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Taking the *Escherichia coli* TonB Transmembrane Domain "Offline"? Nonprotonatable Asn Substitutes Fully for TonB His20[▽]

Cheryl Swayne and Kathleen Postle*

Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

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The TonB system of Gram-negative bacteria uses the proton motive force (PMF) of the cytoplasmic membrane to energize active transport of nutrients across the outer membrane. The single transmembrane domain (TMD) anchor of TonB, the energy transducer, is essential. Within that TMD, His20 is the only TMD residue that is unable to withstand alanine replacement without a loss of activity. H20 is required for a PMF-dependent conformational change, suggesting that the importance of H20 lies in its ability to be reversibly protonated and deprotonated. Here all possible residues were substituted at position 20 (H20X substitutions). The His residue was also relocated throughout the TonB TMD. Surprisingly, Asn, a structurally similar but nonprotonatable residue, supported full activity at position 20; H20S was very weakly active. All the remaining substitutions, including H20K, H20R, H20E, and H20D, the obvious candidates to mimic a protonated state or support proton translocation, were inactive. A second-site suppressor, ExbB(A39E), indiscriminately reactivated the majority of H20 substitutions and relocations, including H20V, which cannot be made protonatable. These results suggested that the TonB TMD was not on a proton conductance pathway and thus only indirectly responds to PMF, probably via ExbD.

The TonB system of Gram-negative bacteria overcomes the limitations of the outer membrane (OM) barrier by providing a means to energize transport of large, scarce, important nutrients across the OM through active transporters (36, 39). The source of energy for this process is the proton motive force (PMF) of the cytoplasmic membrane (CM) (3). This unusual arrangement, whereby electrochemical potential energy from an inner membrane energizes transporters in a concentric outer membrane, was first postulated by Hancock and Braun in their studies of the role of the TonB system in irreversible adsorption of *Escherichia coli* bacteriophages (13).

TonB systems are found throughout Gram-negative bacteria, with the exception of *Francisella* (41). While they serve to mediate transport of iron-siderophores and vitamin B_{12} in *E. coli* K-12, the TonB system supports transport of a diverse range of substrates, ranging from maltose in *Campylobacter* to sucrose in phytopathogenic *Xanthomonas* (2, 28). *Xanthomonas campestris* boasts over 40 genes for TonB-gated transporters in the OM (39). The TonB system can be a virulence factor in human pathogens as well. *Neisseria meningitidis* uses the TonB system to acquire iron from human host proteins transferrin, lactoferrin, hemoglobin, and haptoglobin-hemoglobin (for a review, see reference 33).

Because colicins and bacteriophages also use the TonB system to infect *E. coli*, a variety of different phenotypic assays can be applied to mutant proteins in the TonB system. The assays vary in their sensitivity to TonB levels; thus, the broadest understanding of TonB activity is obtained from use of several. Bacteriophage ϕ80 infection is among the most sensitive of assays to very low levels of activity, but the least able to discriminate among higher levels of activity. Determination of initial rates of [^{55}Fe]ferrichrome transport is the most discriminative and can distinguish among TonB levels from ~10% to 100% of wild-type TonB. Colicin sensitivity is somewhat intermediate between those two extremes (22).

An *E. coli* TonB system consists of the three proteins in the CM and a TonB-gated transporter (TGT) in the OM. ExbB and ExbD appear to harvest the CM PMF and somehow cause TonB to link the harvested energy to a variety of OM active transporters by direct contact (for reviews, see references 5 and 31). ExbB (244 amino acids [aa]) has three transmembrane domains (TMDs), with most of the protein occupying the cytoplasm (18, 20). ExbD (141 aa) has a single TMD, with most of the protein occupying the periplasm (17). TonB (239 aa) has a similar topology to ExbD (14, 38). The three proteins can all formaldehyde cross-link to one another *in vivo*, and they appear to form a complex in the CM (6, 27, 32, 42).

The TonB TMD serves several roles. It plays an essential role in energy transduction, it is the signal sequence for export of TonB to the cytoplasmic membrane, and it appears to anchor TonB in a complex with ExbB and ExbD (16, 19, 20, 26, 37, 44). Histidine 20 is the most highly conserved residue in the TonB TMD (residues 12 to 32), and for *E. coli* TonB, it is the only important residue (8, 23). In *E. coli*, the entire TonB transmembrane domain can be simultaneously substituted for with alanine residues and retain nearly full [^{55}Fe]ferrichrome transport activity, if conserved residues S16 and H20 remain intact. Ser16 appears to be unimportant in the context of the wild-type TMD because the TonB S16A is fully active in [^{55}Fe]ferrichrome transport. In contrast, TonB(H20A) and TonB(H20Y) in the otherwise wild-type TMD are both inactive (23, 24).

Histidine is a protonatable residue on the proton conductance pathway of active secondary transporters such as Lac

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802. Phone: (814) 863-7568. Fax: (814) 863-7024. E-mail: postle@psu.edu.

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permease (12). The field has long thought in terms of a complex of TonB, ExbB, and ExbD, where their respective transmembrane domains form part of a proton conductance pathway. Consistent with that idea, ExbD D25 is also important for TonB-dependent energy transduction (6, 32). Analogous essential aspartate residues also exist in the TMDs of paralogues TolR and MotB, where they are proposed to function in proton conductance (7, 52). Some detailed models of predicted proton pathways through the complex include TonB H20 and ExbD D25 (49). For the MotA/B system, which lacks a TonB homologue, it has been proposed that the first TMD of MotA, which is not present in ExbB or paralogous TolQ, could fill that role.

