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Deletion and Substitution Analysis of the *Escherichia coli* TonB Q160 Region▽

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The active transport of iron siderophores and vitamin B_{12} across the outer membrane (OM) of *Escherichia coli* requires OM transporters and the potential energy of the cytoplasmic membrane (CM) proton gradient and CM proteins TonB, ExbB, and ExbD. A region at the amino terminus of the transporter, called the TonB box, directly interacts with TonB Q160 region residues. R158 and R166 in the TonB Q160 region were proposed to play important roles in cocrystal structures of the TonB carboxy terminus with OM transporters BtuB and FhuA. In contrast to predictions based on the crystal structures, none of the single, double, or triple alanyl substitutions at arginyl residues significantly decreased TonB activity. Even the quadruple R154A R158A R166A R171A mutant TonB still retained 30% of wild-type activity. Up to five residues centered on TonB Q160 could be deleted without inactivating TonB or preventing its association with the OM. TonB mutant proteins with nested deletions of 7, 9, or 11 residues centered on TonB Q160 were inactive and appeared never to have associated with the OM. Because the 7-residue-deletion mutant protein (TonBΔ7, lacking residues S157 to Y163) could still form disulfide-linked dimers when combined with W213C or F202C in the TonB carboxy terminus, the TonBΔ7 deletion did not prevent necessary energy-dependent conformational changes that occur in the CM. Thus, it appeared that initial contact with the OM is made through TonB residues S157 to Y163. It is hypothesized that the TonB Q160 region may be part of a large disordered region required to span the periplasm and contact an OM transporter.

The gram-negative cell envelope consists of an energized cytoplasmic membrane (CM) and a concentric, unenergized outer membrane (OM), separated by the aqueous periplasmic space. The OM is a diffusion barrier, protecting the cell from hydrophobic drugs, detergents, and degradative enzymes present in the environment while allowing the passive diffusion of nutrients smaller than 600 Da through the porins (56). The transport of Fe(III) siderophores or cobalamin across the OM requires the CM protonmotive force to be transduced into high-affinity transporters in the spatially separate OM. The TonB system in the CM (TonB, ExbB, and ExbD) plays a key role in energy transduction events at the OM (for recent reviews, see references 62, 76, and 77).

TonB, ExbB, and ExbD form a complex in the CM through their transmembrane domains (5, 22, 28, 31, 33, 34, 48, 60, 67, 73, 74). TonB is present in the CM as a dimer (19, 68). The TonB/ExbB/ExbD ratio in the cell is 1:7:2, although it is not known if this value reflects the ratio of the three proteins in an energy transduction complex (23, 26). Unlike ExbB and ExbD, approximately one-third of the total cellular TonB is found associated with the OM following sucrose density gradient fractionation (50). Present data suggest that TonB achieves OM association through a shuttling process whereby the entire protein transits out of the CM following initial OM contact and associates entirely with the OM (45, 50). Alternative mechanical models attribute TonB in the OM fractions to the superior strength of the association between the TonB carboxy terminus and OM transporters while in vivo TonB remains tethered to the OM. TonB can be dissociated from OM fractions by high salt concentrations (27). Although existing data support the shuttle model, the question of the TonB mechanism is not resolved, and the oligomeric state of TonB at the OM in vivo is not known. For recent reviews of the issues involved, see references (4, 11, and 63).

TonB transduces the protonmotive force into a different form of energy (chemical, mechanical, or stored conformational change) that enables active transport through OM transporters. The means by which TonB enables active transport through these unusual OM proteins is unknown (3, 66). The TonB-gated OM transporters are 22-stranded β-barrels, with lumens that are occluded by an amino-terminal globular domain (also known as a cork; for reviews, see references 12, 13, 18, 37, 51, and 69). These transporters bind their ligands with subnanomolar affinities, and it is thus most likely the action of TonB that allows the release of the ligands into the periplasmic space. Periplasmic binding proteins retrieve the ligands and deliver them to ABC transporters in the CM (for a review, see reference 39).

At the extreme amino termini of the globular domains of TonB-gated OM transporters is a characteristic, periplasmically accessible sequence known as the TonB box, with which TonB amino acid residues in the region of Q160 can interact directly (8, 9, 57). The TonB box sequence is required for transporter activity (1, 24, 65, 66, 70). The defects in inactive TonB box missense mutants can be suppressed by mutations in TonB (Q160K, Q160L Q160P, or R158L) that do not appear

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to affect TonB activity (2, 6, 25). Group B colicins, protein toxins that kill sensitive strains of *Escherichia coli*, also use the TonB system to translocate across the OM. These colicins also have a TonB box at their amino termini (52).

