Conserved Residues Ser\textsuperscript{16} and His\textsuperscript{20} and Their Relative Positioning Are Essential for TonB Activity, Cross-linking of TonB with ExbB, and the Ability of TonB to Respond to Proton Motive Force* 

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The cytoplasmic membrane protein TonB couples the proton electrochemical potential of the cytoplasmic membrane to transport events at the outer membrane of Gram-negative bacteria. The amino-terminal signal anchor of TonB and its interaction with the cytoplasmic membrane protein ExbB are essential to this process. The TonB signal anchor is predicted to form an \( \alpha \)-helix, with a conserved face comprised of residues Ser\textsuperscript{16}, His\textsuperscript{20}, Leu\textsuperscript{17}, and Ser\textsuperscript{11}. Deletion of either Ser\textsuperscript{16} or His\textsuperscript{20} or of individual intervening but not flanking residues rendered TonB inactive and unable to assume a proton motive force-dependent conformation. In vivo formaldehyde cross-linking experiments revealed that the ability of this subset of mutants to form a characteristic heterodimer with ExbB was greatly diminished. Replacement of residues 17–19 by three consecutive alanines produced a wild type TonB allele, indicating that the intervening residues (Val, Cys, and Ile) contributed only to spacing. These data indicated that the spatial relationship of Ser\textsuperscript{16} to His\textsuperscript{20} was essential to function and suggested that the motif HXXXS defines the minimal requirement for the coupling of TonB to the cytoplasmic membrane electrochemical gradient. Deletion of Trp\textsuperscript{11} resulted in a TonB that remained active yet was unable to cross-link with ExbB. Because Trp\textsuperscript{11} was demonstrably not involved in the actual cross-linking, these results suggest that the TonB/ExbB interaction detected by cross-linking occurred at a step in the energy transduction cycle distinct from the coupling of TonB to the electrochemical gradient.

The outer membrane of Gram-negative bacteria serves as a diffusion barrier, that, by virtue of the lipopolysaccharide in the outer leaflet, presents a polar, negatively charged surface to the external surroundings, hindering the passage of detergents, hydrophobic antibiotics, and other toxic agents soluble in standard phospholipid bilayers. This barrier contains aqueous channels that are formed by general and substrate-specific porin proteins that enable diffusion of small, hydrophilic nutrients (<600 Da in Escherichia coli), across the outer membrane.

Simple diffusion is not sufficient for the acquisition of either the nutrient cobalamin or Fe(III)-siderophore complexes. Instead, these nutrients are efficiently harvested at the outer membrane surface by high affinity receptors, which catalyze the active transport of bound ligand into the periplasmic space (1–3). The release of cobalamin and Fe(III)-siderophores into the periplasmic space is energy-dependent, requiring an intact proton gradient at the cytoplasmic membrane (4–6). The separation of transport events from their energy source suggests the need for an energy transducer; this need is met by the protein TonB (5, 7–9). TonB is anchored to the cytoplasmic membrane presumably by a single transmembrane domain (amino acids 12–32), derived from an uncleaved signal sequence (10), with the bulk of the protein occupying the periplasmic space (11, 12). TonB spans the periplasm and directly contacts outer membrane active transport proteins (13, 14). Roughly one-third to one-half of the total cellular TonB proteins can be found physically interacting with TonB, as evident by in vivo cross-linking (17), which, in the case of ExbB, appears to involve the TonB signal anchor (18).

The TonB residues Ser\textsuperscript{16} and His\textsuperscript{20} occur on a conserved face of the predicted \( \alpha \)-helical TonB transmembrane domain (19). We previously noted that the deletion of a residue (Val\textsuperscript{17}) occurring between the two conserved residues rendered TonB inactive (18), suggesting either that residue Val\textsuperscript{17} made a side chain-specific contribution to energy transduction or conversely that the spatial relationship between Ser\textsuperscript{16} and His\textsuperscript{20} was essential to function. To resolve this dichotomy, we constructed a series of single residue deletions that spanned the amino-terminal two-thirds of the TonB transmembrane domain. Characterization of the resultant deletion mutants and of several substitution mutants suggested by our initial findings indicated that the positional relationship of Ser\textsuperscript{16} to His\textsuperscript{20} was an essential feature for the apparent coupling of TonB to the proton motive force (pmf).\textsuperscript{1}

