Regions of *Escherichia coli* TonB and FepA Proteins Essential for In Vivo Physical Interactions

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The transport of Fe(III)-siderophore complexes and vitamin B12 across the outer membrane of *Escherichia coli* is an active transport process requiring a cognate outer membrane receptor, cytoplasmic membrane-derived proton motive force, and an energy-transducing protein anchored in the cytoplasmic membrane, TonB. This process requires direct physical contact between the outer membrane receptor and TonB. Previous studies have identified an amino-terminally located region (termed the TonB box) conserved in all known TonB-dependent outer membrane receptors as being essential for productive energy transduction. In the present study, a mutation in the TonB box of the ferric enterochelin receptor FepA resulted in the loss of detectable in vivo chemical cross-linking between FepA and TonB. Protease susceptibility studies indicated this effect was due to an alteration of conformation rather than the direct disruption of a specific site of physical contact. This suggested that TonB residue 160, implicated in previous studies as a site of allele-specific suppression of TonB box mutants, also made a conformational rather than a direct contribution to the physical interaction between TonB and the outer membrane receptors. This possibility was supported by the finding that TonB carboxy-terminal truncations that retained Gln-160 were unable to participate in TonB-FepA complex formation, indicating that this site alone was not sufficient to support the physical interactions involved in energy transduction. These studies indicated that the final 48 residues of TonB were essential to this physical interaction. This region contains a putative amphipathic helix which could facilitate TonB-outer membrane interaction. Amino acid replacements at one site in this region were found to affect energy transduction but did not appear to greatly alter TonB conformation or the formation of a TonB-FepA complex. The effects of amino acid substitutions at several other TonB sites were also examined.

*Escherichia coli* and other gram-negative bacteria scavenge Fe(III)-bearing siderophores and vitamin B12 from the environment through a set of ligand-specific, high-affinity transport proteins displayed at the cell surface (outer membrane receptors). The subsequent release of ligand from the periplasmic face of the outer membrane receptor is an active process, dependent upon energy derived from the cytoplasmic membrane (4, 14). The spatial separation of transport events from their energy source necessitates a mechanism for energy transfer. This need is met by TonB, a cytoplasmic membrane protein that spans the periplasmic space to transduce cytoplasmic membrane-derived energy to the outer membrane receptors (most recently reviewed in reference 5).

The requirement of TonB for active transport at the outer membrane was evident in early experiments, in which mutations at the tonB locus were found to abolish transport of Fe(III)-complexed siderophores and vitamin B12, as well as the transport of certain colicins and the irreversible adsorption of bacteriophage that exploit this transport system (5). Studies of *E. coli* and *Salmonella typhimurium* TonB revealed a topology consistent with its role as an energy transducer. Proteinase accessibility studies of *E. coli* and *S. typhimurium* TonB (15, 34) and topology data obtained with TonB-Bla and TonB-PhoA hybrid proteins (15, 36) indicate that TonB is anchored in the cytoplasmic membrane by an uncleaved hydrophobic amino-terminal signal sequence, with the majority of the molecule arrayed in the periplasmic space. Despite the apparent attachment of TonB to the cytoplasmic membrane, the observation that missense mutations in a periplasmically localized region of TonB could weakly suppress a set of mutations involving a region (termed the TonB box) conserved among TonB-dependent outer membrane receptors suggested that TonB may directly interact with these proteins (3, 16, 38). Similarly, a synthetic peptide corresponding to the TonB box inhibits TonB activity (46), and the overexpression of a TonB-dependent outer membrane receptor could diminish the degradation of overexpressed TonB (13). These observations were confirmed by in vivo chemical cross-linking studies, which provided direct evidence for the physical interaction between TonB and the outer membrane receptor for the siderophore enterochelin (also known as enterobactin), FepA (43). FepA protein has a topology consistent with the β-barrel conformation that characterizes outer membrane proteins (2, 31). It has been shown to undergo a change in conformation following the binding of enterochelin (26), which could serve to signal TonB regarding its ligand-occupied status. A rather large region of FepA is exposed to the cell surface and appears to be involved in ligand binding, since deletion of that region converts FepA into an open channel (37).