One of several remaining questions is how the region around TonB Q160 participates in energy transduction. Even though mutations in the TonB box of OM transporter BtuB prevent vitamin B_{12} transport, the mutant BtuB TonB box continues to interact in vivo with residues in the TonB Q160 region; only the nature and efficiency of the interaction change (9). The nature of interactions between the TonB Q160 region and the TonB box also changes depending on the ligand that is being transported (10). In vitro in the absence of the TonB system, the BtuB TonB box becomes disordered and extends into the periplasmic space upon ligand addition (17, 53). In the cocrystal structure of BtuB with the TonB carboxy terminus, the TonB box of BtuB displaces the fourth β-strand in TonB and joins the remaining three β-strands as part of a β-sheet (72). This arrangement brings TonB Q160 into proximity with the BtuB TonB box and provides a rationale for the inactive BtuB TonB box L8P and V10P mutant proteins. The TonB box mutant proteins would not be able to participate in the formation of a β-sheet with TonB. It is less clear how the region around TonB Q160 would play a role, although a potentially important salt bridge between TonB R158 and BtuB D6 in the TonB box was identified. In the cocrystal structure of the ferrichrome OM transporter, FhuA, with the TonB carboxy terminus, the interaction of TonB R166 with FhuA Q56 in the globular domain was deemed to be important and hypothesized to mediate a mechanical shearing or pulling force resulting in the disruption of the globular domain to allow siderophore translocation. In the cocrystal structure, TonB R166 also interacts with FhuA A26 in the nonessential switch helix (15) of the globular domain (58) and two residues in the FhuA barrel, A591 and N594. Still less clear is how TonB suppressors of TonB box mutations may function.

While the importance of the TonB Q160 region seems obvious, it is not readily amenable to a scanning mutagenesis analysis. The replacement of TonB residues 159 to 164 individually with cysteine has no detectable effect on TonB activity (8). To begin to understand the function of this region of TonB, we engineered a series of mutant proteins with nested deletions centered on Q160. Here we show that, as we have observed in a previous study, deletions of residues at the TonB carboxy terminus have differential effects in assays for TonB activity (19, 20). The TonB Q160 region is not required for TonB activity since up to five residues centered on TonB Q160 can be deleted without inactivating TonB. TonB with a deletion of seven residues (S157 to Y163), however, did not associate with the OM and was inactive. Surprisingly, none of the arginyl residues in that region were required for TonB activity. The lowest level of activity observed in iron transport assays of mutants with single, double, or triple R→A substitutions was 70% of wild-type activity. Even the R154A R158A R166A R171A quadruple mutant TonB retained 30% of wild-type activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* KP1406 was constructed by P1*vir* transduction of Tn*10* linked to *aroB* from strain KP1270 (47) into KP1344. Deletions in the *tonB* gene of plasmid pKP325 (carrying *tonB* under the control of the arabinose pBAD promoter) were created using extralong PCR and a pair of divergent primers. Deletions designated Δ1, Δ3, Δ5, and Δ7 were also created in the plasmid backbones pKP472 (TonB C18G W213C) and pKP570 (TonB C18G F230C), and all amplified *tonB* genes were sequenced to rule out unintended base changes. The sequences of the primers are available upon request.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype	Reference
Strains		
W3110	F⁻ IN(*rrnD-rrnE*)*1*	29
KP1270	W3110 *aroB*	47
KP1344	W3110 *tonB*::*blaM*	47
KP1406	KP1344 *aroB*	Present study
Plasmids		
pBAD24	Arabinose promoter, AraC Amp^r	21
PKP325	pBAD24 (pBAD-regulated TonB)	47
pKP325 derivatives		
pKP477	Δ*tonB*	20
pKP489	TonBΔQ160	Present study
pKP490	TonBΔD159-P161	Present study
pKP491	TonBΔR158-Q162	Present study
pKP513	TonBΔS157-Y163	Present study
pKP493	TonBΔL156-P164	Present study
pKP494	TonBΔA155-A165	Present study
pKP813	TonB R154A	Present study
pKP873	TonB R158A	Present study
pKP814	TonB R166A	Present study
pKP815	TonB R171A	Present study
pKP887	TonB R154A R158A	Present study
pKP874	TonB R154A R166A	Present study
pKP875	TonB R154A R171A	Present study
pKP888	TonB R158A R166A	Present study
pKP889	TonB R158A R171A	Present study
pKP876	TonB R166A R171A	Present study
pKP890	TonB R154A R158A R166A	Present study
pKP891	TonB R154A R158A R171A	Present study
pKP892	TonB R154A R166A R171A	Present study
pKP893	TonB R158A R166A R171A	Present study
pKP935	TonB R154A R158A R166A R171A	Present study

TonB single R→A substitutions were generated from pKP325 by 30-cycle ultralong PCR using *Pfu* Ultra DNA polymerase from Stratagene. Forward and reverse primers were designed such that an alanine codon was substituted for the target arginine codon and flanked on either side by 12 to 16 homologous bases (primer sequences are available upon request). The template plasmid was removed by DpnI digestion, and mutations in the *tonB* gene were confirmed by cycle sequencing at The Pennsylvania State University Nucleic Acid Facility, University Park. Double, triple, and quadruple mutant plasmids were created by the same technique utilizing the single, double, and triple mutation-bearing plasmids, respectively, as templates. To avoid mutations occurring in unsequenced regions of the plasmid as a result of multiple PCR amplifications, all *tonB* genes corresponding to multiple amino acid substitutions were subcloned as a 914-bp BamHI fragment into plasmid pKP477 and proper orientation was confirmed by BstEII digestion.

Medium and culture conditions. M9 salts were prepared as described by Miller (54). To prepare minimal media, M9 salts were supplemented with 0.2% Casamino Acids, 40 μg/ml tryptophan, 4 μg/ml thiamine, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1.85 μM Fe (as ferric chloride), and 0.4% glycerol as a carbon source. For the expression of TonB proteins from plasmids, 34 μg/ml chloramphenicol and 0.002% arabinose were added to the medium. Liquid cultures were supplemented with 34 μg/ml chloramphenicol.