Here we present a mutagenic analysis of the H20 residue, replacing it with all other 19 amino acids and also relocating it at every possible position in the TonB TMD (residues 12 to 32). Contrary to expectation, the only substitution that restored full TonB [^{55}Fe]ferrichrome transport activity was Asn, a nonprotonatable residue. The position of His20 within the TMD was also important. When relocated, His could only support detectable ferrichrome transport at one turn higher on the same face of the helix. A previously characterized second-site suppressor in ExbB reactivated most of the substitutions and relocations. Taken together, these results cast strong doubt on a role for H20 and the TonB TMD in proton conductance.

MATERIALS AND METHODS

Strains and plasmids. We used strain W3110 as the parent strain and from it created KP1270 (W3110; *aroB*), KP1344 (W3110; *tonB*::*blaM*), and KP1406 (W3110; *aroB tonB*::*blaM*) (15, 25). Strain KP1353 [W3110; Δ*tonB*::*blaM aroB zhf-3174*::Tn*10kan exbB*(*A39E*) *metC*::Tn*10*] was constructed from KP1344 (W3110; Δ*tonB*::*blaM*) in two steps. First, the *zhf-3174*::Tn*10kan* allele was transduced with P1*vir* into KP1344 by selecting for tetracycline resistance and screening for the linked *aroB* phenotype on CAS plates to create strain KP1351 (40). Second, the *metC*::Tn*10* allele from KP1100 [W3110; *exbB*(*A39E*) *metC*::Tn*10*] (26) was transduced into KP1351 and screened for the closely linked *exbB*(*A39E*) allele by restoration of bacteriophage ϕ80 sensitivity upon transformation of candidate transformants with plasmid pKP341 encoding TonB(H20Y). Strain RA1024 (W3110; Δ*exbBD*::*kan* Δ*tolQRA tonB*::*blaM*) was a kind gift from Ray Larsen. Plasmids used in this study are listed in Table 1. TonB(H20X) substitutions and His relocation mutants were created on either a pKP325 (*tonB*) or pKP381 [*tonB*(*H20A*)] template as previously described (47).

Media. Bacteria were maintained on LB (30) plates containing 34 μg/ml chloramphenicol when appropriate. Bacteria were grown in tryptone broth (T-broth) (30) with 34 μg/ml chloramphenicol for spot titer and steady-state analyses. Plates containing T broth, L-arabinose, and 34 μg/ml chloramphenicol in both top and bottom agar were used for spot titers. For formaldehyde cross-linking studies and [^{55}Fe]ferrichrome transport in KP1406, bacteria were grown in M9 minimal salts medium (30) supplemented as described previously (35). Media were also supplemented with L-arabinose to achieve chromosomal levels of expression, as noted in Table 1.

Spot titer assays. Spot titer assays were performed essentially as described previously (23). Exponential-phase cells were suspended in 3 ml T-top with 34 μg/ml chloramphenicol and L-arabinose as indicated in Table 1. Dilutions (5×) of colicins and dilutions (10×) of bacteriophage ϕ80 in λ-Ca^{2+} buffer were spotted on plates in triplicate up to the 11th dilution. Samples (0.2 A_{550}-ml) were taken to be trichloroacetic acid (TCA) precipitated to determine protein levels by SDS-PAGE and Western blotting. Assays were performed at previously determined chromosomal expression levels of the proteins. Chromosomal levels were verified by immunoblotting of each strain/plasmid immediately prior to the spot titer assay.

Determination of protein levels with and without ExbB. KP1344 (*exbB*$^+$) and RA1024 (Δ*exbB*) carrying TonB(H20X) plasmids were grown to mid-logarithmic phase in M9 mimimal medium in the presence of 0.003% L-arabinose, precipitated with trichloroacetic acid, and processed for immunoblot analysis with anti-TonB antibody 4F1.

Formaldehyde cross-linking. *In vivo* formaldehyde cross-linking was performed as described previously on KP1406 cells grown in M9 medium with 1.85 μM Fe (23). Cross-linked samples were analyzed by SDS-PAGE and Western blotting with anti-TonB antibody 4F1.

Proteinase K accessibility in spheroplasts. Proteinase K accessibility experiments were performed as described previously (25). Whole cells or spheroplasts were subjected to proteinase K treatment to determine whether TonB variants were properly exported.

[^{55}Fe]ferrichrome transport assays. Initial rates of iron transport were determined as described previously (34, 35).

RESULTS

Of all the H20X substitutions, only TonB(H20N) is fully active. To test the idea that TonB H20 was on the proton conductance pathway, the H20 codon was replaced with codons representing each of the other amino acids in an otherwise wild-type plasmid-encoded *tonB* gene (called "H20X" substitutions). The encoded proteins were expressed at chromosomally encoded levels as determined by immunoblotting (data not shown) and characterized by a panel of assays in a Δ*tonB* strain.

Surprisingly, TonB(H20N) was the only fully active substitution (Table 2 and Fig. 1). TonB(H20N) supported full sensitivity to colicins B, D, M, and Ia and bacteriophage ϕ80 and was fully active in [^{55}Fe]ferrichrome transport. None of the other H20X substitution mutants was able to support colicin sensitivity, except TonB(H20S), which was only weakly sensitive to colicins (Table 2) and did not support ferrichrome transport (Fig. 1). Very low levels of sensitivity to ϕ80, which uses the OM transporter FhuA as a receptor, were observed for all H20X substitution mutants. This slight activity could have arisen because the TonB carboxy terminus that contacts FhuA was able to achieve a correct carboxy-terminal conformation a very low percentage of the time (25). This probably accounts also for the previously observed sensitivity of TonB(H20R) to bacteriophages T1 and ϕ80 (46). Initial rates of iron transport were determined only for TonB(H20N) and TonB(H20S), which showed full and slight activities, respectively, in the colicin assays.

