Analysis of *Escherichia coli* TonB Membrane Topology by Use of PhoA Fusions

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Received 13 March 1991/Accepted 20 June 1991

Alkaline phosphatase (PhoA) fusions to TonB amino acids 32, 60, 125, 207, and 239 (the carboxy terminus) all showed high PhoA activity; a PhoA fusion to TonB amino acid 12 was inactive. The full-length TonB-PhoA fusion protein was associated with the cytoplasmic membrane and retained partial TonB function. These results support a model in which TonB is anchored in the cytoplasmic membrane by its hydrophobic amino terminus, with the remainder of the protein, including its hydrophobic carboxy terminus, extending into the periplasm.

The *tonB* gene product is required for energy-dependent transport of large (>600 Da) nutrients across the outer membrane of *Escherichia coli*. TonB couples the electrochemical potential of the cytoplasmic membrane to the active transport of vitamin B₁₂ and ferric siderophores across the outer membrane, to the energy-dependent steps of bacteriophage ϕ 80 and T1 infection, and to the entry of B-group colicins into bacteria. Thus, TonB functions to transduce energy between the cytoplasmic and outer membranes (for a review, see reference 16).

To understand the mechanism of TonB-dependent energy transduction, it is important to know the disposition of TonB within the cell envelope. Hydropathy plots of the TonB amino acid sequence indicate that it is markedly hydrophilic, with the notable exceptions of short hydrophobic regions at both the amino and carboxy termini (17). One or both of these regions could serve as membrane anchors. We recently showed that TonB is membrane associated and that it extends into the periplasm sufficiently to be accessible to proteinase K (18). In addition, we showed that the amino terminus of TonB functions as an export signal but is not proteolytically cleaved (18, 21). Previous studies had shown that TonB expressed during infection of UV-irradiated bacteria with λ tonB transducing phage is associated with the cytoplasmic membrane based on Sarkosyl fractionation (15). These results suggested that the amino terminus of TonB serves to anchor TonB in the cytoplasmic membrane, with the bulk of the polypeptide extending into the periplasm. However, the location of the carboxy terminus remained uncertain. The 11-residue hydrophobic region near the carboxy terminus is shorter than the 21 residues thought to be required to span the lipid bilayer in an α -helix. Nevertheless, the hydropathy profile of this region is similar to that of penicillin-binding protein 5, which is known to be anchored in the cytoplasmic membrane by its carboxy terminus (19). Thus, it seemed possible that the short hydrophobic region at the carboxy terminus of TonB could be a membrane anchor.

To further investigate the membrane topology of TonB, we constructed *tonB-phoA* gene fusions by oligonucleotide-directed deletion mutagenesis (1, 2). To avoid problems with potentially toxic TonB-PhoA fusion proteins (1, 3, 21), the expression of the *tonB* gene was first placed under the

control of the pBR322 *tetA* promoter-operator region in pKP287. Plasmid pKP287 was constructed by inserting a 1,697-bp *HincII tonB*⁺ fragment (17) into the *EcoRV* site in the *tetA* region of pJA113 (1). pJA113 is a derivative of pBR322 in which the *phoA* region of TnphoA (10) is fused to amino acid 45 of *tetA*. Cloning and restriction mapping of the plasmids were aided by use of the UWGCG (4) and CAGE/GEM (5) software packages. Starting with pKP287, a precise deletion of the 5' *tetA* coding region, the *tonB* promoter, and sequences 5' to the *tonB* coding region was made by oligonucleotide-directed mutagenesis. In the resulting plasmid (pKP288), the initiating methionine codon of *tonB* is positioned where the initiating methionine codon of *tetA* is positioned in pBR322. Starting with pKP288, *tonB-phoA* fusions were constructed by additional rounds of oligonucleotide-directed deletion mutagenesis. The details of the mutagenesis procedure will be described elsewhere (1). Fusion plasmids were constructed in the presence of pBST324 (13), which contains the pSC101 *tetR* gene inserted in the *bla* gene of pACYC177. Expression from the *tetA* promoter in pKP288 is repressed in the presence of pBST324 and induced by 5a,6-anhydrotetracycline (12, 13). TonB-PhoA fusion junctions were chosen to bracket the potential membrane-spanning segments of TonB at residues 12 and 32 for the amino-terminal region and residues 207 and 239 for the carboxy-terminal region. In addition, fusion junctions were chosen at residues 60 and 125 within the putative periplasmic region of TonB. All TonB-PhoA fusion junctions were confirmed by DNA sequencing with a primer specific to the 5' end of *phoA* (Table 1). Fusion proteins TonB(60)-PhoA and TonB(207)-PhoA have an Asp→Glu substitution in the second codon of the IS50L region as a result of an error in the synthesis of the mutagenic oligonucleotide.