Steady-state levels of TonB deletion mutant proteins. The concentrations of arabinose required for the induction of TonB deletion mutant proteins to the

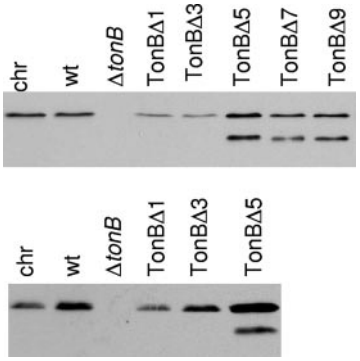

FIG. 1. Steady-state levels of TonB deletion variants. Upper panel: TonB expressed from the wild-type chromosome of W31100 (chr) and from strains KP1344/pKP325 (wild type [wt]), KP1344/pKP477 (TonB$^-$; Δ*tonB*), KP1344/pKP489 (TonBΔ1), KP1344/pKP490 (TonBΔ3), KP1344/pKP491 (TonBΔ5), KP1344/pKP513 (TonBΔ7), and KP1344/pKP493 (TonBΔ9). Samples used for the upper panel were the source of iron transport data for the wild-type, TonBΔ5, TonBΔ7, and TonBΔ9 strains in Table 2. Lower panel: TonB expressed from the wild-type chromosome of W31100 (chr) and from strains KP1344/pKP325 (wt), KP1344/pKP477 (TonB$^-$; Δ*tonB*), KP1344/pKP489 (TonBΔ1), KP1344/pKP490 (TonBΔ3), and KP1344/pKP491 (TonBΔ5). Samples used for the lower panel were the source of iron transport data for the TonBΔ1 and TonBΔ3 strains in Table 2. Samples were resolved on an 11% acrylamide gel and immunoblotted, and TonB was detected with anti-TonB antibodies.

level of chromosomally encoded TonB were determined to be 0.002% for TonBΔ7, TonBΔ9, and TonBΔ11 and 0.0005% for TonBΔ1, TonBΔ3, and TonBΔ5, as described previously (20). Cells were harvested at mid-exponential phase, precipitated with trichloroacetic acid, and analyzed by immunoblotting as described previously (42).

Sensitivity to colicins and bacteriophage. The ability of plasmid-encoded TonB variants to support sensitivity to group B colicins and bacteriophage ϕ80 was assayed with strain KP1406 (*tonB aroB*) as described previously (42). Cells were grown in M9 minimal medium supplemented with 1.85 μM Fe (as ferric chloride), 34 μg/ml chloramphenicol, and 0.002% arabinose and assayed on agar plates on top agar of the same composition. The results obtained were identical to those obtained with cells grown in tryptose broth (T-broth). The duplicate plate assays were repeated several times, and representative data are presented.

Sucrose density gradient fractionation. Strains were grown in M9 minimal medium supplemented with 1.85 μM Fe (as ferric chloride), 34 μg/ml chloramphenicol, and 0.002% arabinose and fractionated as described previously (19). Samples were resolved on 11% sodium dodecyl sulfate (SDS)-polyacrylamide gels (41) and evaluated by immunoblot analysis. The marker for the CM was NADH oxidase activity; the marker for the OM was the presence of a characteristic protein profile in OM fractions as determined by Coomassie blue staining of the polyvinylidene difluoride (PVDF) membrane following immunoblot analysis.

Cross-linking studies. In vivo formaldehyde cross-linking was carried out as described previously (28). Briefly, exponential-phase cultures in T-broth with 0.002% arabinose were harvested and suspended in 100 mM phosphate buffer, pH 6.8. Cells were then treated with 1% monomeric formaldehyde for 15 min and processed for immunoblot analysis. Samples were solubilized at 60°C for 5 min in Laemmli sample buffer to preserve cross-links.

For the detection of spontaneous in vivo disulfide-linked dimers, cells were grown to mid-exponential phase in T-broth supplemented with 0.2% arabinose. Cells were harvested and suspended in Laemmli sample buffer lacking β-mercaptoethanol and supplemented with 50 mM iodoacetamide as described previously (19). Samples were resolved on 11% SDS-polyacrylamide gels (41) and evaluated by immunoblot analysis.

SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Gel-resolved proteins were electrotransferred onto PVDF membranes. Immunoblot analysis was performed using TonB-specific monoclonal antibody 4F1, which recognizes a TonB epitope of residues 120 to 127 (46), and chemiluminescence detection as previously described (73). The loading of equivalent amounts of samples was confirmed by Coomassie staining of the PVDF membrane following immunoblot development.

Transport assays. Initial rates of [^{55}Fe]ferrichrome transport were determined as described previously (40, 61).

RESULTS

Previous studies have demonstrated that the TonB Q160 residue and those around it interact directly with the amino-terminal TonB box of the TonB-gated transporter BtuB in vivo (9, 57). To further define the requirements for TonB activity in that region, a set of nested deletions was generated, centered on TonB Q160. They ranged from Δ1, the deletion of only Q160, to Δ3 (deletion of N159 to P161), Δ5 (deletion of R158 to Q162), Δ7 (deletion of S157 to Y163), Δ9 (deletion of L156 to P164), and Δ11 (deletion of A155 to A165). All of the plasmid-encoded mutant TonB proteins could be expressed to the level of chromosomally encoded TonB in KP1344 (*tonB*::*blaM*) (Fig. 1 and data not shown).

