INFECTION AND IMMUNITY, Sept. 1991, p. 2870–2879
0019-9567/91/092870-10$02.00/0

Vol. 59, No. 9

Effect of Site-Directed Mutagenic Alterations on ADP-Ribosyltransferase Activity of the A Subunit of *Escherichia coli* Heat-Labile Enterotoxin

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Received 12 March 1991/Accepted 5 June 1991

Previous studies of the S1 subunit of pertussis toxin, an NAD^+-dependent ADP-ribosyltransferase, suggested that a small amino-terminal region of amino acid sequence similarity to the active fragments of both cholera toxin and *Escherichia coli* heat-labile enterotoxin represents a region containing critical active-site residues that might be involved in the binding of the substrate NAD^+. Other studies of two other bacterial toxins possessing ADP-ribosyltransferase activity, diphtheria toxin and *Pseudomonas* exotoxin A, have revealed the presence of essential glutamic acid residues vicinal to the active site. To help determine the relevance of these observations to activities of the enterotoxins, the A-subunit gene of the *E. coli* heat-labile enterotoxin was subjected to site-specific mutagenesis in the region encoding the amino-terminal region of similarity to the S1 subunit of pertussis toxin delineated by residues 6 through 17 and at two glutamic acid residues, 110 and 112, that are conserved in the active domains of all of the heat-labile enterotoxin variants and in cholera toxin. Mutant proteins in which arginine 7 was either deleted or replaced with lysine exhibited undetectable levels of ADP-ribosyltransferase activity. However, limited trypsinolysis of the arginine 7 mutants yielded fragmentation kinetics that were different from that yielded by the wild-type recombinant subunit or the authentic A subunit. In contrast, mutant proteins in which glutamic acid residues at either position 110 or 112 were replaced with aspartic acid responded like the wild-type subunit upon limited trypsinolysis, while exhibiting severely depressed, but detectable, ADP-ribosyltransferase activity. The latter results may indicate that either glutamic acid 110 or glutamic acid 112 of the A subunit of heat-labile enterotoxin is analogous to those active-site glutamic acids identified in several other ADP-ribosylating toxins.

Enterotoxigenic *Escherichia coli* produces toxins that are collectively referred to as heat-labile enterotoxins (LT) and include variants derived from strains pathogenic for humans (LT-h) and pigs (LT-p) (reviewed in references 16 and 38). These enterotoxins are members of a family of bacterial protein toxins which includes cholera toxin (CT) and pertussis toxin (PT) and which exert their pathophysiologic effects through the NAD^+-dependent ADP-ribosylation of guanine nucleotide-binding proteins of the adenylate cyclase complex in eukaryotic cell membranes (18, 19, 25, 40). ADP-ribosylation of the stimulatory subunit (G_s) by either CT or LT causes the irreversible activation of adenylate cyclase in target cells, which results in increased intracellular cyclic AMP levels and the subsequent electrolytic imbalance characteristic of bacterial enterotoxin-mediated diarrheal disease (15). LT is an oligomeric toxin composed of an enzymatically active A subunit (M_r, ≈30,000) and five identical B subunits (M_r, 12,000) that constitute the binding portion or B oligomer (16, 17).

Studies have suggested that development of antitoxin immunity through immunization might be useful in the prevention of enteric disease by enterotoxin-producing bacteria (24, 30). To this end, attempts have been made to elicit immunity by using isolated B-subunit preparations of CT which are devoid of toxic activity. However, such preparations appear to be less effective than holotoxins in eliciting protective antibodies (45). More recent efforts have used both synthetic peptides and gene fusions in attempts to produce nontoxic, yet immunogenic LT derivatives (30, 49). A particularly attractive approach to this problem involves the production of an LT holotoxoid-based vaccine through selective mutagenesis of the A-subunit gene of LT to eliminate ADP-ribosyltransferase activity and, therefore, toxic activity (21, 56).