His20X substitutions are correctly assembled in the cytoplasmic membrane. Charged residues in TMD signal sequences can impede proper export across the cytoplasmic membrane via the Sec system (10). To test whether the inactivity of TonB with H20D, -E, -R, or -K substitutions was due to retention in the cytoplasm, proteinase K accessibility in spheroplasts was determined. Each of those substitution mutants was as sensitive to proteinase K in spheroplasts as wild-type plasmid-encoded TonB, exemplified by TonB(H20R) (Fig. 2) (data not shown). Mutants with H20F, -W, or -Y substitutions in TonB, which were tested because of their large size, were also as sensitive to proteinase K as wild-type TonB (data not shown).

Consistent with their correct export, the levels of TonB(H20X) formaldehyde cross-links to ExbB, FepA, Lpp, and OmpA were normal for all of the H20X substitutions, indicating correct assembly (data not shown). TonB is known to be proteolytically unstable in the absence of ExbB (9, 43). Each of the H20X substitution mutants became proteolytically unstable in a Δ*exbB* strain, as indicated by their significantly

TABLE 1. Plasmids used in this study

Plasmid	Description	Source	Required % of arabinose for expression[a]
pKP325	pBAD *tonB*	Larsen et al. (25)	0.00075
pKP477	Empty vector	Larsen et al. (25)	NA[b]
pKP381	pKP325 [TonB(H20A)]	Larsen et al. (23)	0.00075
pKP1196	pKP381 [TonB(H20C)]	Present study	0.0015
pKP1054	pKP325 [TonB(H20D)]	Ollis et al. (32)	0.0015
pKP1207	pKP381 [TonB(H20E)]	Present study	0.002
pKP1235	pKP381 [TonB(H20F)]	Present study	0.001
pKP1208	pKP381 [TonB(H20G)]	Present study	0.0015
pKP1211	pKP381 [TonB(H20I)]	Present study	0.00075
pKP1072	pKP325 [TonB(H20K)]	Present study	0.002
pKP1222	pKP381 [TonB(H20L)]	Present study	0.001
pKP1212	pKP381 [TonB(H20M)]	Present study	0.001
pKP1193	pKP381 [TonB(H20N)]	Present study	0.001
pKP1223	pKP381 [TonB(H20P)]	Present study	0.002
pKP1205	pKP381 [TonB(H20Q)]	Present study	0.0015
pKP1206	pKP381 [TonB(H20R)]	Present study	0.0015
pKP1239	pKP381 [TonB(H20S)]	Present study	0.0015
pKP1240	pKP381 [TonB(H20T)]	Present study	0.001
pKP1241	pKP381 [TonB(H20V)]	Present study	0.001
pKP1242	pKP381 [TonB(H20W)]	Present study	0.001
pKP341	pKP325 [TonB(H20Y)]	Larsen et al. (25)	0.001
pKP1179	pKP381 [TonB(P12H H20A)]	Present study	0.001
pKP1180	pKP381 [TonB(T13H H20A)]	Present study	0.00075
pKP1181	pKP381 [TonB(L14H H20A)]	Present study	0.001
pKP1182	pKP381 [TonB(L15H H20A)]	Present study	0.00075
pKP1187	pKP381 [TonB(S16H H20A)]	Present study	0.001
pKP1192	pKP381 [TonB(V17H H20A)]	Present study	0.0015
pKP1210	pKP381 [TonB(C18H H20A)]	Present study	0.0015
pKP1220	pKP381 [TonB(I19H H20A)]	Present study	0.002
pKP1209	pKP381 [TonB(G21H H20A)]	Present study	0.002
pKP1224	pKP381 [TonB(A22H H20A)]	Present study	0.002
pKP1271	pKP381 [TonB(V23H H20A)]	Present study	0.00075
pKP1230	pKP381 [TonB(V24H H20A)]	Present study	0.001
pKP1280	pKP381 [TonB(A25H H20A)]	Present study	0.00075
pKP1268	pKP381 [TonB(G26H H20A)]	Present study	0.0015
pKP1281	pKP381 [TonB(L27H H20A)]	Present study	0.0015
pKP1283	pKP381 [TonB(L28H H20A)]	Present study	0.00075
pKP1245	pKP381 [TonB(Y29H H20A)]	Present study	0.0015
pKP1266	pKP381 [TonB(T30H H20A)]	Present study	0.001
pKP1289	pKP381 [TonB(S31H H20A)]	Present study	0.00075
pKP1267	pKP381 [TonB(V32H H20A)]	Present study	0.001

[a] Required percentage (wt/vol) of L-arabinose to express TonB proteins at chromosomal levels in T-broth.
[b] NA, not applicable.

reduced steady-state levels, thus confirming that they can each interact with ExbB when it is present (Fig. 3) (data not shown). These data also ruled out the possibility that the H20X substitutions had undergone gross structural distortions.

Of the H20X substitution mutants, only active TonB(H20N) cross-links to ExbD. The ability of TonB to formaldehyde cross-link to ExbD *in vivo* is indicative of its activity and requires the cytoplasmic membrane PMF (32). To determine which H20X substitution mutants retained the ability to cross-link to ExbD, strains with mutant proteins expressed at chromosomal levels were cross-linked with formaldehyde, and the patterns of ExbD cross-linking were examined by immunoblotting (Fig. 4). TonB(H20N) was the only substitution mutant capable of detectably cross-linking to ExbD. The levels of the TonB(H20N)-ExbD cross-links were lower than those seen for wild-type TonB; this was most likely due to the lower steady-state level of TonB(H20N) in that experiment.

TonB H20 relocations define a single face of the transmembrane domain. The importance of the location of the H20 residue within the TonB transmembrane domain was assessed by individually replacing a histidinyl residue at residues 12 to 32 on a template expressing inactive TonB(H20A) (pKP381). The activity of these "His relocation" substitutions was determined by spot titer of colicins and phage and for those exhibiting colicin sensitivity by [^{55}Fe]ferrichrome transport (Table 3). Only TonB(V24H H20A) was active in the spot titer assays. It supported increased sensitivity to colicins B, D, and Ia and full sensitivity to bacteriophage ϕ80. In the more quantitative assay, it supported an initial rate of ferrichrome transport that was ~25% of the wild-type plasmid-encoded rate (Fig. 1). This is a minimum estimate of the activity since the presence of high levels of degradation products may have been inhibitory (29).

