Escherichia coli TonB Protein Is Exported from the Cytoplasm without Proteolytic Cleavage of Its Amino Terminus*

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The requirement for TonB protein in a variety of membrane-related processes suggests that TonB is an envelope protein. Consistent with this suggestion, the deduced TonB amino acid sequence (Postle, K., and Good, R. F., (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5235–5239) contains an amino-terminal region similar to leader (signal) sequences of exported proteins, although its charged region falls out of the hydrophobic core of the fusion protein and presumably TonB is transcribed as a polyacrylamide gel system as TonB synthesized in vitro. We conclude that the amino terminus of TonB is uncleaved following its export from the cytoplasm and that TonB is a membrane-associated protein. Characterization of a tonB gene fusion suggests that the amino-terminal 41 amino acids of TonB are sufficient to promote export of the fusion protein and presumably TonB as well. Models for TonB orientation within the cell envelope are presented.

TonB protein is required during processes in which both outer membrane receptors and active transport proteins in the cytoplasmic membrane participate, such as siderophore-mediated iron transport (Frost and Rosenberg, 1975; Hanke and Braun, 1975; Pugsley and Reeves, 1976; Braun et al., 1982) and vitamin B₁₂ transport (Bassford et al., 1976). TonB is also required for infection by bacteriophage T1 (Anderson, 1946; Gratia, 1966) and φ50 (Matsushima, 1963) and for the action of B-type colicins (Davies and Reeves, 1975). The mechanism of its function in these processes is unknown, but it has been suggested that it serves as an energy transducer, using cytoplasmic membrane potential energy to release transported molecules from their outer membrane receptors into the periplasm (Hancock and Braun, 1976; Holroyd and Bradbeer, 1984). It has also been postulated to cause temporary apposition of the outer and cytoplasmic membranes (Konisky, 1979). The involvement of TonB in these membrane-related phenomena suggests that it is almost certainly an envelope protein.

The DNA sequence of tonB predicts a 26-kDa protein with possible initiating methionines at positions 1, 3, and 6 of the open reading frame defined by S1 analysis of the 5’- and 3’- RNA termini (Postle and Good, 1983). The deduced amino acid sequence contains an amino-terminal hydrophobic region of 19 amino acids. The carboxyl terminus of TonB also contains a hydrophobic region of approximately 14 amino acids that could, by analogy to the penicillin-binding protein D-alanine carboxypeptidase, (Waxman and Strominger, 1981; Pratt et al., 1986), constitute a membrane anchor. The intervening 190 amino acids constitute a significantly hydrophilic region. The deduced amino acid sequence is thus most consistent with the idea that the central region of TonB is in an aqueous compartment of the cell, but that TonB could be anchored in a membrane. A number of studies have attempted to localize TonB function with varied results of periplasmic or outer membrane locations (Weaver and Konisky, 1980; Wookey and Rosenberg, 1978; DiGirolamo et al., 1971; Taylor et al., 1972). On the basis of Sarkosyl fractionation of the Escherichia coli envelope, TonB protein has been localized in the cytoplasmic membrane (Plastow and Holland, 1979); however, the precise location of TonB cannot be considered resolved (Tomassen, 1986).

In this report, we provide direct biochemical evidence that TonB protein is exported from the cytoplasm and that its amino terminus remains intact following export. We also examine the chemical half-life of TonB, determine by partial fractionation of E. coli that TonB is a membrane-associated protein, and present models for TonB orientation in the cell envelope.

MATERIALS AND METHODS

Plasmids, Bacteria, and Bacteriophage Strains — E. coli K12 N4300 (Gottesman et al., 1980), a defective λ lysogen carrying the cI657 allele, CC118 (araD139, Δlara, leu)7897, lacX74, phoAΔ20, gaiE, galK, thi, rpsE, rpoB, argEAm, recA1), and LE392 (supF, supE, hsdR, galK, trpR, metB, lacY, tonA) were the bacterial strains used in these studies. Plasmid pKC30 is an expression vector which carries an Hpal site downstream from the Xp promoter (Rosenberg et al., 1983). Plasmid pKP118 is a clone of a 1697-base pair tonB* HincII fragment from pRZS40 (Postle and Reznikoff, 1979) into pKC30 at the Hpal site such that Xp, and tonB have the same orientation. To construct plasmid pKP161, a 1455-base pair tonB* HaeII fragment was excised from pRZS40, cloned into the HincII site of M13mp7 (Messing et al., 1981), excised as an EcoRI fragment, and cloned into the EcoRI site of pBR322 (Bolivar et al., 1977). In pKP161, tonB is transcribed in the same direction as bla. Plasmid pKP167 is identical to pKP161 except that it is a clone of the 1697-base pair tonB* HincII fragment.