Nucleotide sequence analyses have shown that the enzymatically active S1 subunit of another ADP-ribosylating toxin, PT, exhibits limited amino acid sequence similarity to the enzymatically active A subunits of both CT and LT (LTA) (34, 42). The similarity among the active subunits of these three toxins is confined to two small octapeptide regions which span amino acids 8 through 15, and amino acids 51 through 58 of the S1 subunit of PT. These regions correspond to residues 6 through 13 and 60 through 67 of LTA, respectively. Deletions or substitutions in the sequence encoding the first of these two octapeptides in the S1 subunit of PT, especially at the arginine residue corresponding to arginine 7 of the heat-labile toxin (arginine 9 of S1), dramatically reduce the enzyme activity (3, 5, 11, 31, 32, 46). These observations suggest that these regions, especially the amino-terminal region, are functionally important to enzymatic activity and that alterations within the analogous regions of the A subunits of either LT or CT might have similar effects. In potential support of this prediction is the finding of Harford et al. (21) that substitution of serine 61 within the second region of similarity in LTA results in inactivation of the ability of the enterotoxin to stimulate adenylate cyclase activity in membranes and secretion in rabbit ileal loops.

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Studies using photo-affinity labeling and site-directed mutagenesis have established that diphtheria toxin (DT), *Pseudomonas* exotoxin A, and PT contain glutamic acid residues that are within the active sites of these molecules and are critical to ADP-ribosyltransferase activity (4, 8, 9, 14, 57). These findings suggest that the active A subunits of both LT and CT might possess a similar catalytically functional glutamic acid residue. However, sequence alignments have failed to identify a region of similarity between the enterotoxins and other toxins that would reveal the presence of such a glutamic acid residue (4). Recently, Tsuji et al. (56) described a mutant form of LT in which glutamic acid 112 of the A fragment was changed to lysine. This mutant analog was largely devoid of biologic activity, and this fact was interpreted to indicate the importance of Glu-112 in the active site of LT.

In the present communication, we report the results of experiments employing site-directed mutagenesis that were designed to assess the potential importance of certain residues in the A subunit of *E. coli* heat-labile toxin in enzymatic activity by introducing conservative amino acid substitutions. We focused our attention on the arginine residue at position 7 and two glutamic acid residues (Glu-110 and Glu-112) that are conserved in the active fragments of both LT and CT. The results indicate that all of the substitutions resulted in considerable effects on ADP-ribosyltransferase activity. However, the effects of these mutations on conformation, as judged by trypsin sensitivity, varied markedly.

MATERIALS AND METHODS

Materials. DNA-modifying enzymes were purchased from New England BioLabs, Bethesda Research Laboratories, and International Biotechnologies Inc. The enzymes were used in accordance with the suppliers' recommendations. CT was obtained from List Biologicals. Purified LT (12, 16) was a generous gift of Richard Finkelstein (University of Missouri Medical School, Columbia, Mo.). [*adenylate*-^{32}P]NAD$^+$ (20 to 30 Ci/mmol) was obtained from New England Nuclear, and [*carbonyl*-^{14}C]NAD$^+$ (41 mCi/mmol) was obtained from Amersham Corp. Monoclonal antibodies CP7-3003F7 and CP7-3004G6X1, which are reactive with peptide 6-17 of the A1 subunit of CT, have been described before (7) and were generously provided by James Kenimer (Food and Drug Administration, Bethesda, Md.). Recombinant bovine ADP-ribosylation factor 2 (rARF 2 [48]) was a generous gift of Joel Moss (NHLBI, National Institutes of Health, Bethesda, Md.). Rod outer segment (ROS) membranes (photo bleached) were prepared from frozen bovine retinas by the method of Baehr et al. (2) and stored at −70°C. 5′-Guanylyl-imidophosphate (GMP-PNP) was obtained from Boehringer Mannheim Biochemicals. All other reagents for enzymatic and other biochemical analyses were obtained from Sigma Chemical Co., Boehringer Mannheim, or Calbiochem-Behring.

