Alterations at the Carboxyl Terminus Change Assembly and Secretion Properties of the B Subunit of Escherichia coli Heat-Labile Enterotoxin

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The gene encoding the B subunit of heat-labile enterotoxin (etxB) was mutated at its 3' end by targeted addition of random nucleotide sequences. Gene products from five mutated etxB genes, all of which were shown to encode B subunits with short carboxyl-terminal amino acid extensions, were analyzed with respect to a range of functional and structural properties. One class of altered B subunits, exemplified by EtxB124 and EtxB138, which both have seven extra amino acid residues, were found to be specifically defective in their ability to stably associate with A subunits and form holotoxin. Other altered B subunits were less subtly affected by extensions at their C termini and were, in addition to their failure to associate with A subunits, unable to translocate into the periplasm of Escherichia coli, to pentamelize, or to bind to GM1 ganglioside. This suggests that the carboxy-terminal domain of EtxB mediates A subunit-B subunit interaction.

Heat-labile enterotoxin (LT) from Escherichia coli is a potent toxin which causes severe diarrheal disease in humans and certain domestic animals (for a review, see references 25). It is a multimeric protein consisting of a single A subunit of 28 kilodaltons (kDa) and five identical B subunits of 12 kDa each (8). Its structure, immunological properties, and toxic activity resemble very closely those of the cholera enterotoxin produced by Vibrio cholerae (4, 8, 15, 16, 22, 30). The genes specifying the two subunit polypeptides of LT (etxA and etxB) are normally found on large conjugal plasmids and have been cloned from a variety of enterotoxigenic E. coli strains (4, 5, 19, 28, 34, 36, 37). LT genes from E. coli of human and porcine origin have been given various mnemonic abbreviations, including elt, etfl, eltP, and rox (3, 4, 19, 37). Since these genes are allelic, we have chosen to adopt etx as a common designation to describe all LT genes of E. coli. The etxA and etxB genes are part of a contiguous operon which encodes a single polycistronic mRNA specifying synthesis of A- and B-subunit precursor polypeptides that contain N-terminal hydrophobic signal sequences (3, 5, 23, 29, 35). The precursors are proteolytically processed during export across the E. coli cytoplasmic membrane, and the mature subunits (EtxA and EtxB) are assembled within the periplasm into holotoxin (9, 11, 13, 14). A protease-susceptible site in the A subunit, which may be cleaved by a variety of proteases including trypsin, is probably nicked by intestinal proteases during a natural infection with enterotoxigenic E. coli. This yields an enzymatically active A2 peptide of 21 kDa linked by a disulfide bridge to a smaller A3 peptide (1). Binding of the toxin to eucaryotic cell membranes is mediated by interaction of the B subunits with GM1 ganglioside, which is followed by translocation of the A3 peptide across the membrane and the activation of adenylate cyclase (2, 17, 22, 33). The resulting elevated concentration of cyclic AMP and concomitant change in ion fluxes across the cell membrane bring about the loss of fluid and electrolytes characteristic of cellular dehydration and diarrhea.

In addition to the importance of LT as a pathogenicity factor of enterotoxigenic E. coli, the subunits of the toxin exhibit several distinctive properties which make them convenient models for studying the relationship between protein structure and function. Of particular interest are the properties exhibited by the B subunit of LT, including their ability to interact with one another to form stable pentamers, the association of A and B subunits to form holotoxin, translocation of B subunits across the outer membranes of certain gram-negative bacteria such as V. cholerae (10, 12), and interaction of B subunits with GM1 ganglioside.

In this paper we examine the effect of introduced sequence alterations at the C terminus of the B subunit on its export, assembly, and receptor-binding properties. We show that the addition of just a few amino acids at this region can alter drastically the ability of the B-subunit polypeptide to form oligomers, associate with A subunits, and be translocated through membranes.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli strains were CC118 [araD139 ara leu-7697 lacX74 phoA20 sulE galK thi rpsE rpoB argE(Am) recA1] (20) and CSR603 (recA1 uvrA6 phr-l) (26). V. cholerae TRH7000 is a thy derivative of strain JBK70, which lacks the cholera toxin genes as a result of a chromosomal deletion (12).

The genes for heat-labile enterotoxin were obtained from an E. coli H74-114 isolate of human origin. Recombinant plasmids carrying these genes were pWD605 (etxA+ etxB) and pWD615 (etxA etxB+) (3). The controlled expression vector plasmid was pMB66EH (7).

