Deletion mutations in N-terminal α1 helix render heat labile enterotoxin B subunit susceptible to degradation

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Heat-labile enterotoxin (LT) from enterotoxigenic *Escherichia coli* is a heterohexameric protein consisting of an enzymatically active A subunit, LTA, and a carrier pentameric B subunit, LTB. It is clear from the crystal structure of LTB that the N-terminal α1 helix lies outside the core structure. However, the function of the N-terminal α1 helix of LTB is unknown. The present work was carried out to investigate the effect of site-directed mutagenesis of the α1 helix on LTB synthesis. Six amino acids (PQSITE) located at positions 2–7 from the N terminus, including 4 aa from the α1 helix, were deleted by site-directed mutagenesis. The deletion resulted in complete inhibition of LTB expression in *E. coli* when expressed along with its signal sequence. A single amino acid deletion within the α1 helix also resulted in loss of expression. However, a single amino acid deletion outside the α1 helix did not affect LTB synthesis. Mutant proteins, whose synthesis was not detected *in vivo*, could be successfully translated *in vitro* by using the coupled transcription–translation system. Immunoblot analysis, Northern blot analysis, and *in vitro* transcription–translation data collectively indicate that the lack of synthesis of the mutant proteins is caused by the immediate degradation of the expressed product by cellular proteases rather than by faulty translation of mutant LTB mRNA. Coexpression of the LTA could not rescue the degradation of LTB mutants.

gene expression | site-directed mutagenesis | coupled transcription–translation

Diarrhea caused by the enterotoxigenic *Escherichia coli* (ETEC) is a major cause of death in developing countries, especially among children, with an estimated mortality of 1.5 million cases every year (1–3; for review, see ref. 4). Approximately 20% of cases of traveler's diarrhea are caused by ETEC, and thus the organism spreads to the developed countries (4, 5). ETEC produces a number of virulence factors, such as enterotoxins and colonization factors. Of these, the heat-labile (LT) and heat-stable enterotoxins produced by the ETEC are the major virulence factors responsible for its pathogenicity (5, 6). The LT belongs to a family of bacterial proteins designated heat-labile enterotoxins and shares phenotypic and genotypic similarities with other members of the family such as cholera toxin produced by *Vibrio cholerae* (7, 8; for review, see ref. 9).

The mature toxin consists of a single A polypeptide (LTA) and five B polypeptides (LTB) (10–12). LTB and cholera toxin B show a high degree of homology, with 85% conservation of amino acids (13). The genes of the two LT subunits, *elt*A and *elt*B, are transcribed as a single polycistronic mRNA (14) and are expressed with signal peptides. The two subunits are synthesized as a precursor protein, and each subunit has its own ribosome-binding site (15–17). After cleavage of the signal peptide, the two subunits of LT are released into the periplasmic space where they spontaneously assemble into a mature holotoxin (18, 19). LTA is known to influence LTB oligomerization. In the course of LTB pentamerization, LTA associates noncovalently with the B oligomer. However, LTA cannot associate with fully assembled LTB subunits. Because of defects in folding, the unassembled B monomers are rapidly degraded (20). LTB is responsible for delivering catalytically active LTA to target cells by binding to GM1 ganglioside receptors and thus acts as a carrier molecule (10, 21–26). The strong GM1 ganglioside receptor-binding activity demonstrated by LTB makes it an important mucosal adjuvant, and thus LTB shows great promise for developing oral vaccines.

The crystal structure of LT has been solved and can serve as an excellent model system to study the structure–function relationship of different regions. The N-terminal α1 helix of LTB is a structure whose function is unknown. The crystal structure shows that the α1 helix lies outside the core structure and interacts with the β5 strand through a disulfide bond between Cys^9 and Cys^{86} (27). However, a crystal structure by itself is not enough to define the role of a domain or an amino acid in protein expression and function.

The N terminus of several polypeptides has been shown to serve multiple functions, including protein expression, folding, and interaction with other molecules (28–31). The N-terminal region of ribosomal protein S7 is crucial for its interaction with the 3′ major domain of 16S rRNA (28). The α helix present at the N terminus of rhodanese is known to participate in initial folding, in the global stability of the protein, and in providing resistance to degradation (29). In the case of aldehyde dehydrogenase-1 and -2, the N terminus helps in folding and maintaining protein stability (31). The N- and C-terminal helices of cytochrome *C* are the first to form and serve as a docking surface to guide subsequent folding of the protein (32). Because a specific role for the N-terminal α1 helix of LTB has not yet been established, the present work was undertaken to understand its role in LTB expression and secretion.

Results

Deletion of the N-Terminal 6 Amino Acids from the α1 Helix of LTB Impairs Expression in *E. coli*. To study the role of the N-terminal α1 helix in structure–function analysis, 6 N-terminal aa (PQSITE) from positions 2–7, including 4 aa from the α1 helix, were deleted (Fig. 1). It was anticipated that the mutant protein would have a better chance of achieving stable expression in its native environment through the secretory pathway. Therefore, the mutant *ltb* gene, along with its natural N-terminal signal sequence, was cloned in pMMB vector to study the effect of the mutation under its

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Abbreviations: IPTG, isopropyl β-D-thiogalactoside; LT, heat-labile enterotoxin; LTA, heat-labile enterotoxin A subunit; LTB, heat-labile enterotoxin B subunit.