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To isolate a tonB-phoA fusion, $\alpha$TnphoA (b221, c857, Pcon) was grown on strain LE392 and used to infect CC118 carrying pKP161 as described (Mznol and Beckwith, 1985). Colonies expressing high levels of alkaline phosphatase activity were detected by blue colonies on TYE agar containing 40 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 100 mg/ml ampicillin, 300 mg/ml kanamycin.

medium was M9 minimal salts (Shedlovsky and Brenner, 1963) supplemented with 0.4% D-glucose, 4 mg/ml thiamine hydrochloride, 1 mM magnesium sulfate, 100 mg/ml bixin, and a 30 mg/ml concentration of all t-aa acids except methionine.

Enzymes and Biochemicals—Phenylmethylsulfonyl fluoride, 5-bromo-4-chloro-3-indolyl phosphate p-toluene sulfonic acid, bovine serum albumin (A5503), p-nitrophenyl phosphate disodium-hexahydrate, protein A covalently coupled with Sepharose CL-4B, diethyl pyrocarbonate, sodium deoxycholate, and kenzidine hydrochloride were purchased from Sigma. Proteinase K and aprotinin were from Boehringer Mannheim. DNA-directed in vitro transcription/translation kits and [35S]methionine (~400 Ci/mmol) were purchased from Amersham Corp. Triton X-100 was purchased from Aldrich. T4 DNA ligase and restriction enzymes were purchased from Bethesda Research Laboratories and New England Biolabs. SDS (specially pure) was purchased from BDH Pharmaceuticals LTD, Autofluor was purchased from National Diagnostics, Inc. The synthetic peptide was purchased from Bachem.

Synthesis in Vitro—A standard reaction (5 ml of S30 cell extract) contained 25 mg of purified plasmid DNA as template along with 18 mg of [35S]methionine, according to the instructions provided with the kit. Reactions were incubated at 37 °C for 1 h and terminated by addition of methionine chase solution (provided with the kit) and by addition of an equal volume (35 ml) of sample buffer (Laemmli, 1970). Samples were heated to 95 °C for 3 min, electrophoresed on 11% SDS-polyacrylamide slab gels (0.5 mm thick) (Laemmli, 1970), dried, and autoradiographed to locate TonB protein.

Analysis of [35S]Methionine in Amino-terminal TonB Amino Acid Sequence—Regions of the dried acrylamide gel containing [35S]methionine-labeled TonB protein were excised and rehydrated in sterile distilled water.

To determine TonB half-life, aliquots were precipitated with methionine at 42 °C for 10 min and labeled with [35S]methionine (60 mg/ml) of cells. After 1 min, a 600-fold excess of cold methionine was added. Aliquots of 500 ml were precipitated with an equal volume of cold acetone, suspended in sample buffer, heated, and electrophoresed as described above.

To determine TonB half-life, aliquots were removed and acetone-pelletized at 0, 5, 10, 15, 30, 60, and 90 min following the addition of unlabeled methionine. Samples were suspended in sample buffer, heated, and either electrophoresed or immunoprecipitated (see below) and electrophoresed. Autoradiographs were scanned by a Helena Laboratories gel scanner. For the estimation of TonB chemical half-life, the amount of TonB in Fig. 6 was normalized to the amount of all t-aa acids except methionine and high percentage of proline.

Analysis of the peptide and its highly charged nature of the peptide and its high percentage of proline suggested that it was likely to correspond to a solvent-exposed portion of the protein and therefore to be antigenic. The peptide was coupled to the bovine serum albumin using bis-diazotized benzidine (Walter et al., 1980). Antiserum to the synthetic peptide was obtained from a 5- to 6-month-old New Zealand female rabbits with 1.5 mg of peptide-bovine serum albumin conjugate emulsified in 2.0 ml of Freund's complete adjuvant. Intradermal injection was carried out at 13 sites and cutaneous injection (0.5 ml) at three sites. The animals were boosted four times, approximately 4 weeks apart, and bled 10 days after each boost. Antiserum was characterized by enzyme-linked immunosorbent assay using peroxidase-linked goat anti-rabbit IgG (Qualex) and by immunoprecipitation of TonB synthesized in vitro.