Plasmids and bacterial strains. Plasmid EWD299 (13, 52, 59) carries the operon encoding the heat-labile enterotoxin from *E. coli* of porcine origin and was obtained from Yankel Kuperstzoch (University of Texas Southwestern Medical Center, Dallas, Tex.). The expression plasmid pT7-7 (53) contains an ampicillin resistance gene, the bacteriophage T7 promoter (ϕ10), and the translational start site for the T7 gene 10 protein located 5′ to a multiple cloning site. Plasmid pGP-1-2 (53) contains a kanamycin resistance gene, the T7 RNA polymerase gene under control of the leftward promoter of bacteriophage lambda (p_L), and the gene for the temperature-sensitive lambda repressor *c*Its857. *E. coli* K38, plasmid pT7-7, and plasmid pGP-1-2 were kindly provided by Stanley Tabor (Harvard Medical School, Boston, Mass.). Phagemid pTZ18R, which contains an f1 origin and the T7 promoter adjacent to a multiple cloning site, was obtained from Pharmacia/LKB.

Recombinant LTA genes and site-specific mutagenesis. Manipulations of DNA were performed by using standard methods (37). The nucleic acid sequence of the complete LT gene from EWD299 has been reported by Yamamoto et al. (59) and was used as the basis for all sequence manipulations and alterations. All plasmid vectors were treated with calf intestinal alkaline phosphatase after digestion with appropriate enzymes and prior to insertion of heterologous DNA. To construct the full-length LTA expression clone (pT7-7/rLTA), plasmid EWD299 was digested with *Sfa*NI and *Cla*I. The 0.87-kb *Sfa*NI-*Cla*I fragment containing the coding sequence for the mature A subunit and part of the B subunit (Fig. 1) was isolated by agarose gel electrophoresis, and the protruding ends were filled in by using the Klenow fragment of DNA polymerase I. The blunt-ended fragment was inserted into the unique *Sma*I site of plasmid pT7-7. This construct was designed to encode the mature form of the A subunit with nine amino acids fused to the amino terminus that are derived from both the vector and the signal peptide (Fig. 1). A gene encoding an A subunit that possesses a 10-amino-acid amino-terminal truncation (rLTA/t10) was constructed by digesting plasmid EWD299 with *Xba*I and filling in the protruding ends with Klenow fragment. The truncated gene fragment was excised by digestion with *Cla*I, isolated by agarose gel electrophoresis, and then inserted into plasmid pT7-7 that had been digested with *Cla*I and *Sma*I.

To construct the substitution mutants of the LTA gene encoding A subunits in which arginine 7 was either replaced with lysine (rLTA/R7K) or deleted (rLTA/R7Δ), plasmid EWD299 was first digested with *Xba*I and *Cla*I. The following double-stranded oligonucleotide linkers that incorporate the substitution or deletion of the codon for arginine 7 (CGT to AAA or deletion of CGT, respectively) with *Nde*I and *Xba*I cohesive ends were synthesized: 5′-TATGGCTAGA ATTCGCGCCCTATATGCAAATGGCGACAGATTA TACAAAGCTGAC-3′ and 5′-TATGGCTAGAATTCGCG CCCTATATGCTGGCGACAGATTATACAAAGCTGA C-3′. The linkers were phosphorylated and ligated to the *Xba*I-*Cla*I fragment of EWD299. The resultant products which contain *Nde*I and *Cla*I cohesive ends were then ligated to pT7-7 that had been digested with *Nde*I and *Cla*I.

The recombinant plasmids containing the constructs rLTA, rLTA/t10, rLTA/R7K, and rLTA/R7Δ were transformed (20) into *E. coli* JM101. In each case, several transformants were screened for the presence and orientation of the inserts by using restriction enzyme digestion. The 5′ end of inserts from plasmids from each of the selected transformants was subjected to nucleotide sequence analysis to verify that no unintended alterations occurred at the ligation sites and, in the case of rLTA/R7K and rLTA/R7Δ, to verify the presence of the mutagenic substitutions. Inserts in each of the expression plasmids were also subjected to 3′ sequence analysis to assess the integrity of the 3′ region of the inserts and to verify the presence of an in-frame translational stop codon (TGA).