Media and growth conditions. LB medium, liquid or solidified with 1.5% Difco agar (21), supplemented with 1.0 mM thymine was used for subculturing bacteria. Synthesis medium (6) supplemented with 1.0 mM thymine was used for cultivating V. cholerae strains. M9 medium (21) supplemented with 0.4% glucose, 1.0 mM thymine, 18 L-amino acids (all except methionine and tryptophan) (40.0 mM each), and

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0.01% ampicillin was used for induction of the cloned etxB gene and for radioactive labeling of proteins. Cultures were grown at 37°C on a gyratory shaker. At an optical density of 0.2 at 650 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM, and after a 2-h incubation, 100 µCi of [35S]methionine (1,000 Ci/mmol) was added for 10 min. Plasmid-encoded proteins were detected by the maxi-cell method with strain CSR603 (26).

**Toxin and enzyme assays.** Proteins were released from the periplasm by treatment of the cells with lysozyme and EDTA (11). The concentration of EtxB and EtxA protein in medium or cell fractions was determined by a GM1 ganglioside–enzyme-linked immunosorbent assay (GM1-ELISA) (30). The activity of β-lactamase was assayed by measuring the hydrolysis of nitrocefin as described above (10).

**Immunoprecipitation of enterotoxin subunits.** Centrifuged cells (from 0.2 ml of labeled culture) were suspended in 0.2 ml of 0.75 M sucrose–10 mM Tris hydrochloride (pH 7.6), and then 20 µl of lysozyme (2 mg/ml) was added, followed by 0.4 ml of 1.5 mM EDTA–0.3 M KCl. After 10 min at 0°C, the cells were sonicated (two 10-s bursts of the Branson Sonifier B-12) at full power. To this suspension, 0.8 ml of 1.0% Triton X-100–0.1 mM phenylmethylsulfonyl fluoride was added, and the mixture was centrifuged for 60 min at 80,000 × g in an Eppendorf centrifuge. Supernatants were mixed with 2.5 µl of polyclonal rabbit anti-cholera toxin antiserum (αR420) or with 5.0 µl of rabbit anti-cholera toxin A-subunit antiserum (αR833). After incubation at 0°C for 60 min, 25.0 mg of rehydrated protein A-Sepharose was added, and incubation was continued at room temperature for a further 60 min. The suspension was then centrifuged, and the pellet was washed with 0.15 M NaCl–0.5% Triton X-100–5.0 mM EDTA–10.0 mM Tris hydrochloride (pH 7.6)–0.1 M PMSF, then with the same buffer containing 0.5 M NaCl, and finally with 10 mM Tris hydrochloride (pH 7.6)–0.1 M PMSF. Washed protein A-Sepharose was mixed with 20 µl of 2× electrophoresis sample buffer (18), incubated at 100°C for 5 min, and centrifuged, and 20 µl of the supernatant was loaded onto a sodium dodecyl sulfate (SDS)–polyacrylamide gel (15% acrylamide) prepared as described by Laemmli (18). Molecular weight standards and their respective molecular masses (in kilodaltons) were as follows: bovine serum albumin (68.0) and carbonic anhydrase (29.0) (Sigma Chemical Co.) and E. coli alkaline phosphatase (49.0) (Pharmacia, Inc.). Prestained molecular range markers (Bethesda Research Laboratories, Inc.) were as follows: ovalbumin (43.0), chymotrypsigenin (25.7), β-lactoglobulin (18.4), lysozyme (14.3), and bovine trypsin inhibitor (6.2). For autoradiography of dried gels Cronex no. 4 film (E. I. du Pont de Nemours & Co., Inc.) was used.

**Localization of EtxB during growth of V. cholerae.** V. cholerae TRH7000 harboring different plasmids were grown with aeration at 37°C in Synacore broth. At an optical density of 0.2 at 650 nm, IPTG (1.0 mM) was added. Samples were removed at various times and centrifuged (8,000 × g for 2 min in an Eppendorf centrifuge). Cells were washed once with the ice-cold medium, suspended in the same volume of medium, and disrupted by sonication. Samples of cell-free medium and sonicated cells were analyzed for EtxB concentration and β-lactamase activity. In all experiments the activity of β-lactamase was >98% cell associated.

**Insertion of the etxB gene under tac promoter control.** The smaller EcoRI–HindIII fragment (0.59 kilobases) was excised from plasmid pWD615 and inserted between the same sites of plasmid pMMB66EH. The resulting recombinant plasmid, pMMB68 (Fig. 1), contains the entire coding sequence and the ribosome-binding site of the etxB gene and only six codons from the 3′ end of the etxA gene, including its stop codon (19, 36). The etxB gene in the recombinant plasmid pMMB68 was inserted downstream of the inducible tac promoter.