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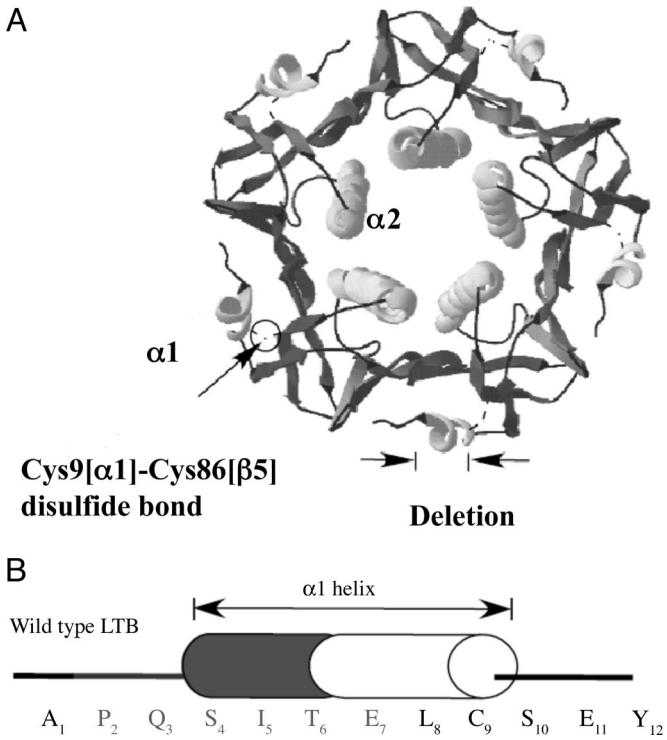

Fig. 1. Ribbon structure of LTB pentamer and sequence of N-terminal α1 helix. (*A*) Ribbon representation of LT of enterotoxigenic *E. coli*. The structure is generated by using program SWISS-PDB Viewer (52). The position of the N-terminal deletion is marked by two arrows. The Cys^{9}[α1]–Cys^{86}[β5] disulfide bond is shown by a circle. Two helices (α1 and α2) are shown. (*B*) Amino acid sequence of the N terminus of the *ltb* gene. Cylinder represents the α1 helix. Corresponding amino acids represented by single letter code are given. The deleted residues are shown in gray.

normal secretory pathway in *E. coli* DH5α. Total cell extract, culture supernatant, and periplasmic fractions were analyzed for expression of mutant LTB by immunoblotting with polyclonal anti-LTB antibody (Fig. 2). As evident from the figure, no expression of mutant protein Δ6 (N-terminal 6-aa deletion) from plasmid pLTBΔ6 was detected in the supernatant, the periplasmic fraction, or the total cell extract of induced cells of *E. coli* (lanes 1, 2, and 4,

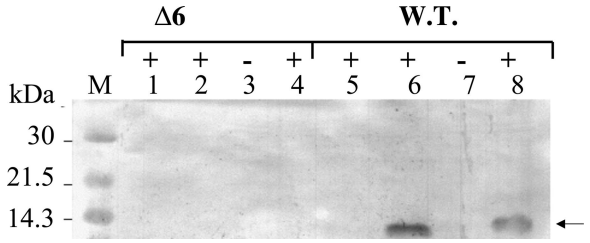

Fig. 2. Analysis of expression of mutant LTB protein. Immunoblot analysis of expression of mutant LTB clone, pLTBΔ6. *E. coli* DH5α cells harboring pMMB68 [containing the wild-type (W.T.) LTB gene] and mutant LTB plasmid pLTBΔ6 were induced with IPTG. Different fractions of *E. coli* cell lysates were analyzed for expression with anti-LTB antibodies. Lanes 1 and 2 represent culture supernatant and periplasmic fraction of induced cells harboring mutant plasmid pLTBΔ6. Lanes 3 and 4 represent total cell extract from the induced cells harboring the pLTBΔ6. Lanes 5 and 6 represent culture supernatant and periplasmic fraction from cells harboring wild-type plasmid pMMB68 grown in the presence (+) of IPTG. Lanes 7 and 8 represent total cell extract of the same. + and − denote cells grown in the presence and absence of IPTG, respectively. Arrow points to the expressed protein. The migration of the molecular mass marker (kDa) is shown on the left.

