In the study of TolR TMH, Zhang et al assayed the disulfide cross-linking of residues from 18 to 39, and found that cross-linking of several residues occurred along one side of the TMD bordered by the two strongest cross-linkings L22 and V24. The strongest cross-linking observed in the absence of oxidative catalyst was ~3:1 dimer: monomer. In contrast, ExbD had only rather low levels of disulfide dimer formation through the corresponding residues. In my study the last four residues in the TMD showed very high cross-linking. These residues were not assayed by Zhang et al, making comparisons more difficult.

Additionally, it has been demonstrated that the dimer formation of ExbD was independent of the presence of TonB and ExbB proteins (Ollis A. et al., 2009). Similar results have been found in MotB dimer formation as well (Braun T. F. and Blair D.F., 2001). However, formaldehyde cross-linking analysis has been shown that wild-type ExbD had physical interaction with TonB and ExbB via their periplasmic domains in vivo (Ollis A. et al., 2009) which suggested that these three proteins were in close contact in the TonB complex. Therefore, the steric effect and states of TonB or ExbB are likely to affect the organization of ExbD.
In the *aroB* strains, the endogenous synthesis of enterochelin is prevented, resulting in the division of TonB into two states: charged and discharged states (Larsen et al., 1999). Using KP1526 (RA1045, *aroB*) as the host strain to study the disulfide cross-linking of ExbD TMD, only ExbD T42C dimerization was affected in the *aroB* strains, which suggested TonB states slightly influenced ExbD TMD organization on some specific position, and the residue on this position may play some important role in the TonB energy transduction cycle.

Furthermore, we found that the disulfide cross-linking of ExbD cysteine substitutions was more efficient in the absence of TonB, and less efficient in the absence of ExbB. The different patterns may reflect their relative position in the complex. These results suggested one possible model for TonB–ExbB–ExbD organization. In this model, TonB competes with one ExbD protein to interact with another ExbD. If so, in the absence of TonB, the ExbD proteins could bind with each other more tightly and form the dimer more efficiently. However, since the ExbB is the scaffold of the TonB complex, I still don’t have a proper interpretation about the effect or ExbB on the ExbD dimerization.

**Asp 25 is the sole functionally important side chain in the ExbD TMD**

The Asp25 is the only charged amino acid in ExbD TMD, near to the inner face of the cytoplasmic membrane (Eick-Helmerich, K. and Braun V., 1989; Kampfenkel, K. and Braun V., 1992). Interestingly, the only charged and essential amino acid in the TonB TMD, His20, is also located close to the inner side of the cytoplasmic membrane, which makes it more likely that two amino acids can collaborate with each other during the energy transduction (Traub, I., et al, 1993). The interaction of aspartate residue and histidine was ever found in the M2 proton channel of influenza A virus, which is modulated by pH level (Schnell J. R. and Chou J. J. 2008).
While it had been previously demonstrated that the conserved Asp25 was important for ExbD function (Braun et al., 1996), I discovered in this study that Asp25 was the only functionally important side chain in the ExbD TMD since all of the other residues are tolerant to cysteine replacement in the spot titer assay. The homologous proteins of ExbD, TolR and MotB, also have the corresponding aspartate residue in their TMDs, D23 and D32. Both of them are also essential for their respective systems function, and it has been proposed that they form proton pathways with the other essential residues in TolQA or MotA (Zhou et al., 1998; Cascales et al., 2001), suggesting maybe ExbD D25 also plays the similar role in the TonB system, sensing the electrochemical potential of the cytoplasmic membrane, and transduce the energy to TonB.