ExbB(A39E) suppresses both H20X and His relocation substitutions. We had previously isolated a set of second-site

TABLE 2. Colicin and bacteriophage ϕ80 sensitivities of H20X substitutions

Mutant type[a]	Sensitivity to[b]:				
	Colicin B	Colicin D	Colicin M	Colicin Ia	ϕ80
Chromosomal	8, 8, 8	7, 7, 7	4, 3, 4	8, 8, 8	8, 8, 8
pKP325	9, 9, 8	7, 7, 7	5, 4, 4	8, 8, 8	8, 9, 9
Δ*tonB*	T, T, T	T, T, T	T, T, T	T, T, T	T, T, T
H20A	T, T, T	T, T, T	T, T, T	T, T, T	0, 0, 1
H20C	T, T, T	T, T, T	T, T, T	T, T, T	0, 1, 1
H20D	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 1
H20E	T, T, T	T, T, T	T, T, T	T, T, T	2, 3, 2
H20F	T, T, T	T, T, T	T, T, T	T, T, T	0, 0, 0
H20G	T, T, T	T, T, T	T, T, T	T, T, T	0, 0, 0
H20I	T, T, T	T, T, T	T, T, T	T, T, T	0, 0, 0
H20K	T, T, T	T, T, T	T, T, T	T, T, T	0, 0, 0
H20L	T, T, T	T, T, T	T, T, T	T, T, T	0, 0, 0
H20M	T, T, T	T, T, T	T, T, T	T, T, T	0, 0, 0
H20N	8, 8, 8	7, 7, 7	4, 5, 5	8, 8, 8	9, 8, 9
H20P	T, T, T	T, T, T	T, T, T	T, T, T	1, 2, 1
H20Q	T, T, T	T, T, T	T, T, T	T, T, T	2, 2, 2
H20R	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 0
H20S	4*, 4*, 4*	1*, 1*, 2*	T, T, T	4*, 4*, 4*	2, 2, 3
H20T	T, T, T	T, T, T	T, T, T	T, T, T	0, 0, 0
H20V	T, T, T	T, T, T	T, T, T	T, T, T	0, 0, 0
H20W	T, T, T	T, T, T	T, T, T	T, T, T	3, 3, 2
H20Y	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 0

[a] Chromosomal indicates the wild-type strain W3110 carrying the vector plasmid pKP477. Δ*tonB* indicates strain KP1406 (Δ*tonB aroB*), which served as the background in which all plasmids were analyzed. Wild-type TonB from pKP325 and the TonB substitutions were expressed at chromosomal levels using the L-arabinose levels shown in Table 1.

[b] Colicins were spotted in 5-fold dilutions in λ-Ca^{2+} buffer, and bacteriophage ϕ80 was spotted in 10-fold dilutions in λ-Ca^{2+} buffer. Results of triplicate assays for the highest dilution at which clearing was observed are shown. T, tolerance (insensitivity) to the agent tested; *, clearing was faint at all dilutions. A result of 0 indicates that clearing was seen only with undiluted agent.

ExbB suppressors that restore partial colicin sensitivity to TonB with inactivating mutations in its transmembrane domain [TonB(ΔV17), TonB(S16L), or TonB(H20Y)]. The ExbB(V35E), ExbB(V36D), and ExbB(A39E) suppressors each change an uncharged residue at the cytoplasmic edge of the first transmembrane domain to a negatively charged residue. They are not allele specific (25, 26).

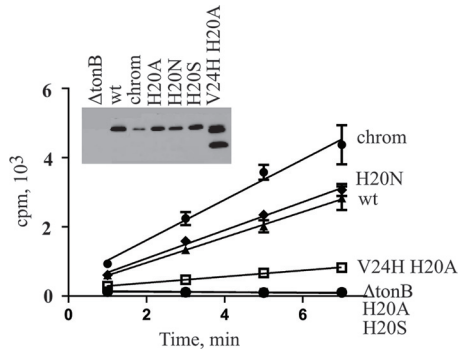

FIG. 1. TonB(H20N) supports full activity. Strains expressing the indicated versions of TonB at chromosomal levels were grown and assayed for initial rates of [^{55}Fe]ferrichrome transport as described in Materials and Methods. All time points were sampled in triplicate, with transport rates calculated by linear regression of the entire data set collected for each strain-plasmid combination for the time frame indicated. Relative rates of transport expressed in cpm per 0.2 A_{550}-ml per min are as follows: KP1270/pKP477 (chrom), 583.2 ± 67.05 (r = 0.8832); KP1406/pKP477 (Δ*tonB*), −8.733 ± 3.666 (r = 0.3620); KP1406/pKP325 (wild type [wt]), 366.9 ± 39.20 (r = 0.8976); KP1406 H20A, −9.067 ± 2.441 (r = 0.5797); KP1406 H20N, 406.7 ± 22.11 (r = 0.9713); KP1406 H20S, −4.083 ± 2.088 (r = 0.2766); and KP1406 V24H H20A, 89.84 ± 4.697 (r = 0.9734). (Inset) Steady-state levels of TonB in the strains assayed as determined by immunoblotting with anti-TonB (α-TonB) antiserum of samples precipitated with trichloroacetic acid at time zero (t = 0).