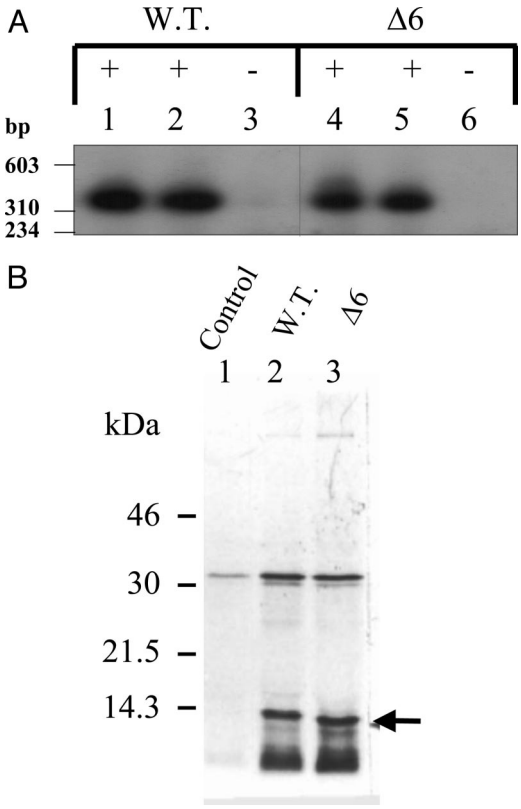

Fig. 3. Northern blot analysis and *in vitro* coupled transcription–translation analysis of mutant pLTBNΔ6 clone. (*A*) Northern blot analysis of total RNA isolated from *E. coli* DH5α cells harboring LTB plasmids, using a radiolabeled *ltb* gene probe. Lanes 1–3 represent RNA isolated from cells harboring wild-type (W.T.) LTB plasmid pMMB68. Lanes 4–6 represent RNA isolated from cells harboring mutant plasmid pLTBΔ6 (Δ6). + and − denote cells grown in the presence and absence of IPTG, respectively. Lanes 1 and 2, and 4 and 5 represent the respective samples in duplicate. Molecular mass markers (HaeIII-digested ϕX174 bacteriophage DNA) are shown on the left. (*B*) *E. coli* S30 extract for linear template was used for *in vitro* synthesis. Protein labeling was carried out in the presence of 1.85×10^6 Bq of [^{35}S]methionine. Equimolar concentrations of wild-type and mutant Δ6 clones were used. The translated product was acetone-precipitated, separated on SDS/15% PAGE, and visualized by autoradiography. Lane 1, plasmid pMMB66EH (control); lane 2, pMMB68 (W.T.); and lane 3, pLTBΔ6 (Δ6 mutant). Arrow indicates translated LTB protein. The migration of the molecular mass marker (kDa) is shown on the left.

respectively). Expression of wild-type (WT) LTB from plasmid pMMB68 in the periplasmic fraction and total cell extract (lanes 6 and 8, respectively) could be detected. The recombinant LTB, when expressed as inclusion body, could be detected in immunoblot analysis (data not shown), suggesting that the deletion mutation of LTB did not affect its reactivity with polyclonal anti-LTB antibody used in this work.

WT and Mutant LTB mRNAs Are Efficiently Transcribed in *E. coli*. The loss of expression could be caused either by faulty transcription or by faulty translation. It is likely that the mRNA of mutant LTB was not being transcribed at all or was rapidly degraded. To check whether the loss of expression was the result of an effect of the N-terminal deletion on *ltb* gene transcription, total RNA isolated from pMMB68 (WT) and deletion mutant (Δ6) clones expressed in *E. coli* DH5α was subjected to Northern blot analysis with radiolabeled *ltb* gene as a probe. As evident from Fig. 3*A*, the LTB mRNA transcript could be detected in the induced cultures of both the WT and the mutant clones (lanes 1 and 2, and 4 and 5, respectively). These results indicate that the transcription of *ltb* was

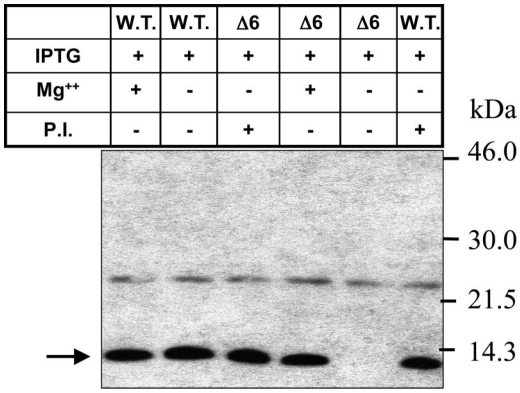

Fig. 4. Expression of mutant lone pLTBΔ6 in *E. coli* BL21(DE3)pLysS cells. *E. coli* BL21(DE3)pLysS cells harboring pMMB68 [wild-type (W.T.), lanes 1 and 2] and mutant pLTBΔ6 (Δ6, lanes 3–5) were grown in LB medium with or without 100 mM $MgCl_2$ or protease inhibitor mixture; they were induced with IPTG for 2 h. P.I. indicates the addition of complete protease inhibitor mixture (Roche, Mannheim, Germany) to the LB-ampicillin medium (2 tablets per 20 ml of medium). Total cell extract was immunoblotted with anti-LTB polyclonal antibody. + and − denote the presence and absence of the respective reagent to the medium. Uninduced or induced cultures are indicated by the absence (−) or presence (+) of IPTG, respectively. Arrow points to the expressed protein. The migration of the molecular mass marker (kDa) is shown on the right.

not affected by the deletion. The mRNA appears to be highly stable as evident from the Northern blot as well as from its efficient translation by using an *in vitro* translation system described below.

Mutant LTB Is Expressed in the *E. coli in Vitro* Translation System. It is possible that the presence of the mutation at the 5′ end of ORF changed the secondary structure of the mRNA to the extent that it was no longer suitable for initiating translation. To address this question, an *E. coli* S30 extract system was used for *in vitro* coupled transcription–translation of linear WT pMMB68 and mutant (pLTBΔ6) templates (Fig. 3*B*). pMMB66EH vector alone (lane 1) was included as a negative control. *In vitro* translation analysis revealed that like the WT LTB (lane 2), the mutant Δ6 (lane 3) protein could also be synthesized by using this system. Because the *E. coli* cell extract does not have an intact periplasmic space, the expressed protein may not process for signal peptide removal from the mutant or WT LTB. That is why the *in vitro* translated protein was ≈13 kDa instead of 11.6 kDa, like *in vivo* expressed LTB. These findings indicate that the mutation did not block translation of the *ltb* mRNA. Based on these data, the lack of expression of the mutant protein *in vivo* was probably the result of a posttranscriptional and posttranslational defects and may be attributed to protein degradation.