TonB deletion mutants display assay-dependent phenotypes. The deletion of Q160 (TonBΔ1) had various effects on colicin sensitivity phenotypes. While retaining approximately 70% of wild-type ferrichrome transport rates and sensitivity to colicin Ia and bacteriophage ϕ80, cells expressing TonBΔ1 became completely insensitive to colicin D and were nearly insensitive to colicins B and M, with incomplete zones of clearing at every dilution after the fourth (Table 2). These results were reminiscent of those obtained with alanyl substitutions at aromatic residues in the TonB carboxy terminus, when differential phenotypes were observed depending on the assay used (20). In addition, changes in this region have previously been associated with phenotypic decreases specific to colicin B or colicin D sensitivity (7, 55). This result indicated that Q160 was not essential for TonB activity, consistent with previous results (78).

The deletion of residues 159 to 161 (TonBΔ3) greatly decreased TonB activity in every assay and resulted in complete

TABLE 2. Phenotypic characterization of mutants with TonB Q160 region deletions

Deletion	% of wild-type rate of Fe transport[a]	Sensitivity[b] of strain to:				
		ColB[c]	ColD[c]	ColIa[c]	ColM[c]	ϕ80[d]
Entire *tonB* gene	0	R	R	R	R	R
None (wild-type *tonB*)	100 ± 8	8, 8	7, 7	7, 8	7, 7	8, 8
Q160	56 ± 4	4, 4[e]	R, R	7, 7	4, 4[e]	8, 8
N159 to P161	0 ± 1	R, R	R, R	3, 3[e]	R, R	2, 2[f]
R158 to Q162	60 ± 4	6, 6	0, 0[g]	7, 7	5, 5	8, 8
S157 to Y163	0 ± 1	R, R	R, R	R, R	R, R	R, R
L156 to P164	0 ± 1	R, R	R, R	R, R	R, R	R, R
A155 to A165	0 ± 1	R, R	R, R	R, R	R, R	R, R

[a] Results ± standard errors are shown.
[b] Numbers are reciprocals of the highest dilution that showed evidence of killing or plaque formation. The results of two independent experiments are shown. R, insensitive to undiluted colicin.
[c] Fivefold serial dilutions of colicin B (ColB), colicin D (ColD), colicin Ia (ColIA), and colicin M (ColM).
[d] Tenfold serial dilutions.
[e] Fuzzy zones of killing at all dilutions where sensitivity was observed.
[f] Fuzzy zones of killing with all dilutions were observed, and no plaques formed.
[g] Undiluted.