One candidate for the TonB site that mediates direct interaction with outer membrane transporters is the region surrounding *E. coli* residue Gln-160, to which the suppressors of TonB box mutations map (3, 16, 38). Interestingly, while this residue itself is not conserved among the TonB molecules of other species, it is adjacent to a region that is highly conserved (residues 163 to 167) (23, 33). Other possible candidates include (i) a conserved region (residues 199 to 216 in *E. coli*)
with the predicted features of an amphipathic helix (20, 25, 33) and (ii) the extreme carboxyl terminus itself. Loss of the final eight carboxyl-terminal residues does not render TonB inactive, whereas loss of the final fifteen residues does (1). The interpretations of these results are complicated by the inherent instability of TonB molecules with carboxyl-terminal truncations (1, 7; also, the present study). Replacement of the final 32 residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein that was unable to transduce energy, while residues of TonB with a functional PhoA protein resulted in a more stable protein th...
cotransduction with tcpB::Tn10, with screening for kanamycin susceptibility. Strains bearing mutations in periplasmic protease-encoding genes or exbB::Tn10 were constructed by infection with P1::lac on donor strains, with selection by the appropriate antibiotic.

Strains carrying ΔompT::fepA-entF were derived by transduction of W3110 and MC4100 with P1::lac grown on UT5600 and then were recovered by being cultured in Luria-Bertani (LB) broth containing 100 mM sodium citrate for 5 h prior to selection in the presence of colicin B. Transduction of the deletion was confirmed by observing resistance to colicin D and the absence of reactivity on Chromzeurol S (CAS) indicator plates. These strains were the basis for the construction of all other ΔompT::fepA-entF-bearing strains used in this study.

Culture conditions. Tryptrone (T) plates, T top agar, and LB broth and agar were made as described in reference 30, and CAS agar was made as described in reference 40. Supplemented M9 broth was made as previously described (24, 25).

Strains were maintained on LB agar. Liquid cultures were grown with aeration at 37°C, except as noted otherwise, in either LB broth or M9 minimal salts. Antibiotics were used at the following concentrations: ampicillin at 100 μg ml⁻¹, chloramphenicol at 34 μg ml⁻¹, kanamycin at 20 μg ml⁻¹ (for transductions) or at 50 μg ml⁻¹ (for plasmid selections), pirazomam at 0.25 μg ml⁻¹, and tetracycline at 20 μg ml⁻¹. Strains carrying plasmids that encoded RNA suppressors were grown in ampicillin-containing media supplemented with 0.5 μg of freshly prepared clavulanic acid per ml.

Assays. The relevant phenotypes of putative TonB mutants and strains during their construction were determined by cross-streaking against undiluted bacteriophage and colicins as previously described (25). A quantitative analysis of tonB amber and ochre mutants in the presence and absence of their suppressors was performed by a modification of the spot titer technique previously described by Gudmundsdottir et al. (12). Cells, grown to an A500 of 0.4 (as determined with a Spectronic 20 spectrophotometer; path length = 1.5 cm), were harvested in 100-μl aliquots, suspended in 3 ml of molten T top agar (60°C), and overlaid onto T plates. Serial dilutions of bacteriophage φ80 (10-fold dilutions) or colicins (5-fold dilutions) were applied as 5-μl aliquots, and then the cells were incubated for 16 h at 37°C. Results were scored as the reciprocal of the highest dilution at which clearing of the lawn occurred.

Sequence determinations. The sequences of the tonB genes from selected mutants were determined either by the double-stranded sequencing (with a version 2.0 Sequenase kit) of pooled plasmid sets derived by PCR cloning as previously described (25) or by sequencing, without a plasmid intermediate, with a dsCycle sequencing kit and templates that were PCR amplified by using the methods and primers described previously (24).