Expression of Mutant LTB in the Presence of Mg^{2+} Ions Rescues Its Degradation. To address the possibility of degradation of the mutant protein soon after its synthesis in *E. coli*, the mutant plasmids were transformed into *E. coli* BL21(DE3)pLysS cells (*lon* and *ompT* protease-deficient) and analyzed for expression after induction with isopropyl β-D-thiogalactoside (IPTG) (Fig. 4). It is to be noted that the *ltb* is under the control of *Tac* promoter in pMMB68, and the T7 promoter/polymerase system is not used for the expression of *ltb* in *E. coli* BL21(DE3)pLysS. These cells were used solely for being *lon* and ompT protease-deficient. Like DH5α cells, no expression of the mutant LTB (Δ6) could be detected in these cells (lane 5). Because Mg^{2+} ions at higher concentrations (≥80 mM) are known to inhibit most of the proteases (33, 34), an attempt was made to express the mutant protein in the presence of 100 mM Mg^{2+} ions. The expression of mutant LTB could be detected in the induced *E. coli* BL21(DE3)pLysS cells in the presence of Mg^{2+} ions (lane 4) and in the medium supplemented with protease inhibitor mixture (lane 6). No significant change in the expression of WT LTB was observed in the presence of Mg^{2+} (lane 1) or protease inhibitor mixture (lane 3) in the medium (compare with lane 2). A similar effect of Mg^{2+} was observed on the expression of mutant LTB (Δ6) in *E. coli* DH5α cells (data not shown). These data suggest that failure to detect the mutant protein in the induced *E. coli* cells was indeed caused by degradation of the newly synthesized mutant protein by cellular proteases.

Analysis of Additional Deletion Mutations in the α1 Helix of LTB. To identify the amino acids important for the structure–function relationship, amino acids from positions 2–7 were sequentially deleted from the N terminus of LTB to generate different mutants. Total cell extracts of *E. coli* DH5α cells transformed with the mutant LTB plasmids were analyzed for the expression of mutant proteins (Fig. 5*A*). Deletion mutant proteins ΔS4 (deletion of serine, lane 7), ΔI5 (deletion of isoleucine, lane 8), ΔT6 (deletion of threonine, lane 9), ΔE7 (deletion of glutamic acid, lane 10), and ΔT6E7 (double deletion of threonine and glutamic acid, lane 11) failed to express. However, deletion mutants ΔP2 (deletion of proline, lane 5), ΔQ3 (deletion of glutamine, lane 6), substitution mutant E7G (substitution of glycine for glutamic acid, lane 12) and E7D (substitution of aspartic acid for glutamic acid, lane 13) were expressed.

As per earlier observations, it was suspected that the lack of expression in the various mutants was caused by degradation of the expressed protein product immediately after its synthesis. To rule out the possibility of faulty transcription and translation, Northern blot analysis was carried out with total RNA isolated from the induced *E. coli* DH5α cells harboring mutant LTB plasmids. The mutant proteins ΔS4 (lane 3), ΔI5 (lane 4), ΔT6 (lane 5), ΔE7 (lane 6), and ΔT6E7 (lane 7), whose expression was not detected by Western blotting, revealed the presence of abundant mRNA (Fig. 5*B*). The *E. coli* S30 extract system for linear template was used for *in vitro* coupled transcription–translation of all mutant LTB constructs in the presence of [^{35}S]methionine. It was observed that the mRNAs for mutant proteins, ΔS4, ΔI5, ΔT6, ΔE7, ΔT6E7, and Δ6, whose expression was not detected *in vivo*, could be successfully translated by using the *E. coli in vitro* translation system (Fig. 5*C*, lanes 1–6, respectively). These results further confirm that the failure to express the mutant proteins was not caused either by faulty transcription or by faulty translation.

Coexpression of LTA and Mutant LTB to Generate Holotoxin in *E. coli*. To check whether coexpression of LTA could alter the expression pattern of mutant LTB proteins, the LTA gene was excised out of the plasmid pLTA-LTB and cloned into plasmids harboring mutant LTB genes. Periplasmic fractions of induced *E. coli* DH5α cultures were analyzed by immunoblotting with anti-LTA antibody (Fig. 6*A*). Expression of LTA (lanes 1 and 3–10) was detected in all of the induced recombinant clones. The same blot, when stripped and reblotted with anti-LTB antibody, did not result in the detection of the mutant Δ6 (lane 3), ΔT6 (lane 5), ΔE7 (lane 6), and ΔT6E7 (lane 7) (Fig. 6*B*). However, expression of WT (lane 1), ΔQ3 (lane 4), E7G (lane 8), E7D (lane 9), and ΔP2 (lane 10) could be detected. Thus, coexpression of the LTA did not alter the expression pattern of the various mutant LTB proteins (compare Figs. 5*A* and 6).

