JOURNAL OF BACTERIOLOGY, Apr. 1994, p. 2326–2338
0021-9193/94/$04.00+0

Vol. 176, No. 8

Role of the TonB Amino Terminus in Energy Transduction between Membranes

JACQUELINE C. JASKULA,[1]† TRACY E. LETAIN,[1] STEVEN K. ROOF,[1]‡ JONATHAN T. SKARE,[1]§ AND KATHLEEN POSTLE[1,2]*

Department of Microbiology[1] and Department of Biochemistry and Biophysics,[2] Washington State University, Pullman, Washington 99164-4233

Received 14 October 1993/Accepted 6 February 1994

***Escherichia coli* TonB protein is an energy transducer, coupling cytoplasmic membrane energy to active transport of vitamin B_{12} and iron-siderophores across the outer membrane. TonB is anchored in the cytoplasmic membrane by its hydrophobic amino terminus, with the remainder occupying the periplasmic space. In this report we establish several functions for the hydrophobic amino terminus of TonB. A G-26→D substitution in the amino terminus prevents export of TonB, suggesting that the amino terminus contains an export signal for proper localization of TonB within the cell envelope. Substitution of the first membrane-spanning domain of the cytoplasmic membrane protein TetA for the TonB amino terminus eliminates TonB activity without altering TonB export, suggesting that the amino terminus contains sequence-specific information. Detectable TonB cross-linking to ExbB is also prevented, suggesting that the two proteins interact primarily through their transmembrane domains. In vivo cleavage of the amino terminus of TonB carrying an engineered leader peptidase cleavage site eliminates (i) TonB activity, (ii) detectable interaction with a membrane fraction having a density intermediate to those of the cytoplasmic and outer membranes, and (iii) cross-linking to ExbB. In contrast, the amino terminus is not required for cross-linking to other proteins with which TonB can form complexes, including FepA. Additionally, although the amino terminus clearly is a membrane anchor, it is not the only means by which TonB associates with the cytoplasmic membrane. TonB lacking its amino-terminal membrane anchor still remains largely associated with the cytoplasmic membrane.**

Gram-negative bacteria are surrounded by a dual-membrane system. The cytoplasmic membrane contains the proteins necessary to generate and maintain a proton motive force, as well as proteins that use the proton motive force to obtain nutrients via active transport. ATP hydrolysis occurring in the cytoplasm is also used to energize active transport across the cytoplasmic membrane. A second external membrane acts as a diffusion barrier, protecting the bacterium from noxious agents such as detergents, some hydrophobic antibiotics, and proteases. This outer membrane can be traversed in three ways. Nutrients can (i) diffuse through nonspecific pores (formed by porin proteins), (ii) diffuse through stereospecific pores such as LamB, or (iii) be actively transported across the outer membrane via high-affinity outer membrane receptors (32).

Active transport requires input of energy in some form. The presence of porins in the outer membrane allows free diffusion of protons and other small ions, thus preventing the establishment of a significant electrochemical potential (47). In addition, the outer membrane lacks access to the ATP-generating reactions of the cytoplasm and cytoplasmic membrane. However, the electrochemical potential of the cytoplasmic membrane can be harnessed to drive active transport of iron-siderophores and vitamin B_{12} across the outer membrane by means of the cytoplasmic membrane protein TonB (21, 36, 37). Without TonB, there is no active transport of nutrients across the outer membrane. Similarly, collapse of the cytoplasmic membrane electrochemical potential by protonophores prevents active transport across the outer membrane. Cyanide can have the same effect in *atp* strains, in which alternative means of generating the proton motive force are not operational (5, 40).

TonB protein is anchored in the cytoplasmic membrane by its uncleaved amino terminus, with the bulk of the protein occupying the periplasmic space, the aqueous compartment bounded by the outer and cytoplasmic membranes (14, 39, 41). Chromosomally encoded TonB can be cross-linked by formaldehyde in vivo into complexes with the cytoplasmic membrane protein ExbB, with the outer membrane receptor for the siderophore enterochelin, FepA, and with at least two other, unidentified proteins (26, 43). While these results are all consistent with the role of TonB as an energy transducer, first proposed by Hancock and Braun (13), the mechanism of energy transduction is not yet apparent.

A question central to identification of the mechanism of energy transduction is the role of the TonB amino terminus, TonB's sole direct connection to the cytoplasmic membrane electrochemical potential. To establish the mechanism, it is necessary to understand whether the amino terminus serves merely to position the periplasmically accessible domain or whether it plays an active role in transducing energy to the outer membrane receptors. In these studies, which confirm and extend similar studies on *Salmonella typhimurium* TonB (19), we show that, in addition to serving as an export signal, the amino terminus does indeed play a direct role in energy transduction. Like *S. typhimurium* TonB, *Escherichia coli* TonB requires the specific sequences of its amino terminus to transduce energy. Furthermore, by use of an engineered leader peptidase cleavage site in TonB, we show that synthesis of

* Corresponding author. Phone: (509) 335-5614. Fax: (509) 335-3517.

† Present address: Department of Biological Sciences, University of Idaho, Moscow, ID 83843.

‡ Present address: Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4965.

§ Present address: Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA 90024.

TABLE 1. Bacterial strains, phages, and plasmids

Bacterium, phage, or plasmid	Description	Source or reference
Bacteria		
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17* ($rK^- mK^+$) *supE44 relA1 deoR* Δ(*argF-lacZYA*)U169 φ80d*lacZ*Δ*M15*	Gibco-BRL
KBT001	*leu pro lysA purE metE str lac tonA*	40
CH483	*thi pro lac galE* Δ(*trp-tonB-opp-ana*)*467*	15
KP1083	KBT001 Δ(*trp-tonB-opp-ana*)*467*	This study
MC4100	*araD rpsL150 deoC1 ptsF25 flbB5301* Δ(*argF-lacZYA*)U169 *relA1 thi*	8
KP1039	MC4100 Δ(*trp-tonB-opp-ana*)*467*	41
GM1	*ara* Δ(*lac-pro*) *thi* F′(*lac-pro*)	48
KP1037	GM1 *exbB*::Tn*10*	45
KP1060	GM1 Δ(*ompT-fepA-entF*)	43
KP1082	GM1 Δ(*trp-tonB-opp-ana*)*467*	43
KP1119	KP1082 *exbB*::Tn*10*	This study
KP1120	KP1060 Δ(*trp-tonB-opp-ana*)*467*	This study
TG1	Δ(*lac-pro*) *supE thi hsd*Δ*5* F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*	Amersham
CJ236	*dut ung thi relA1*(pCJ105) Cm^r	Bio-Rad
MV1190	Δ(*lac-pro*) *thi supE* Δ(*srl-recA*) *306*::Tn*10* F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*	Bio-Rad
Phages		
R408	f1; IR1, Δpackaging signal, *gtrcA*	42
φ80vir		30
pKP100	*M13 mp7 clone of tonB*	This study
Plasmids		
pKP210	TonBG26D expressed from the *tonB* promoter	This study
pKP292	TonB expressed from the *tetA* promoter	41
pKP301	TonBE37A,P39A expressed from the *tetA* promoter	This study
pKP302	Clone of the 1,697-bp *tonB*$^+$ fragment into the *Eco*RV site of pJA113	This study
pKP321	TetA-TonB expressed from the *tetA* promoter	This study
pBST324	*tetR* plasmid, Kan^r	2
pJA113	*tetA-phoA* plasmid	2

TonB amino-terminal sequences is not sufficient; TonB must also be connected to its membrane anchor. In addition, the amino terminus is required for detectable cross-linking to ExbB, a participant with TonB in energy transduction. Finally, the amino terminus is necessary for TonB to enter into a detectable association with a membrane fraction having a density intermediate to those of the cytoplasmic and outer membranes. It is not, however, required for association with either the cytoplasmic membrane fraction or the outer membrane receptor FepA.

MATERIALS AND METHODS

Bacteria, phages, and plasmids. The strains used in this study are listed in Table 1. Strain KP1083 [Δ(*trp-tonB-opp-ana*)] was constructed by P1 transduction of strain KBT001 with a lysate made on strain KP1039 (41). The *tonB* phenotype was selected by plating on Luria-Bertani medium overlaid with colicin B, followed by screening for loss of CO_2 production by glucose fermentation (*ana* mutation). KP1119 was constructed by P1 transduction of *exbB*::Tn*10* from KP1037 into strain KP1082 and selection for tetracycline resistance. KP1120 was constructed by P1 transduction of Δ(*trp-tonB-opp-ana*) from KP1082 into KP1060 and selection for resistance to φ80 and colicin Ia. All strains bearing pKP292, pKP301, or pKP321 also contain the *tetR* plasmid pBST324 to control expression of the *tonB* genes.

Growth of bacteria. Bacterial strains were grown aerobically on Luria-Bertani medium at 37°C (except *degP* strains, which were grown at 30°C) or maintained as 25% glycerol stocks at −70°C (31). Labelling medium consisted of M9 minimal medium supplemented with 0.2% D-glucose, 1 μg of thiamine hydrochloride per ml, 1 mM magnesium sulfate, 0.1 mM calcium chloride, and 30 to 40 μg of all L-amino acids (except methionine) per ml (also referred to as growth medium [39]). For the growth of strain KBT001 and its derivatives, growth medium was additionally supplemented with 50 μg of L-methionine and 30 μg of adenine hydrochloride per ml. Bacteria containing plasmids were grown in the presence of 100 μg of ampicillin or 50 μg of kanamycin or both per ml. Expression from the *tetA* promoter was fully induced with 0.2 to 0.3 μg of 5a,6-anhydrotetracycline (AT) per ml.