Preparation of Blocked Protein A-Sepharose—N4830 carrying pKP118 was grown at 30 °C in 50 ml of minimal labeling medium to OD900 = 1.0, centrifuged, and 10 ml was added to 25 ml of Triton X-100, 0.9 M sucrose, 0.1% SDS, 0.001% aprotinin, 0.15 M sodium chloride, 0.01 M Tris-HCl, pH 7.4, followed by 10 ml of sucrose or, as a control, no sucrose (Semler et al., 1986). After incubation on ice for 30 min, the cell fraction of A-Sepharose was added to collect immune complexes, and the samples were incubated on ice for 30 min with occasional mixing. Protein A-Sepharose beads were washed vigorously three times with RIPA buffer and once with 0.05 M sodium chloride, 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA. After the final wash was completed, beads were resuspended in 60 ml of RIPA sample buffer, heated to 95 °C for 3 min, electrophoresed on a polyacrylamide gel, treated with autoradiography, and exposed to film.

Proteinase K Accessibility—TonB synthesis was induced, and the culture was pulse-labeled as described above. Proteinase K accessibility experiments were carried out essentially according to the method of Randall and Hardy (1986), except that whole cells or spheroplasts were centrifuged for 1 min rather than 40 s, and fractions were precipitated with acetone rather than trichloroacetic acid. Whole cell pellets were suspended in 0.2 M Tris acetate, pH 8.2, 20 mM magnesium sulfate; and divided into two samples, one of which was treated with proteinase K.

Conversion to Spheroplasts and Cell Fractionation—TonB synthesis was induced, and the culture was pulse-labeled as described above. Cells were transferred to spheroplasts by a variation of the procedure of Witholt et al. (1976) as follows. After 1 min of labeling, 1 ml of cells was removed to prechilled microcentrifuge tubes and pelleted for 5 min at 39,000 × g in a Sorval RC-5B centrifuge. The pellet was suspended in 250 μl of 0.2 M Tris-HCl, pH 8.0, 0.5 M sucrose, 0.5 mM EDTA. Lysozyme was added to a final concentration of 160 μg/ml, followed by the immediate addition of 250 μl of 0.2 M Tris-HCl, pH 7.4, 1 mM EDTA. Cultures were shaken for 3 min at 37 °C. Cultures were then resuspended in 10 mM MgSO4 and then precipitated with an equal volume of cold acetone for 30 min. Acetone pellets from both fractions were suspended in 100 μl of Laemmli (1970) sample buffer, heated for 10 min at 95 °C for proteinase K accessibility and electrophoresed.

In order to examine fractionation of a known periplasmic protein (maltose-binding protein), N4830 carrying pKP118 was grown at 30 °C to OD900 = 0.25 in minimal labeling medium containing 0.4% maltose in place of glucose. Cells (2 ml) were pulse-labeled at 30 °C for 1 min with 120 μCi of [35S]methionine and fractionated as described above.

RESULTS

Identification of TonB Synthesized in Vitro—[35S]Methionine-labeled proteins encoded by plasmids pBR322 and its tonB" derivative, pKP167, are shown in Fig. 1 (upper). Com-
**Fig. 1.** Upper, identification of TonB protein synthesized in vitro. An autoradiogram of \[^{35}S\]methionine-labeled proteins is shown. Lane a, template DNA is pBR322; lane b, template DNA is pKP167, a tonB\(^+\) derivative of pBR322. Lower, comparison of TonB protein synthesized in vitro and in vivo. An autoradiogram of proteins labeled with \[^{35}S\]methionine either in vivo (lanes a and b) or in vitro (lane c) as described under “Materials and Methods” is shown. Lane a, N4830 carrying plasmid pKC30; lane b, N4830 carrying plasmid pKP118, a tonB\(^+\) derivative of pKC30; lane c, template DNA is pKP118. The arrows indicate the position of TonB protein.