**Introduction of carboxy-terminal extensions into the EtxB protein.** The etxB gene contains a recognition site for SpeI endonuclease, which spans the stop codon (Fig. 2). Ligation of random nucleotide sequences at this site would introduce short extensions to the coding sequence of the gene until a new stop codon was encountered.

Plasmid pMMB68 was digested with SpeI, and then the single-strand protruding ends were removed with mung bean nuclease. The DNA was further digested with HindIII, and the recessed ends were filled in with the aid of T4 DNA polymerase. The resulting blunt ends were religated. Determination of the nucleotide sequence in the newly created clones revealed the presence of two nucleotide insertions, one of which was 76 bases long.

FIG. 1. Construction of plasmid pMMB68. Open boxes represent coding portions of the etx region. The arrowheads indicate the direction of transcription. No vector portions of the plasmids are shown. Restriction endonuclease-sensitive sites are indicated as follows: C, Clal; E, EcoRI; H, HindIII; P, PstI; Sa, SmaI; Sa, SalI; Sp, SpeI.

FIG. 2. Nucleotide sequences of the 3′ portions of the engineered etxB genes and of the wild-type parental gene present in plasmid pMMB68. Nucleotides are numbered starting from the guanine nucleotide in the EcoRI recognition site upstream of the ribosome-binding site of the etxB gene (19). Numbers above the predicted amino acid sequence indicate the number of the amino acid residue in the mature molecule of the wild-type EtxB.
junction revealed two classes of recombinant plasmids. In one of them, exemplified by pMMB113, four adenyl residues were present, at positions 384 to 387, instead of the expected five. In the other, exemplified by pMMB141, only three adenyl residues remained, at positions 384 to 386 (Fig. 2).

Using a similar strategy, we constructed plasmid pMMB117 from pMMB113 by digestion with HindIII, filling in the recessed ends with T4 DNA polymerase, and religation. We obtained plasmid pMMB124 from pMMB113 by HindIII digestion, filling in the recessed ends with T4 DNA polymerase, digestion at the XmnI site present in the vector sequences (7), and religation. Plasmid pMMB138 was saved as a by-product of an attempted fusion between two genes. In this construction a HindIII DNA fragment containing the coding sequences of the mature portion of \( \beta \)-lactamase, from the plasmid pKTH74 (24), was inserted into the HindIII site of pMMB113. The fusion between the two coding sequences was out of frame, however, owing to a loss of one adenyl residue. In the resulting plasmid, pMMB112 (structure not shown), the HindIII site was recreated upon ligation. DNA of pMMB112 was digested with EcoRI and XmnI, and the smaller fragment was isolated by electrophoresis in an agarose gel and inserted between the EcoRI and Smal sites of pMMB66. The plasmid thus obtained was called pMMB138.

Nucleotide sequences at the 3' ends of the newly created genes were verified by the dideoxy chain termination method (27). The determined nucleotide sequences, together with the predicted amino acid sequences, are shown in Fig. 2.

RESULTS

Short amino acid extensions at the C terminus of the B subunit of LT were engineered so that the possible contribution of this region to the various properties of the subunit could be assessed. Extensions were introduced onto the B subunit by manipulating the etxB gene. First, the gene was recloned from plasmid pWD615 and introduced into a controlled expression vector, pMMB66EH, so that the new recombinant plasmid, pMMB68, carried the etxB gene under the control of the tac promoter and the lacF gene (Fig. 1; see Materials and Methods). B-subunit production in E. coli harboring plasmid pMMB68 was inducible in the presence of IPTG (Fig. 3). A single, conveniently situated recognition site for SpeI in pMMB68 made it possible to introduce random nucleotide sequences at the 3' end of the etxB gene. Using this approach, we generated a variety of nucleotide alterations at this site, which changed the coding sequence of the gene and gave rise to novel EtxB polypeptides with C-terminal extensions (Fig. 2).