Enzymes and chemicals. Dimethyl sulfoxide, Kodak XAR-5 X-ray film, Tween 20, polyethylene glycol (8000), Triton X-100, adenine hydrochloride, L-amino acids, antibiotics, sodium deoxycholate, Coomassie brilliant blue, proteinase K, leupeptin, pepstatin, and sucrose were purchased from Sigma Chemical Company (St. Louis, Mo.). Bacto yeast extract, tryptone, and agar were purchased from Difco (Detroit, Mich.). Trichloroacetic acid, methanol, and acetic acid were purchased from the J. T. Baker Chemical Company (Phillipsburg, N.J.). Acrylamide was purchased from Fisher Scientific (Fair Lawn, N.J.). Bisacrylamide was purchased from Aldrich Chemical Company (Milwaukee, Wis.). Ammonium persulfate and TEMED (*N,N,N′,N′*-tetramethylethylenediamine) were purchased from Bio-Rad (Richmond, Calif.). Cytoscint ES was purchased from ICN Biomedicals, Radiochemical Division (Irvine, Calif.). ^{35}S-dATP (1,200 Ci/mmol) and [^{35}S]methionine (1,000 Ci/mmol) were purchased from DuPont NEN Research Products (Boston, Mass.). The ECL Western blotting (immunoblotting) kit and [^{57}Co]vitamin B_{12} (10 μCi/μg) were purchased from Amersham Corporation (Arlington Heights, Ill.). T4 polynucleotide kinase and gene 32 protein were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). Phenylmethylsulfonyl fluoride, T4 DNA ligase, and T4 DNA polymerase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Mung bean nuclease was purchased from New England Biolabs (Beverly, Mass.). The Sequenase kit (version 2.0) was purchased from U.S. Biochemical (Cleveland, Ohio). The Immobilon-P transfer membrane was purchased from Millipore (Bedford, Mass.). Monoclonal antibodies (MAb) were prepared as described previously (43). Anti-maltose-binding protein antibody was the generous gift of Linda Randall. AT was the generous gift of Kevin Bertrand.

Oligonucleotide-directed in vitro mutagenesis. A mutant gene encoding the G-26→D (G26D) substitution in TonB was created by mutagenesis (7) of pKP100 with the oligonucleotide 5′-GTT GTG GCG GAT CTG CTC TAT-3′ (the mutagenic change is underlined). Plasmid pKP100 is a clone of the 1,697-bp *tonB*$^+$ *Hin*cII fragment into the *Hin*cII site of M13mp7. The target mutation was confirmed by dideoxy DNA sequence analysis of a region of *tonB* including the *Bgl*II-*Bst*EII fragment (bp 169 to 774 [38]), which was subsequently cloned into pKP166 by replacement of the corresponding fragment to

create plasmid pKP210. pKP166 is a clone of the 1,697-bp *tonB*$^+$ *Hin*cII fragment with *Eco*RI ends into pWU5 (50), such that it replaces the *trp* terminator fragment. A mutant gene encoding TonBE37A,P39A was constructed by oligonucleotide-directed in vitro mutagenesis (2) of plasmid pKP292 (41) with the mutagenic oligonucleotide 5′-AAT CGG CTG CGC AGG CGC AGC TAG TGC AAT AAC CTG ATG TAC CGA-3′ (changes are underlined), resulting in plasmid pKP301. A hybrid gene encoding amino acids 1 to 28 of pBR322 TetA protein fused to amino acids 34 to 239 of TonB was constructed by oligonucleotide-directed in vitro mutagenesis of plasmid pKP302 with the mutagenic oligonucleotide 5′-AGG CGC AGG TAG TTC AAT AAC CTG **CAG TAC CGG CAT AAC CAA GCC TAT GCC TAC AGC AGC CAG GGT**-3′ (the *tetA* sequence is in boldface), generating plasmid pKP321. The mutagenic oligonucleotide also included the D17A mutation (underlined) in the first transmembrane region of TetA that enhances export (2). Mutations were confirmed by dideoxy sequencing. Plasmid pKP302 is a clone of the 1,697-bp *tonB*$^+$ *Hin*cII fragment into the *Eco*RV site of pJA113 (2) such that the *tetA* promoter and the *tonB* promoter are in the same orientation.

Localization of mutant TonB proteins. Strain DH5α/pKP210 (encoding TonBG26D) was grown in M9-based labelling medium containing 40 μg of tryptophan per ml and 100 μg ampicillin of per ml to an optical density at 550 nm (OD_{550}) of 0.3, at which time it was pulse-labelled for 1 min with [^{35}S]methionine, converted to spheroplasts, treated with proteinase K (39), and immunoprecipitated with polyclonal anti-TonB antiserum as described previously (45). Chromosomally encoded TonB, which is sensitive to proteinase K that is added to spheroplasts, was not detected in this experiment.

KP1039/pKP301 (encoding TonBE37A,P39A) and KP1083/pKP321 (encoding TetA-TonB) were grown in labelling medium with 0.2% maltose or 0.2% D-glucose, respectively. At an OD_{550} of 0.5, bacteria were separated into spheroplast and periplasmic fractions and treated with proteinase K as described previously (39). Fractionation of TonBE37A,P39A was performed in the presence of 200 mM phenymethylsulfonyl fluoride, 100 mM EDTA, 1 mM leupeptin, and 1 mM pepstatin. Under these conditions, maltose-binding protein was associated entirely with the periplasmic fractions, as expected (data not shown). Membrane fractions were electrophoresed on sodium dodecyl sulfate (SDS–11% polyacrylamide gels (24) and immunoblotted with MAb 1C11.

Δ*tonB* bacteria carrying either pKP292 or pKP301 and the *tetR* plasmid pBST324 were grown under noninducing conditions, converted to spheroplasts, and fractionated on sucrose density gradients (34). Refractive indices and NADH oxidase activity were determined for each fraction. Equal volumes of each fraction were also precipitated with an equal volume of 10% trichloroacetic acid (TCA), washed with 10 mM Tris-HCl (pH 8.0), suspended and boiled in Laemmli sample buffer, resolved on SDS–11% polyacrylamide gels, and examined by immunoblotting with MAb 1C11.

In vivo cross-linking. In vivo cross-linking experiments were performed essentially as described previously (43) with 1% formaldehyde, except that after suspension of cross-linked cells in sample buffer, samples were heated at 60 instead of 37°C. To compare chromosomally encoded GM1 TonB protein with mutant TonB proteins expressed from the *tetA* promoter, Δ*tonB* strains expressing either TonBE37A,P39A or TetA-TonB were partially induced at an OD_{550} of 0.2 with only 0.2 ng of AT per ml. Under these conditions, levels of proteins expressed from plasmids were similar to levels of chromosomally encoded TonB (data not shown). For cells expressing TonBE37A,P39A, chloramphenicol was added at an OD_{550} of 0.5 to 100 μg/ml 1 h prior to cross-linking to allow complete processing of TonBE37A,P39A to its 32-kDa cleaved form. Cross-linked complexes were detected with MAb 4H4 at a 1:5,000 dilution (43).

Immunoblot analysis. Bacterial cultures were either cross-linked in vivo as described above or harvested by precipitation with equal volumes of 10% TCA, washed twice with 10 mM Tris-HCl (pH 8.0), and suspended in 20 μl of Laemmli sample buffer (24). Following electrophoresis on SDS–11 or 14% polyacrylamide gels, proteins were electroblotted to polyvinylpyrrolidone Immobilon-P membrane, and TonB-specific epitopes were detected by enhanced chemiluminescence as described previously (43). Primary antibodies were MAb 4H4, 1C11, and 1D3, which recognize epitopes between residues 60 and 95, between residues 125 and 193, and between residues 207 and 239, respectively, of TonB (44). Preparation of MAb has been described previously (43). Primary antibodies were used at a dilution of 1:10,000. Membranes were stained with Coomassie blue following blotting to confirm equivalent loading of gel lanes.

Vitamin B_{12} transport assay. Vitamin B_{12} transport assays were performed essentially as described previously (40) with the following modifications. Cultures grown in Luria-Bertani broth overnight were subcultured 1:100 in growth medium supplemented with methionine and adenine and grown aerobically in sidearm flasks at 37°C to an OD_{550} of 0.4. Cells expressing TonBE37A,P39A were induced with 0.3 μg of AT per ml at an OD_{550} of 0.3 and grown to an OD_{550} of 0.4. After being pelleted, cells were washed once with magnesium-free labelling medium containing 0.5% D-glucose and preincubated for 5 min with shaking at 275 rpm in a 37°C water bath. To stop synthesis of full-length TonBE37A,P39A and allow its complete conversion into the cleaved form, 100 μg of chloramphenicol per ml was added during preincubation, which was extended to 15 min. After preincubation, [^{57}Co]vitamin B_{12} was added to a final concentration of 10 nM, 1-ml aliquots were filtered through membranes, washed once, and dried, and ^{57}Co was determined by liquid scintillation counting. Background levels were established by binding of [^{57}Co]vitamin B_{12} to cells at 4°C.

Assay of irreversible φ80 adsorption. φ80 adsorption assays were performed as described previously (45). In brief, bacteriophage and cells were mixed together and incubated at 37°C. At various times, samples were diluted into growth medium, vortexed vigorously to remove reversibly adsorbed bacteriophage, and centrifuged to pellet bacteria, and titers in supernatants containing unadsorbed bacteriophage were determined.