Plasmids containing LTA genes in which the codons for either glutamic acid 110 or glutamic acid 112 were replaced with the codon for aspartic acid were obtained by using the site-directed mutagenesis procedure of Kunkel (26). Briefly,

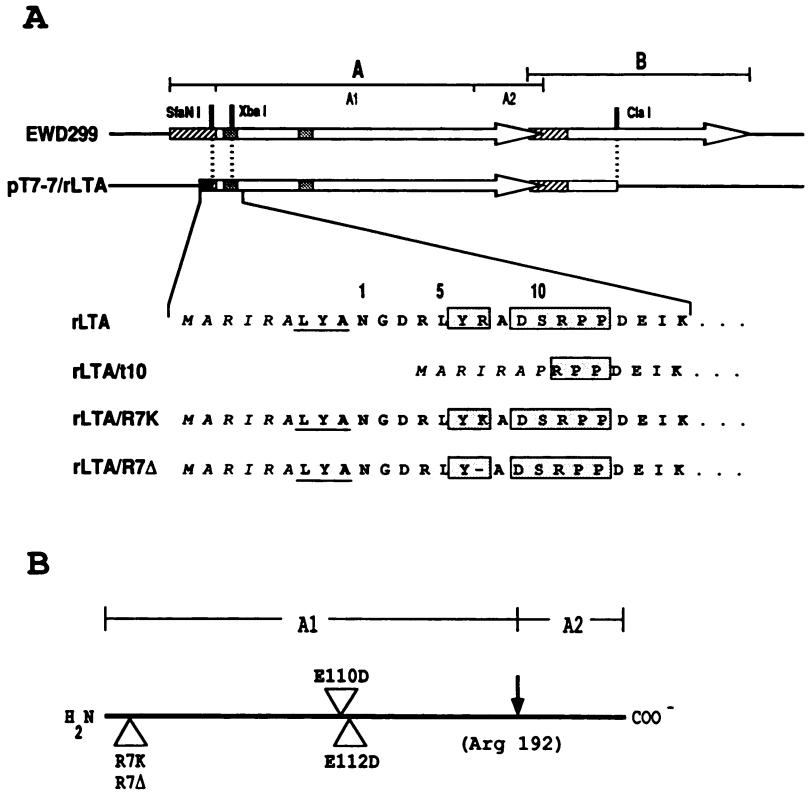

FIG. 1. Schematic representation of the recombinant LTA constructs. (A) Genes encoding the A and B subunits of LT and relative position of the *Sfa*NI-*Cla*I fragment that was subcloned into pT7-7. The boundary of the putative A1 and A2 fragments of LTA is also indicated. The hatched and cross-hatched regions indicate sequences encoding the signal peptides of the A and B subunits. The stippled areas indicate the two regions of amino acid sequence similarity between the S1 subunit of PT and the A subunits of CT and LT. The filled-in region in pT7-7/rLTA indicates a sequence encoding a fusion peptide derived from the polylinker of vector pT7-7. An expanded version of the amino acid sequence encoded by the 5′ region of the parental construct (pT7-7/rLTA) and the three constructs resulting in amino-terminal mutations is also shown. The amino acids in the first region of sequence similarity are denoted with stippled boxes. Amino acids derived from the signal peptide sequence are underlined, and the vector-derived fusion peptides are shown in italics. (B) Relative position of the amino-terminal mutations and the mutations at Glu-110 and Glu-112 relative to the mature LT A-subunit protein. The boundary of the A1 and A2 fragments formed by cleavage after Arg-192 is also indicated.