Discussion

Our data indicate that the deletion of 6 aa from the N-terminal α1 helix of LTB resulted in a complete loss of expression. Immunoblotting and Northern blot analysis, together with *in vitro* coupled transcription–translation data, suggest that the loss of expression was not because of faulty transcription and translation but because of degradation of the expressed protein. Mg^{2+} ions at a higher concentration (≥80 mM) are known to inhibit most of the proteases (33, 34), and therefore, the presence of Mg^{2+} could prevent the

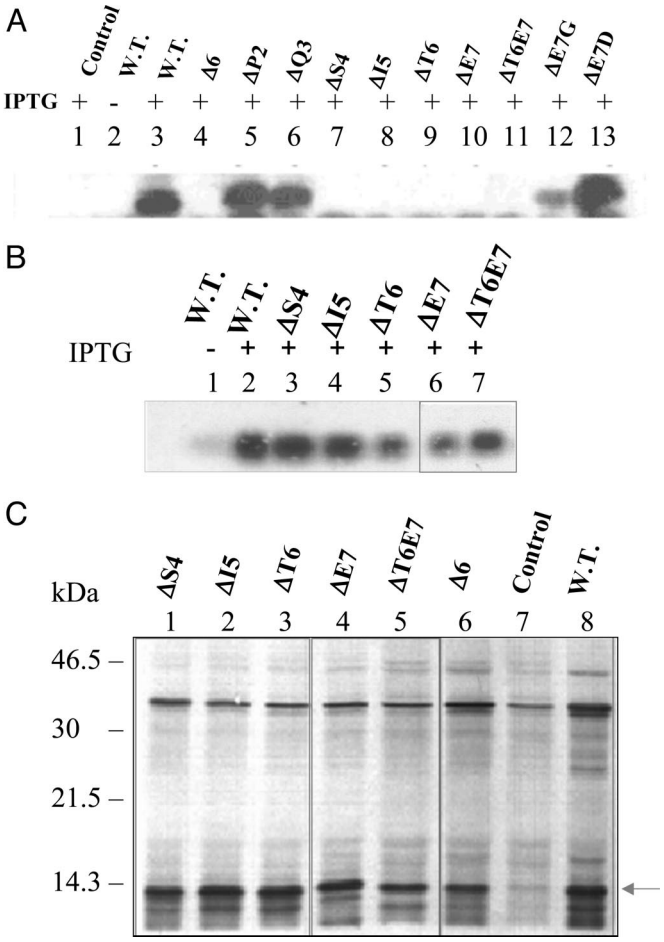

Fig. 5. Analysis of expression of other LTB mutants. (*A*) Immunoblot analysis of expression. The *E. coli* DH5α cells harboring various mutant plasmids were induced at A_{600} = 0.4 for 5 h. The total cell extracts from different cultures were analyzed by Western blotting using anti-LTB polyclonal antibodies. Lane 1, control pMMB66EH; lanes 2 and 3, wild-type (W.T.) pMMB68; lane 4, pLTBΔ6; lane 5, pLTBΔP2; lane 6, pLTBΔQ3; lane 7, pLTBΔS4; lane 8, pLTBΔI5; lane 9, pLTBΔT6; lane 10, pLTBΔE7; lane 11, pLTBΔT6E7; lane 12, pLTBE7G; lane 13, pLTBT7D. + and − denote cells grown in the presence and absence of IPTG, respectively. (*B*) Northern blot analysis of LTB mutants. *E. coli* DH5α cells harboring the respective plasmids were induced for 5 h. Total RNA was isolated and subjected to Northern blot analysis using a radiolabeled *ltb* gene probe. Lanes 1 and 2, W.T. pMMB68; lane 3, pLTBΔS4; lane 4, pLTBΔI5; lane 5, pLTBΔT6; lane 6, pLTBΔE7; lane 7, pLTBΔT6E7. + and − denote cells grown in the presence and absence of IPTG, respectively. (*C*) *In vitro* translation of LTB mutants. *E. coli* S30 extract for linear template was used for *in vitro* synthesis. Protein labeling was carried out in the presence of 1.85×10^6 Bq of [^{35}S]-methionine. Equimolar concentrations of control and deletion plasmid DNA were used. The translated product was acetone-precipitated, separated on SDS/15% PAGE, and visualized by autoradiography. Lane 1, pLTBΔS4; lane 2, pLTBΔI5; lane 3, pLTBΔT6; lane 4, pLTBΔE7; lane 5, pLTBΔT6E7; lane 6, pLTBΔ6; lane 7, pMMB66EH (negative control); lane 8, pMMB68 (W.T.). The translated product is shown by an arrow.

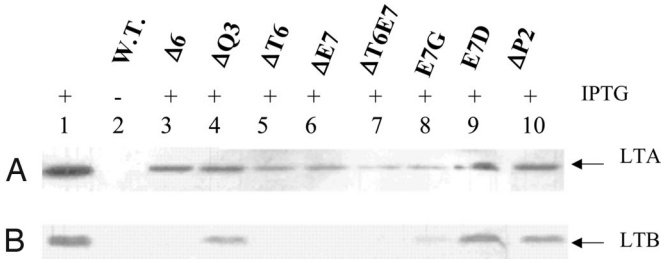

Fig. 6. Immunoblot analysis of periplasmic expression of LTA-mutant LTB holotoxin in *E. coli*. *E. coli* cells harboring plasmids containing both the LTA and LTB chains were induced at A_{600} = 0.4 for 5 h. Periplasmic fractions were analyzed for the presence of LTA or LTB subunit of holotoxin by using anti-LTA antibody and anti-LTB antibody, respectively. (*A*) Western blot analysis with anti-LTA antibody. (*B*) The same blot was then striped, washed, and immunoblotted with anti-LTB antibody. Lanes 1 and 2, pLTA-LTB; lane 3, pLTA-LTBΔ6; lane 4, pLTA-LTBΔQ3; lane 5, pLTA-LTBΔT6; lane 6, pLTA-LTBΔE7; lane 7, pLTA-LTBΔT6E7; lane 8, pLTA-LTBE7G; lane 9, pLTA-LTBE7D; lane 10, pLTA-LTBΔP2. + and − denote cells grown in the presence and absence of IPTG, respectively. Arrows indicate LTA (*A*) and LTB (*B*).

degradation of mutant LTB in *E. coli* cells. Thus, restoration of expression of mutant LTB in *E. coli* cells in the presence of Mg^{2+} ions further confirms that the mutant LTB was getting degraded soon after its synthesis (in the absence of Mg^{2+} ions).