**Fig. 2.** 5' region of tonB gene. The 5' end of tonB RNA (Postle and Good, 1983) is indicated by an asterisk. Probable –35 and –10 regions of the tonB promoter and the Shine-Dalgaro sequence are indicated by overlining. The amino-terminal amino acid sequence of TonB deduced from partial amino acid sequence results is shown above the DNA sequence. Underlining indicates the amino-terminal hydrophobic region of TonB. The region of the fusion junction formed by TnphoA insertion is indicated by overlining.

parison of two profiles reveals in pKP167 (lane b) a single strongly expressed band of 36 kDa, which we identify as TonB protein. The aberrant behavior of TonB protein in SDS-polyacrylamide gels has been observed previously and is probably due to the high proline content (16%) of TonB (Postle and Reznikoff, 1979; Postle and Good, 1983).

Partial Amino-terminal Amino Acid Sequence for TonB Synthesized in Vitro—The amino terminus of TonB is unknown. Methionine codons exist at positions 1, 3, 6, 54, 206, and 215 of the tonB open reading frame localized between the 5' and 3' termini of tonB RNA (Postle and Good, 1983). In addition, there are potential initiating valyl codons (encoded by GUG in the mRNA) at positions 29, 73, 92, 108, 154, 193, 211, 230, and 231 of the tonB open reading frame. However, since in-frame fusion of codons 1–26 of trpC to codons 17–244 of the tonB open reading frame encodes a single fusion polypeptide of expected size, only the methionine codons at positions 1, 3, and 6 (Fig. 2) are actual candidates for the initiation codon.

To determine a partial sequence of the TonB amino terminus, \[^{35}S\]methionine-labeled TonB protein synthesized in vitro was purified by gel electrophoresis, and radioactive TonB was analyzed by use of an automated gas-phase amino acid sequenator. The amounts of \[^{35}S\] radioactivity in the sequenator cycles are shown in Fig. 3. A single \[^{35}S\]methionine-labeled peak appears in cycle 1, consistent with initiation at the third methionine (codon 6 of the open reading frame). All further references in this paper to amino acid positions are based on the assignment of codon 6 as the initiating methionine (Fig. 2).

Identification of TonB Synthesized in Vivo—In order to express high levels of TonB in a regulated manner, the tonB gene was placed under control of the bacteriophage XPL promoter in plasmid pKP118 and the cI857 temperature-sensitive repressor in strain N4830. TonB expression can be induced by denaturing the repressor during a temperature shift from 30 to 42 °C. Fig. 1 (lower) shows an autoradiogram comparing proteins from N4830/pKP118 grown at 30 °C (lane a) or at 42 °C (lane b) labeled during a 1-min incubation with \[^{35}S\] methionine. A unique protein band with an apparent molecular mass of 36 kDa is induced by growth at 42 °C compared to 30 °C. This protein (lane b) has an identical mobility to TonB protein synthesized in vitro (lane c) and is not synthesized at 42 °C in N4830/pKC30 (data not shown). On the
basis of its inducibility at 42 °C and its apparent molecular mass, it is possible to identify the unique protein band as the TonB protein. The same result was obtained when the samples were run on a polyacrylamide gel (Laemmli, 1970) with 7 M urea or on a phosphate-buffered SDS-polyacrylamide gel (Randall and Hardy, 1977) (data not shown). These results suggest that the amino terminus of TonB is not cleaved in vivo.

Chemical Half-life of TonB—Our failure to detect TonB among Coomassie Blue-stained total cellular proteins synthesized following temperature induction of N4830/pKP118 (data not shown), as well as a published report of a short functional half-life for TonB (Bassford et al., 1977), prompted us to examine the chemical half-life of TonB. Fig. 4 shows results of a pulse-chase experiment performed on N4830/pKC30 (lanes 1–7) and on N4830/pKP118 (lanes 8–14). A unique 36-kDa protein band is present in N4830/pKP118 following a 1-min pulse with [35S]methionine (compare lanes 1 and 9), but disappears rapidly. In order to determine the TonB half-life, a portion of the samples from the pulse-chase experiment (Fig. 4) were immunoprecipitated using antitonalB peptide antiserum. Comparison of immunoprecipitates of the 0-min chase of N4830 carrying either pKC30 or pKP118 (Fig. 5, lanes b and i) serves to identify TonB protein (which also co-migrates with TonB protein synthesized in vitro; data not shown). Immunoprecipitation of the 5-, 10-, 15-, 30-, 60-, and 90-min samples (lanes c–h, respectively) demonstrates degradation of TonB protein with time, whereas the bands immunoprecipitated by preimmune control serum (lane a) remain stable. The estimated chemical half-life for TonB under these conditions appears to be biphasic. The majority of TonB appears to be degraded with a half-life of approximately 10 min, whereas a small proportion appears to be degraded more slowly.