**EXPERIMENTAL PROCEDURES**

Bacterial Strains and Plasmids—All bacteria used in this study were derived from the *E. coli* K-12 strain W3110 (20). The majority of experiments used the ampicillin-resistant tonB deletion strain KP1344 and its exbB::Tn10 derivative, KP1347 (16). Transport assays used an *aroB* derivative of KP1344, KP1351 (16). When necessitated by the use of ampicillin-selected plasmids, experiments were performed with the ampicillin-sensitive tonB deletion strains KP1229 (21) and KP1304 (con-
The resultant proteins identified as TonBL14/15 and TonBV23/24, respectively. This fashion are described in Table I.

The inserts ultimately verified by sequencing. Plasmids generated in altered characteristic restriction patterns, products bearing desired genic primers were designed to also introduce silent mutations that produced to the parent vectors cut at these same sites. Because the mutagenic Bam primers used for amplification flanked two mutation that includes a thermostabile ligase, such that the mutagenic a method adapted from that described by Michael (27), wherein a third, ExbB. The second gene in the operon, encoding ExbD, was unaltered by phosphorylated. Constructs were mapped for orientation, and inserts which resulted in steady state TonB levels near that of chromosomal vector (26) that had been restricted at the unique Sma I site and de

Resultant changes in the restriction profile to facilitate mutant identification. Amino acid residue changes in the encoded protein. Note that consecutive identical residues occur in TonB at positions 14 and 15 and positions b

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| Phenotypea | Restriction featuresa |
|-------------|----------------------|
| Acl site introduced |        |
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TonB Transmembrane Domain Mutants

| Plasmid | Genotypea | Phenotypea | Restriction featuresa |
|---------|-----------|------------|----------------------|
| pKP325 derivatives | | | |
| pKP411 | Δ364–366, G372A | TonBΔ14/15 | Acl site introduced |
| pKP412 | Δ367–369, G372A | TonBΔ14/15 | Acl site introduced |
| pKP409 | Δ370–372, C366A | TonBΔ15/16 | Acl site introduced |
| pKP404 | Δ373–375, C366A | TonBΔ15/16 | Acl site introduced |
| pKP402 | Δ379–381, C366A | TonBΔ15/16 | Acl site introduced |
| pKP405 | Δ382–384, C366A | TonBΔ15/16 | Acl site introduced |
| pKP419 | Δ388–390, T393C | TonBΔ15/16 | Acl site introduced |
| pKP420 | Δ391–393 | TonBΔ15/16 | Acl site introduced |
| pKP440 | G368T, G390T, G372A | TonBΔ15/16 | Acl site introduced |
| pKP441 | G384T, T386C, C387A | TonBΔ15/16 | Acl site introduced |
| pKP368 derivatives | | | |
| pKP421 | Δ394–396 | TonBΔ15/16 | Acl site introduced |
| pKP423 | Δ397–399 | TonBΔ15/16 | Acl site introduced |
| pKP424 | Δ400–402, G411A, T414C | TonBΔ15/16 | Acl site introduced |
| pKP422 | Δ403–405, G411A, T414C | TonBΔ15/16 | Acl site introduced |
| pKP390 derivatives | | | |
| pKP439 | G694T, G695C, C701A | ExbBΔ1510 | SspI site introduced |

a Base changes by position, numbered as per original tonB (28) and exbB (25) sequence.
b Amino acid residue changes in the encoded protein. Note that consecutive identical residues occur in TonB at positions 14 and 15 and positions 23 and 24. Because deletion of either residue in a given pair will result in the same protein, only a single deletion was made for each pair, with the resultant proteins identified as TonBΔ14/15 and TonBΔ23/24, respectively.

Resultant changes in the restriction profile to facilitate mutant identification.
room temperature 100 mM phosphate buffer (pH 6.8), followed by the addition of 62 μl of fresh 16% formaldehyde monomer. Suspensions were incubated for 15 min at room temperature, centrifuged, suspended in 50 μl of Laemmli sample buffer, incubated for 5 min at 60 °C, and then stored at −20 °C until analysis.