Electrophoretic analysis of TonB proteins. Cells were grown in LB to an A500 of 0.4, precipitated with 1% formaldehyde, and incubated for 15 min at 22°C (43). Cells were pelleted and then suspended in Laemmli sample buffer at 0.005 A550/ml equivalent and solubilized by incubation at 97°C for 5 min. Samples containing 0.025 A550/ml equivalent were resolved by SDS-11% polyacrylamide gel electrophoresis (PAGE), electrottransferred to Immobilon-P membranes, probed with the TonB-specific MAB 4H4, and visualized by enhanced chemiluminescence as previously described (43).

In vivo chemical cross-linking. Cells were grown in LB to an A500 of 0.4, harvested in 1-ml aliquots, pelleted, suspended in 1 ml of 100 mM sodium phosphate buffer (pH 8.5) adjusted to 1% formaldehyde, and incubated for 15 min at 22°C (43). Cells were pelleted and then suspended in Laemmli sample buffer at 0.01 A550/ml equivalent μl⁻¹ and solubilized by incubation at 60°C for 5 min. Samples containing 0.20 A550/ml equivalent were resolved by SDS-PAGE and then electrotransferred, probed, and visualized as described above. Specific detection of FepA and FepA-containing complexes was performed in an identical manner with an anti-FepA MAB at a 1:25,000 dilution.

Whole-cell protease digestion of FepA. Approximately 6 × 10⁶ LB-grown mid-log-phase cells of E. coli ΔfepA strains carrying pITS249, pITS249-I14P, or pITS249-I229-332 were suspended in 100 μl of M9 salts and treated with proteases for 90 min at 25°C (or 37°C for staphylococcal V8 protease). Relative sensitivity was measured over the following range of protease concentrations: proteinase K, 0.1 to 40 mg ml⁻¹; trypsin, 12.5 to 200 μg ml⁻¹; and staphylococcal V8 protease, 50 to 250 μg ml⁻¹. The reactions were stopped by the addition of Pefabloc to 2.0 mM. Cells were harvested, washed with M9 salts containing 2.0 mM Pefabloc, suspended in SDS-PAGE loading buffer, boiled for 10 min, and electrophoresed on 7 to 18% Laemmli gradient gels. Proteins were electrottransferred onto nitrocellulose and immunoblotted with a 1:1,000 dilution of anti-FepA MAB IB9C4 that recognizes an epitope near the FepA amino terminus.

RESULTS

In vivo chemical cross-linking of wild-type TonB with FepA (Ile14Pro). In previous studies, treatment of live cells with 1% formaldehyde generated a characteristic set of TonB-containing complexes (23, 43). In addition to monomeric TonB (36-kDa apparent mass), four major complexes are identified by SDS-PAGE immunoblots, migrating with apparent molecular masses of 43, 59, 77, and 195 kDa. The compositions of the 43- and 77-kDa complexes remain unclear, whereas the 59-kDa complex contains TonB and the cytoplasmic membrane protein ExbB (24). The 195-kDa complex contains TonB and the outer membrane transporter FepA (43). In the present study, the ΔfepA strain MT912 was transformed with plasmids bearing either wild-type fepA or a fepA derivative encoding a prolyl substitution at Ile-14 in the TonB box that resulted in a complete loss of transport function for both ferric enterochelin and colicin B (data not shown) and the cells were chemically cross-linked in vivo with 1% formaldehyde. Samples were resolved in duplicate on the same gel, blotted, and then separated and probed with either an anti-TonB or an anti-FepA MAB (Fig. 1). In the absence of plasmid, MT912 probed with anti-TonB gave a standard cross-linking profile but lacked a band corresponding to the 195-kDa FepA-TonB complex. This band was restored by the plasmid encoding wild-type FepA but not by the plasmid encoding FepA(Ile14Pro). The identity of the TonB-FepA complex was confirmed in the duplicate blot probed with anti-FepA MAB. In the absence of the plasmid, FepA was not detected, whereas in cells bearing the plasmid encoding wild-type FepA, an abundance of both FepA monomer and degradation product were evident, as well as an apparent FepA dimer and a band that migrated at 195 kDa, corresponding to the TonB-FepA complex. This complex was not detected for cells encoding FepA(Ile14Pro). These results
indicated that the prolyl substitution in the TonB box of FepA resulted in a molecule unable to form the close physical associations with TonB normally detected by in vivo formaldehyde cross-linking and suggested that such close associations are required for the activity of a TonB-coupled receptor.