RESULTS

The uncleaved amino terminus of TonB is an export signal. The uncleaved amino terminus of TonB (39) serves to anchor TonB in the cytoplasmic membrane, with the remainder of the protein occupying the periplasmic space (14, 41). We have previously shown that a Gly→Asp substitution in the hydrophobic amino terminus of a hybrid TrpC-TonB protein (corresponding to amino acid 26 of the TonB protein [39]) prevents export of the hybrid protein from the cytoplasm (46). In order to determine whether that mutation would also affect export of TonB protein, the G26D mutation was constructed by oligonucleotide-specific mutagenesis and cloned on a multicopy plasmid. Under conditions in which plasmid-encoded TonB is sensitive to proteinase K in spheroplasts (39), plasmid-encoded TonBG26D was resistant to externally added protein-

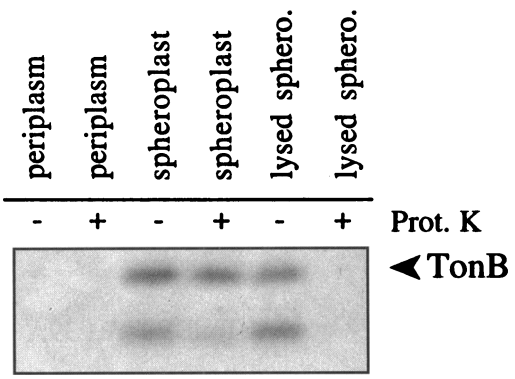

FIG. 1. Proteinase K accessibility of TonBG26D. DH5α carrying pKP210 was pulse-labelled with [^{35}S]methionine and fractionated as described in Materials and Methods. Periplasmic, spheroplast, and lysed spheroplast fractions were divided and either left untreated or treated with proteinase K. Samples reflecting equivalent amounts of cells were electrophoresed and autoradiographed. The arrowhead indicates the position of full-length TonB protein. The unlabelled band is a TonB degradation product.

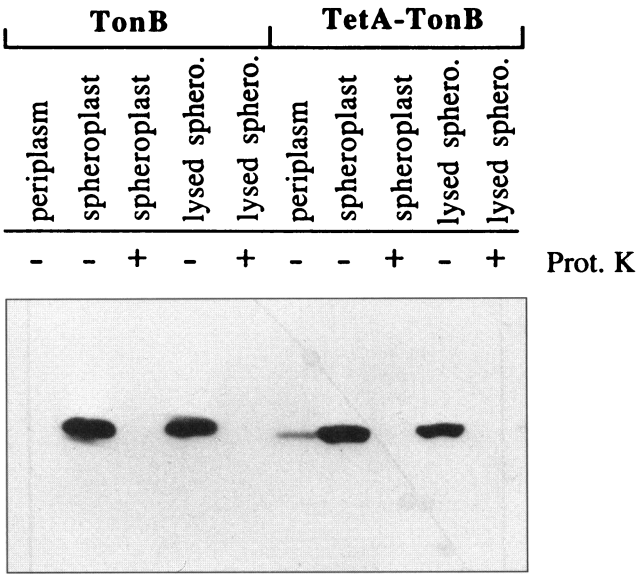

FIG. 3. Proteinase K accessibility of TetA-TonB fusion protein. KP1083 (Δ*tonB*) carrying either plasmids pKP292 (TonB) and pBST324 or plasmids pKP321 (TetA-TonB) and pBST324 was grown without AT induction and fractionated as described in Materials and Methods. Periplasmic, spheroplast, and lysed spheroplast fractions were divided and either left untreated or treated with proteinase K. Samples reflecting equivalent amounts of cells were electrophoresed, and TonB-specific protein was detected by immunoblotting with MAb. The same results were also seen following induction of the proteins with AT (data not shown).

ase K in spheroplasts and became sensitive when the spheroplasts were lysed, indicating that the G26D substitution prevented TonB export from the cytoplasm (Fig. 1). Given the similarity of the amino-terminal region to other signal sequences (49), it is reasonable to conclude that it serves as the export signal for TonB. Deletion of amino acids 1 to 32 of *S. typhimurium* TonB has also been shown to prevent export (19).

An alternative membrane anchor cannot energize vitamin B_{12} transport. TonB protein is an energy transducer, coupling cytoplasmic membrane energy to active transport across the outer membrane (5, 13, 40). Since TonB is anchored in the cytoplasmic membrane by its uncleaved amino terminus, we wanted to determine the role of the membrane anchor in energy transduction. Initial studies involved determining the activity of a hybrid protein generated by replacement of amino acids 1 to 33 of TonB with the first transmembrane segment of the pBR322-encoded cytoplasmic membrane protein, TetA (amino acids 1 to 28) (Fig. 2). Expression of the resulting gene fusion, encoded on plasmid pKP321, is under the control of the *tetA* promoter and can be induced by the addition of the gratuitous inducer AT.

To determine whether the TetA-TonB hybrid protein was appropriately exported, its susceptibility to externally added proteinase K was assessed. Like wild-type TonB, the TetA-TonB hybrid protein was sensitive to proteinase K in spheroplasts and lysed spheroplasts, indicating that it was exported (Fig. 3). The small amount of TetA-TonB hybrid appearing in the periplasmic fraction most likely reflected a portion of spheroplasts not pelleted, since it contained full-length TetA-TonB.

The inability of the TetA-TonB hybrid to energize vitamin B_{12} transport in Δ*tonB* strains is shown in Fig. 4. Unlike wild-type TonB, the TetA-TonB protein was entirely unable to energize vitamin B_{12} transport, indicating that attachment to the cytoplasmic membrane alone is not sufficient to support TonB activity. Strains expressing TetA-TonB instead of TonB were also completely resistant to bacteriophage ϕ80 (data not shown). Similar results have been obtained for *S. typhimurium* TonB with amino-terminal substitutions of OmpA or BlaM sequences (19).

The amino terminus of TonB can be cleaved following creation of a leader peptidase recognition site. While substitution of the TetA amino terminus inactivated TonB, the question of whether, if present, the TonB amino terminus must be attached to the periplasmic domain to transduce energy has not been addressed. To answer that question, a leader peptidase cleavage site was engineered in TonB.

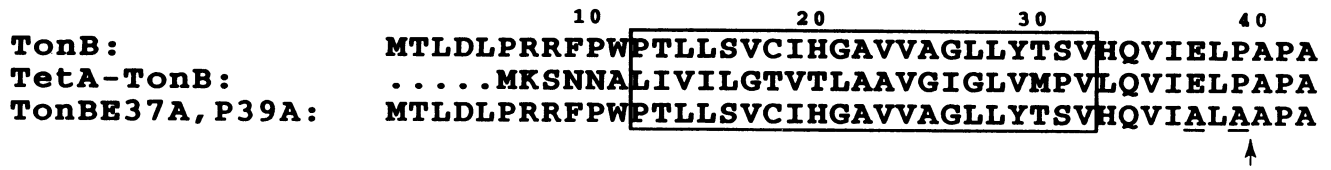

FIG. 2. Comparison of deduced amino-terminal amino acid sequences of TetA-TonB and TonBE37A,P39A with those of wild-type TonB. The predicted hydrophobic regions of the three proteins are boxed. For the three proteins the carboxy-terminal sequences not shown are identical. The TetA sequence includes a D17A substitution that enhances export of TetA-PhoA fusion protein (2). For the TonBE37A,P39A mutant, E37 and P39 are underlined and the predicted leader peptidase cleavage site is indicated by an arrow. The cleavage site was designed on basis of the (−3,−1) rule, which states that only small, nonpolar amino acids should be found in these positions (33).

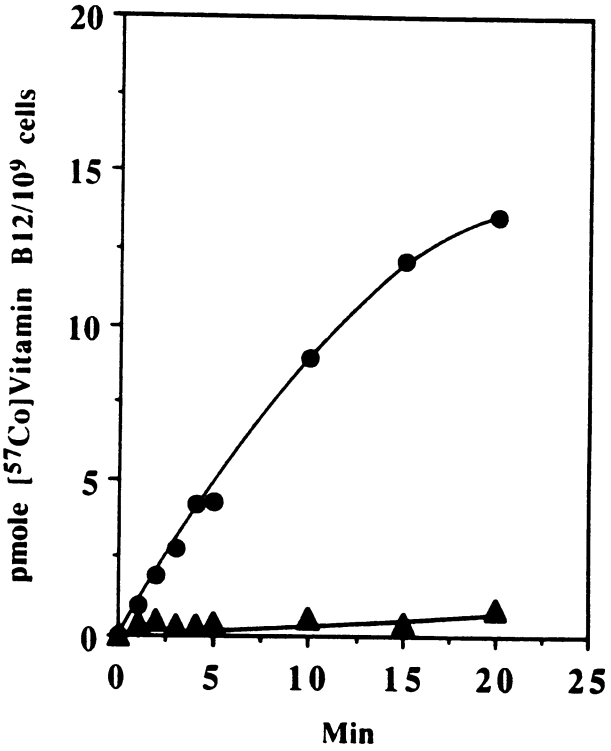

FIG. 4. Vitamin B_{12} transport assays of TetA-TonB activity. KP1083 (Δ*tonB*) carrying either plasmids pKP292 (TonB) and pBST324 (●) or plasmids pKP321 (TetA-TonB) and pBST324 (▲) was grown without AT induction and assayed as described in Methods and Materials.