To learn more about the nature of the H20X substitutions, plasmid-encoded H20X substitutions were transformed into a strain expressing ExbB(A39E), the most effective suppressor, and assayed for degree of sensitivity to colicins and bacteriophage ϕ80 (Table 4). The results obtained depended upon the particular H20X substitution and, unexpectedly, upon the assay used to detect suppression. While only some H20X substitutions could be suppressed with respect to colicin sensitivity

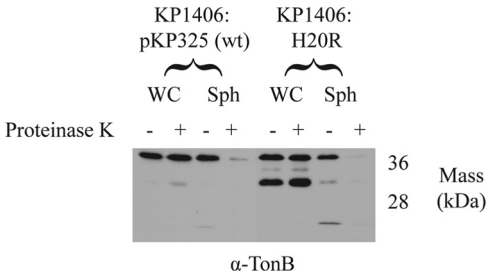

FIG. 2. H20X substitutions are correctly exported. Immunblots of strains expressing the TonB wild type (wt) or the TonB(H20R) substitution mutant in whole cells (WC) or spheroplasts (Sph) are shown. Samples were generated for TonBs expressed at chromosomal levels and treated with proteinase K (+) or left untreated (−) as described previously (25). Samples were resolved on 11% polyacrylamide–SDS gels, and immunoblots were developed with anti-TonB (α-TonB) antibody 4F1. Positions of mass markers are indicated on the right. Identical results were obtained for substitutions of K, D, E, F, W, and Y at H20 (data not shown).

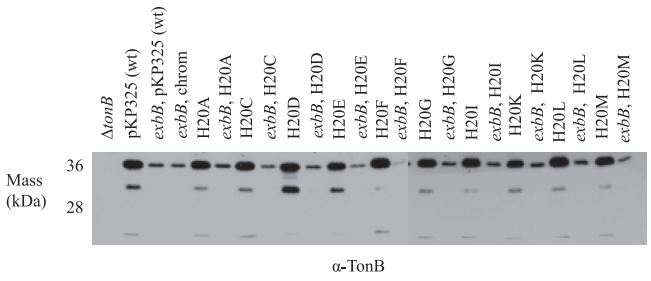

FIG. 3. ExbB stabilizes the H20X mutants. Expression of all H20X substitutions was induced in KP1344 (*exbB*$^+$) or RA1024 (Δ*exbB*) with 0.003% L-arabinose in M9 minimal medium. Cells were precipitated with TCA in mid-exponential phase and identical A_{550}-ml equivalents were analyzed by immunoblotting of 11% polyacrylamide–SDS gels and developed with anti-TonB (α-TonB) 4F1 antiserum. Positions of mass markers are indicated on the left. Two immunoblots with identical exposures were combined for this figure. Results for TonB(H20A) to TonB(H20M) are shown. Results for TonB(H20N) to TonB(H20Y) were identical (data not shown).

(H20C, -D, -E, -M, -Q, -R, -S, -T, -V, -W, and -Y), all of the H20X substitution mutants could be rendered more sensitive to ϕ80. That result could reflect the relative strength of the colicin preparations compared to the phage preparation. That interpretation seems unlikely since some of the strains expressing TonB(H20X) substitution mutants were insensitive to all colicins, but showed a high degree of sensitivity to ϕ80 [e.g., TonB(H20A) or TonB(H20L)].

Effects of the ExbB(A39E) suppressor on the activity supported by His relocation mutants were also determined (Table 5). Here again, all of the His relocation mutants were restored to increased ϕ80 sensitivity in the presence of ExbB(A39E). Most of the relocation mutants exhibited increased sensitivity to one or more colicins, while a few remained insensitive to all colicins (H20A with T13H, V23H, and S31H). Interestingly, TonB(V24H H20A) became almost completely inactive in the presence of ExbB(A39E), with only slight sensitivity to bacteriophage ϕ80 observed. The meaning of this inactivation was not clear.

DISCUSSION

The TonB system uses PMF of the cytoplasmic membrane to energize active transport across the outer membrane. The PMF is required for a formaldehyde cross-link between TonB and ExbD periplasmic domains. The presence of mutations in the TMD of either TonB(H20A) or ExbD(D25N) prevents formaldehyde cross-linking between them, but preserves detectable interaction of either mutant protein with ExbB (32). ExbB is apparently a scaffold on which TonB and ExbD assemble since it is the only protein in the complex that is proteolytically stable in the absence of the other two (1, 9; unpublished data). Consistent with that idea, ExbB is also required for formation of the TonB-ExbD formaldehyde cross-link (32). Thus, we hypothesized that TonB, ExbB, and ExbD form a complex in the cytoplasmic membrane such that the H20 residue of TonB was on the proton conductance pathway. That H20 is the sole important residue in the TonB transmembrane domain underscored that idea (23).