Alkaline phosphatase activities in the fusion strains were determined under inducing and noninducing conditions (Table 1). The induction ratio for strains encoding active fusion proteins is 10 to 20, indicating that fusion protein synthesis is indeed under control of the *tetA* promoter-operator. The shortest fusion protein (TonB amino acid 12) is completely inactive, consistent with the TonB amino terminus being anchored on the cytoplasmic side of the cytoplasmic membrane. All the longer fusion proteins (TonB amino acids 32, 60, 125, 207, and 239) have significant PhoA activity (71 to 97 units). In particular, the PhoA fusion to the extreme carboxy terminus [TonB(239)-PhoA] is as active as other active fusions, suggesting that TonB amino acids 32 to 239, includ-

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TABLE 1. Properties of *tonB-phoA* fusion plasmids^a

Plasmid	Fusion protein	Fusion junction	Alkaline phosphatase activity ^b			Growth inhibition ^c
			Uninduced	Induced	Ratio	
pKP297	TonB(12)-PhoA	12 TGG CCG ACT GAC TCT TAT Trp Pro Thr Asp Ser Tyr	2	2	1	1.02
pKP294	TonB(32)-PhoA	32 TCG GTA CCT GAC TCT TAT Ser Val Pro Asp Ser Tyr	4	83	21	0.82
pKP291	TonB(60)-PhoA	60 CAA GCC GCT GAG TCT TAT Gln Ala Ala Glu Ser Tyr	10	97	10	0.98
pKP293	TonB(125)-PhoA	125 CCG TTT GCT GAC TCT TAT Pro Phe Ala Asp Ser Tyr	6	71	12	0.97
pKP290	TonB(207)-PhoA	207 GTG AAA ACT GAG TCT TAT Val Lys Thr Glu Ser Tyr	5	84	17	0.59
pKP289	TonB(239)-PhoA	239 ATT CAG CCT GAC TCT TAT Ile Gln Pro Asp Ser Tyr	5	95	19	0.60

^a Fusion proteins are designated by their carboxy-terminal TonB amino acid. The two amino acids of TonB before the fusion junction and the four amino acids of the IS50L region of *TnphoA* following the fusion junction are shown.

^b Alkaline phosphatase activities (11) were determined in *E. coli* TG1 [$\Delta(lac-pro)$ *supE* *thi* *hsd-5* (*F'* *traD36 proAB lacI^q lacZ* Δ M15)] carrying both the *tonB-phoA* plasmid and the *tetR* repressor plasmid pBST324. Induced cultures were assayed 1 h after the addition of 5a,6-anhydrotetracycline (0.3 μ g/ml).

^c The effect of fusion protein synthesis on growth was determined by measuring the optical densities at 600 nm of parallel uninduced and induced cultures 1 h after induction. Growth inhibition is expressed as a ratio of the optical densities (induced/uninduced).

ing the hydrophobic carboxy terminus, are located in the periplasm. This analysis does not rule out the possibility that the carboxy terminus of TonB could be localized in the outer membrane; however, this seems unlikely. Comparison of the growth rates of induced and uninduced cultures shows that longer TonB-PhoA fusion proteins slow the growth of induced cultures (Table 1). These results suggest that repression of fusion protein expression during plasmid construction was an important feature of the mutagenesis strategy.