Electrophoretic Analysis of TonB Protein—Samples prepared as above were subjected to electrophoresis on SDS 11% polyacrylamide gels, and the resolved proteins were electrotransferred to polyvinylidene fluoride membranes. Immunoblot analyses were subsequently performed using TonB-specific mAbs and ECL, as previously described (30).

Transport of [55Fe]Ferrichrome—Ferrichrome transport assays were adapted from a study by Köster and Braun (31). The strain KP1351 (ΔtonB, aroB), carrying either a control plasmid (pACYC184), or plasmids encoding either wild type TonB (pKP325) or TonBΔ17-20μAA (pKP441) was grown to an A590 of 0.5 in supplemented M9 salts with chloramphenicol, defined amino acids, and 67 μg/L L-arabinose. Cells were centrifuged and then suspended to 2 × 106 colony-forming units/ml in M9 salts containing 0.1 mM nitritotriacetate, followed by incubation with shaking for 5 min. at 30 °C. Samples of 0.5 ml were removed and precipitated with trichloroacetic acid preparative to electrophoresis. Transport was initiated by the addition of 150 pmol of [55Fe]ferrichrome (generated by preincubation of deferriferrichrome with [55Fe]Cl3 at a 6.7:1 molar ratio in 10 mM HCl at 37 °C for 15 min) to 2 × 106 colony-forming units, with incubation continued by shaking at 30 °C. Samples of 0.5 ml were harvested at the indicated time points by filtration onto glass filters, which were then washed three times with 5 ml of 0.1 M LiCl and dried. Incorporated [55Fe] was determined by liquid scintillation counting.

RESULTS

Single Residue Deletions at Positions 16–20 Result in the Loss of TonB Activity—Certain bacteriophage and colicins have evolved to exploit the TonB-dependent transport system to gain entry into E. coli (32, 33), and their toxicity provides indirect assays for TonB function. In this study, the activity of TonB proteins carrying single residue deletions in the transmembrane domain was assayed by spot titer against the TonB-dependent bacteriophage φ80. Every attempt was made to express the plasmid-encoded proteins at chromosomal levels. TonB-encoding plasmids and controls were carried in the tonB deletion strain KP1344, with TonB expression induced by the presence of 1.3 mM L-arabinose in the top agar. All assays were performed in triplicate. Because this is a solid phase assay, direct determination of expression levels is problematic; however, this concentration of L-arabinose resulted in a level of activity and pmf responsiveness assays, with the ability of the entire set of single residue deletions (Fig. 3). Several minor deviations in the cross-linking profile were evident, including variations in the signal intensity of the several bands comprising the 77-kDa cluster of the TonBΔ17Δ19 mutants, increased signal intensity of the 43.5-kDa complex for the Δ17Δ19 mutants, and the appearance of a novel 66-kDa complex (indicated by the arrow in the right panel) for the Δ17Δ19 mutants. Because the non-TonB components of these complexes are not as yet determined, the implications of these differences were unclear. More readily interpretable were the effects of the mutations on the ability of TonB to form the 59-kDa heterodimer with ExbB. These results paralleled the results of the activity and pmf responsiveness assays, with the ability of the Δ16Δ20 mutants to form the characteristic cross-linked complex with ExbB greatly diminished or absent. This phenotype expressed relative to chromosomally encoded TonB.

Single Residue Deletions at Positions 16–20 Result in the Loss of TonB pmf Responsiveness—We had previously demonstrated that the conformation of TonB varies with the presence or absence of pmf (16). Comparison of spheroplasts in the presence and absence of a protonophore (CCCP or dinitrophenol) revealed that upon collapse of the cytoplasmic membrane proton gradient, wild type TonB acquires a distinct conformation wherein the amino-terminal 152–156 residues exhibit an enhanced resistance to digestion by proteinase K. This ability to respond to pmf changes is dependent upon the presence of Exb/B/D and is lost upon mutation of either TonB residues Ser16 or His20 or the deletion of residue Val17 (16). Here, identical patterns of pmf responsiveness were performed on the present deletion series (Fig. 2). The results obtained paralleled those of the φ80 spot titer assays (Fig. 1), with proteins bearing single residue deletions involving positions 16–20 rendered unresponsive to pmf, whereas proteins with deletions on either flank of this region (residues 10–14/15 and 21–23/24) retained pmf responsiveness (Fig. 2). In addition, each TonB derivative examined was susceptible to proteinase K in spheroplasts, indicating that negative phenotypes did not merely reflect improper secretion.