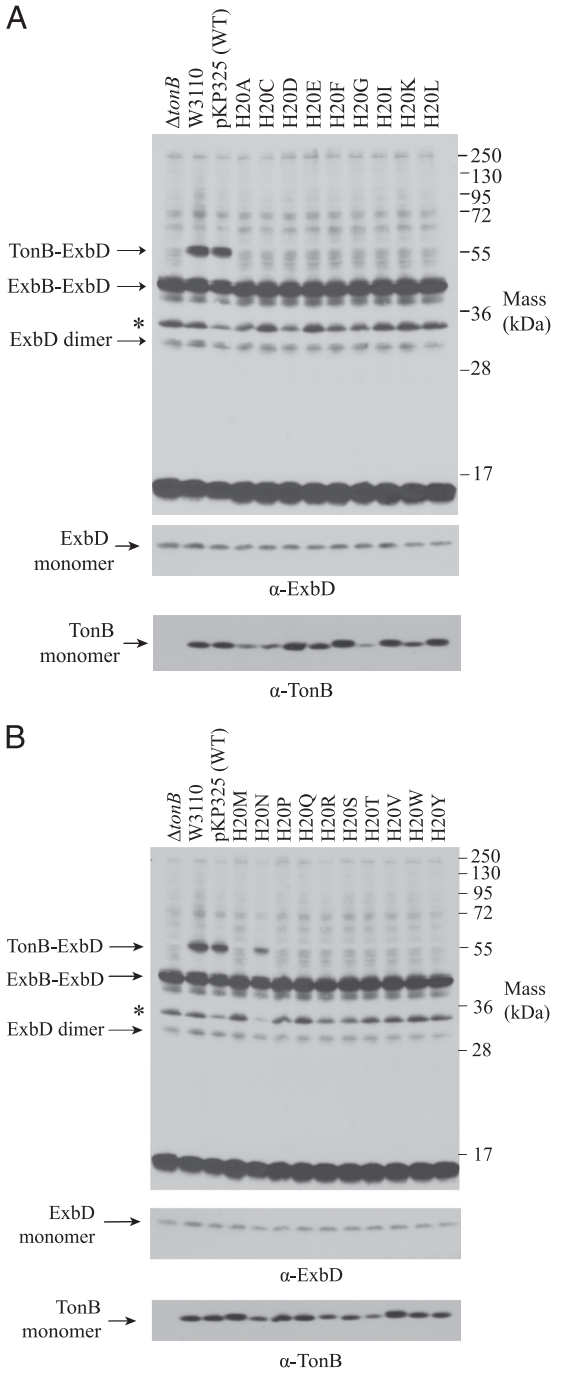

FIG. 4. Of the H20X substitution mutants, only TonB(H20N) retains the ability to cross-link to ExbD. KP1344 (Δ*tonB*), W3110 (wild type [WT]), KP1344/pKP325 (plasmid-encoded TonB), and KP1344 with plasmids expressing the H20X substitutions at chromosomal levels are shown. Strains were cross-linked with formaldehyde as described previously (34), and resolved on a 13% polyacrylamide–SDS gel. ExbD was visualized with anti-ExbD (α-ExbD) polyclonal antiserum. Positions of TonB-ExbD complex, the ExbB-ExbD complex, and the ExbD dimer are shown on the left. Shorter exposures showing relative ExbD and TonB monomer levels are immediately below the formaldehyde cross-linking blot. The positions of the mass markers are shown on the right. The asterisk indicates the position of an uncharacterized ExbD complex. Panel A shows results for TonB(H20A) to TonB(H20L), and panel B shows results for TonB(H20M) to TonB(H20Y).

TABLE 3. Colicin and bacteriophage ϕ80 sensitivities of His relocation mutants

Mutant type[a]	Sensitivity to[b]:				
	Colicin B	Colicin D	Colicin M	Colicin Ia	ϕ80
Chromosomal	9, 9, 9	8, 8, 8	4, 5, 3	9, 9, 9	9, 8, 9
pKP325 (wild type)	9, 9, 9	7, 7, 7	3, 3, 5	9, 9, 9	8, 8, 8
Δ*tonB*	T, T, T	T, T, T	T, T, T	T, T, T	T, T, T
P12H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 0, 0
T13H H20A	T, T, T	T, T, T	T, T, T	T, T, T	0, 0, 1
L14H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 0
L15H H20A	T, T, T	T, T, T	T, T, T	T, T, T	2, 2, 1
S16H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 1
V17H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 1
C18H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 1
I19H H20A	T, T, T	T, T, T	T, T, T	T, T, T	2, 1, 2
G21H H20A	T, T, T	T, T, T	T, T, T	T, T, T	2, 3, 4
A22H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 2
V23H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 1
V24H H20A	6, 6, 6	3, 3, 3	T, T, T	7, 7, 7	8, 9, 8
A25H H20A	T, T, T	T, T, T	T, T, T	T, T, T	3, 3, 3
G26H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 2
L27H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 2, 3
L28H H20A	T, T, T	T, T, T	T, T, T	T, T, T	2, 1, 1
Y29H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 0
T30H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 1
S31H H20A	T, T, T	T, T, T	T, T, T	T, T, T	2, 1, 1
V32H H20A	T, T, T	T, T, T	T, T, T	T, T, T	1, 1, 1

[a] Chromosomal indicates the wild-type strain W3110 carrying the vector plasmid pKP477. Δ*tonB* indicates strain KP1406 (Δ*tonB aroB*), which served as the background in which all plasmids were analyzed. Wild-type TonB from pKP325 and TonB mutants were expressed at chromosomal levels using the L-arabinose levels shown in Table 1.

[b] Colicins were spotted in 5-fold dilutions in λ-Ca^{2+} buffer, and bacteriophage ϕ80 was spotted in 10-fold dilutions in λ-Ca^{2+} buffer. Results of triplicate assays for the highest dilution at which clearing was observed are shown. T, tolerance (insensitivity) to the agent tested; *, clearing was faint at all dilutions. A result of 0 indicates clearing was seen only with undiluted agent.

The TonB TMD does not appear to be part of the proton conductance pathway. In this study, Asn, a nonprotonatable residue, was the only amino acid substitution out of all 19 substitutions tested at TonB H20 that restored full activity, even in the most discriminative assay, [^{55}Fe]ferrichrome transport.

What were the properties of Asn that allowed it to substitute for His? Aromaticity and charge were not important since Phe, Tyr, Trp, Lys, Arg, Glu, and Asp substitutions did not restore function. It was unlikely that TonB(H20N) could be participating in a water wire for proton conductance since other amino acid substitutions, possibly glycine or alanine, should also support that function but did not. There is a report that Asn can translocate protons as part of a hydrogen bond network (48). Glutamine might be expected to work equally well but supported no activity. Instead, Asn is the only amino acid with a structure similar to His, although it is somewhat smaller. For both His and Asn, the R group is a methylene CH_2 followed by a carbon that is singly bonded to N and doubly bonded to either O (asparagine) or C (histidine).