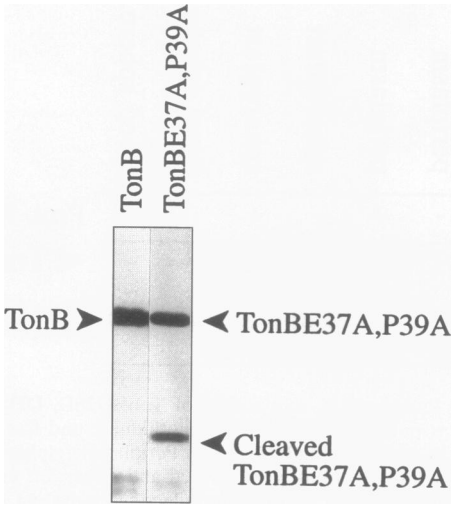

FIG. 5. Steady-state levels of TonB and TonBE37A,P39A. KP1083 (Δ*tonB*) carrying either plasmids pKP292 (TonB) and pBST324 or plasmids pKP301 (TonBE37A,P39A) and pBST324 was grown and induced with AT as described in Materials and Methods. Samples were precipitated with TCA, electrophoresed, and immunoblotted with TonB-specific MAb. The positions of full-length TonB and TonB E37A,P39A, both at 36 kDa, and cleaved TonBE37A,P39A at 32 kDa are indicated by arrowheads.

Like the consensus prokaryotic cleaved signal sequence, the deduced amino-terminal TonB amino acid sequence has a charged region preceding a 20-amino-acid stretch of hydrophobic residues (38, 49). The absence of a consensus leader peptidase cleavage site (Ala-X-Ala) six residues beyond the hydrophobic amino terminus is consistent with the lack of TonB cleavage normally observed (39). To engineer a leader peptidase cleavage site, E37A and P39A substitutions were simultaneously introduced by oligonucleotide-directed mutagenesis into a *tonB*$^+$ plasmid in which *tonB* expression is under the control of the *tetA* promoter (Fig. 2).

Processing of the TonB leader peptidase cleavage mutant was expected to occur following Ala-39, generating an approximately 32-kDa cleavage product consisting of amino acids 40 to 239 of TonB. To identify the 32-kDa cleavage product, steady-state levels of proteins from Δ*tonB* strains with plasmids expressing either wild-type TonB or TonBE37A,P39A were compared by immunoblot analysis (Fig. 5). A 32-kDa cleaved form of TonBE37A,P39A was present that was not seen in the strain expressing TonB. This 32-kDa product was detectable with MAb specific for the carboxy terminus of TonB (data not shown), suggesting that cleavage had occurred at the amino terminus. The 32-kDa product was still present in *degP* and *ompT* strains, suggesting that neither of these proteases was responsible for formation of the product (data not shown). Further cleavage of the 32-kDa product was sometimes observed in immunoblots and may reflect processing during handling, even though samples are always precipitated with TCA. Together, these results suggested that the 32-kDa cleavage product was created by leader peptidase-specific cleavage.

To determine the half-life of processing, proteins from the same strains were analyzed after addition of chloramphenicol to prevent further protein synthesis (Fig. 6). Consistent with our earlier results on the half-life of chromosomally encoded TonB (45), immunoblotted TonB protein was essentially a stable protein whether or not chloramphenicol was present, although a small amount of 30-kDa degradation product was detectable. In contrast, strains expressing TonBE37A,P39A exhibited two forms of TonB, one at the apparent molecular mass of full-length TonBE37A,P39A and one at the apparent molecular mass of 32 kDa predicted for the leader peptidase-specific cleavage product. After addition of chloramphenicol, the full-length TonBE37A,P39A was rapidly processed to the 32-kDa product, although darker exposures revealed the presence of full-length TonBE37A,P39A until approximately 5 min (data not shown). Chloramphenicol had no effect on the degradation of wild-type TonB, as we had observed previously (45).

The TonB membrane anchor must remain attached to the periplasmic domain to transduce energy. To determine the effect of leader peptidase cleavage on the ability of TonB to energize transport of vitamin B_{12} across the outer membrane, the cultures used for Fig. 6 were assayed for ability to transport vitamin B_{12} (Fig. 7). The total accumulation of [^{57}Co]vitamin B_{12} by the strain expressing TonBE37A,P39A in the absence of chloramphenicol was similar to that by the strain expressing wild-type TonB. Pretreatment of cultures expressing wild-type TonB with chloramphenicol resulted in approximately 90% of the vitamin B_{12} accumulation observed in the absence of chloramphenicol. These results were consistent with the known decay of TonB activity in the absence of protein synthesis (16, 45). The effect of chloramphenicol on the ability of strains expressing TonBE37A,P39A to transport vitamin B_{12} was more pronounced. The strain encoding TonBE37A,P39A accumulated vitamin B_{12} at an appreciably slower rate for the first 5 min of the assay, after which accumulation was no longer

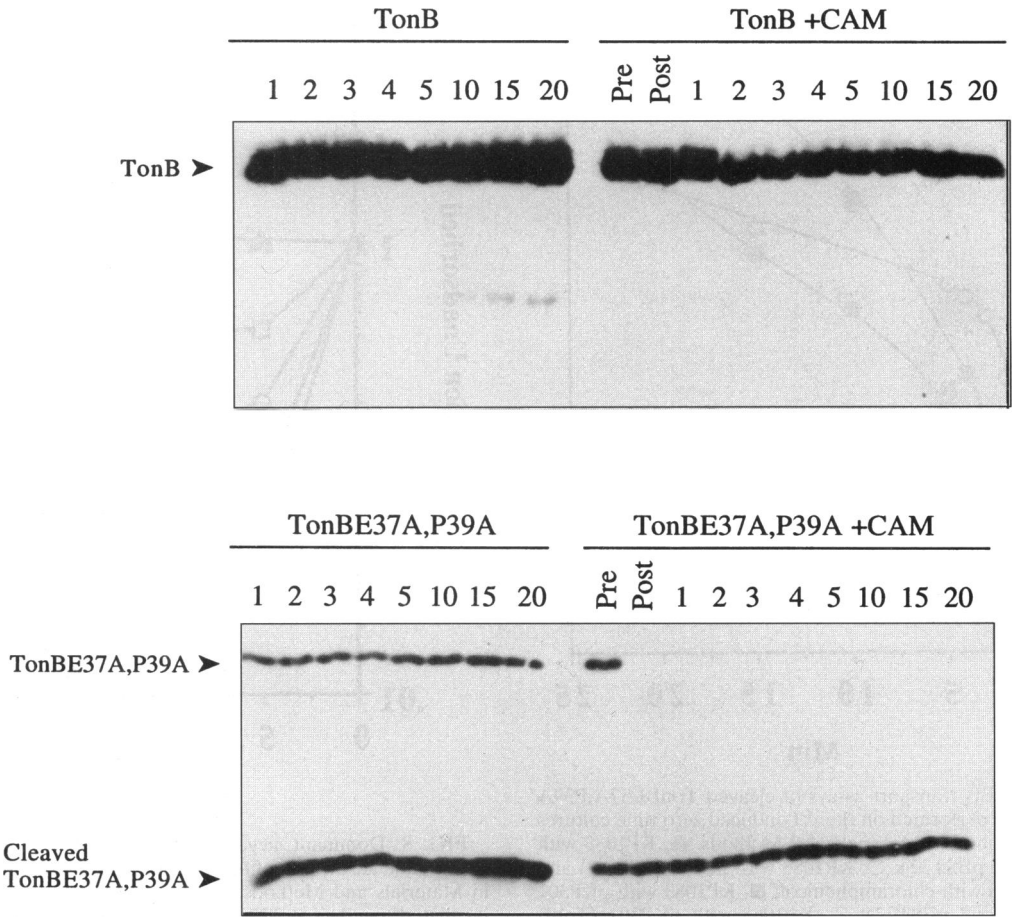

FIG. 6. Processing of TonBE37A,P39A. KP1083 (Δ*tonB*) carrying either plasmids pKP292 (TonB) and pBST324 or plasmids pKP301 (TonBE37A,P39A) and pBST324 was grown and induced with AT as described in Materials and Methods. Duplicate subcultures from the same saturated starter culture were either treated with chloramphenicol (CAM) or left untreated. Samples were removed at the times indicated (minutes), precipitated with TCA, and processed for polyacrylamide gel electrophoresis. TonB-specific proteins were detected by immunoblot analysis and MAb. Samples were also removed at zero time and assayed for vitamin B_{12} transport activity (Fig. 7). The positions of TonB and full-length TonBE37A,P39A, both at 36 kDa, and cleaved TonBE37A,P39A at 32 kDa are indicated by arrowheads. The samples on the two panels were electrophoresed simultaneously and developed on the same piece of film.

evident. The ability to transport vitamin B_{12} (Fig. 7) correlated positively with the presence of full-length TonB or full-length TonBE37A,P39A (Fig. 6) during the assay. The conversion of full-length TonBE37A,P39A into cleaved TonBE37A,P39A resulted in the inability to transport vitamin B_{12}. These results suggested that the amino terminus of TonB must be attached to the periplasmic domain to transduce energy.