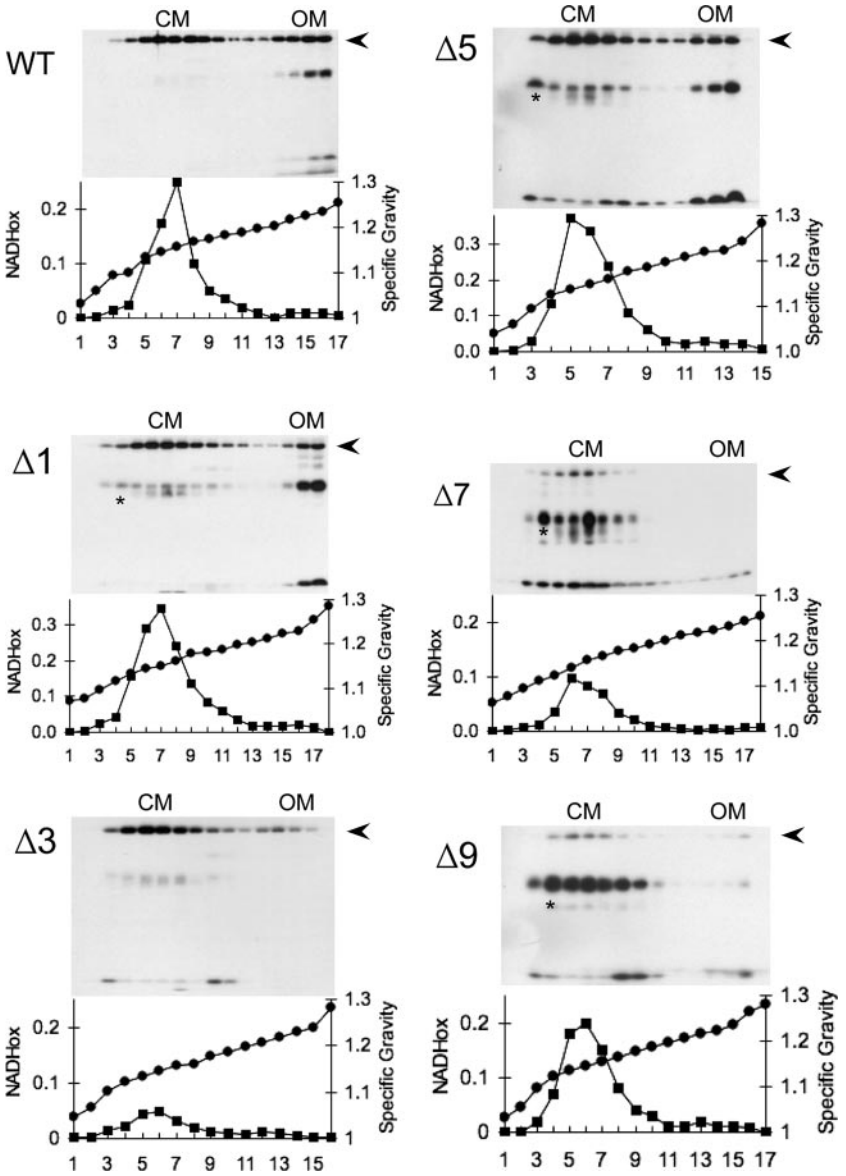

FIG. 2. Inactive TonB variants with deletions in the Q160 region do not associate with the OM. Immunoblots of sucrose density gradient fractionations are shown, each labeled with respect to the bacterial strain fractionated. WT, wild type (KP1344/pKP325); Δ1, KP1344/pKP489; Δ3, KP1344/pKP490; Δ5, KP1344/pKP491; Δ7, KP1344/pKP513; and Δ9, KP1344/pKP493. Samples were resolved on an 11% acrylamide gel and immunoblotted, and TonB was detected with anti-TonB antibodies. The positions of CM and OM fractions are indicated. The position of monomer TonB on each immunoblot is indicated by an arrowhead. Fractions containing soluble TonB degradation products are indicated by an asterisk. NADH oxidase activities (NADHox), in units (1 unit = 1 μmol/min NADH oxidized), and specific gravities of each fraction are indicated below each immunoblot.

sensitivity to colicins B, D, and M, a 10^{-6}-fold decrease in ϕ80 sensitivity, incomplete zones of colicin Ia clearing at all dilutions where sensitivity was observed, and an inability to support ferrichrome transport (Table 2). The deletion of residues 158 to 162 (TonBΔ5) unexpectedly restored sensitivity to the sixth fivefold dilution of colicin B, undiluted colicin D, and the fifth fivefold dilution of colicin M and full sensitivity to colicin Ia and bacteriophage ϕ80, while the initial rate of ferrichrome transport increased to 60% of the wild-type rate. Plasmid-expressed TonB proteins with deletions of seven or more amino acids (TonBΔ7, TonBΔ9, and TonBΔ11) were inactive in all assays.

The Q160 region is involved in OM association in sucrose density gradients. The deletion mutations affected a region of TonB known to interact with the OM transporters through the TonB box. The inactivity of deletion mutant proteins TonBΔ3 and TonBΔ7, TonBΔ9, and TonBΔ11 suggested that, unlike wild-type TonB, they would not be able to associate with the OM. To test this idea, cells expressing mutant proteins were fractionated on sucrose density gradients, with the result that only the relatively active mutant proteins TonBΔ1 and TonBΔ5 were able to associate significantly with the OM (Fig. 2). The TonBΔ11 mutant protein had the same frac-

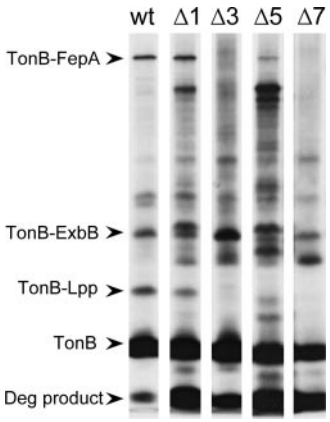

FIG. 3. Inactive TonBΔ3 and TonBΔ7 deletion variants do not cross-link to FepA or Lpp. Strains expressing wild-type (wt) TonB (KP1344/pKP325), TonBΔ1 (Δ1; KP1344/pKP489), TonBΔ3 (Δ3; KP1344/pKP490), TonBΔ5 (Δ5; KP1344/pKP491), and TonBΔ7 (Δ7; KP1344/pKP513) were cross-linked with formaldehyde as described in Materials and Methods. Samples were resolved on an 11% acrylamide gel and immunoblotted, and TonB was detected with anti-TonB antibodies. Positions of characteristic cross-linked complexes of TonB with Lpp, ExbB, and FepA are indicated by arrowheads on the left. The positions of monomeric TonB and a degradation (Deg) product are also indicated.

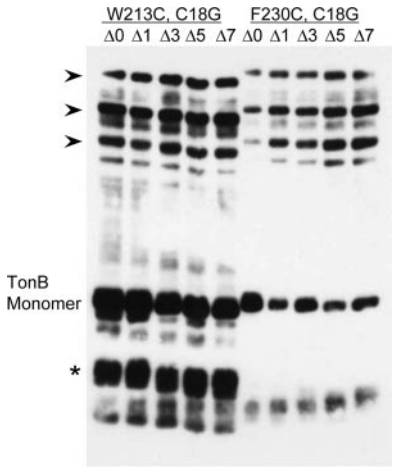

FIG. 4. Both active and inactive TonB variants with deletions in the Q160 region can form disulfide-linked dimers through the TonB carboxy-terminal Cys substitutions at aromatic residue W213 or F230. Strains expressing wild-type TonB (Δ0; KP1344/pKP325), TonBΔ1 (Δ1; KP1344/pKP489), TonBΔ3 (Δ3; KP1344/pKP490), TonBΔ5 (Δ5; KP1344/pKP491), and TonBΔ7 (Δ7; KP1344/pKP513) were harvested at mid-exponential phase and processed by boiling in gel sample buffer that lacked a reducing agent and contained 50 mM iodoacetamide. Samples were resolved on an 11% acrylamide gel and immunoblotted, and TonB was detected with anti-TonB antibodies. Identities of the TonB variants in each sample are indicated above each lane. The positions of the three characteristic disulfide-linked dimers are indicated by arrowheads. The position of monomeric TonB is indicated. Degradation products are indicated by an asterisk.

tionation profile as the TonBΔ7 and TonBΔ9 mutant proteins (data not shown).