To examine the rates of synthesis of the TonB-PhoA

fusion proteins, cells were induced and pulse-labeled for 1 min, and the fusion proteins were immunoprecipitated with anti-PhoA antiserum (Fig. 1). All the fusion proteins appear to be synthesized at roughly equal levels.

Western blot (immunoblot) analysis of cultures induced for 1 h showed that all the active fusion proteins accumulate to comparable levels (Fig. 2). However, the inactive TonB(12)-PhoA protein accumulated to a somewhat lower level. This last result is consistent with other reports that cytoplasmically localized inactive PhoA fusion proteins are often unstable (3). In fact, the TonB(12)-PhoA protein was

FIG. 1. Pulse-labeling and immunoprecipitation of TonB-PhoA fusion proteins. TG1 carrying *tonB-phoA* plasmids was induced for 10 min with 5a,6-anhydrotetracycline, pulse-labeled for 1 min with [³⁵S]methionine, and immunoprecipitated with anti-PhoA antiserum (5Prime $\rightarrow$ 3Prime) as described elsewhere (1). A fluorogram of the immunoprecipitates electrophoresed on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel is shown.

FIG. 2. Western blot analysis of TonB-PhoA fusion proteins. An SDS-10% polyacrylamide gel of total cellular protein from uninduced (U) and induced (I) cultures was blotted onto nitrocellulose filters (22). Anti-PhoA antiserum was used to detect TonB-PhoA fusion proteins. The position of wild-type PhoA (Sigma) is shown in the leftmost lane. The numbers indicate the carboxy-terminal TonB amino acid in each fusion protein. Positions of molecular size standards are indicated.

FIG. 3. Sucrose density gradient fractionation of TonB(239)-PhoA. The upper panel shows a Western blot analysis of fractions from a sucrose density gradient (14). The arrow marks the position of the TonB(239)-PhoA fusion protein detected with anti-PhoA antiserum; the position of wild-type PhoA is shown in the leftmost lane. The lower panel shows the NADH oxidase activity and density of the fractions.

synthesized in an amount greater than or equal to that of the other fusion proteins (Fig. 1). The Western blot analysis also showed that there is little accumulation of PhoA-sized proteins, suggesting that the alkaline phosphatase activities that we measured were derived primarily from fusion proteins as opposed to PhoA-sized degradation products. For fusions to TonB amino acids 60, 125, 207, and 239, near-equal amounts of two TonB-PhoA species accumulated in the induced samples (Fig. 2). In each case, the smaller fusion protein presumably resulted from proteolytic degradation of the full-length fusion protein and may well reflect the documented instability of plasmid-encoded TonB (6, 18). The fact that this degradation product is present in the TonB(60)-PhoA sample suggests that proteolytic cleavage occurs within the amino-terminal 60 amino acids of the TonB portion of the fusion proteins.

To confirm that TonB is anchored in the cytoplasmic membrane, we fractionated lysed spheroplasts of cells carrying plasmids encoding the full-length TonB(239)-PhoA fusion protein by sucrose density gradient centrifugation (14). Western blot analysis of the gradient fractions (Fig. 3) showed that most of the TonB(239)-PhoA fusion protein fractionates with the cytoplasmic membrane as judged by fraction density ($\rho = 1.14$ and 1.16) and NADH oxidase

FIG. 4. TonB function of TonB(239)-PhoA. Irreversible adsorption of bacteriophage $\phi 80$ was assayed as previously described (9, 20). Bacteria were grown to the mid-logarithmic phase in M9 minimal medium (18), bacteriophage were added at a multiplicity of infection of 0.5, and the mixture was incubated at 37°C . At the times indicated, samples were taken, diluted 100-fold to remove reversibly adsorbed bacteriophage, and centrifuged, and the supernatants containing unadsorbed bacteriophage were then titrated. Higher titers indicate lower levels of TonB function. ■, KP1039 [MC4100 $\Delta(trp tonB opp galU ana)$]; ○, KP1039 carrying the TonB(239)-PhoA plasmid pKP289, uninduced; ●, KP1039 carrying pKP289, induced with 5a,6-anhydrotetracycline ($0.3 \mu\text{g/ml}$ for 1 h); ◇, KP1039 carrying the *tonB*⁺ plasmid pKP292, uninduced. Results shown are averages of triplicate assays on three separate cultures.