Inactive cleaved TonBE37A,P39A interferes with the activity of wild-type TonB. In the vitamin B_{12} assays, the presence of cleaved TonBE37A,P39A at steady-state conditions did not seem to diminish the ability of cells to transport vitamin B_{12} compared with that of cells expressing wild-type TonB from the same promoter. However those assays, which are not the most sensitive for TonB activity, were performed under inducing conditions in which full-length TonBE37A,P39A was almost certainly not the limiting protein in energy transduction. To carefully test the possibility of interference by cleaved TonBE37A,P39A, sensitive φ80 adsorption assays were performed on uninduced cultures that also expressed wild-type TonB (Fig. 8). Cells expressing TonB and TonBE37A,P39A adsorbed φ80 as well as those expressing only TonB. Under these conditions, there was slightly less full-length TonBE37A,P39A than TonB present (data not shown). While chloramphenicol pretreatment for 15 min would be expected to diminish the ability of TonB to energize adsorption of bacteriophage because of its short functional half-life under those circumstances, the effect of chloramphenicol addition on the strain expressing both TonB and TonBE37A,P39A was significantly more severe. Curiously, there was less cleaved TonBE37A,P39A present in these cultures than expected (data not shown). These results suggested that either the amino terminus or the periplasmic domain, both inactive, could compete with full-length TonB for access to other proteins involved in energy transduction. As expected, chloramphenicol treatment of cells expressing TonBE37A,P39A alone rendered them essentially inactive, because of conversion of full-length TonBE37A,P39A to the cleaved 32-kDa form as shown in Fig. 6.

Fractionation of wild-type TonB and TonBE37A,P39A. Release of the periplasmic domain of TonB from its membrane anchor could cause the periplasmic domain to float free in the periplasm or, alternatively, to remain associated with either the

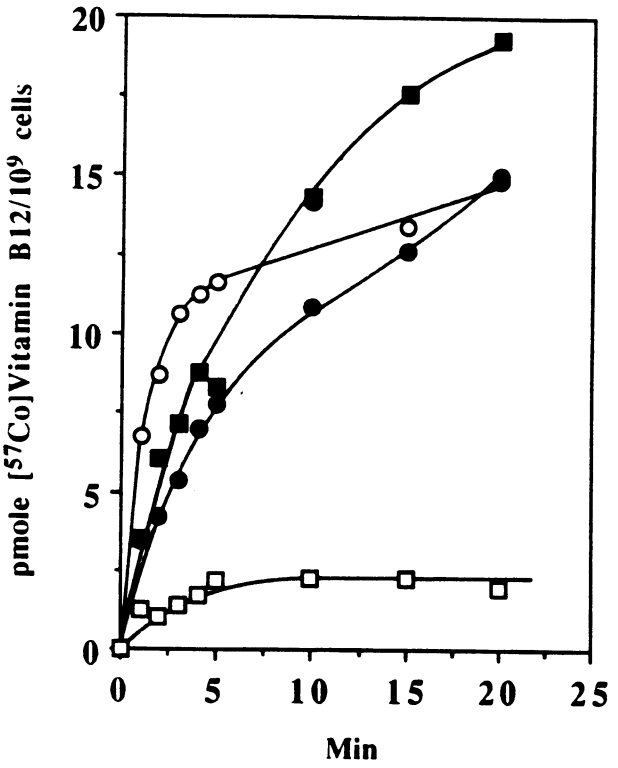

FIG. 7. Vitamin B_{12} transport assays of cleaved TonBE37A,P39A activity. Assays were performed on the AT-induced zero time cultures from Fig. 6 as described in Materials and Methods. ●, KP1083 with pKP292 (TonB) and pBST324; ○, KP1083 with pKP292 (TonB) and pBST324, pretreated with chloramphenicol; ■, KP1083 with pKP301 (TonBE37A,P39A) and pBST324; □, KP1083 with pKP301 (TonB E37A,P39A) and pBST324, pretreated with chloramphenicol. Chloramphenicol pretreatment lasted for 15 min.

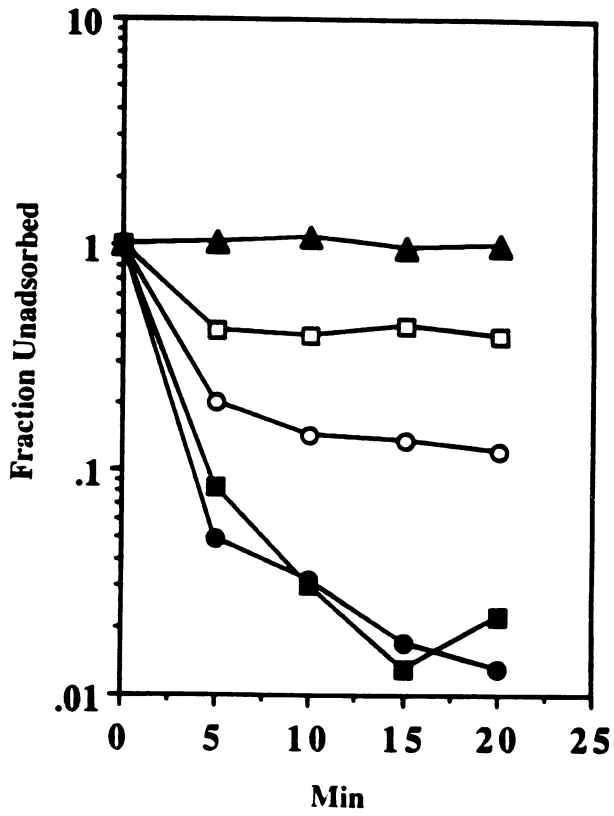

FIG. 8. Dominant negative effect of cleaved TonBE37A,P39A. Irreversible adsorption of bacteriophage ϕ80 was assayed as described in Materials and Methods. Briefly, bacteria and bacteriophage were incubated together at 37°C, and at the times indicated, samples were diluted to remove reversibly adsorbed bacteriophage. After centrifugation to pellet bacteria with irreversibly adsorbed bacteriophage, titers of bacteriophage remaining in the supernatant were determined, and the results are expressed as the fraction of bacteriophage unadsorbed. The higher the fraction unadsorbed, the lower the relative level of TonB function. ●, MC4100; ○, MC4100, pretreated with chloramphenicol; ■, MC4100 carrying pKP301 (TonBE37A,P39A) and pBST324; □, MC4100 carrying pKP301 and pBST324, pretreated with chloramphenicol; ▲, KP1039 (Δ*tonB*) carrying pKP301 and pBST324, pretreated with chloramphenicol. Choramphenicol pretreatment lasted for 15 min.

cytoplasmic or outer membrane because of associations with other proteins (11, 12, 43, 45). In initial experiments to determine the localization of the 32-kDa soluble domain of cleaved TonBE37A,P39A, cells were fractionated into samples containing periplasm and spheroplasts (Fig. 9). As expected (39), wild-type TonB remained spheroplast associated, as did full-length TonBE37A,P39A. However, while approximately one-third of the cleaved TonBE37A,P39A was located in the periplasm, fully two-thirds remained associated with the spheroplasts.

Sucrose density gradient fractionation of chromosomally encoded TonB indicated that TonB could be found in the cytoplasmic membrane fraction (ρ = 1.13), as expected, and in a fraction of intermediate density (ρ = 1.19) (Fig. 10). Both fractions had NADH oxidase activity, indicating that they both contained cytoplasmic membranes. In these gradients, the outer membrane, which does not exhibit NADH oxidase activity, fractionates with ρ = 1.22 (34). The intermediate-density fractions most likely represent regions where cytoplasmic and outer membranes are in contact (34), perhaps mediated by the association of TonB with the outer membrane receptor FepA (43). Alternatively, the intermediate-density fraction may represent a collection of particularly protein-rich regions in the cytoplasmic membrane. Surprisingly, while full-length TonBE37A,P39A was also found in both the cytoplasmic membrane fraction and the intermediate-density fraction, the 32-kDa cleaved form of TonBE37A,P39A was associated only with the cytoplasmic membrane (Fig. 11). These results suggested that the amino terminus of TonB plays a role in the ability of TonB to stably associate with an additional component of the cell envelope.

Both the cleaved form of TonBE37A,P39A and TetA-TonB can be cross-linked to FepA but not to ExbB. One explanation for the lack of association of cleaved TonBE37A,P39A with the intermediate-density fraction is that it might not be associating with the outer membrane receptor, FepA. To test that hypothesis, strains expressing TonBE37A,P39A at chromosomal levels were treated with chloramphenicol to allow complete conversion to the 32-kDa cleaved form and were cross-linked in vivo with formaldehyde (Fig. 12). Since the cleaved form of TonBE37A,P39A is smaller than TonB, we would expect the complexes formed by this protein to be smaller than those observed for TonB. Comparison of the *fepA*$^+$ and Δ*fepA* strains expressing cleaved TonBE37A,P39A showed that an approximately 160-kDa TonB-specific band present in the *fepA*$^+$ strain was absent from the Δ*fepA* strain.

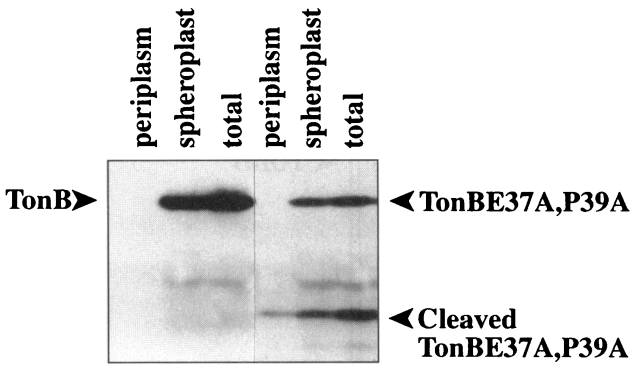

FIG. 9. Localization of cleaved TonBE37A,P39A. KP1039 (Δ*tonB*) carrying either pKP292 (TonB) and pBST324 or pKP301 (TonBE37A, P39A) and pBST324 was grown and fractionated into periplasmic and spheroplast fractions as described in Materials and Methods. Samples were precipitated with TCA, electrophoresed on polyacrylamide gels, and immunoblotted with TonB-specific MAb. Positions of the various TonB species are indicated by labelled arrowheads.