Some Single Residue Deletions Alter the in Vivo Chemical Cross-linking Profile of TonB—Wild type TonB can be cross-linked in vivo with formaldehyde to form a set of characteristic higher molecular mass complexes that includes a TonB–FepA complex that migrates with an apparent mass of 195 kDa, a cluster of uncharacterized complexes that migrate at 77 kDa, an additional unidentified complex that migrates at 43.5 kDa (13), and a TonB–ExbB heterodimer with an apparent molecular mass of 59 kDa (13, 17). Initial studies with TonBΔ17 indicated that the mutant could not form a detectable cross-linked complex with ExbB upon formaldehyde treatment yet retained the ability to form the other characteristic complexes (18). These experiments were extended here to include the entire set of single residue deletions (Fig. 3). Several minor deviations in the cross-linking profile were evident, including variations in the signal intensity of the several bands comprising the 77-kDa cluster of the Δ14/15Δ20 mutants, increased signal intensity of the 43.5-kDa complex for the Δ13Δ20 mutants, and the appearance of a novel 66-kDa complex (indicated by the arrow in the right panel) for the Δ17Δ19 mutants. Because the non-TonB components of these complexes are not as yet determined, the implications of these differences were unclear. More readily interpretable were the effects of the mutations on the ability of TonB to form the 59-kDa heterodimer with ExbB. These results paralleled the results of the activity and pmf responsiveness assays, with the ability of the Δ16Δ20 mutants to form the characteristic cross-linked complex with ExbB greatly diminished or absent. This phenotype
was also evident with the Δ14/15 mutant and, surprisingly, with the Δ11 mutant. Preliminary studies \(^2\) suggest the inability to detect cross-linked complexes of the Δ14/15 mutant with ExbB may be related to its apparent instability (see “Experimental Procedures”). Because tryptophan 11 is theoretically formaldehyde-reactive, the inability to detect cross-linked complexes of the Δ11 mutant with ExbB suggested the possibility that the actual cross-linking occurred through this residue. To test this possibility, two additional mutations were generated. First, the Trp\(^{11}\) residue of TonB was conservatively replaced by phenylalanine, a residue nonreactive with formaldehyde. Second, Trp\(^{38}\) of ExbB was replaced with a phenylalanine, because topological comparisons suggested that this was the nearest formaldehyde-reactive residue with which TonB Trp\(^{11}\) could interact. The TonB activity of strains carrying one or both of these alleles, as determined by spot titer assays, was indistinguishable from wild type (data not shown). Alleles were paired and evaluated by in vivo formaldehyde cross-linking (Fig. 4). Unexpectedly, replacement of TonB Trp\(^{11}\) with a nonreactive residue did not result in the loss of formaldehyde-mediated cross-linking of TonB to ExbB. Similarly, ExbB\(^{W38F}\) did not lose the ability to cross-link to TonB; however, the relative amount of complex formed appeared to be diminished with both wild type TonB and TonB\(^{W11F}\).

**Alanine Replacement of Residues 17–19 Does Not Alter the TonB Phenotype**—The deletion of either residue 16 or 20 or of the individual amino acids that reside between these positions resulted in essentially the same phenotype; inability to support 1,000 cpm/1 × 10^8 cells throughout the experiment (data not shown). Immunoblot analysis of TonB and the ferrichrome receptor FhuA levels in the assayed cultures indicated that the levels of both were essentially identical in cells expressing either wild type TonB or TonB\(^{VC117–19AAA}\) (data not shown).