**Analysis of FepA(Ile14Pro) conformation.** Since the uncoupled phenotype of TonB box mutants is dependent upon substitution with amino acids (Pro and Gly) that may alter regional protein conformation, the mutations may not precisely define residues involved in the interaction but rather may reflect regional perturbations that affect the primary contact regions. To assess whether such conformational changes could be detected in FepA(Ile14Pro), its accessibility to externally supplied proteases was analyzed. By using a MAb that recognizes a FepA epitope near the amino terminus, the position of each proteolytic cleavage was mapped and identified on a FepA topology model (Fig. 2 and data not shown). The cleavage patterns from proteinase K were distinct between wild-type FepA and the TonB box mutant. Although the same proteolytic fragments (cleavage at sites P1 to P5) were produced, FepA(Ile14Pro) was more resistant at all concentrations, particularly at sites P2 and P5, which resulted in significant underrepresentation of the fragments of 35 and 24 kDa, respectively (Fig. 2). While site P2 was sensitive to proteinase K at 0.1 mg ml$^{-1}$, little if any cleavage at this site was detected in the Ile14Pro mutant even at 0.5 mg ml$^{-1}$. Additionally, the P5 fragment detected at high proteinase K concentrations in the wild-type receptor was not produced by cleavage of the TonB box mutant. Both sites P2 and P5 are predicted to be located in external FepA domains, with P2 in the ligand-binding gatekeeper domain (2). In contrast, both the wild-type and mutant receptors yielded equivalent levels of a 41-kDa fragment from cleavage at site S1 with staphylococcal V8 protease (Fig. 2). Neither receptor was very sensitive when whole cells were exposed to trypsin (data not shown); however, at high concentrations, cleavage at two sites, T1 and T2 (Fig. 2), could be detected, producing amino-terminal fragments of 28 and 39 kDa, respectively, but the FepA(Ile14Pro) cleavage pattern was indistinguishable from that of FepA (data not shown). Both the proteinase K cleavage site P1 (Fig. 2) and the trypsin site T1 (data not shown) were “unmasked” in FepA(Δ259-332), a deletion mutant constructed by the removal of the large external FepA domain involved in ligand binding. FepA(Δ259-332) is completely sensitive to proteinase K (0.1 and 0.5 mg ml$^{-1}$), producing amino-terminal 27- and 24-kDa fragments (Fig. 2) and is totally cleaved by trypsin (at 50 μg ml$^{-1}$) at site T1 to produce the 28-kDa fragment (data not shown).

The changes observed in the proteinase K cleavage patterns between wild-type FepA and the TonB box mutant could represent structural differences due to an intrinsic folding property of the proteins, but the differences could have been caused by TonB uncoupling. If there is an intrinsic difference, then the cleavage pattern for FepA(Ile14Pro) should remain distinc-
The ability to stabilize some of the truncated TonB proteins allowed us to determine the role of the carboxyl terminus in cross-linking to FepA. Strains encoding TonBam175 and TonBoc192 were cross-linked in vivo with formaldehyde, revealing abbreviated sets of TonB-containing complexes in all cases (Fig. 4). In the absence of Tsp protease, both TonBam175 and TonBoc192 were stabilized at the appropriate molecular mass, as we had seen before (Fig. 3). In the case of TonBam175 (Fig. 4A), successful cross-linking to ExbB was identified on the basis that the putative TonBam175-ExbB complex was smaller than the TonB-ExbB complex and was absent in an exbBD background. At higher masses, no other complexes were apparent, suggesting that no cross-linking to FepA had occurred. The ability of TonBam175 to cross-link to ExbB was not surprising since the amino-terminal signal anchor of TonB has been clearly identified as being responsible for that interaction (24, 43).