Previously, we found that a 195-kDa complex requires both TonB and FepA for its formation and can be detected by MAb to either TonB or FepA (43). While the molecular mass shift of the FepA complex with cleaved TonBE37A,P39A (to 160 kDa) was greater than expected, these data strongly suggested that the cleaved TonBE37A,P39A can interact with FepA.

If cleaved TonBE37A,P39A could be cross-linked to ExbB, we would expect to find an ExbB-specific complex at an apparent molecular mass somewhat less than 59 kDa (the apparent molecular mass of a TonB-ExbB complex [26, 43]). Because there are no prominent bands visible with the predicted apparent molecular mass and because protein profiles of *exbB*$^+$ and *exbB*::Tn*10* strains expressing cleaved TonB E37A,P39A showed no difference in their cross-linking patterns, we conclude that cleaved TonBE37A,P39A could not be detectably cross-linked to ExbB. Obvious candidates for a complex corresponding to the "43.5-kDa" complex detected for wild-type TonB were also lacking. Furthermore, it was surprising that the 77-kDa cluster appeared to exist at the same apparent molecular mass seen for the complex with wild-type TonB and that a new complex appears at approximately 61 kDa. The significance of these last observations was unclear.

The associations detected during in vivo cross-linking of cleaved TonBE37A,P39A might have occurred prior to cleavage of the amino terminus while TonBE37A,P39A was still intact. To test whether TonB that is missing its amino terminus from the onset can interact with other proteins, a Δ*tonB* strain expressing TetA-TonB was cross-linked in vivo (Fig. 13). Since TetA-TonB has only a slightly lower apparent molecular mass than TonB, cross-linking patterns of the two proteins could be compared directly. The 59-kDa TonB-ExbB complex was not present, but the other three complexes of 195 kDa (TonB plus FepA), 77 kDa, and 43.5 kDa were all present. The extra bands immediately above and below the position for TonB plus ExbB were also present in an *exbB*::Tn*10* strain expressing TetA-TonB (data not shown), indicating that they did not contain ExbB protein. These results indicated that the amino terminus of TonB is not required for interaction with the outer membrane receptor FepA or for interaction with proteins in the 77- and 43.5-kDa complexes but that it is required for cross-linking to ExbB protein. These results supported the idea that TonB interacts with ExbB through its amino-terminal membrane-spanning domain, as previously suggested (20, 22).

DISCUSSION

One of the central questions in studies of energy transduction is the role of the TonB amino terminus. Since the source of energy for active transport across the outer membrane appears to be the electrochemical potential of the cytoplasmic membrane, the location and membrane topology of TonB suggest that the single amino-terminal cytoplasmic membrane anchor of TonB might be important for energy transduction. Alternatively, like many eukaryotic proteins (10) and apparently *E. coli* leader peptidase (23), the membrane anchor might serve only to position TonB on the periplasmic face of the cytoplasmic membrane without participating directly in energy transduction. Under those circumstances, the energy source for TonB-dependent phenomena would be questionable. Recently, it has been shown that the amino terminus of *S. typhimurium* TonB plays an essential role in energy transduction (19). To confirm those results and to more broadly define the role of the TonB amino terminus, we constructed and characterized a series of mutations in that region.

Substitution of the first membrane-spanning domain of tetracycline efflux protein, TetA, for the hydrophobic membrane anchor of TonB resulted in an inactive protein that was efficiently exported but could not energize vitamin B_{12} transport across the outer membrane. These results indicate that the specific amino acid sequence of the amino terminus is important for TonB activity. Similar results have been reported for *S. typhimurium* TonB, where substitution of either the amino terminus of β-lactamase or OmpA for the TonB amino terminus prevented TonB-dependent φ80 adsorption (19).

However, even though the energy transduction activity of TonB was absent, the ability of TetA-TonB to be cross-linked in vivo to FepA and at least two other uncharacterized proteins remained completely intact. These results indicate that the energy of the cytoplasmic membrane is not required for interactions that can be monitored by the in vivo cross-linking assay. The one protein to which TetA-TonB could not be cross-linked was ExbB. Rather than reflecting a requirement for cytoplasmic membrane energy, this probably reflects the fact that, on the basis of their respective topologies (14, 18, 41), TonB and ExbB interactions are restricted to the transmembrane domains and small amino-terminal regions of each protein that could dangle into the cytoplasm (amino acids 1 to 12 of TonB) or periplasm (amino acids 1 to 15 [18] or 1 to 25 [20] of ExbB). Substitution of the TetA amino terminus removes amino acids 1 to 33 of TonB and prevents detectable cross-linking with ExbB. TrpC-TonB protein, missing the presumably cytoplasmic amino-terminal amino acids 1 to 12 of TonB, can still be cross-linked to ExbB (25). Furthermore, a deletion of one codon in the transmembrane domain of TonB prevents cross-linking to ExbB (26). Together, these data strongly suggest that the sites through which TonB and ExbB can be cross-linked are within their transmembrane domains. It seems likely, therefore, that the complete inactivity of TetA-TonB was due to the inability to interact with either ExbB or TolQ, its crosstalk analog (6). ExbB participates directly in TonB-dependent energy transduction (43). Just as the absence of ExbB and TolQ results in the complete inactivity of TonB (6), replacement of the TonB transmembrane region through which ExbB and, by analogy, TolQ can interact with TonB should have the same effect.

Results from the TetA-TonB substitution experiments did not address the question of whether a connection to the cytoplasmic membrane is required for TonB activity if both the TonB amino terminus and the periplasmically exposed domain are synthesized. To address that question, a leader peptidase

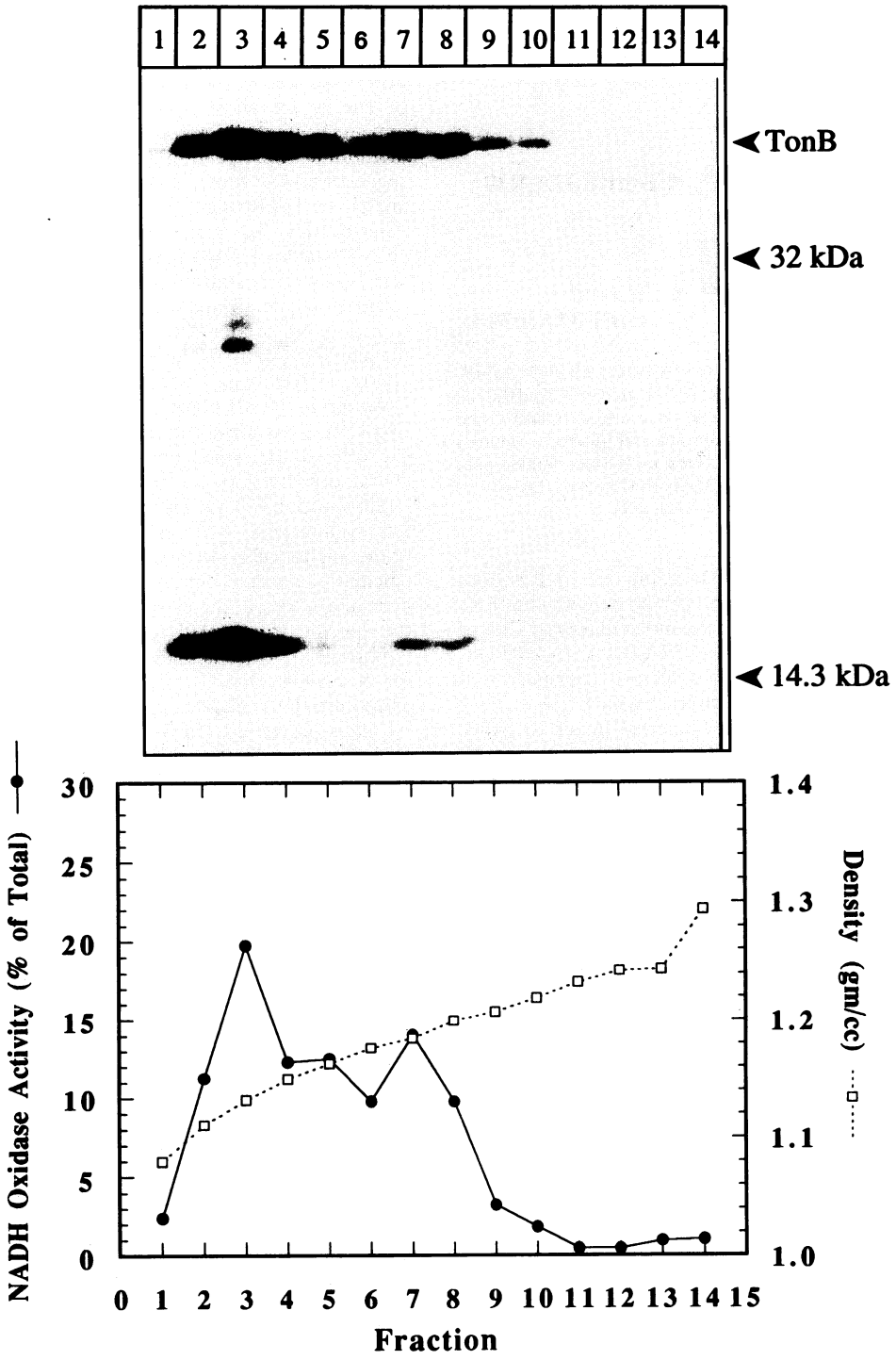

FIG. 10. Sucrose gradient fractionation of TonB protein. KP1039 (Δ*tonB*) carrying plasmids pKP292 (TonB) and pBST324 was grown to an OD_{550} of 0.5 without the inducer AT, converted to spheroplasts, lysed, and layered onto a sucrose density gradient as described in Methods and Materials. Fractions of approximately 1 ml were collected. The upper panel shows an immunoblot of the fractions, developed with a TonB-specific MAb. The lower panel shows refractive indices and NADH oxidase activities of the corresponding fractions. The position of wild-type TonB is indicated along with those of 32- and 14.3-kDa markers. The two degradation products at approximately 28 and 16 kDa were not characterized further.