**DISCUSSION**

TonB functions as an energy transducer, apparently harvesting the pmf of the cytoplasmic membrane to drive active transport at the outer membrane of Gram-negative bacteria. \(^{24–36}\) TonB-dependent processes can be blocked by protonophores (4–6) and, in udc strains (which lack membrane-bound ATP synthase), by cyanide (6). Together these results suggest pmf is necessary, but whether it is the direct, or even the sole energy source, is unresolved. Regardless of the energy source, recent observations suggest a dynamic model of energy transduction, wherein TonB transits through a set of conformations that alternately store and release potential energy, with transition to a higher energy isomer coupled to the energy source and transition to a lower energy isomer coincident to energy release to a ligand-occupied outer membrane receptor (16). The means by which TonB is coupled to the pmf of the cytoplasmic membrane is unknown, but it is clear that the transmembrane signal anchor of TonB resides 12–32 and its ability to interact with ExbB are required. Beyond targeting and tethering TonB to the energy source (10), the TonB signal anchor is essential for both activity (37, 38) and formation of the formaldehyde cross-linked TonB-ExbB heterodimer (38). More recent observations suggest a dynamic model of energy transduction, wherein TonB transits through a set of conformations that alternately store and release potential energy, with transition to a higher energy isomer coupled to the energy source and transition to a lower energy isomer coincident to energy release to a ligand-occupied outer membrane receptor (16). The means by which TonB is coupled to the pmf of the cytoplasmic membrane is unknown, but it is clear that the transmembrane signal anchor of TonB resides 12–32 and its ability to interact with ExbB are required. Beyond targeting and tethering TonB to the energy source (10), the TonB signal anchor is essential for both activity (37, 38) and formation of the formaldehyde cross-linked TonB-ExbB heterodimer (38). More recent observations suggest a dynamic model of energy transduction, wherein TonB transits through a set of conformations that alternately store and release potential energy, with transition to a higher energy isomer coupled to the energy source and transition to a lower energy isomer coincident to energy release to a ligand-occupied outer membrane receptor (16). The means by which TonB is coupled to the pmf of the cytoplasmic membrane is unknown, but it is clear that the transmembrane signal anchor of TonB resides 12–32 and its ability to interact with ExbB are required. Beyond targeting and tethering TonB to the energy source (10), the TonB signal anchor is essential for both activity (37, 38) and formation of the formaldehyde cross-linked TonB-ExbB heterodimer (38).
recently, we noted that TonB could assume a pmf-sensitive conformation. This required both a competent signal anchor and ExbB/D, with mutants such as TonB<sub>S16L</sub> and TonB<sub>H20Y</sub> unable to achieve the conformation unless an ExbB suppressor allele was present (16).

Further evidence for the importance of the TonB signal anchor stems from the ability of TolQ and TolR to substitute in part for ExbB and ExbD (39–41). These respective protein sets were all expressed in the Exb<sup>B</sup> strain KP1344. To normalize small differences in expression level, samples were first run in 10-μl aliquots, and the relative amount of TonB monomer was estimated visually (gel not shown). Samples were then loaded on a second gel in volumes of 5, 10, or 15 μl (except for TonB<sub>314/15</sub> for which 25 μl was loaded) to provide equivalent TonB signals for each sample. Samples were resolved and visualized as with Fig. 2, except that probing was performed with 1,2,500 nM 4F1 (specific for TonB residues 120–128; Ref. 29). The position of TonB monomer and of the standard TonB-containing complexes are indicated on the left. The position of the novel 66-kDa complex present in samples ΔV17ΔI19 is indicated by the arrow on the right.

The conserved face common to the signal anchors of TonB and TolA consists of the <i>E. coli</i> TonB residues Ser<sup>16</sup>, His<sup>20</sup>, Leu<sup>27</sup>, and Ser<sup>31</sup> (depicted in red, Fig. 6). Of these residues, mutation of Ser<sup>31</sup> did not appear to overly affect TonB activity (49). In our hands, mutations involving either Leu<sup>27</sup> or Ser<sup>31</sup> have not been recovered in extensive mutant hunts using both high and low stringency selections for TonB activity (16, 18, 50). Similar mutations of the corresponding residues in TolA do not disrupt function (51). Thus, residues Leu<sup>27</sup> and Ser<sup>31</sup> do not appear to be essential for energy transduction. Conversely, mutations involving either Ser<sup>16</sup> or His<sup>20</sup> have a profound impact on TonB activity. When expressed at wild type levels, TonB<sub>S16L</sub>, TonB<sub>H20Y</sub> (16), and TonB<sub>H20A</sub> lack demonstrable activity, whereas for TonB<sub>H20R</sub> (assayed under conditions of overexpression) activity was greatly diminished (49). Similarly, substitutions at the corresponding S and H residues of TolA render it inactive (51).