activity (14). A small amount of the TonB(239)-PhoA fusion protein was in fractions with densities characteristic of the outer membrane ($\rho = 1.22$). However, these fractions also contained low levels of NADH oxidase activity and were therefore presumably contaminated with cytoplasmic membrane. The degradation products seen in Fig. 2, all of which were presumably missing the amino-terminal transmembrane anchor, were nevertheless present in the membrane fractions. This could be due to artifactual association of the hybrid proteins with the membrane or association through TonB-PhoA dimers in which only one TonB-PhoA monomer has been proteolytically cleaved and the other remains associated with the membrane.

To determine whether the full-length TonB(239)-PhoA fusion protein retains TonB function, we examined the ability of strains to adsorb bacteriophage $\phi 80$. Bacteriophage $\phi 80$ initially adsorbs to the FhuA receptor (23) in the outer membrane in an energy-independent, TonB-independent step (7). To proceed to the second and irreversible step, both TonB and cytoplasmic membrane electrochemical potential are required (7). Measurement of irreversible $\phi 80$ adsorption thus provides a measure of TonB function (9, 20). An appropriate *tonB*⁺ control plasmid was constructed by introducing two nonsense codons (TAATGA) at the end of the *tonB* coding region in the TonB(239)-PhoA fusion plasmid. The resulting plasmid (pKP292) expressed wild-type TonB under the control of the *tetA* promoter-operator. Using cross-streaks against $\phi 80$ and colicin B, the uninduced TonB(239)-PhoA fusion strain had a low but detectable level of TonB function compared with the *tonB* deletion strain (data not shown). It also grew faster on plates and in liquid

culture. TonB function in the TonB(239)-PhoA fusion strain increased upon induction (Fig. 4) but not to the level seen for the *tonB*⁺ control (pKP292). Perhaps the hydrophobic carboxy terminus of TonB is normally buried and its conformation is altered in the TonB(239)-PhoA fusion protein. In any case, this result showed that the TonB(239)-PhoA protein retains some function and lent confidence to the assumption that the periplasmic localization of the PhoA moiety reflects the normal localization of the TonB carboxy terminus. However, the possibility that the TonB activity observed reflects a small fraction of the fusion proteins from which the PhoA moiety has been clipped, thereby activating the TonB carboxy terminus, cannot be ruled out. None of the shorter fusion proteins confers sensitivity to $\phi 80$ or colicin B (data not shown), indicating that the carboxy-terminal 32 amino acids of TonB are necessary for function.

Taken together with previous work (15, 18, 21), the data presented here support a model in which *E. coli* TonB is anchored in the cytoplasmic membrane by its uncleaved hydrophobic amino terminus, with the remainder of the protein, including the hydrophobic carboxy terminus, extending into the periplasmic space. Such a location is consistent with its role as an energy transducer, coupling cytoplasmic membrane energy to active transport across the outer membrane. In a recent report, Hannavy et al. (8) came to the same conclusion regarding the membrane topology of *Salmonella typhimurium* TonB, based on the properties of TonB fusions to the periplasmic protein β -lactamase.

We thank Whyshen Liao for constructing strain KP1039 and Linda Randall for assistance with the sucrose gradient fractionation. We also thank the staff of the Center for Visualization, Analysis and Design in the Molecular Sciences (VADMS), a computer resource at Washington State University.

This work was supported by NSF grant DCB8903334 and NSF career advancement award DCB9009013 to K.P., Public Health Service grant AI16735 from the NIH to K.P.B., and a Battelle Pacific Northwest Laboratories energy fellowship to S.K.R. J.D.A. is an NIH predoctoral trainee (GM08336).

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