a *Pst*I-*Bgl*II fragment containing the LT A-subunit gene was excised from plasmid pT7/rLTA and isolated by gel electrophoresis. The *Pst*I-*Bgl*II fragment was then inserted into phagemid pTZ18R that had been digested with *Pst*I and *Bam*HI. The resulting recombinant phagemid, pTZ18R/rLTA was then transformed into *E. coli* CJ236 (*dut ung*); transformants were selected with ampicillin and chloramphenicol. Selected transformants were then grown with helper phage (R408; Stratagene) in the presence of uridine. The single-stranded, uridine-containing template DNA was extracted and then incubated with M13 reverse primer, T4 DNA ligase, and one of two oligonucleotides encoding the mutations to allow synthesis of hybrid duplexes containing the mutant sequences. The two oligonucleotide primers used were as follows (mutant codons underlined): 5′-CCATAT GACCAGGAGGTTTCT-3′ (Glu-110 to Asp) and 5′-ATG AACAGGATGTTTCTGCGTTA-3′ (Glu-112 to Asp). The double-stranded DNA was then transformed into *E. coli* DH5α. Ten to twenty transformants from each reaction were isolated; their plasmid DNA was extracted and subjected to DNA sequence analysis to confirm the presence of the desired mutation. The relative positions of the single-substitution mutations in the LT A subunit are illustrated in Fig. 1.

Expression of recombinant LT A-subunit genes in *E. coli* and extraction of recombinant proteins. pT7-7 or pTZ18R and derivatives containing the various recombinant LTA constructs were transformed into *E. coli* K38 harboring plasmid pGP1-2. Recombinant gene expression under control of the T7 promoter was induced essentially as described by Tabor and Richardson (53). Briefly, cells were grown to a density of 1.5 U/ml (optical density at 600 nm) at 30°C in 100 ml of liquid medium containing kanamycin and ampicillin. The cultures were incubated at 42°C for 30 min to permit synthesis of the T7 polymerase. Rifampin (200 μg/ml) was then added to the cultures to suppress host cell transcription, and incubation was continued for 2 h at 37°C with shaking. The cells (optical density at 600 nm, approximately 150 U) were washed once by centrifugation and suspended in 1 ml of 50 mM Tris HCl, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride. The cells were placed on ice and then disrupted by sonication, using five 15-s cycles of a Branson Sonifer cell disruptor with 1-min alternate cooling intervals in an ice-water bath. The cell extracts were then fractionated by centrifugation at 12,000 × *g* and 4°C, and the pellet fractions were washed once in ice-cold disruption buffer. The pellet fractions were then extracted in 1 ml of 8 M urea–1 mM phenylmethylsulfonyl fluoride with gentle agitation for 2 h at room temperature and then centrifuged at 12,000 × *g* for 20

min to remove insoluble debris. The supernatant fractions were dialyzed exhaustively against 50 mM Tris HCl, pH 8.0. The dialyzed extracts were centrifuged at 12,000 × *g* for 20 min at 4°C to remove any remaining insoluble material. The relative amounts of recombinant analogs in the preparations were determined by quantitative densitometric scanning of proteins separated by electrophoresis and stained with Coomassie blue or by densitometry of immunoblots with various amounts of the extracts. Whole-cell pellets and the dialyzed sonic extract fractions were stored at −70°C.

Immunoblotting. The urea-extracted fractions from recombinant cell sonic extracts were diluted with an equal volume of double-strength electrophoresis sample buffer (27) and heated to 95°C for 5 min. Aliquots containing 15 μg of total protein were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% minigels, using the discontinuous buffer system described by Laemmli (27), and then transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore Corp.), using the buffers described by Towbin et al. (54). The blots were then incubated with a mixture of two monoclonal antibodies to peptide 6-17 of the A1 subunit of CT (7). Bound antibodies were detected with peroxidase-conjugated goat or rabbit anti-mouse immunoglobulin G and 4-chloro-1-naphthol as the chromogenic substrate.