It is evident from this work that the N-terminal α1 helix plays an important role in LTB expression in its natural environment. It is well established that LTB precursors are translocated to the periplasm in an unfolded state and fold into native structure once the export is completed (19, 20, 35, 36). Because the export is directed by the signal sequence, the deletion of α1 helix of LTB is unlikely to affect its translocation to the periplasm. It is, therefore, expected that the mutant protein lacking the α1 helix is unable to assemble into appropriately folded structure in the periplasm and is subsequently degraded. Crystal structure data suggest that the N-terminal α1 helix interacts with the β5 strand through a Cys^9–Cys^{86} disulfide bond (27). The exposed hydrophobic surface of the β barrel (β2, β3, β4 and β1, β5, β6) is masked by the solvent-exposed N-terminal α1 helix. It is likely that in the absence of the α1 helix, this property remains unsatisfied, making mutant LTB unable to fold into its native structure and thus rendering LTB susceptible to degradation by cellular proteases.

LTB is synthesized by membrane-associated polysomes, where the nascent chain is inserted into the membrane before the termination of polypeptide synthesis (37). Thus, the N terminus of the protein is the first to come in contact with the periplasmic milieu and has a better chance of folding after the removal of the signal peptide. No proteins are known to associate with LTB during the process of folding. It is believed that LTB is not assisted by folding factors or chaperones in *in vivo* folding (38). Hence, the information carried by the primary amino acid sequence should accomplish proper folding of the protein. However, DsbA protein does help in disulfide bond formation (20, 39). Refolding studies on cytochrome *C* (32) and RNase A (40) indicate that the N-terminal or C-terminal helices are the first to fold and serve as a docking surface (template) to guide subsequent folding reactions. Likewise, the N-terminal α1 helix may be providing structural stability for the nucleation of protein folding, and in its absence, the mutant Δ6 LTB is targeted for degradation. On the other hand, one can argue that it is the intrachain disulfide bond between Cys^9 and Cys^{86} that is important for maintaining the integrity of the structure. The major deletion of the α1 helix may make the disulfide bond formation difficult because Cys^9 is part of the α1 helix, which would make it impossible for the protein to fold into its native structure, and thus the misfolded mutant Δ6 LTB gets degraded. However, if the disruption of the α1 helix only affected disulfide bond formation and not subsequent protein folding, the mutant protein would have been present in the total cell extract. Earlier studies carried out in the presence of reducing agents also suggest that disulfide bond formation is not necessary for maintaining the integrity of the α1 helix (20, 41). The failure to observe the presence of the mutant protein in the total cell extract indicates that the α1 helix deletion possibly heightens the protease sensitivity of mutant LTB. Degradation of proteins resulting from misfolding is widely acknowledged (42–44). Our results are in line with the earlier reports on bovine rhodanese, wherein the N-terminal α helix of the enzyme was reported to

contribute to the global stability of the protein when expressed in *E. coli* (29).

Studies with LTB mutants with a single amino acid deletion from positions 2–7 from the N-terminal region of LTB indicate that the amino acids at positions 4–6 are crucial for protein stability. Deletion of Pro^2 or Gln^3 that lie outside the N-terminal α1 helix (at the N″ and N′ positions, respectively) did not affect expression. However, deletion of amino acids Ser^4, Ile^5, Thr^6, or Glu^7, which are part of the α1 helix, completely abolished expression. Because the amino acids Ser^4–Ser^{10} are involved in hydrogen bonding, any single deletion would be expected to cause disruption of hydrogen bonding and would thus destabilize the helix. As expected, substitution of Glu^7 with Asp within the α1 helix did not abolish the expression because the same amino acid is present at the 7th position in cholera toxin B (13). Although glycine is known to destabilize the helix (45), no effect on the expression was observed when Glu^7 was substituted with Gly. The data clearly indicate that any single amino acid deletion within the α1 helix is not tolerated. It is logical to consider that the deletion of a single amino acid from a given position within the helix should be compensated by an adjacent amino acid, restoring the necessary main-chain hydrogen bonds. However, this may not be the case with LTB. The adjacent amino acid may not be favorable at that position because certain amino acids prefer to stay at specific positions, such as at the N terminus, at the C terminus, or in the middle of the helix (45).

LT is transcribed as a single polycistronic mRNA (14). In *E. coli*, both the subunits are synthesized and exported to the periplasm, where the LTB binds to the LTA to form the holotoxin (18, 19). Streatfield *et al.* (46) have suggested that an "intramolecular folding factor" mediates coordinate assembly of A and B subunits of the toxin. Although the presence of LTA is not obligatory for LTB pentamerization, because *E. coli* strains devoid of LTA are capable of assembling LTB into a pentamer (47, 48), LTA accelerates LTB subunit pentamerization *in vivo* (20). The C terminus of the LTA interacts with the LTB subunit and stabilizes the assembly intermediate. Therefore, coexpression of LTA and LTB was performed to check whether LTA could prevent the degradation of a folding-defective mutant LTB. LTA is known to assist folding and assembly of LTB (20, 44). However, this too, could not prevent the degradation of mutant LTB proteins in *E. coli*.