The patterns of degradation products in the OM fractions shown in Fig. 2 correlated with significant levels of TonB activity, as seen in Table 2. Of the mutant proteins, only active TonBΔ1 and TonBΔ5 exhibited the OM degradation pattern characteristic of wild-type TonB, with significant levels of degradation products that were slightly smaller than full-length TonB and significant accumulation of degradation products at the dye front (50). Consistent with that idea, the TonBΔ3 mutant showed little evidence of functional interaction with the OM in that it entirely lacked the OM degradation products. The small amount of full-length TonBΔ3 at the OM may reflect the small amount of CM contamination that occurs in this procedure. The inactive mutant proteins TonBΔ7, TonBΔ9, and TonBΔ11 were unstable, as evidenced by the high proportion of degradation products relative to the full-length protein in the CM. Most importantly, due to the absence of any degradation products in the OM beyond levels that would arise from contamination with the CM, there is no evidence that they could ever associate with the OM. The TonBΔ1, TonBΔ5, TonBΔ7, TonBΔ9, and TonBΔ11 mutants had the unusual feature of degradation products that appeared in the soluble fractions of the gradient, as well as the CM (indicated by the asterisks in the relevant lanes of Fig. 2). The proportion of apparently soluble degradation products appeared to increase as greater portions of TonB were deleted.

Inactive TonB deletion mutant proteins do not cross-link to FepA. To determine whether the deleted regions were required for TonB interaction with various proteins, strains expressing the TonB deletions were cross-linked in vivo with formaldehyde, and their cross-linking profiles were compared to that of the wild type (Fig. 3). It has recently been shown that the cross-links of TonB to OM proteins Lpp and FepA represent unenergized TonB (19). Here, the results tended to mirror the transport results rather than those of the colicin and phage sensitivity assays, with TonBΔ1 and TonBΔ5 forming cross-linked complexes with FepA (73). TonBΔ1 formed complexes with Lpp and ExbB; however, it was not clear whether TonBΔ5 also formed these complexes since the candidate bands appeared at slightly different molecular masses (27, 28). The nature of the interactions of TonBΔ3, TonBΔ7, TonBΔ9, and TonBΔ11 with the OM proteins Lpp and FepA was changed such that no characteristic cross-links were detectable (Fig. 3 and data not shown). TonBΔ3 and TonBΔ7 apparently retained the ability to cross-link to ExbB. Unidentified complexes appeared in several of the mutants.

TonB mutant proteins with nested deletions formed spontaneous C-terminal disulfide cross-links. The in vivo formaldehyde cross-linking data suggested that deletions of the TonB Q160 region prevented contact with OM proteins. This result may be due to the deletion of an important region of direct contact between TonB and a TonB-gated transporter. Alternatively, because the Q160 region is part of the TonB carboxy terminus, the result may be due to an inability of the TonB carboxy terminus to assume an energized conformation. We have recently demonstrated that TonB mutant proteins with a cysteine substitution at carboxy-terminal aromatic residue F202, W213, Y215, or F230 spontaneously form disulfide-linked TonB dimers in the CM in vivo (19). These dimers form only if TonB can be energized through its transmembrane domain and ExbB/ExbD association. Thus, the ability to form disulfide-linked dimers can serve as an assay of the conformational fitness of the TonB carboxy terminus because dimer formation occurs prior to OM association. To

FIG. 5. Amino acid sequences of TonB variants with deletions in the Q160 region. Arg residues are underlined. Residue numbers are indicated at the end of each line. The types of deletions and the relative activities of the variants are indicated on the left.

determine the effect of Q160-centered deletions Δ1 through Δ7 on disulfide dimer formation by the TonB carboxy terminus, the deletions were engineered in plasmid-carried *tonB* W213C C18G and *tonB* F230C C18G genes and the expressed proteins were analyzed by immunoblotting on nonreducing SDS-polyacrylamide gels (Fig. 4). The protein variants with Q160-centered deletions retained the ability to form disulfide-linked dimers, indicating that none of the TonB Q160 region deletions interfered with the ability to achieve a conformation associated with an energetically competent TonB protein.

Arginyl residues in the Q160 region of TonB are not important for activity. The restoration of significant TonB activity in the TonBΔ5 mutant compared to the TonBΔ3 mutant suggested that the deletion of five residues restored an important sequence or relationship that was lacking when three residues were deleted. A comparison of the sequences remaining after deletion suggested that a pair of arginyl residues spaced six to seven residues apart might be important for TonB activity (Fig. 5). In addition, arginyl residues at TonB 158 and 166 were recently identified as making important contacts with BtuB and FhuA, respectively, in cocrystals with the TonB carboxy-terminal domain (58, 72).

TABLE 3. Arginyl residues in the TonB Q160 region are not essential for TonB activity

TonB substitution(s)[a]	% of wild-type Fe transport[b]
None (wild-type TonB)	100 ± 6
R154A	87 ± 8
R158A	89 ± 9
R166A	78 ± 6
R171A	110 ± 10
R154A, R158A	96 ± 6
R154A, R166A	79 ± 6
R154A, R171A	123 ± 10
R158A, R166A	97 ± 7
R158A, R171A	112 ± 10
R166A, R171A	81 ± 4
R154A, R158A, R166A	69 ± 7
R154A, R158A, R171A	75 ± 7
R154A, R166A, R171A	76 ± 6
R158A, R166A, R171A	72 ± 9
R154A, R158A, R166A, R171A	32 ± 6

[a] All TonB variants were expressed to the level of chromosomally encoded TonB.
[b] Results ± standard deviations are shown.