ADP-ribosyltransferase assays. ADP-ribosyltransferase activity was determined as the ability to catalyze the transfer of [^{32}P]ADP-ribose from [*adenylate*-^{32}P]NAD^+ to the alpha subunit of transducin (Tα) in bovine ROS membranes. The conditions used were similar to those described for determination of CT-catalyzed ADP-ribosylation of transducin in ROS described by Abood et al. (1). Unless otherwise indicated, preparations containing the recombinant molecules and control preparations of CT or purified LT were incubated with 10 mM dithiothreitol (DTT) for 15 min at room temperature prior to assay. Reaction mixtures (40 μl) containing 100 μM [*adenylate*-^{32}P]NAD^+ (1 to 2 μCi), 10 mM thymidine, 4 mM $MgCl_2$, 100 μM GPP-PNP, 50 μg of ROS, 100 mM sodium phosphate (pH 6.8), and 0.5 to 25 μg of the recombinant extracts or purified toxins were incubated at 30°C for 2 to 6 h. The reactions were terminated by the addition of 0.2 ml of ice-cold 10% (wt/vol) trichloroacetic acid. The reaction tubes were incubated on ice for 1 h, and the precipitated proteins were collected by centrifugation. The precipitates were washed once with 1 ml of ice-cold water and then solubilized in 30 μl of electrophoresis treatment buffer. The labeled proteins were analyzed by SDS-PAGE in 12.5% gels and autoradiography, using Cronex Lightning-Plus intensifying screens. Autoradiographic exposure times ranged from 2 to 24 h.

NAD^+:agmatine ADP-ribosyltransferase activity was measured as the release of [*carbonyl*-^{14}C]nicotinamide from radiolabeled NAD^+ in the absence or presence of bovine rARF 2 (39, 55). Reaction mixtures (0.05 ml) containing 10 mM $MgCl_2$, 20 mM DTT, 100 μM GTP, 10 mM agmatine, 0.2% sodium cholate, 3 mM dimyristoylphosphatidylcholine, 100 μM [*carbonyl*-^{14}C]NAD^+, 100 mM potassium phosphate (pH 7.5), 1 μg of rARF 2, and 40 μg of the recombinant extracts were incubated at 30°C for 4 h. The amount of nicotinamide released was determined by using Dowex AG1-X2 ion-exchange resin as described previously (33).

Other analytical procedures. DNA sequencing was performed by the primer-extension, dideoxynucleotide chain termination method (10, 50), using modified T7 DNA polymerase (Sequenase; United States Biochemical Corp.). Protein determinations were done by the bicinchoninic acid method (51), using a kit from Pierce Chemical Co. and bovine serum albumin as the standard. Quantitative densitometric analyses of stained proteins in polyacrylamide gels and of immunoblots were done with an LKB Ultroscan XL enhanced laser densitometer.

RESULTS

Expression of the rLTA genes in *E. coli*. The recombinant LTA expression plasmid (pT7-7/rLTA) contains the coding sequence for the entire A subunit and part of the B subunit which overlaps the coding sequence of the A subunit, as shown in Fig. 1. The construct also encodes a nine-amino-acid fusion peptide at the amino terminus. The LT A-subunit genes encoding rLTA and the other mutant derivatives were expressed under control of a T7 promoter in *E. coli* K38. Fractionation of induced sonicated cells by differential centrifugation and analysis by SDS-PAGE and immunoblotting revealed that the wild-type protein, rLTA, was almost entirely associated with the insoluble pellet fractions (not shown). SDS-PAGE and Coomassie blue staining of the urea-solubilized pellet fraction revealed an intense but diffuse band in the rLTA preparation that was not present in extracts of cells containing the parent plasmid pT7-7 (Fig. 2). The increase in staining intensity in this area of the gel corresponding to M_r ≈31,000 and immunoblot analysis with monoclonal antibodies prepared against the amino-terminal peptide of the A subunit of CT that contains the region of similarity shared by the enzymatically active subunits of LT, CT, and PT revealed a broad but specifically immunoreactive band at the same relative position (Fig. 2). The M_r (≈31,000) is similar to that of the A subunit of authentic LT and consistent with the presence of a nine-amino-acid fusion peptide at the amino terminus (Fig. 2, LT lanes). Preparations of rLTA/t10 contained a novel protein species that migrated slightly faster than rLTA, an observation consistent with the reduction in size via amino-terminal truncation (Fig. 2). This protein did not react appreciably with the monoclonal antibodies directed against the region of similarity in the A subunit of CT corresponding to residues 6 through 17. The lack of reactivity was not surprising in view of the fact that only three of the amino acids in the region of similarity are retained in rLTA/t10. Preparations containing proteins rLTA/R7K, rLTA/R7Δ, rLTA/E110D, and rLTA/E112D yielded intensely stained novel protein bands (Fig. 2) and a pattern of immunoreactivity on immunoblots that was identical to that exhibited by the A subunit of authentic LT but with slightly reduced electrophoretic mobility. Quantitative densitometric analyses of the stained proteins and immunoblots indicated that the relative amounts of recombinant product in the various preparations did not differ routinely by more than a factor of 2.