In the case of TonBoc192 (Fig. 4B), a complex of approximately 160 kDa was observed in the tsp strain at a sufficiently high molecular mass to potentially include FepA. To test that hypothesis, strains bearing a fepA deletion were cross-linked. Since the existence of the 160-kDa band was independent of the presence of FepA in cross-linkings of both TonBam175 and TonBoc192, we conclude that residues beyond 191 were required for the formation of the TonB-FepA complex.

**Informational suppression of tonB amber and ochre mutations.** Three of the TonB truncations examined in this study resulted from premature termination of protein synthesis at an amber codon (at positions 162, 175, and 215). Strains bearing these mutations were transformed with a set of nine plasmids encoding synthetic tRNA suppressors (21, 27, 32). An immunoblot analysis found these suppressors to be highly efficient, with the majority of TonB occurring as a full-length protein for all amino acid substitutions except proline, provided that cultures were supplemented with clavulanic acid to maintain plas-
mid carriage (data not shown). The phenotypes of \( \text{tonB}_{\text{am}} \) strains carrying these plasmids were determined by spot titer assays against TonB-specific colicins and bacteriophage \( \phi 80 \) (Table 2). All substitutions at positions 162 and 175 resulted in TonB proteins with essentially wild-type levels of activity, whereas the phenotypes of cells carrying substitutions at TonB residue 215 varied greatly. Although all substitutions at this position restored some degree of TonB activity; only those involving His or Phe restored activity approaching wild-type levels. To expand the range of amino acid substitutions, involving His or Phe restored activity approaching wild-type levels, whereas the phenotypes of cells carrying substitutions at TonB positions 104 and 177 and the Lys replacements of Gln at position 192 and Ser at 195 (data not shown).

The phenotypes of \( \text{tonB}_{\text{am}} \) alleles were transduced with \( P1 \) vir into strains carrying chromosomally encoded ochre suppressors and were evaluated as described above. In these strains the degree of suppression obtained was weaker than that seen with chromosomally encoded ochre suppressors and varied greatly between certain \( \text{tonB} \) alleles (data not shown). For some allele pairings, weak or absent TonB activity was noted; however, each such strain produced little or no demonstrable full-length TonB and was as such not informative. Substitutions at each of the four sites that did restore some level of activity were found. These included the Gln replacements of Lys at positions 104 and 177 and the Lys replacements of Gln at position 192 and Ser at 195 (data not shown).

**In vivo chemical cross-linking of termination-suppressed \( \text{TonB}_{\text{am215}} \).** Because data from substitution experiments indicated that Tyr 215 was important for TonB function and because Tyr is a formaldehyde-reactive residue (28), the in vivo chemical cross-linking profiles of strains encoding TonB molecules carrying termination suppressor-generated substitutions at position 215 were compared to that obtained with wild-type TonB (Fig. 5). The majority of unsuppressed \( \text{TonB}_{\text{am215}} \) oc-

### Table 2. Phenotypes of informationally suppressed \( \text{tonB}_{\text{am}} \) strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Substitution</th>
<th>Activity* in the presence of:</th>
<th>( \phi 80 )</th>
<th>colM</th>
<th>colla</th>
<th>colB</th>
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<tr>
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<td>( \text{tonB}_{\text{am162}} )</td>
<td>Gln→Ala</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Gln→Glu</td>
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<td></td>
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*Activity of TonB derivatives encoded from \( \text{tonB} \) amber mutations in the presence of plasmid-encoded suppressors relative to that of wild-type TonB.

*Activities were determined by spot titer assay as described in Materials and Methods and are expressed as the reciprocal of the highest dilution of agent at which clearing of the cell lawn was evident. Bacteriophage \( \phi 80 \) was assayed as serial 10-fold dilutions; colicins M (colM), Ia (colla), and B (colB) were assayed as serial 5-fold dilutions.