Interestingly, in all of the ExbD TMD Cys-substitution mutants, D25C is the only one to be highly overexpressed. This phenomenon was also found in the other substations of this residue, such as D25A, D25H and D25N (Ollis, Xie, data not shown). We have confirmed that this overexpression was not due to enhanced stability of the mutated protein, since the half-life of ExbD D25C was approximately 60 minutes which was very close to chromosomally encoded wild-type ExbD. As verified previously, ExbB and ExbD are tandemly encoded on a single operon separated by only 9 bp, but surprisingly, ExbB exists in the cells at a 3.5 molar excess relative to ExbD (Higgs et al., 2002). In the pKP999 plasmid, the \textit{exbD} gene is encoded following the last 30 bp of \textit{exbB}. Therefore, we propose that the mRNA of ExbD D25C probably formed a special secondary structure with the upstream sequence, which caused its high transcription. Further study is needed to verify this hypothesis. However, in order to decrease the expression of ExbD D25C, 0.05\% (v/v) was added into the medium, since the \textit{prpB} promoter is activated by CAP and the addition of glucose suppresses the production of cyclic AMP (cAMP), which activates catabolite activator protein (CAP).
Previously, it has been known that functional transmembrane domain is not required for the ExbD homodimer formation via their periplasmic domains (Ollis A. et al, 2009). In my study, we found the functional transmembrane domain is also not necessary for the ExbD TMD dimer formation, since in the presence of D25N, ExbD mutants, L40C, A 41C, T42C and V43C, still formed prominent disulfide cross-linking. Interestingly, the levels of these disulfide-linked dimers in presence of D25N were even higher than their single mutants. Since the presence ExbD(D25N) prevented efficient formaldehyde cross-linking between ExbD and TonB via their periplasmic domain (Ollis A. et al., 2009), it’s quite possible the interaction of ExbD and TonB via periplasmic domain configured the ExbD TMDs in the presence of ExbD(D25N).

Effect of different disulfide bonds on the functioning of ExbD

In the case of MotAB complex, it is proposed that two proton channels lie between the A3 and A4 segments of a MotA subunit and face to MotB subunit, so that most of the conserved residues are in the channel including D32 (Timothy F., et al. 2004). We hypothesized that channel-like structure also existed in the TonB system and D25 was required to be in this area for the functioning of ExbD.

Although ExbD L40C and A41C formed high levels of disulfide cross-linking, they still maintained most of ExbD functions (colicins import and iron transport activities). Even in the presence of copper-1, 10-phenothaline, almost all of these two ExbD mutants were in the dimer state, they still retained about 60% iron transport activities (data not shown). However, the other two ExbD mutants, T42C and V43C, were specifically blocked in their iron transport activities in vivo due to disulfide bond formation. Interestingly, their iron transport activities were restored near to the plasmid-encoded ExbD level after treated with DTT (Figure 3-6). As I demonstrated here that D25 is the only essential residue in the ExbD TMD for the its function. Therefore we proposed that the disfunction of the mutants, T42C and V43C, might be related to the D25.
Whereas L40C and A41C also formed high level of disulfide cross-linking, and their disulfide bonds have no significant effect on the functioning of ExbD. These data seem contradictory, and at present I still don’t figure out a proper explanation.

**Stability of ExbD in the absence of ExbB**

Normally, before the detection of ExbD dimer formation, the proper concentration of sodium propionate was determined to make sure the expression of protein in the chromosomal level. However, because ExbD is proteolytically unstable in the absence of ExbB (Fischer et al., 1989), ExbD monomers were rapidly degraded in the absence of ExbB on the non-reducing gel even after TCA precipitation and boiled at 95°C. Commonly, TCA precipitation is considered the most popular and efficient method for precipitating proteins. Although the real mechanism of TCA precipitation is still unclear, it’s proposed that TCA caused protein to precipitate by sequestering the protein-bound water or inducing the protein conformational changes (Kumar T.K.S. et al., 1994).

In order to minimize the influence of ExbD degradation, the protein samples precipitated by TCA were duplicated. One set of sample was treated with reducing LSB for the detection of overall expression level, and another set of sample was treated with non-reducing LSB for the detection of ExbD dimer formation. These two detections were done at the same time. The results showed that this method was very effectively used to compare the dimer formation of ExbD in three different background strains (Figure 3-9).

In summary, D25 was the only essential residue in the ExbD TMD. The cysteine replacement of this residue abolished all ExbD functions (colicins import and iron transport activities). Cysteine scanning study was shown that six residues in the ExbD TMD formed disulfide cross-linking spontaneously. T42C and V43C specifically suppressed the iron transport
activity. We confirmed that the disulfide bonds formed by them took the major responsibility to
the loss of ExbD function. Furthermore, we also demonstrated that absence of ExbB favors ExbD
monomer, while absence of TonB facilitates the dimer formation of ExbD.
REFERENCES


Figure 4-1 Alignment of ExbD and TolR. Protein sequence alignment, produced by ClustalX, showed that the ExbD TMD was highly conserved since 12 residues were identical to TolR TMD. The yellow frame represents the position of the TM domain. Red: basic amino acids; Blue: aromatic amino acids; Green: hydrophobic amino acids; "*" indicates identical; "." indicates conserved substitutions; ":" indicates semi-conserved substitution (similar shapes). "|", low levels of dimerization; "▼", high levels of dimerization. The residues in the red-dotted frame are not assayed by Zhang et al.