The results from the H20X substitutions suggested that, surprisingly, the important feature of H20 was its structure and not its ability to be protonated. The effect of the second-site suppressor ExbB(A39E) on the H20X substitutions also supported that idea. ExbB(A39E) indiscriminately suppressed H20X substitutions with large, small, polar, nonpolar, positively charged, negatively charged, or aromatic side chains to significant levels of TonB activity, as evidenced by nearly wild-type colicin sensitivity in many instances. These included TonB(H20W) and TonB(H20V), neither of which could somehow become protonatable.

We previously demonstrated that the conformation of the TonB carboxy terminus changes, depending on whether the PMF is present or absent (25). TonB is completely degraded by proteinase K in spheroplasts. If protonophores are added to collapse the PMF prior to addition of proteinase K, the amino-terminal ~150 amino acids of TonB become resistant to degradation. Consistent with that, inactive TonB(H20Y) does not form the proteinase K-resistant conformation. The results in this study suggest that TonB does not itself sense and conformationally respond to PMF because it lacks any protonatable residues in its TMD with which to do so. In that case, the likely mediator of the PMF-dependent conformational change is ExbD, which would transmit the conformational change to TonB through periplasmic domain contact. While it is not known whether ExbD can conformationally respond to the absence of PMF, it seems likely since PMF is required for TonB-ExbD interactions through their periplasmic domains (32).

H20 is required to correctly position TonB in the TonB/ExbB/ExbD complex. Taken together, these results suggested that TonB H20 has unique structural features. Previously, replacement of all residues in the TonB TMD with Ala except H20 and S16 still supported full TonB activity in the iron transport assay. However, the TonB TMD consisting entirely of Ala residues except H20 is essentially inactive, indicating that, within that context, S16 is required. In the context of a wild-type TMD, TonB S16 is not required. The cognate residues in the wild-type TonB TMD can apparently

TABLE 4. Suppression of the H20X substitutions by ExbB(A39E)

Mutant type[a]	Sensitivity to[b]:				
	Colicin B	Colicin D	Colicin M	Colicin Ia	φ80
Chromosomal	9, 9, 9	7, 7, 7	6, 5, 5	9, 9, 9	8, 8, 8
pKP325 (wild type)	8, 8, 7	6, 7, 6	3, 5, 5	7, 7, 7	8, 7, 7
Δ*tonB*	T, T, T	T, T, T	T, T, T	T, T, T	T, T, T
ΔV17	4*, 4*, 4*	3, 3, 3	T, T, T	T, T, T	3, 3, 5
H20A	T, T, T	T, T, T	T, T, T	T, T, T	6, 7, 8
H20C	3*, 4*, 4*	T, T, T	T, T, T	4*, 3*, 3*	7, 6, 6
H20D	4*, 4*, 5*	4, 4, 3	0*, 2*, 2*	T, T, 2	3, 5, 3
H20E	7, 7, 7	5, 6, 7	3, 3, 4	6, 6, 6	8, 7, 7
H20F	T, T, T	T, T, T	T, T, T	T, T, T	5, 5, 5
H20G	T, T, T	T, T, T	T, T, T	T, T, T	2, 3, 3
H20I	T, T, T	T, T, T	T, T, T	T, T, T	4, 5, 6
H20K	T, T, T	T, T, T	T, T, T	T, T, T	6, 5, 5
H20L	T, T, T	T, T, T	T, T, T	T, T, T	6, 6, 6
H20M	4*, 3*, 4*	3, 3, 3	T, T, T	3*, 2*, 2*	6, 7, 8
H20N	8, 9, 8	5, 6, 6	4, 5, 5	7, 7, 7	8, 7, 7
H20P	T, T, T	T, T, T	T, T, T	T, T, T	5, 7, 7
H20Q	7, 7, 7	3, 3, 4	2*, 3*, 3*	4, 5, 5	4, 4, 5
H20R	7, 7, 7	5, 5, 5	5, 5, 5	6, 6, 6	7, 8, 8
H20S	4*, 4*, 4*	3*, 3*, 2*	2*, 3*, 3*	3*, 2*, 3	7, 8, 7
H20T	5*, 6*, 7*	2*, 3*, 4*	3*, 3*, 3*	3, 3, 3	9, 8, 8
H20V	6, 6, 6	3, 4, 4	3*, 3*, 4*	4, 5, 4	7, 8, 8
H20W	7, 7, 7	4, 4, 4	5, 4, 4	5, 5, 4	8, 8, 7
H20Y	6, 6, 6	3, 3, 4	4, 4, 3	4, 4, 4	6, 7, 7

[a] Chromosomal indicates the wild-type strain W3110 carrying the vector plasmid pKP477. Δ*tonB* indicates strain KP1353 [*tonB aroB exbB*(*A39E*)] which served as the background in which all plasmids were analyzed. Wild-type TonB from pKP325 and TonB substitutions were expressed at chromosomal levels using the L-arabinose levels shown in Table 1.

[b] Colicins were spotted in 5-fold dilutions in λ-Ca^{2+} buffer, and bacteriophage φ80 was spotted in 10-fold dilutions in λ-Ca^{2+} buffer. Results of triplicate assays for the highest dilution at which clearing was observed are shown. T, tolerance (insensitivity) to the agent tested; *, clearing was faint at all dilutions. A result of 0 indicates clearing was seen only with undiluted agent.

collectively compensate for the absence of S16 such that TonB(S16A) is fully active in an iron transport assay. TonB S16 only becomes important in the absence of the cognate residues or if, in their presence, S16 is converted to Leu, a bulkier residue that likely inactivates TonB through steric hindrance (23, 26). The paralogous TolA TMD has a similar arrangement of H22 and S18 that probably explains why there is cross talk between the TonB and Tol systems (4, 11, 43).