———, complete resistance.
curred as a 27-kDa degradation product that, when cross-linked, formed a 50-kDa TonB-ExbB heterodimer and a 34-kDa band corresponding to the 43-kDa TonB complex, while bands corresponding to the 77-kDa complex and the TonB-FepA complex were not evident. Full-length products of termination suppression were able to form all of the complexes normally detected for wild-type TonB (despite the fact that some substitutions involved aminoacyl groups not reactive with formaldehyde), with band intensities corresponding to the efficiencies of individual termination suppressors. This indicated that, although involved in TonB activity, Tyr 215 did not play an essential role in the formation of the 195-kDa TonB-FepA cross-linked complex.

**DISCUSSION**

Genetic studies have implicated one region of the outer membrane receptors as a potential site of interaction with TonB. This region, located near the amino terminus as a highly conserved seven-residue motif, occurs in all described TonB-dependent outer membrane receptors and has been termed the TonB box (39). Certain mutations in the TonB box abolish the transport of TonB-dependent ligands without altering the ability of the receptor to bind lipid (16, 35). Mutations in TonB involving the Gln-160 residue result in partial restoration of these transport functions (3, 16, 29, 38). At issue is whether this suppression is allele specific, indicating direct TonB-outer membrane receptor interactions, or is not allele specific, leaving the question of direct sites of interaction open. The outer membrane receptor TonB box appears to be quite plastic, as only a subset of the substitutions with potentially major conformational consequences affect ligand transport (11, 19, 38). Such plasticity is not consistent with the requirements of allele-specific interactions.

In the present study, the TonB box mutant FepA(Ile14Pro) did not interact with TonB in a manner that afforded either the transport of ligand or the detection of an in vivo chemically cross-linked complex. Although the wild-type TonB box of FepA was required for cross-linking with TonB, the replaced amino acid (Ile) could not be the residue through which this cross-linking occurred, since Ile is not formaldehyde reactive (28). This result suggested that the cross-linking occurred elsewhere and therefore involved a conformation of FepA influenced by the TonB box. A role for the TonB box in FepA conformation was confirmed by in vivo proteolysis studies of FepA and FepA(Met1-Pro). The Ile14Pro substitution altered the susceptibilities of distant, surface-exposed FepA regions to protease K, indicating that this mutation did have significant effects on FepA conformation. These effects occurred independent of the presence of TonB; thus, the distinct conformation detected reflected an intrinsic difference in the mutant FepA secondary structure rather than being the result of an inability to couple with TonB. These results were consistent with the previous suggestion that the TonB box carries primarily conformational information (11) and suggest that other regions of the outer membrane receptor contain sites through which interaction with TonB occurs but do not exclude the possibility that other interactions, not detected by cross-linking, directly involve the TonB box.

Cumulative data indicate that at least three different domains of the TonB molecule contribute in distinct ways to its function as an energy transducer. (i) The amino terminus (residues 1 to 32 in *E. coli*), in addition to providing an export signal and anchorage to the energy source (18), is also involved in an interaction with ExbB as well as in the mediation of conformational changes through which energy appears to be propagated (24). (ii) The proline-rich region [residues 63 to 102 in *E. coli*, consisting of a (Glu-Pro), and a (Lys-Pro), repeat separated by a short spacer] is not essential for activity but does enhance the ability of TonB to productively span the periplasm (25). As for mutations in the signal anchor, the deletion of this region does not block the ability of TonB to form cross-linked complexes with FepA in vivo. (iii) The carboxy terminus (residues 102 to 239 in *E. coli*) would certainly be the most likely candidate for interaction with the outer membrane receptors. However, the important residues within that 137-amino-acid region have not been completely defined. Thus, the characterization of the third relevant TonB region, through which the outer membrane connection is probably made, remains far from complete.