To test this hypothesis, TonB mutant proteins with substitutions for arginyl residues at positions 154, 158, 166, and 171 were examined for their ability to support ferrichrome transport (Table 3). Iron transport assays demand high levels of TonB activity for a positive result and can distinguish levels of TonB activity between 100 and ~20%. In contrast, colicin and phage sensitivity assays can overestimate the levels of TonB activity (42). Both single R→A substitutions and all combinations of double R→A substitutions had little effect on iron transport rates. The four triple substitutions retained 70 to 75% of wild-type TonB activity. Surprisingly, the quadruple R154A R158A R166A R171A TonB mutant protein still supported an iron transport rate of 30% of the wild-type rate.

DISCUSSION

The TonB Q160 region has been a subject of great interest ever since it was discovered to be a site where suppressors of TonB box mutations in the TonB-gated OM transporters occur (25). The genetic data were later supported by results from biochemical experiments demonstrating disulfide cross-linking between cysteinyl substitutions at TonB amino acids Q160, Q162, and Y163 and cysteinyl substitutions at residues 6 to 12 in the TonB box of the OM transporter BtuB (9). Somewhat surprisingly, the presence of uncoupling BtuB mutations L8P and V10P did not prevent interactions with the TonB cysteinyl substitutions but rather changed the degree of and the partners involved in disulfide cross-linking between TonB and BtuB. The picture that emerged was one of flexible interactions where, even though interactions between TonB and BtuB were preserved, the nature of the interactions varied depending on the mutations introduced into the TonB box of BtuB. Somehow the subtle changes in contacts between TonB and the transporter TonB box determine whether or not transport occurs.

To explore TonB-transporter interactions from the TonB perspective, we began a nested deletion analysis of the region centered on TonB Q160 to create TonBΔ1, TonBΔ3, TonBΔ5, TonBΔ7, TonBΔ9, and TonBΔ11. Sensitivity to bacteriophage ϕ80 requires as little as 1 molecule of TonB per cell for detection (42). In the bacteriophage sensitivity assay, TonBΔ5 (lacking residues 158 to 162) was fully as sensitive as wild-type TonB, while TonBΔ7, TonBΔ9, and TonBΔ11 were entirely insensitive to ϕ80 and inactive in all the other assays. Sensitiv-

ity to group B colicins combines two variables: first, the ability of TonB to interact with the OM transporter that serves as the colicin receptor, and second, most likely the ability to interact with the colicin itself, since group B colicins also have an essential TonB box at their amino termini (52). TonBΔ5 was severely impaired in colicin D sensitivity but retained full sensitivity to colicin Ia and slightly reduced sensitivity to colicins B and M. The test of the ability to transport iron is an assay that can discriminate between levels of TonB in the 20 to 100% range and thus provides the most accurate, time-sensitive estimate of TonB impairment (42). In this assay, TonBΔ5 was still able to support ferrichrome transport at 60% of the wild-type rate. Taken together, these results led to the surprising possibility that, in spite of its apparent importance in suppressing TonB box mutations, the five-residue region centered on TonB Q160 (TonBΔ5) was not essential to wild-type TonB activity. To put this in perspective, a single H20A substitution in the amino-terminal transmembrane domain of TonB eliminates all the TonB activities assayed as described above (43). Thus, the TonB suppressor alleles most likely represent novel gain-of-function mutations, although it is not clear what that function may be.

The ability of residues in the region surrounding TonB Q160 to discriminate between colicin D and colicin B has been observed previously (55). The two colicins are 96% identical throughout the first 313 amino acids that constitute the amino-terminal translocation domain (including the TonB box) and a central receptor-binding domain. They diverge in their carboxy-terminal killing domains, where colicin B has a pore-forming toxicity domain (64) and the colicin D toxicity domain is a tRNase (75). One difference between the translocation domains of these two colicins occurs in the TonB box, where colicin B has aspartyl and threonyl residues and colicin D has histidyl and seryl residues. Whereas wild-type TonB confers sensitivity to both colicins, in the work of Mora et al., TonB R158S and TonB P161L mutants were shown to be specifically insensitive to colicin D while retaining sensitivity to colicin B (55). Because these two colicins use the same OM receptor, FepA, the difference in the patterns of sensitivity of the TonB R158S and P161L mutants must be due entirely to interactions with the colicins themselves. The interactions of TonB R158S and TonB P161L with colicins are not, however, confined to the colicin TonB box regions. The TonB R158S and TonB P161L mutants exhibited only slightly decreased sensitivity to colicin B after the conversion of the colicin B TonB box into a colicin D TonB box (D17, T18→H17, S18). By analogy, it seems likely, therefore, that the interaction of TonB with OM transporters may not be confined solely to the transporter TonB box.