The diffuse electrophoretic appearance of rLTA appears to represent altered electrophoretic migration due to self-ADP-ribosylation of rLTA rather than a flaw in the recombinant gene construct. Evidence for this phenomenon is presented below in the context of the enzymatic activity of rLTA and the mutant analogs.

ADP-ribosyltransferase activity of rLTA. The urea-solubilized, high-speed pellet fraction of an *E. coli* sonic extract containing rLTA was assayed for the ability to catalyze the ADP-ribosylation of the 39,000-M_r Tα in bovine ROS membranes. Purified authentic LT and CT were included in the analysis for comparative purposes. To establish conditions that would result in readily detectable enzyme activity, a number of preliminary assays were performed. It was deter-

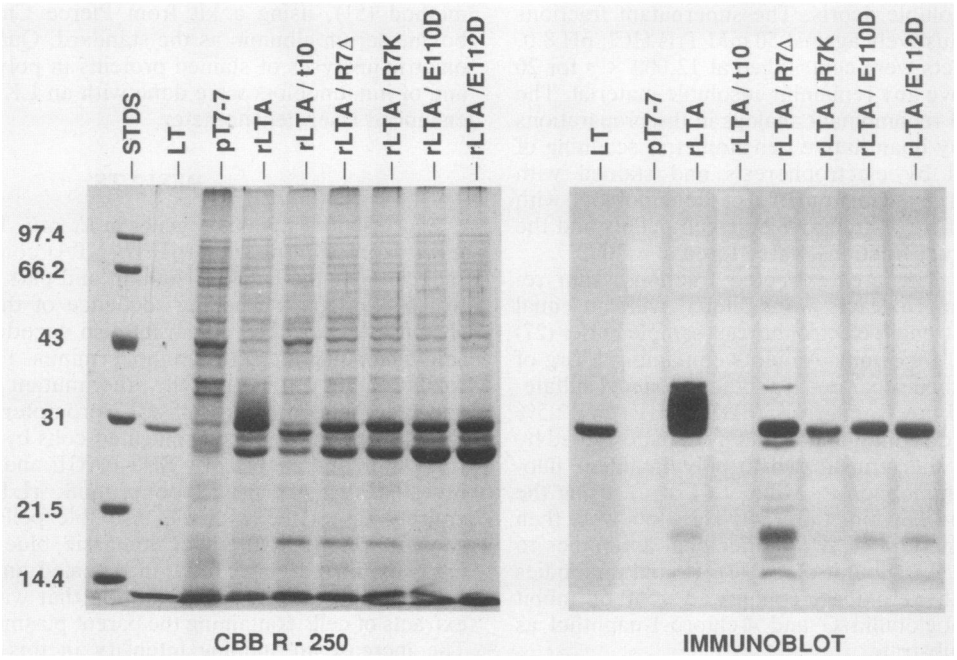

FIG. 2. Recombinant LT A subunits in urea-solubilized, high-speed pellet fractions of recombinant *E. coli* sonic extracts. Aliquots of dialyzed fractions containing 15 μg of total protein and 2.5 μg of purified authentic LT were electrophoresed in 12.5% SDS–polyacrylamide minigels. (Left) Proteins were stained with Coomassie brilliant blue R-250. pT7-7 represents an extract from cells containing the expression vector with no insert. The B subunit of LT is visible as a band near the dye front at the bottom of the gel. (Right) Proteins in a parallel gel were electroblotted, and immunoreactive proteins were detected with monoclonal antibodies to peptide 6-17 in the A subunit of CT as described in Materials and Methods. The positions of the molecular mass standards are indicated on the right in kilodaltons.