When the authentic unaltered B subunits were synthesized in the maxicell strain E. coli CS603 carrying plasmid pMMB68 and then analyzed by SDS-polyacrylamide gel electrophoresis, the B subunits were found to migrate with an apparent molecular mass of approximately 12 kDa (Fig. 3, lane 2). An identical analysis of the altered B subunit EtxB113, which has an additional six amino acid residues, revealed that it had an electrophoretic mobility of only 11 kDa (lane 4). The lower apparent molecular mass of EtxB113 may be due to the influence of the extra amino acids on SDS binding and migration. EtxB117, which contains 17 additional amino acid residues, had an apparent molecular mass of 13.2 kDa (lane 6). EtxB124 and EtxB138, both of which have seven extra amino acids at their C termini, had electrophoretic mobilities similar to that of the authentic B subunit (lanes 8 and 10). EtxB141, which carries an addi-
released from the periplasm may occur because the additional amino acids cause the polypeptides either to aggregate or to be anchored to cell membranes.

**Recognition of altered B subunits by anti-cholera toxin antibodies.** All altered B subunits, except EtxB141, were immunoprecipitated by anti-cholera toxin antiserum (Fig. 5). Thus the amino acid extensions on EtxB113, EtxB117, EtxB124, and EtxB138 do not prevent the subunits from displaying native epitopes. However, for both EtxB113 and EtxB117, the quantity of B subunits immunoprecipitated was consistently lower than for EtxB124 and EtxB138. The reason for this became apparent when we analyzed the clarification pellets from the preparatory steps of the immunoprecipitation and found that they contained approximately 90% of the total EtxB113 and EtxB117 polypeptides (data not shown). Thus the majority of these polypeptides were absent from the extracts used for immunoprecipitation.

**Binding of altered B subunits to GM1.** The above data showed that except for EtxB141, the altered B subunits were recognized by anti-cholera toxin antibodies. We therefore used a GM1-ELISA technique, with either the polyclonal antisera or monoclonal antibodies to EtxB, to determine whether the subunits had retained their ability to bind to GM1. We found that EtxB124 and EtxB138 were both detectable in the GM1-ELISA assay, whereas EtxB113 and EtxB117 were not (Table 1). We conclude that the extra amino acid residues on EtxB124 and EtxB138 do not interfere with the GM1-binding domain of the subunit.

**Oligomerization of altered B subunits.** The B subunits of LT ordinarily exist as pentamers in the periplasm of *E. coli*, and when they are analyzed on SDS-polyacrylamide gels they migrate as stable oligomers (9). It is only after heat treatment at >70°C that the pentamers dissociate into their constituent monomers.

When periplasmic fractions containing either authentic B subunit, EtxB124, or EtxB138 were analyzed with or without heat treatment, it was found that the altered as well as the wild-type B subunits migrated as oligomers (Fig. 6). Interestingly, the electrophoretic mobilities of the EtxB124 and EtxB138 oligomers were slower than those of the authentic B oligomer, thereby unequivocally demonstrating

![FIG. 4. Export of the wild-type and altered EtxB polypeptides in *E. coli.* Strain CC118, containing plasmids indicated below, were exposed to 1.0 mM IPTG for 2 h and then labeled with [35S]methionine for 10 min. Periplasmic and spheroplast fractions were then isolated, and equivalent amounts were subjected to SDS-polyacrylamide electrophoresis and autoradiography. Lanes: 1 and 2, pMMB68; 3 and 4, pMMB113; 5 and 6, pMMB117; 7 and 8, pMMB124; 9 and 10, pMMB138; 11 and 12, pMMB141. Arrows indicate the positions of B-subunit polypeptides. Numbers on the right side of the gel show the positions of molecular mass markers.

![FIG. 5. Recognition of the wild-type and altered B subunit by the anti-cholera toxin antibodies. *E. coli* CC118, containing the plasmids indicated below, were labeled as described in the legend to Fig. 4. Labeled cells were disrupted by sonication, and the B-subunit polypeptides were immunoprecipitated with anti-cholera toxin antiserum αR420 as described in Materials and Methods. Lanes: 1, pMMB68; 2, pMMB113; 3, pMMB117; 4, pMMB124; 5, pMMB138; 6, pMMB141.