cleavage site was engineered (TonBE37A,P39A), such that, according to the $(-3,-1)$ rule (49), the sequence Ala-X-Ala follows the hydrophobic membrane-spanning domain. These substitutions resulted in the accumulation of a 32-kDa TonB-specific product as expected. While it is not certain that cleavage resulted from leader peptidase activity, it clearly occurred in response to the introduction of a leader peptidase cleavage site. In addition, it occurred at the amino terminus of

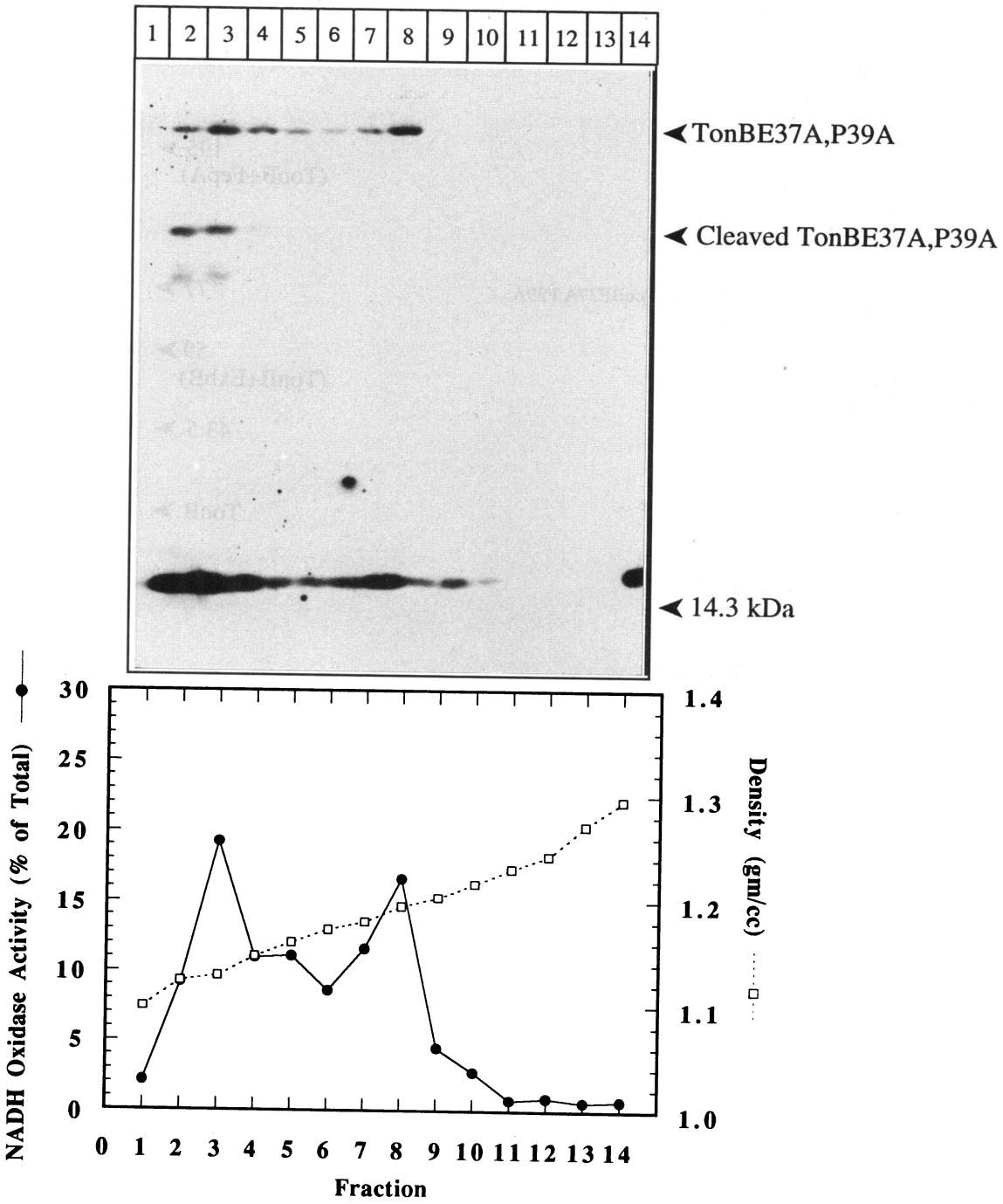

FIG. 11. Sucrose gradient fractionation of TonBE37A,P39A protein. KP1039 (Δ*tonB*) carrying plasmids pKP301 (TonBE37A,P39A) and pBST324 was grown to an OD_{550} of 0.5 without the inducer AT, converted to spheroplasts, lysed, and layered onto a sucrose density gradient as described in Materials and Methods. Fractions of approximately 1 ml were collected. The upper panel shows an immunoblot of the fractions, developed with a TonB-specific MAb. The lower panel shows refractive indices and NADH oxidase activities of the corresponding fractions. The positions of TonBE37A,P39A and cleaved TonBE37A,P39A are indicated along with that of the 14.3-kDa marker.

TonBE37A,P39A, since the 32-kDa cleavage product could be detected by immunoblotting with a MAb specific for the TonB carboxy-terminal 32 amino acids. Furthermore, the 32-kDa species was not generated by the envelope protease OmpT or DegP, since it was also detected in *ompT* and *degP* strains. Under inducing conditions, cleavage of TonBE37A, P39A was rather slow but was essentially complete within 5 min. Slow cleavage of an engineered leader peptidase recognition site has also been observed previously (33). Both full-length and cleaved TonBE37A,P39A accumulated at steady state, to approximately equal extents. Vitamin B_{12} assays indicated that under those conditions, the combination of full-length

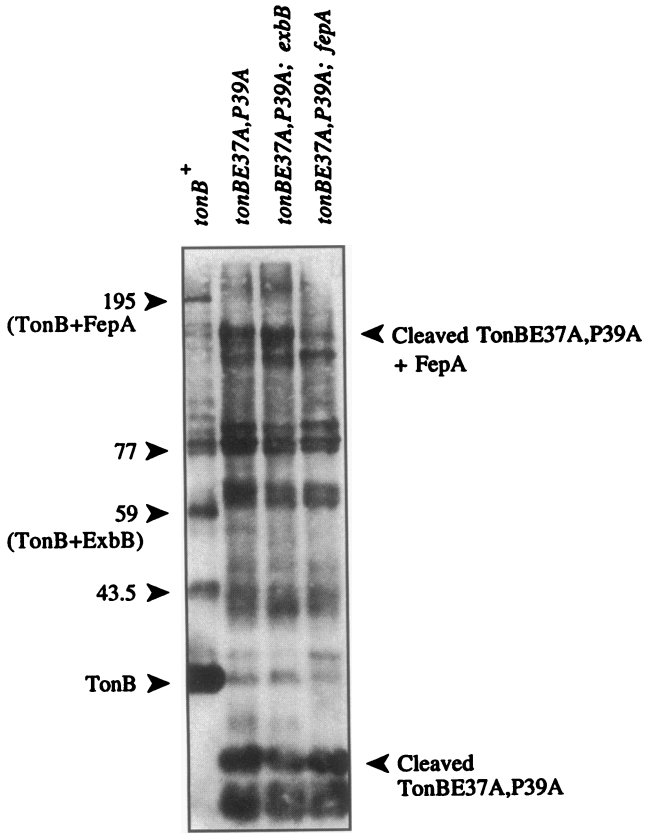

FIG. 12. In vivo cross-linking of cleaved TonBE37A,P39A. From left to right, strain GM1 (*tonB*$^+$), strain KP1082 (Δ*tonB*) carrying plasmids pKP301 (*tonBE37A,P39A*) and pBST324, strain KP1119 (Δ*tonB exbB*::Tn*10*) carrying plasmids pKP301 (*tonBE37A,P39A*) and pBST324, and strain KP1120 (Δ*tonB* Δ*fepA*) carrying plasmids pKP301 (*tonBE37A,P39A*) and pBST324 were cross-linked in vivo as described in Materials and Methods. Samples representing equal cell numbers (except for the KP1119 sample, which contained twice as many cell equivalents) were electrophoresed on SDS-polyacrylamide gels, and TonB protein was detected by immunoblot analysis with TonB-specific MAb. On the left are noted the positions, apparent molecular masses (in kilodaltons), and, when possible, the compositions of TonB-specific bands previously observed in cross-linking wild-type TonB. On the right are noted the positions of cleaved TonBE37A,P39A monomer and a complex containing cleaved TonBE37A,P39A and FepA.