Thus, the present work has clearly demonstrated that the integrity of the N-terminal α1 helix of LTB is essential for its stability and may play a role in the initial folding of the protein, thus protecting it from degradation by cellular proteases. It is likely that the folding of LTB starts at the N terminus because of the formation of the α1 helix, which provides structural stability for the folding of the protein into a ternary structure. Further studies are needed to assess directly the role of the N-terminal α1 helix in the folding of LTB into a stable conformation.

Methods

Bacterial Strains and Plasmids. *E. coli* DH5α cells [F^- *SupE*44, ΔlacU169 (ϕ80*lacZ*ΔM15) hsdR17 *recA*1 *endA*1 *gyrA*96 *thi*-1] and *E. coli* BL21(DE3)pLysS cells [F^- *ompT hsdS*$_B$ (r_B^- m_B^-)*gal dcm* (DE3) pLysS(Cm^R), *lon* and *omptT* protease-deficient] were purchased from Novagen (San Diego, CA). Plasmid pMMB68 was kindly provided by J. Holmgren (University of Goteborg, Sweden). Plasmid pDF82 harboring the LTA gene, *lta* of heat-labile enterotoxin, was a gift from H. S. Mason (Boyce Thompson Institute, New York, NY).

Bacterial Medium and Chemicals. All of the chemicals were of molecular biology grade. Bacto tryptone, yeast extract, and Bacto agar were procured from Difco Laboratories (Sparks, MD). Ampicillin was purchased from Sigma (St. Louis, MO). All restriction enzymes, DNA-modifying enzymes, and DNA markers were purchased from New England Biolabs (Ipswich, MA) and used as recommended by the suppliers. [α-^{32}P]dCTP (specific activity, 1.11×10^4 Bq/mmol at 3.7×10^8 Bq/ml) and [^{35}S]methionine (specific activity, 4.3475×10^{13}Bq/mmol at 4.07×10^8 Bq/ml) were purchased from PerkinElmer (Waltham, MA). The ECL-Western blotting kit and multiprime DNA labeling kit were procured from Amersham (Piscataway, NJ). The TRIzol-RNA isolation kit and *E. coli* S30 cell extract were obtained from Invitrogen (Carlsbad, CA) and Promega (Madison, WI), respectively.

PCR Amplification. Mutations at the N terminus of LTB were introduced by PCR using the following oligonucleotides as forward primers: Δ6, 5′-CCAGAGCTCTATGTTCGGAATATCAC-3′; ΔP2, 5′-CCAGAGCTCAGTCTATTACAGAACTATGTTC-3′; ΔQ3, 5′-CCAGAGCTCCTTCTATTACAGAACTATGTTCG-3′; ΔS4, 5′-CCAGAGCTCCTCAGATTACAGAACTATGT-TCGGAATATCAC-3′; ΔI5, 5′-CCAGAGCTCCTCAGTCTA-CAGAACTATGTTCGGAATATCAC-3′; ΔT6, 5′-CCAGAGC-TCCTCAGTCTATTGAACTATGTTCGGAATATCAC 3′; ΔE7, 5′-CCAGAGCTCCTCAGTCTATTACACTATGTTCG-GAATATCACAAC-3′; ΔT6E7, 5′-CCAGAGCTCCTCAGTC-TATTCTATGTTCGGAATATCACAACACAC-3′; E7G, 5′-CCAGAGCTCCTCAGTCTATTACAGGCCTATGTTCGG-AATATCACAC-3′; E7D, 5′-CCAGAGCTCCTCAGTCTATTA-CAGATCTATGTTCGGAATATCACAAC-3′.

The oligonucleotide 5′-TATAAAGCTTCCTAGCATTAGAC 3′ was used as a reverse primer. The oligonucleotides were synthesized by Rama Biotechnologies (Hyderabad, India) and BioSynthesis, Inc. (Lewisville, TX). The restriction sites SacI and HindIII (underlined sequences) were introduced in the primers for cloning purposes. Specific mutations were introduced into the LTB gene by PCR amplification using the above-mentioned primers, with *Taq* polymerase enzyme (Promega) in 1× reaction buffer and 1.5 mM $MgCl_2$. PCR involved denaturation at 92°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min for 30 cycles with a thermal cycler 480 (PerkinElmer). Sequencing of the mutant *ltb* gene was performed on both of the strands to confirm the induced mutations. Sequencing primers 5′-GTGTGGAATTGTGA-GCGG-3′ and 5′-TGATAACCATTTCTCTTT-3′ were used to sequence the cloned gene from the 5′ and 3′ ends, respectively.

Cloning Strategies. Plasmid DNA isolation, ligation, and bacterial transformation were carried out essentially as described by Sambrook *et al.* (49).

Cloning of mutant* ltb *gene into pMMB vector. pMMB68 is a derivative of pMMB66EH vector (GenBank accession no. X15234) in which *ltb* gene has been cloned at EcoRI–HindIII sites. The PCR-amplified product and the pMMB68 vector DNA were digested with SacI and HindIII restriction enzymes. Digestion of the plasmid pMMB68 with the above enzymes resulted in the removal of the *ltb* gene, leaving its signal sequence intact. SacI–HindIII-digested, PCR-amplified mutant *ltb* gene was then ligated with the digested plasmid containing the LTB signal sequence and transformed into *E. coli*-competent cells.