Proteinase K Accessibility and Partial Fractionation of TonB—The TonB hydropathy plot (Postle and Good, 1983) predicts that, if TonB is an envelope protein, the central hydrophobic region of TonB will extend into an aqueous compartment, either cytoplasm or periplasm. To determine whether TonB protein extends into the periplasm, the proteinase K sensitivity of TonB in whole cells, spheroplasts, and lysed spheroplasts was determined. As shown in Fig. 6, TonB is insensitive to proteinase K in whole cells (lanes 1 and 2). The majority of TonB (~90%) is sensitive in spheroplasts (lanes 3 and 4) and is completely sensitive to proteinase K in the lysed spheroplasts (lanes 5 and 6). These results suggest that TonB is periplasmically exposed.

To test whether TonB is free in the periplasm or membrane-associated, cells synthesizing TonB under AP box control were converted to spheroplasts and separated into a periplasmic fraction and a spheroplast fraction containing outer membrane, cytoplasmic membrane, and cytoplasm. The periplasmic and spheroplast fractions from cells induced at 42 °C in glucose/minimal labeling medium are shown in Fig. 7 (lanes c and d, respectively). The spheroplast fraction in lane d contains essentially all of the TonB protein. As a control, the same bacteria were grown in maltose/minimal labeling medium at 30 °C, pulse-labeled for 1 min, and fractionated. Under these conditions, TonB protein is not detectable, but the 38-kDa maltose-binding protein is induced and fractionates, as expected, with the periplasm (compare lanes a and b). These results suggest that TonB is membrane-associated.
Characterization of Active tonB-phoA Gene Fusion.—To determine whether the TonB amino terminus is involved in its export, we isolated a tonB-phoA gene fusion and characterized the level of alkaline phosphatase which it synthesized. Since the amino termini of exported proteins can promote export of alkaline phosphatase lacking its own leader sequence, TnphoA insertions into genes encoding exported proteins synthesize high levels of alkaline phosphatase whenever the protein fusion boundary is periplasmically localized (Manoil and Beckwith, 1986). Insertions into genes encoding cytoplasmic proteins have little or no activity since cytoplasmically localized alkaline phosphatase appears to be enzymatically inactive (Michaelis et al., 1983). TnphoA insertions were isolated in pKP161, a pBR322 derivative carrying the tonB gene. Most of the TnphoA insertions resulting in high levels of alkaline phosphatase activity were in the pBR322 blu gene. One insertion expressing high levels of activity was correctly oriented in tonB as determined by restriction enzyme analysis. The position of the tonB::TnphoA fusion junction was shown to occur between TonB amino acids 39 and 41 (Fig. 2). Strains carrying this plasmid-borne gene fusion synthesize 180 units of alkaline phosphatase, suggesting that the region following TonB amino acids 39-41 is periplasmically exposed and that the TonB amino terminus can function in export (Manoil and Beckwith, 1985).

DISCUSSION

To investigate TonB export and localization, we began by characterizing the amino terminus of TonB protein synthesized in vitro. The DNA sequence of tonB specifies an open reading frame whose location was consistent with the location of the 5’ and 3’ termini of tonB RNA (Postle and Good, 1983) and with the location of four tonB::IS1 insertions (Postle and Reznikoff, 1979). The candidates for initiation codons are 3 methionines occurring at positions 1, 3, and 6 of the open reading frame (Fig. 2). To determine which of the 3 methionines was utilized in vitro, the initiating methionine for TonB was determined by 10 cycles of automated Edman degradation of [35S]methionine-labeled TonB protein synthesized in vitro. The recovery of [35S]radioactivity in a single peak corresponding to cycle 1 is consistent with in vitro initiation of TonB protein synthesis at the sixth codon, methionine, of the open reading frame (Figs. 2 and 3). The tonB DNA sequence also argues strongly for initiation at codon 6 of the open reading frame. Of the 3 potential initiating methionines, only the
The deduced amino acid sequence of the amino terminus codon 6 methionine is associated with a ribosome-binding site that is similar to the 3' end of 16S RNA (Fig. 2). These results suggest that TonB protein synthesized in vitro has not been proteolytically processed at its amino terminus.