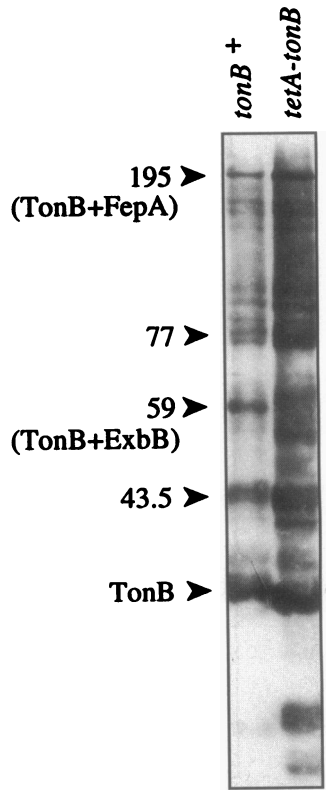

FIG. 13. In vivo cross-linking of TetA-TonB. Strains GM1 (*tonB*$^+$) and strain KP1082 (Δ*tonB*) carrying plasmids pKP321 (*tetA-tonB*$^+$) and pBST324 were cross-linked in vivo as described in Materials and Methods. Samples representing equal cell numbers were electrophoresed on SDS-polyacrylamide gels, and TonB protein was detected by immunoblot analysis with TonB-specific MAb. On the left are noted the positions, apparent molecular masses (in kilodaltons), and, when possible, the compositions of TonB-specific bands previously observed in cross-linking wild-type TonB.

TonBE37A,P39A and cleaved TonBE37A,P39A had TonB activity. Addition of chloramphenicol stopped synthesis of full-length TonBE37A,P39A, allowed conversion entirely to its cleaved form, and resulted in the loss of TonB activity, demonstrating that even if both the amino terminus and the periplasmically accessible domain are synthesized, TonB must remain connected to its energy source to remain active.

Under noninducing conditions, cleaved TonBE37A,P39A could interfere with normal TonB function. These results suggest that the inactive portions of the cleaved TonBE37A, P39A, either amino terminal or carboxy terminal, can displace functional TonB interactions with other proteins (4). In the TonB system, TonB proteins inactivated by carboxy-terminal deletions showed no dominant negative effect, although the assays used to detect it were not described (3). In contrast, fusion of 34 or 71 TonB amino-terminal amino acids to BlaM (19) interferes with the ability of ϕ80 to adsorb irreversibly to cells; this is probably the most sensitive assay for TonB activity and the one whereby the interference of cleaved TonBE37A, P39A was detected. In the somewhat analogous Tol system (28), the carboxy-terminal 131 amino acids of TolA can interfere with wild-type TolA function. Therefore, it may be that either the amino terminus or the periplasmically accessible domain of cleaved TonBE37A,P39A, or both, could interfere with wild-type TonB activity. Alternatively, the cleaved amino terminus might be rapidly degraded by signal peptide peptidase. The observed interference is consistent with previously reported dominant negative effects of overproduction of TonB protein (9, 29). In this situation, overexpression of TonB protein ironically results in decreased activity, most likely because overexpressed TonB exceeds the capacity of chromosomally encoded ExbB to stabilize it. Excess TonB is then degraded, with the degradation products interfering with wild-type TonB function. The inability to detect competition by cleaved TonBE37A,P39A in the vitamin B_{12} transport assays in which both full-length TonBE37A,P39A and cleaved TonBE37A,P39A are present could be due to the overproduction of those TonB species to a level at which they are not limiting for vitamin B_{12} transport.

Also consistent with the interference is the observation that cleaved TonBE37A,P39A could be cross-linked in vivo to several proteins, one of which appears to be the outer membrane receptor FepA. The putative complex of cleaved

TonBE37A,P39A with FepA was absent from the Δ*fepA* strain. Although the apparent mass of the FepA complex with cleaved TonBE37A,P39A was smaller than predicted (160 versus 190 kDa), this may reflect cross-linking at different sites which could then greatly change the migration of the complex in the gel. Such large, position-dependent molecular mass shifts have been observed in cross-linking studies with aspartate chemoreceptor dimers (35). It is puzzling that the 77-kDa cluster seen with full-length TonB did not appear to shift to a correspondingly lower molecular mass, as the FepA complex did. The 77-kDa complex does shift to an appropriately lower apparent molecular mass in TonB with the proline-rich region deleted (27). It may be that the 77-kDa complex in cells expressing wild-type TonB contains TonB that has lost its amino terminus because of membrane proteases. When we have identified the components of the 77-kDa complex, we will hopefully be in a position to understand this result. In any case, a similar number of complexes were formed upon cross-linking of cleaved TonBE37A,P39A.

Consistent with the results from cross-linking TetA-TonB, one of the proteins to which cleaved TonBE37A,P39A could not be detectably cross-linked was ExbB. While some or all of the cross-links may have formed prior to its cleavage, this result nonetheless strongly suggests that TonB cannot be cross-linked to ExbB through any of the periplasmic-domain amino acids subsequent to the putative cleavage site at amino acid 39. A MAb specific for the periplasmic domain was used in detection of cross-linked complexes so that any complexes consisting of ExbB and cleaved TonBE37A,P39A would have been detected at an apparent mass of 55 kDa.

Sucrose density gradient analysis indicated that full-length, wild-type TonB was associated both with the cytoplasmic membrane, as expected, and with a membrane fraction of intermediate density that clearly also contained cytoplasmic membrane by virtue of its NADH oxidase activity. The density of that intermediate fraction ($\rho = 1.19$) correlates well with the M-band fraction previously described as containing both cytoplasmic and outer membranes (34), which would be consistent also with our ability to detect TonB cross-linked to FepA outer membrane receptor. While this may indeed be the case, results obtained with the cleaved TonBE37A,P39A complicated the interpretation. Rather than being free in the periplasm, as assumed but not demonstrated for cleaved *S. typhimurium* TonB (19), the cleaved TonBE37A,P39A was primarily localized with the spheroplast fraction, with about one-third of the protein fractionating with the periplasm. Sucrose density gradient fractionation of strains expressing TonBE37A,P39A suggested that while the full-length TonBE37A,P39A was distributed between both the cytoplasmic membrane and the intermediate-density membrane fraction, cleaved TonBE37A, P39A was found only in the cytoplasmic membrane fraction. This result suggests that the amino terminus of TonB is required for association with that denser fraction. One possible explanation is that the cleaved TonBE37A,P39A is preferentially degraded in the intermediate-density fraction. If this explanation is correct, it would suggest that cleaved TonB E37A,P39A associated with the intermediate-density fraction has a different, protease-sensitive conformation compared with TonB fractionating with the cytoplasmic membrane at $\rho = 1.13$. This explanation is also most consistent with detection of cross-links between cleaved TonBE37A,P39A and FepA in whole cells. It may be that disruption of cells in preparation for sucrose gradients releases proteases to which cleaved TonB E37A,P39A is now susceptible. Alternatively, it may be that cleaved TonBE37A,P39A readily associates with FepA, in a fashion that can be detected by cross-linking in whole cells, but this does not reflect the stable energy-transduced interaction that leads to transport of ligands across the outer membrane and that can be detected by association of wild-type TonB with the intermediate-density membrane fraction. This idea is supported by the ability of TetA-TonB protein (which is not functional) to be cross-linked to FepA.

It is also interesting that the majority of cleaved TonBE37A, P39A remained associated with cytoplasmic membrane rather than being released into the periplasm. This result suggests that interactions, potentially with cytoplasmic membrane proteins, retain cleaved TonBE37A,P39A with the cytoplasmic membrane fraction. This would certainly be consistent with our earlier observations that TonB can be cross-linked to at least two uncharacterized proteins in 43.5- and 77-kDa complexes, in addition to FepA and ExbB (43). Since cleaved TonBE37A, P39A could not cross-link to ExbB, it may be that its association with proteins in the 43.5- or 77-kDa complex could account for fractionation with the cytoplasmic membrane. Alternatively, this fractionation could be due to association with ExbD, which extends into the periplasmic space (17). However, to date, we have not detected any TonB-ExbD interactions (1).

A final role for the amino terminus is that it serves as the export signal for TonB, since a Gly→Asp substitution at amino acid 26 prevents export of TonB from the cytoplasm. The same mutation prevents export of a hybrid TrpC-TonB protein (46), as does deletion of the amino-terminal 32 amino acids (19).

This study demonstrates that the amino terminus of TonB plays many roles. It serves as the signal for TonB export, it is required for TonB-dependent energy transduction, it is required for cross-linking to ExbB, and it is required for detectable TonB association with a fraction of the *E. coli* cell envelope that is denser than the cytoplasmic membrane but not for that with the cytoplasmic membrane itself. It is also interesting that neither the TonB amino terminus nor, by implication, the proton motive force is required for TonB cross-linking to FepA or to two other unknown proteins. These results suggest that the proton motive force plays a different role, perhaps in directly energizing the active transport of ligands through the outer membrane receptors once contact with TonB has been established (37). The fact that both the amino terminus and its connection to the periplasmically accessible domain of TonB are required for energy transduction, but not for association with envelope proteins, strengthens the idea that the source of energy is indeed the cytoplasmic membrane electrochemical potential and suggests that further roles for the amino terminus remain to be discovered. A key to elucidating the mechanism of energy transduction almost certainly lies in the complete understanding of all TonB amino-terminal interactions.

ACKNOWLEDGMENTS

We thank Linda Randall for helpful discussions and anti-maltose-binding protein antibody, Kevin Bertrand for bacterial and phage strains and AT, and Ray Larsen for critical reading of the manuscript.

This work has been supported by NIH grant GM42146 and NSF grant DCB89-03334.

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