The majority of mutations that partially suppress TonB box mutations of outer membrane receptors and group B colicins involve replacement of *E. coli* TonB residue Gln-160 by any of three chemically dissimilar (Leu, Lys, or Pro) residues (3, 16, 29, 38). If the role of the TonB box is primarily conformational, this variability and the absence of absolute allele specificity both suggest that suppression is conformationally based. Supporting evidence that the suppression of TonB box mutations involves TonB conformation is provided by a study of TonB from *Enterobacter aerogenes* (7). This TonB bears an Asp residue at the site corresponding to *E. coli* Gln-160 and when expressed in *E. coli* can suppress a BtuB TonB box mutation, but only if a Gly residue near the extreme TonB carboxyl terminus (corresponding to *E. coli* residue 234) is replaced by Val. One interpretation of this result is that residue 160 affects TonB conformation at a distal site which is involved in productive interaction with outer membrane receptors (7).

The requirement for a specific residue near the carboxyl terminus of *E. aerogenes* TonB to suppress a BtuB TonB box mutant suggests that the extreme carboxyl terminus interacts with outer membrane transport proteins. This is difficult to reconcile with the apparent lack of sequence conservation in this region of TonB (23). Deletion of the eight carboxyl-terminal residues from *E. aerogenes* TonB removed residue 234 but did not inactivate TonB, consistent with a conformational effect rather than a direct interaction with BtuB. A larger deletion (13 residues) resulted in a complete loss of TonB function (7). Similar observations have been made with *E. coli* TonB, for which removal of the final 15 carboxyl-terminal residues deletes function, while removal of the final 8 carboxyl-terminal residues only diminishes function (1). In those studies, the carboxyl-terminally modified TonB proteins were proteolytically unstable, so it is unclear whether the essential nature of the region is based on a role in interaction with outer membrane receptors or simply in maintaining TonB integrity. Conversely, replacement of the final 32 *E. coli* TonB residues by the fusion of the PhoA polypeptide following TonB residue 207 results in a stable, membrane-anchored chimera. Despite its stability, this fusion protein lacks TonB function, whereas the fusion of PhoA to the intact carboxyl terminus of TonB does not disrupt function (36). Thus, it is clear that the carboxyl terminus of TonB is important, but there is no concrete information regarding the precise role of the carboxyl terminus in energy transduction.

In this study, the series of carboxyl-terminally truncated TonB molecules resulting from nonsense mutations were analyzed for their abilities to cross-link to FepA. It was initially important to stabilize these deleted variants, which were otherwise degraded to a common 140- to 145-residue membrane-anchored fragment, as seen in previous studies (1). In the absence of periplasmic protease Tsp, polypeptides that terminated at or before residue 191 were stable. Both 174- and
191-residue TonB derivatives retained the ability to chemically cross-link with ExbB, suggesting proper cytoplasmic membrane insertion, but were inactive and could not be chemically cross-linked to FepA. These results indicate that the final 48 residues of TonB are essential for cross-linking with FepA to occur. It is unclear whether the absence of cross-linking by truncated TonB resulted from a loss of the actual physical point at which cross-linking occurs or simply reflected the inability of truncated TonB to assume a conformation that supported cross-linking. In either case, it was apparent that the inability of truncated TonB to assume a conformation that cross-linked to FepA. These results indicate that the final 48 residues of TonB are essential for cross-linking with FepA to facilitate the association of TonB with the outer membrane receptor. It was thus not surprising that the spontaneous tonB mutations were initially isolated by Gwenda Wood. The tonB sequence for KP172 was determined by Camari Ferguson. We thank Penelope Higgs and Tracy Letain for their creative criticisms and discussions, Phil Youdarian for suggesting the use of clavulanic acid, and Mike Konkel for his critical review of the manuscript. This work was supported by grants awarded to K.P. (NIH-GM42146 and NSF-MCB08006) and to M.A.M. (NIH-GM54243). D.F.-H. was supported in part by an NRS postdoctoral fellowship (F32 AI09449) from the NIH.

REFERENCES

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