![FIG. 6. Oligomerization of wild-type and altered EtxB polypeptides. Strain CC118 containing plasmids indicated below were cultured in the presence of 1.0 mM IPTG for 2 h and then labeled with [35S]methionine for 10 min. Periplasmic fractions were isolated and mixed with an equal volume of 2× concentrated sample buffer (18). Equal portions were incubated at 100°C for 5 min or kept at room temperature and then subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Lanes: 1 and 2, pMMB68; 3 and 4, pMMB124; 5 and 6, pMMB138. Samples in lanes 1, 3, and 5 were heated to 100°C, whereas those in lanes 2, 4, and 6 were kept at room temperature before being loaded onto the gel.

| TABLE 1. GM1 ganglioside-binding property of altered B subunits determined by GM1-ELISA |
|-----------------|-----------------|-----------------|
| E. coli strain(plasmid) | Subunit produced | Subunit concn (ng/ml) |
|                 |                  | Expt I*          | Expt II*         |
| CC118(pMMB68)   | EtxB             | 1,060           | 177              |
| CC118(pMMB113)  | EtxB113          | 0               | 0                |
| CC118(pMMB117)  | EtxB117          | 0               | 0                |
| CC118(pMMB124)  | EtxB124          | 300             | 60               |
| CC118(pMMB138)  | EtxB138          | 360             | 55               |

* B subunit detected with monoclonal antibody LT-39.
* B subunit detected with anti-cholera toxin antiserum αR420.
that the amino acid extensions on EtxB124 and EtxB138 are not removed prior to oligomerization and that such extensions do not interfere with B-subunit–subunit interaction.

A similar analysis of the clarification pellets obtained during the preparatory steps for immunoprecipitation of EtxB113 and EtxB117 showed that >70% of the EtxB113 and >90% of the EtxB117 polypeptides migrated as monomers on SDS-polyacrylamide when kept at room temperature, in comparison with the percentage migrating as monomers when the samples were boiled (data not shown). This suggests that the amino acid extensions on these subunits may be interfering in either a direct or indirect manner with the domains involved in B-subunit assembly.

**Mediation of subunit A-subunit B interaction by the EtxB carboxy-terminal domain.** We constructed E. coli strains which harbored pWD605 (which encodes the A subunit of LT), together with the plasmids that code either for authentic B subunit or for EtxB124 or EtxB138. This was done to assess whether EtxB124 and EtxB138 can assemble into holotoxin.

Immunoprecipitates from cell extracts of these strains were prepared by using anti-cholera toxin A-subunit antiserum (Fig. 7, lanes 1 to 5) or anti-cholera toxin A/B-subunit antiserum (lanes 6 to 10) and analyzed by SDS-polyacrylamide gel electrophoresis. Authentic EtxB was found to be immunoprecipitated by both anti-A-subunit and anti-A/B-subunit antiserum (compare lanes 3 and 8). Therefore authentic B subunits are assembled into a holotoxin complex that is immunoprecipitable by antiserum raised against cholera toxin A subunit.

When similar analyses were performed on extracts containing both the A subunit and either EtxB124 or EtxB138, none of the B subunits were immunoprecipitated by anti-A-subunit antiserum (Fig. 7, lanes 4 and 5), whereas they were precipitated by the anti-A/B-subunit antiserum (lanes 9 and 10). This shows that the seven extra amino acids at the C termini of EtxB124 and EtxB138 prevent the subunits from stably interacting with the A subunit of LT.

When sonic lysates of E. coli strains harboring the various plasmid combinations shown in Fig. 7 were assayed by GM1-ELISA with monoclonal antibody LT-39, we found that only the A subunits from the strain producing authentic B subunits could be detected (data not shown). This confirms that the C-terminal region of EtxB defines a domain that is important for A-subunit association.

**Secretion of altered B subunits from V. cholerae.** V. cholerae can secrete both assembled holotoxin and LT B subunits into the extracellular milieu (12). Therefore we constructed V. cholerae strains encoding either EtxB124, EtxB138, or authentic B subunits and then assayed cultures for the distribution of the subunits in the medium and cells. We found that EtxB124 and EtxB138 were secreted from V. cholerae as efficiently as, if not more so than, authentic B subunits (Table 2). We therefore conclude that the extra amino acids on the C terminus of EtxB124 and EtxB138 do not interfere with subunit translocation from V. cholerae.

### DISCUSSION

In this paper we describe the use of a new strategy for studying the structure and function of a potent diarrheagenic enterotoxin. It involves the addition of short amino acid extensions onto the carboxy terminus of the B subunit. This approach left unchanged 102 of the 103 amino acids that constitute the mature EtxB subunit (19). We therefore expected that the extensions might cause only subtle perturbations in B-subunit structure and trap the molecule at some intermediate step of export, assembly, or secretion. In this way we hoped to be able to define the role of the C-terminal domain of EtxB in contributing to the various properties of the molecule.