Cloning of* lta *gene in translation frame with mutant* ltb *gene. *lta* gene was amplified with pDF82 as a template. Oligonucleotides (containing an EcoRI site, underlined) 5′-CAGAATTCCGATGAA-AAATATAACTT-3′ and 5′-CCGAATTCTGTTATATATG-3′ were used as forward and reverse primers, respectively. The EcoRI-digested, PCR-amplified product was cloned at EcoRI site in plasmid pMMB68 (harboring WT *ltb* gene). This cloning resulted in the LTA and LTB subunits to be in the same translational frame. The resultant plasmid was named pLTA-LTB.

SacI–MluI-digested product from pLTA-LTB vector containing *Tac* promoter, LTA with its own signal sequence, and the LTB signal sequence, was ligated with the SacI–MluI-digested vector of different LTB mutants generated earlier (pLTBΔ6, pLTBΔP2, pLTBΔQ3, pLTBΔT6, pLTBΔE7, pLTBΔT6E7, pLTBE7G, and pLTBE7D). The recombinant plasmids were transformed into *E. coli*-competent cells. The recombinant clones thus obtained were

named pLTA-LTBΔ6, pLTA-LTBΔP2, pLTA-LTBΔQ3, pLTA-LTBΔT6, pLTA-LTBΔE7, pLTA-LTBΔT6E7, pLTA-LTBE7G, and pLTA-LTBE7D.

Expression of Mutant *ltb* Gene and Preparation of Cellular Fractions. Expression of the recombinant LTB and preparation of different cellular fractions were carried out as described earlier (50). *E. coli* cells harboring mutant or WT plasmids were grown in 50 ml of LB medium in the presence of ampicillin (50 μg/ml) at 37°C in a gyratory shaker until A_{600} reached 0.4. The cells were then induced with 1 mM IPTG and grown for 5 h at 37°C. The culture was chilled on ice, and the cells were pelleted at 4,000 $\times$ *g* for 10 min at 4°C (Sorvall centrifuge, SS34 rotor; Thermo, Waltham, MA). The cells were directly suspended in reducing sample buffer and boiled for 5 min before loading onto SDS/PAGE. Periplasmic fraction was prepared by adding 5 μl of lysozyme (2 mg/ml) and 100 μl of ice-cold buffer (100 mM sodium phosphate buffer, pH 7.6/12.5 mM EDTA/0.3 M sucrose) to the cells. The fraction was incubated on ice for 20 min with occasional gentle shaking and then centrifuged at 8,000 $\times$ *g* for 10 min in a microcentrifuge at 4°C (220.59v rotor; Hermle Labortechnïk, Wehingen, Germany). The collected supernatant represented the periplasmic fraction.

SDS/PAGE and Western Blotting. The samples were analyzed on 12% or SDS/15% polyacrylamide gel as described by Laemmli (51). The proteins were electrotransferred onto nitrocellulose membrane at 35 mA overnight. Nonspecific sites were blocked by incubating the membrane in 1% nonfat milk powder in 50 mM PBS (pH 7.4) for 1 h. The blot was then incubated with goat anti-LTB polyclonal antibodies (Reagent Bank, National Institute of Immunology, New Delhi, India) followed by washing with PBS buffer containing 0.05% Tween 20. The blot was then incubated with anti-goat IgG horseradish peroxidase conjugate (Vector Laboratories, Burlingame, CA) for 1 h followed by three washes with 0.05% Tween 20/PBS buffer. The immunoreactive bands were visualized by using 4-chloro-1-naphthol (5 mg/ml) and hydrogen peroxide (1 μl/ml) or by the ECL-Western blotting kit. Stripping buffer (100 mM β-mercaptoethanol/2% SDS/62.5 mM Tris·Cl, pH 6.7) was used when immunoblotting with a different antibody was to be carried out. The blot was washed three times with 80 mM PBS (pH 7.3) after stripping.

Northern Blot Analysis. Uninduced and induced cultures of *E. coli* cells harboring mutant or WT plasmid were grown for 5 h, and total RNA was prepared by using an RNA midi kit (Qiagen, Valencia, CA) or TRIzol reagent. The RNA was electrophoresed in a 1.2% formaldehyde gel at 50 mA for 2 h and transferred to nylon membrane (GeneScreen; PerkinElmer) by the capillary transfer method as described by Sambrook *et al.* (49). The *ltb* probe was prepared by radiolabeling the *ltb* fragment with [α-^{32}P]dCTP, using a random primer labeling kit (Amersham). The blot was hybridized with radiolabeled probe for 2 h at 68°C followed by washes with 2$\times$ SSC/0.5% SDS at room temperature for 5 min, 2$\times$ SSC/0.1% SDS at room temperature for 15 min, 0.1$\times$ SSC/0.5% SDS at 67°C for 1 h, and 0.1$\times$ SSC at room temperature for 5 min. The blot was then subjected to autoradiography.

***In Vitro* Translation.** *E. coli* S30 extract for the linear template from Promega was used for *in vitro* translation. S30 extract was mixed with template DNA ($\approx$4 μg), and protein labeling was carried out by using 1.5 μl of [^{35}S]methionine (4.3475 $\times$ 10^{13} Bq/mmol at 4.07 $\times$ 10^{8} Bq/ml) in a 50-μl reaction mix at 37°C for 2 h. Five microliters of the reaction mix was then precipitated with 20 μl of acetone, centrifuged at 10,000 $\times$ *g* for 10 min, air-dried, electrophoresed on SDS/polyacrylamide gel, and visualized by autoradiography.

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