We previously isolated a <i>tob</i> mutation where loss of function resulted from the deletion of a single residue (Val<sup>17</sup>) in the region separating Ser<sup>16</sup> and His<sup>20</sup> (18). The phenotype of this mutant was similar to that of the later isolated Ser<sup>16</sup> and His<sup>20</sup> mutations, to the extent that ExbB suppressors isolated for Ton<sub>ΔV17</sub> and TonB<sub>S16L</sub> were essentially interchangeable (16, 18). When considered together, these and the above-cited results led us to consider whether or not transmembrane domain residues exclusive of positions 16–20 were of direct consequence to the coupling of TonB to the cytoplasmic membrane proton gradient. That hypothesis was examined using a set of single residue deletions, the phenotypes of which are summarized in Fig. 6.

Not surprisingly, deletion of either Ser<sup>16</sup> or His<sup>20</sup> resulted in a negative phenotype by all measures, similar to the results obtained with point mutations involving these residues (16). Deletion of any single intervening residue also produced a negative phenotype, consistent with the previous ΔV17 results (18). Deletion of single residues to the carboxyl-terminal flank of His<sup>20</sup> generated a wild type TonB, indicating that amino acids 21–24 have no role in energy transduction and suggesting that the position of Ser<sup>16</sup> and His<sup>20</sup> relative to the other conserved residues (Leu<sup>27</sup> and Ser<sup>31</sup>) was not essential to function. Deletion of single residues to the amino-terminal flank of Ser<sup>16</sup> also generated essentially wild type TonB (except for formaldehyde-mediated cross-link formation with ExbB by...
TonB<sub>17-19AAA</sub> was wild type by all measures, indicating that residues 17–19 by three consecutive alanines. The resultant energization mechanism. This was resolved by the substitution of one or more intervening residues contributed to the motif S<sub>16</sub>H<sub>20</sub> that occurs prior to the stage marked by cross-linking to ExbB. Thus, the reduction or absence of cross-linking to ExbB by TonB mutants unable to support energy transduction might reflect the fact that they are blocked in a portion of the cycle that occurs prior to the stage marked by cross-linking to ExbB.

Studies concerning the contribution of the TonB signal anchor to the recycling phase of the shuttling cycle are ongoing. The present study has addressed the role of the TonB signal anchor in the energization step of the cycle and has defined what appears to be the minimal structural requirement of TonB for efficient coupling to the cytoplasmic membrane proton gradient. It is evident that this process is facilitated by ExbB and ExbD; however, the means by which this occurs remain obscure. ExbB and ExbD can interact in vitro (52), they may occur as a heterohexamer, structurally consistent with the idea that they could be a proton translocator. They are also required for TonB to respond to the presence or absence of pmf (16). Understanding ExbB and ExbD will be essential for elucidating the mechanism by which TonB be-

**FIG. 5.** Transport of <sup>55</sup>Fe<sub>ferrichrome</sub>. KP1351 carrying plasmids encoding either wild type TonB or TonB<sub>VC17-19AAA</sub> were grown and assayed for uptake of <sup>55</sup>Fe<sub>ferrichrome</sub>, as described under “Experimental Procedures.” Data are expressed as cpm/cells. In four separate experiments, counting efficiency ranged from 23.5 to 34.6% (data not shown). Combined with a radiochemical purity of 99.9%, this predicts that 10,000 cpm corresponds to 0.63–0.93 pmol of <sup>55</sup>Fe<sub>ferrichrome</sub> transported. Values for wild type TonB are indicated by the filled triangles, whereas values for TonB<sub>VC17-19AAA</sub> are indicated by the open squares.

TonB<sub>3L14/15</sub> (which may be unstable) and TonB<sub>W11</sub>, discussed below), indicating that amino acids 10–15 have no role in energy transduction and suggesting that the position of Ser<sup>16</sup> and His<sup>20</sup> relative to the TonB cytoplasmic domain and the first several residues of the signal anchor was also not essential to function.