The most striking observation obtained by this approach was that the addition of seven extra amino acid residues onto the C terminus of EtxB prevented stable interaction and assembly with EtxA. Two of the altered B subunits with this defect, EtxB124 and EtxB138, were normal with respect to a range of other properties, including export to the periplasm of E. coli, oligomerization into pentamers, secretion from V. cholerae, recognition by anti-toxin antibodies, and binding to GM1 gangloside. The amino acid sequences of the extensions in EtxB124 and EtxB138 differ from one another in the preponderance of negatively and positively charged residues, and both extensions have a single proline residue.

Chou-Fasman analyses of EtxB124 and EtxB138 predict that the extensions have a strong tendency to adopt an α-helical conformation and would thereby extend the α-helix predicted to be present in the authentic carboxy-terminal region (our unpublished results). Our data suggest that the exten-

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<th>TABLE 2. Secretion of B subunits from V. cholerae</th>
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<td>Subunit concn (ng/ml)</td>
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<td>-----------------------</td>
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<tr>
<td>V. cholerae strain</td>
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<tr>
<td>TRH70000(pMMB68)</td>
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<tr>
<td>TRH70000(pMMB124)</td>
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* Determined by GM1-ELISA with monoclonal antibody LT-39.
* Percentage of total subunit secreted.

**FIG. 7.** Inability of altered B subunits to associate with A subunits. E. coli CC118 containing plasmids indicated below were induced with IPTG and labeled as described in the legend to Fig. 4. Cells were disrupted, immunoprecipitated, and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Samples in lanes 1 through 5 were precipitated with antiserum aR833 directed against subunit A of cholera toxin, and those in lanes 6 through 10 were precipitated with antiserum aR420 against the cholera holotoxin. Lanes: 1 and 6, pMMB68; 2 and 7, pWD605; 3 and 8, pMMB124; and 9, pMMB138 and pWD605; 5 and 10, pMMB138 and pWD605. Positions of the A and B subunits are indicated by the arrowheads.
sions on EtxB124 and EtxB138 cause negligible alterations to the B-subunit overall structure. We therefore conclude that the amino acid extensions mask a site, at or close to the C terminus, which is required for stable interaction of the A subunit.

The extensions on EtxB113 and EtxB117 interfere with a range of properties normally exhibited by the authentic B subunit. It is unclear why this should be, although one possible explanation is that the extra carboxy-terminal amino acids trap the polypeptides in folded states that have not yet attained the tertiary or quaternary structures required to express its various properties. Both EtxB113 and EtxB117 fold into conformations recognized by anti-cholera toxin antibodies. However, the proportion immunoprecipitated from cells expressing these subunits was only a small fraction of the total amount present. Most of the EtxB113 and EtxB117 subunits were found in sedimentation pellets after extraction of cells with lysozyme and EDTA, sonication, and addition of low levels of Triton X-100. Thus it is not clear whether only a fraction of the subunits fold correctly and are therefore extractable and immunoprecipitatable, or whether all of the EtxB113 and EtxB117 subunits would have immunoprecipitated if the extraction procedure had been more efficient.

One interpretation of the sedimentation characteristics of EtxB113 and EtxB117 would be that the molecules aggregate. It is known, for example, that authentic LT and B pentamers tend to aggregate under certain physicochemical conditions (11, 31). Alternatively, the carboxy-terminal extensions, which include a stretch of six hydrophobic residues, may anchor the molecules in the membrane, thereby preventing extraction. A further observation that EtxB113 and EtxB117 migrate mainly as monomers on SDS-polyacrylamide gels could indicate that the extensions in these subunits interfere with the process of B-subunit-B-subunit oligomerization. The extension on EtxB141 contains a stretch of six hydrophobic amino acids followed by four polar residues. We were unable to detect EtxB141 in E. coli CC118, even though it could be observed in maxicells irradiated with UV light. We suspect that the molecule is unstable and rapidly degraded.

Previous studies by Tsuji et al. (32) have resulted in the isolation of a mutant enterotoxigenic E. coli strain that produces an EtxB subunit that does not bind to GM1 ganglioside. Other defective B subunits, altered in export, assembly, or secretion, have not been reported. The work described here, involving targeted mutagenesis on the 3' end of the etxB gene, has enabled us to obtain several different EtxB subunits that are defective in different properties. One class, represented by EtxB124 and EtxB138, is specifically defective in B-subunit-A-subunit association. Thus the approach we have used can generate subtle as well as major perturbations in structure and may therefore be of more general use in analyzing the structural and functional properties of other complex proteins.

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LITERATURE CITED