To further understand the TonB Q160 region, we attempted to discover why, when TonBΔ3 was almost entirely inactive, activity could be largely restored by the deletion of two additional residues. Our hypothesis was that the maintenance of correct spacing between arginyl residues previously suggested to be important in cocrystal structures of the TonB carboxy terminus with BtuB or FhuA was the key. The number of residues between R158 and R166 is seven. TonBΔ1 and TonBΔ5 both had a space of six to seven residues between arginyl residues (Fig. 5) and were both highly active. TonBΔ3, TonBΔ7, TonBΔ9, and TonBΔ11 had fewer numbers of residues between the arginyl residues, which correlated with their lack of activity. This hypothesis was refuted: TonB with alanyl substitutions at any three of four arginyl residues retained between ~70 and 75% of wild-type TonB activity. Even TonB with substitutions at all four arginyl residues retained ~30% of wild-type activity. Although charged residues can be important in protein-protein interactions, we conclude that TonB R154, R158, R166, and R171 are not essential for TonB-mediated energy transduction.

The phenotypic activities of the TonB variants with deletions centered on Q160 correlated best with their abilities to associate with the OM in sucrose density gradients, where only TonBΔ1 and TonBΔ5 associated with the OM and gave rise to the same proteolysis pattern in the OM as wild-type TonB. TonBΔ3 associated only slightly with the OM and had very little detectable activity. In addition, it lacked the proteolytic products that characterize active TonB. TonBΔ7, TonBΔ9, and TonBΔ11 in the CM fractions degraded significantly. There were also no full-length or proteolytic degradation products to suggest that they ever associated with the OM. Additionally, even though the in vivo formaldehyde cross-links between TonB and FepA represent interactions made by unenergized TonB (19), they at least represent a capacity for some sort of interaction between the two proteins. TonBΔ3 and TonBΔ7 (as well as TonBΔ9 and TonBΔ11) (data not shown) lacked even this capacity. We have previously shown that TonB Q160 is not in itself sufficient for OM association (44).

The deletion mutations yielded variants that were successively more sensitive to proteases in the cytoplasmic fractions, with TonBΔ7, TonBΔ9, and TonBΔ11 being so sensitive that very little full-length TonB was detectable. Although we have never previously detected soluble forms of TonB, here, soluble degradation products appeared in all the mutants except the TonBΔ3 mutant. These soluble fragments may arise from either the amino or carboxy termini of the deletion mutant proteins. If they arose from the carboxy terminus, they may represent a stable form of TonB similar to that observed in the crystal and nuclear magnetic resonance (NMR) structures. In the monomeric NMR structure, the TonB residues from 103 to 151 are unstructured (59). If the fragments contained the extreme amino terminus, they could have arisen by dissociation after shuttling to the OM (45). We have been unsuccessful in our efforts to determine the origin of these important soluble pieces of TonB.

The inability of TonB mutant proteins with Q160 region deletions to interact with OM proteins was not due to an inability of the carboxy terminus to form an energized conformation at the CM. At the initiation of energy transduction in the CM, the TonB carboxy terminus apparently has to fold correctly through an intermediate where the five aromatic residues F180, F202, W213, Y215, and F230 come into sufficiently close contact that disulfide-linked dimers form when cysteine is substituted for F202, W213, Y215, or F230 (19, 20). The dimers must be resolved into an unknown conformation for TonB to proceed to associate with the OM. When coupled with TonB W213C or F230C, the tested deletion mutant proteins (TonBΔ1, TonBΔ3, TonBΔ5, and the inactive TonBΔ7) were not impaired in the formation of disulfide-linked dimers, indicating that the region centered on TonB Q160 is not essential for that energy-dependent conformational change.

Taken together, the data suggest that residues centered on TonB Q160 constitute the region through which TonB forms an initial, stable association with OM transporters. This idea raises the question of how the Q160 region transits across the ~200-Å periplasmic space to contact OM transporters. While the proline-rich domain of TonB (residues 70 to 102) that precedes Q160 may serve as a 100-Å arm to facilitate that contact (16), it is not essential for TonB activity (49, 71). In the cocrystal structure with OM transporters FhuA and BtuB, the TonB carboxy terminus (residues 153 to 235) occupies only ~25 Å (58, 72). Presumably, a different structure is required to initiate contact with OM transporters.

One hypothesis is that the (broadly defined) TonB carboxy terminus is held in an unstructured or disordered conformation by ExbB/ExbD to allow the induced-fit recognition of ligand-bound OM transporters (43, 63). This hypothesis is consistent with much of our knowledge about protein disorder (14) and about TonB protein. First, disordered proteins are often modular, with independent domains. The TonB amino terminus can undergo a protonmotive force-dependent conformational change in vivo in the absence of the carboxy terminus (47), the carboxy terminus can bind independently to OM transporters (30, 35, 36, 38), and the intervening proline-rich region between the two domains can be deleted without affecting TonB activity. Second, the independent disordered domains act like beads on a string where the linker serves to allow a search by the attached domain. TonB searches for a ligand-bound OM transporter and does not transduce energy unless the search is successful (47). Third, protein ligands for binding can also be disordered prior to binding. In vivo, the BtuB TonB box appears to be disordered (10). Fourth, coupled folding and binding of the disordered domain often result in high specificity and low affinity, which are important for dissociation and reassociation at a new site. TonB must interact with numerous different ligands and dissociate after each interaction in order to be recharged for a subsequent round of energy transduction (32).

Consistent with these ideas, large regions of TonB are predicted to be disordered (43), and in the NMR structure of the monomeric TonB carboxy terminus (residues 101 to 239), residues from 101 to 151 are indeed disordered (59). It seems reasonable to speculate that ExbD, which has the same topology as TonB, may serve as the chaperone to manage the various conformational changes (possibly disorder-order transitions) that TonB goes through during an energy transduction cycle (47).

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