These results suggested two alternatives, either the position of Ser<sup>16</sup> and His<sup>20</sup> relative to each other was sufficient for effective coupling of TonB to the energy gradient or the side chains of one or more intervening residues contributed to the energization mechanism. This was resolved by the substitution of residues 17–19 by three consecutive alanines. The resultant TonB<sub>VC17-19AAA</sub> was wild type by all measures, indicating that the intervening residues did not make side chain-specific contributions essential for the energization of TonB. Beyond supporting the hypothesis that the positional relationship of Ser<sup>16</sup> to His<sup>20</sup> is necessary to function, these results suggest that the motif XXXH (where residues S and H occupy a common face of the α-helix) may define the minimal structural requirement for the coupling of TonB to the cytoplasmic membrane proton electrochemical gradient. It should be noted that the initial suspicion that Ser<sup>16</sup> and His<sup>20</sup> were of consequence came from their conservation in TolA and the suggestion that these residues would occupy the same face of an α-helix (19). In the absence of a solved crystal structure, the present data represent the strongest advocacy to date for such a structure.

The inability of TonB<sub>W11</sub> to form formaldehyde-mediated cross-links with ExbB, while otherwise remaining ostensibly wild type, is intriguing. Our initial suspicion that the explanation for this observation would prove to be trivial (i.e. the deleted tryptophan was the site of cross-link formation) was not borne out. Thus, we are left with the more exciting interpretation that this lack of detectable cross-linking could be indicative of an altered TonB conformation wherein the actual cross-linking site on TonB is no longer proximal to a formaldehyde-reactive site on ExbB. This suggests that the TonB/ExbB interaction detected by cross-linking is not essential to energy transduction. One possibility is that cross-linking detects an interaction that defines the recycling process by which spent TonB is shuttled back to a form competent to store energy. Thus, the reduction or absence of cross-linking to ExbB by TonB mutants unable to support energy transduction might reflect the fact that they are blocked in a portion of the cycle that occurs prior to the stage marked by cross-linking to ExbB.

Studies concerning the contribution of the TonB signal anchor to the recycling phase of the shuttling cycle are ongoing. The present study has addressed the role of the TonB signal anchor in the energization step of the cycle and has defined what appears to be the minimal structural requirement of TonB for efficient coupling to the cytoplasmic membrane proton gradient. It is evident that this process is facilitated by ExbB and ExbD; however, the means by which this occurs remain obscure. ExbB and ExbD can interact in vitro (52), they may occur as a heterohexamer, structurally consistent with the idea that they could be a proton translocator. They are also required for TonB to respond to the presence or absence of pmf (16). Understanding ExbB and ExbD will be essential for elucidating the mechanism by which TonB be-

**FIG. 6.** Thermodynamically minimized α-helical (3.6 residues per turn, 5.4 angstrom pitch) prediction for the TonB aminoterminal transmembrane domain region (residues 9–32), modeled in and adapted from Ref. 18. Residues 12–32 are predicted by average hydrophobicity (48) and topology (12) to represent the transmembrane domain. The orientation of the structure relative to the cytoplasm and the periplasmic space is indicated. Residues comprising the conserved face are indicated in red and identified by label. The data presented in Figs. 2–4 are summarized at the right of the figure at individual positions corresponding to the position of the involved residue depicted in the modeled structure. Activity is scored as the highest log dilution of $\Delta S_{\text{m}}$, at which clearing or plaque formation was evident, – indicates no clearing with undiluted $\Delta S_{\text{m}}$. For pmf sensitivity, the ability to form the pmf-dependent proteinase K-resistant product is indicated by +, whereas the apparent absence of such a product is indicated by –. For in vitro cross-linking to ExbB, the ability to form an identifiable TonB/ExbB heterodimer is indicated by +, whereas the apparent inability to efficiently form such a product is indicated by (–).
comes energized. A likely first step would involve determining whether or not ExbB (or ExbD) directly interacts with the TonB SXXXH motif and, if so, which features of ExbB participate in such an interaction.

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