The idea that H20 and S16 play structural roles was consistent with the fact that, while they are highly conserved, there are TonB transmembrane domains from other species that lack either or both of them (8). However, by themselves, the bioinformatics data did not rule out the requirement for His as a proton translocator in the *E. coli* TonB TMD. TonB systems of different species might transduce energy somewhat differently. An elegant study characterized a TMD His residue in *Pseudomonas* TonB1 that was important in *E. coli*, but not in *Pseudomonas* (51). However, *Pseudomonas* TonB1 differs from *E. coli* TonB in that it has a long N-terminal extension, and it does not appear to work with ExbB or ExbD (50). As another example, a fourth protein, TtpC, has been found to be essential for iron transport mediated by TonB2 of *Vibrio anguilarum* (21). Finally, for some species of TonB, the conserved His or other protonatable residue might have been moved to a TMD on the cognate ExbB or ExbD. In the *Vibrio alginolyticus* Pom flagellar system, the essential D24 in the PomB TMD could be moved to position 194 in PomA and retain activity (45).

The His20 or Ser16 can be moved one turn of the helix toward the periplasm. A His residue was relocated to each position on the TonB(H20A) transmembrane domain. The only active His relocation mutant was TonB(V24H H20A), which supported ~25% of the wild-type initial rate of [^{55}Fe]ferrichrome transport, a significant degree of activity. If, as is likely, the TonB TMD is an α-helix, it means that His must remain on the same face of the helix, but can relocate to a higher turn of the spiral at position 24 for activity. Likewise, TonB(H20S), with a Ser residue one turn higher on the helix in addition to position 16, was able to support low but detectable sensitivity to colicins B and Ia, even though the His residue was absent. Neither TonB(H20T) nor TonB(H20C) supported sensitivity to colicins, suggesting that the polarity of the Ser residue was not important. Alternatively, there could be hydrogen-bond-forming propensities and size prerequisites specific to the Ser residue.

His on a lower turn of the spiral at position 16, or any other position, was inactive. It has previously been speculated that His20 and ExbD D25 might form a salt bridge. We tested that possibility and obtained a negative result that was deemed uninterpretable (32). Results obtained here suggest that may not be the case since the only active His relocation substitution moved it further away from ExbD D25.

The results in these studies suggest that we must look elsewhere for the proteins that directly use PMF. ExbB and ExbD are the obvious candidates.

TABLE 5. Suppression of the His relocation mutants by ExbB(A39E)

Mutant[a]	Sensitivity to[b]:				
	Colicin B	Colicin D	Colicin M	Colicin Ia	ϕ80
Chromosomal	9, 9, 9	7, 7, 7	5, 4, 5	9, 9, 8	8, 8, 8
pKP325 (wild type)	8, 8, 8	6, 6, 5	5, 5, 5	6, 7, 7	8, 8, 8
Δ*tonB*	T, T, T	T, T, T	T, T, T	T, T, T	T, T, T
ΔV17	4*, 4*, 4*	3, 3, 3	T, T, T	T, T, T	3, 3, 5
P12H H20A	T, T, T	T, T, T	T, T, T	2*, 2*, 2*	6, 5, 6
T13H H20A	T, T, T	T, T, T	T, T, T	T, T, T	3, 4, 5
L14H H20A	4*, 4*, 4*	3, 2, 3	T, T, T	4, 4, 3	7, 6, 6
L15H H20A	T, T, T	T, 2*, T	T, T, T	2, 2, 3	5, 5, 6
S16H H20A	T, 1*, 3*	T, T, T	T, T, T	T, T, T	7, 6, 6
V17H H20A	T, T, T	T, T, T	T, T, T	2*, T, T	7, 5, 7
C18H H20A	5*, 2*, 3*	1*, 2*, 1*	T, T, T	2*, 3*, 1*	6, 6, 7
I19H H20A	T, T, T	T, T, T	T, T, T	T, T, T	5, 5, 6
G21H H20A	5, 5, 6	2*, 2*, 2*	4, 3, 3	4, 4, 3	7, 8, 8
A22H H20A	3*, 4*, 5*	2, 2, 3	T, T, T	4, 5, 5	4, 4, 5
V23H H20A	T, T, T	T, T, T	T, T, T	3, 3, 3	6, 7, 7
V24H H20A	T, T, T	T, T, T	T, T, T	T, T, T	3, 3, 4
A25H H20A	7, 7, 7	4, 5, 5	2, 2, 3	6, 6, 6	7, 8, 9
G26H H20A	T, T, T	2, 1, 1	T, T, T	5, 5, 6	4, 4, 5
L27H H20A	5, 5, 6	2, 3, 3	T, T, T	3, 4, 4	5, 5, 6
L28H H20A	5, 5, 5	T, T, T	3, 4, 4	4, 4, 4	7, 8, 8
Y29H H20A	3, 4, 5	2, 3, 2	T, T, T	3, 4, 5	7, 8, 8
T30H H20A	T, T, T	1*, 1*, 1*	T, T, T	2*, 2*, 3*	7, 7, 8
S31H H20A	T, T, T	T, T, T	T, T, T	T, T, T	5, 5, 5
V32H H20A	5, 6, 5	3, 3, 3	2, 2, T	T, T, T	3, 3, 3

[a] Chromosomal indicates the wild-type strain W3110 carrying the vector plasmid pKP477. Δ*tonB* indicates strain KP1353 [*tonB aroB exbB*(*A39E*)] which served as the background in which all plasmids were analyzed. Wild-type TonB from pKP325 and TonB substitution mutants were expressed at chromosomal levels using the L-arabinose levels shown in Table 1.

[b] Colicins were spotted in 5-fold dilutions in λ-Ca^{2+} buffer and bacteriophage ϕ80 was spotted in 10-fold dilutions in λ-Ca^{2+} buffer. Results of triplicate assays for the highest dilution at which clearing was observed are shown. T, tolerance (insensitivity) to the agent tested; *, clearing was faint at all dilutions.

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