TonB protein synthesized in vivo has been detected in minicells (Postle and Reznikoff, 1979) and in UV-irradiated cells infected with a λtonB transducing phage (Plastow and Holland, 1979). Minicells have the disadvantage that they are generally defective in the proteolytic processing of leader sequences (Silhavy et al., 1983), whereas decreased expression or accumulation of TonB in the λtonB-infected, UV-irradiated bacteria made detection a problem. We have developed an improved system for detecting TonB protein synthesized in vivo. In this system, TonB is expressed from a plasmid carrying the tonB gene cloned downstream from the bacteriophage λP1 promoter. TonB expression is induced following heat inactivation of host-encoded cI857 repressor protein. By inducing at 42 °C and pulse labeling with [35S]methionine for 1 min, detection of TonB in an unfractionated protein profile of whole cells is straightforward (Fig. 4). Comparison of the relative gel mobilities of TonB synthesized in vitro and in vivo, using three different polyacrylamide gel electrophoresis systems, indicates that they are identical. In addition, we have been unable to detect the in vitro cleavage of TonB by purified leader peptidase (a generous gift of W. Wickner) under conditions where pre-b-lactamase is cleaved (data not shown). On the basis of these results and other results to be discussed below, we conclude that TonB protein is not proteolytically processed in vivo at its amino terminus. This result is not surprising since the deduced TonB amino acid sequence lacks a consensus leader peptidase cleavage site (von Heijne, 1983; Perlman and Halvorson, 1983) in the region 6–7 amino acids carboxyl-terminal to the hydrophobic region (Fig. 2). A notable consensus cleavage site (Val-Val-Ala-Gly; amino acids 23–26) does exist, but it is within the hydrophobic region, making cleavage at this site unlikely according to current models for leader peptidase action (Dalbey and Wickner, 1985; von Heijne, 1985).

The functional half-life of TonB protein in vitamin B12 transport had been previously determined to be 15 min at 42 °C (Bassford et al., 1977). Since temperature induction of the pKP118 clone did not result in accumulation of TonB relative to other Coomassie Blue-stained cellular proteins (data not shown), we began to suspect that TonB protein might have a short chemical half-life as well. A pulse-chase experiment indicated that the chemical half-life of TonB at 42 °C is approximately 10 min, in good agreement with the TonB functional half-life and consistent with our failure to accumulate TonB protein in vivo. The results of the half-life determination also have a bearing on the interpretation of Fig. 1 (lower), a comparison of TonB synthesized in vitro and in vivo. Immunoprecipitation of the pulse-chase samples indicates that TonB is not processed to a "mature form" typical of many exported proteins, thus confirming the suggestion that the amino terminus of TonB is not proteolytically cleaved following its export. The fact that antibodies directed against a synthetic TonB peptide can immunoprecipitate TonB protein confirms our original assignment of the TonB open reading frame (Postle and Good, 1983).

The phenotypes of tonB mutants involve defects in membrane-related processes and thus predict that TonB is an exported protein. The results of proteinase K accessibility experiments support this hypothesis. The majority of TonB protein synthesized in pKP118 is proteinase K-sensitive in spheroplasts but not whole cells, indicating that a portion of TonB is periplasmically exposed and therefore exported. A small fraction of TonB induced from pKP118 appears to be cytoplasmically localized, perhaps as a result of its overexpression. This finding is consistent with the apparent biphasic half-life of TonB, but will require further investigation.

Either the uncleaved hydrophobic TonB amino terminus or the hydrophobic carboxyl terminus, which resembles the carboxyl-terminal membrane anchor of the penicillin-binding protein D-alanine carboxypeptidase (Waxman and Strominger, 1981; Pratt et al., 1986), could serve as a membrane anchor. The results of fractionation indicate that TonB fractionates with spheroplasts under conditions where maltose-binding protein fractionates with the periplasm. These results suggest that TonB is membrane-associated and, together with the proteinase K accessibility of TonB in spheroplasts, allow us to conclude that the amino terminus of TonB is uncleaved following its export from the cytoplasm.

The deduced amino acid sequence of the amino terminus

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**Fig. 7. Determination of cellular location of TonB and maltose-binding protein.** Lanes a and b, periplasmic and spheroplast fractions of N4830/pKP118 grown at 30 °C in maltose medium and pulse-labeled for 1 min with [35S]methionine, respectively. The position of maltose-binding protein (MBP) is indicated. Lanes c and d, periplasmic and spheroplast fractions of N4830/pKP118 grown in glucose medium, thermally induced at 42 °C, and pulse-labeled for 1 min with [35S]methionine, respectively. Lane e, in vitro transcription/translation of pKP118. The position of TonB protein is indicated.

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**Fig. 8. Models for cellular location of TonB protein.** The amino and carboxyl termini of TonB are designated N and C, respectively. Hydrophobic regions are indicated as black rectangles. OM, outer membrane; CM, cytoplasmic membrane. The rationale for each model is presented under "Discussion."
of TonB (Postle and Good, 1983) is similar to those of prokaryotic exported proteins (Michaels and Beckwith, 1982). It has a charged region (Asp×Arg×Arg×), followed by a 19-aminoc acid hydrophobic region (aminoc acids 14-32; Fig. 2). It differs from common leader sequences in that the charged region contains a negatively charged amino acid (Asp) and is longer (13 amino acids) than expected. To determine whether the amino terminus of TonB is involved in export, a tonB-phoA gene fusion was isolated which fused the first 39–41 TonB amino acids to an alkaline phosphatase lacking its own amino-terminal leader sequence. Surprisingly, only one tonB-phoA fusion was isolated, compared to approximately 20 bla-phoA fusions. A possible explanation is that fusions carboxy-terminal to TonB amino acids 39-41 may be more likely to result in rapid degradation of TonB-PhoA hybrid proteins, with a concomitant decrease in detectable alkaline phosphatase activity. Nevertheless, the one tonB-phoA fusion isolated encoded substantial alkaline phosphatase activity. Nevertheless, the one tonB-phoA fusion isolated encoded substantial alkaline phosphatase activity (180 units), indicating that the first 39–41 amino acids of TonB contain information capable of directing the export of alkaline phosphatase and, by implication, TonB as well. These results also suggest that the TonB-PhoA fusion boundary is periplasmically localized.

The data in this paper suggest the models shown in Fig. 8 as possibilities for TonB orientation in the cell envelope. The features of the models are as follows. 1) The amino-terminal hydrophobic sequence is a membrane anchor. By analogy to the chemotaxis chemotransducers (Kriks et al., 1983; Bollinger et al., 1986; Mowbray et al., 1985; Manoil and Beckwith, 1986) and leader peptidase (Moore and Miura, 1987), it is unlikely that such structures exist within the central region of TonB. This speculation is consistent with the proteinase K accessibility results and with the analysis of the tonB-phoA gene fusion. 2) The carboxy-terminal hydrophobic sequence may be free in the periplasm (model I), anchored in the cytoplasmic membrane (model II), or anchored in the outer membrane (model III). Models have been proposed where TonB protein functions by interacting with outer membrane receptors (Hancock and Braun, 1976; Konisky, 1979; Holroyd and Bradbeer, 1984). In support of this hypothesis, amino acid sequences of TonB-dependent outer membrane receptors examined to date appear to have similar amino-terminal regions (Krone et al., 1985; Coulton et al., 1986; Heller and Kadner, 1986; Lundrigan and Kadner, 1986). A mutation in the amino-terminal region of the vitamin B₁₂ outer membrane receptor (BtuB) causes the receptor to function as if it failed to interact productively with TonB: the mutant BtuB can bind vitamin B₁₂ but cannot transport it across the outer membrane (Reynolds et al., 1980). Each of the three models in Fig. 8 is consistent with current hypotheses for TonB function; determining which model is correct will provide insight into the mechanism of its function. We are presently pursuing experiments to differentiate among these models.

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