mined that neither rLTA, purified LT, nor CT would ADP-ribosylate purified transducin; a similar finding has been reported for CT previously (1). In the presence of the nonhydrolyzable analog of GTP, GMP-PNP, both LT and rLTA, as well as CT, were able to ADP-ribosylate Tα in ROS membranes (Fig. 3). GMP-PNP was used because this nucleotide analog has been reported to enhance CT-catalyzed ADP-ribosylation of Tα in ROS membranes, while GTP was found to be inhibitory (1). This nucleotide requirement likely reflects the activation of another guanine nucleotide-binding protein in the ROS membrane preparations that, in turn, activates LT (e.g., ADP-ribosylation factor [22, 55]). Maximal activity was found to occur at a pH of approximately 6.8. As also shown in Fig. 3, ADP-ribosylation of Tα by either rLTA or purified LT did not require preincubation with DTT. However, labeling of a protein corresponding to an M_r of ≈31,000 was enhanced significantly by preexposure of the preparations to DTT. This band represents self-ADP-ribosylation of the A subunit since it appears in reaction mixtures containing either rLTA or purified LT, and this labeled species also appears when these preparations are incubated with radiolabeled NAD^+ in the absence of ROS, although with diminished intensity (not shown).

The enzymatic activity of both LT and rLTA also exhibited heat lability (5 to 10% residual activity after incubation at 95°C for 5 min). The labeling of Tα and that of the 31,000-M_r rLTA were inhibited (approximately 50 to 60%) when 100 mM nicotinamide was added to the assay, and the labeling of both species was reduced by approximately 50% when incubation was with 20 U of snake venom phosphodiesterase I per ml. These results indicate that the labeling was a result of ADP-ribosylation and not a result of incorporation of breakdown products (e.g., ortho- or pyrophosphate) of NAD^+. No detectable ADP-ribosylation was observed when either buffer or identically prepared extracts from the parental *E. coli* strain K38 harboring vector pT7-7 were used (Fig. 3).

It is noteworthy that the self-ADP-ribosylation of authentic LT results in the appearance of a labeled A subunit that exhibits a more diffuse appearance than is observed after SDS-PAGE followed by protein staining or detection by immunoblotting and, upon close inspection, appears to represent two predominant species or a doublet (cf. Fig. 2 and 3). This alteration in electrophoretic migration suggested conformational or other changes induced by ADP-ribosylation and further suggested that the diffuse or broad appearance of rLTA in SDS-PAGE was similarly a result of this reaction. To examine this possibility, purified LT was activated with DTT and incubated for 15 h in ADP-ribosyltransferase assay buffer, with or without added NAD^+ (1 mM), and then analyzed by SDS-PAGE and immunoblotting. The analysis revealed a NAD^+-dependent, partial shift of the A subunit towards slower electrophoretic mobility with a resultant appearance that was very similar to that of immunoreactive rLTA after extraction from *E. coli* (not shown). This result further indicates that self-ADP-ribosylation is responsible for observed altered electrophoretic migration. Such altered electrophoretic migration of ADP-ribosyl–protein conjugates has been noted by others (23, 41).

Collectively, these analyses demonstrate that the rLTA molecule, despite the presence of an amino-terminal fusion peptide and self-ADP-ribosylation, possesses enzymatic properties that are qualitatively indistinguishable from those exhibited by the A subunit of authentic LT, under the conditions used.

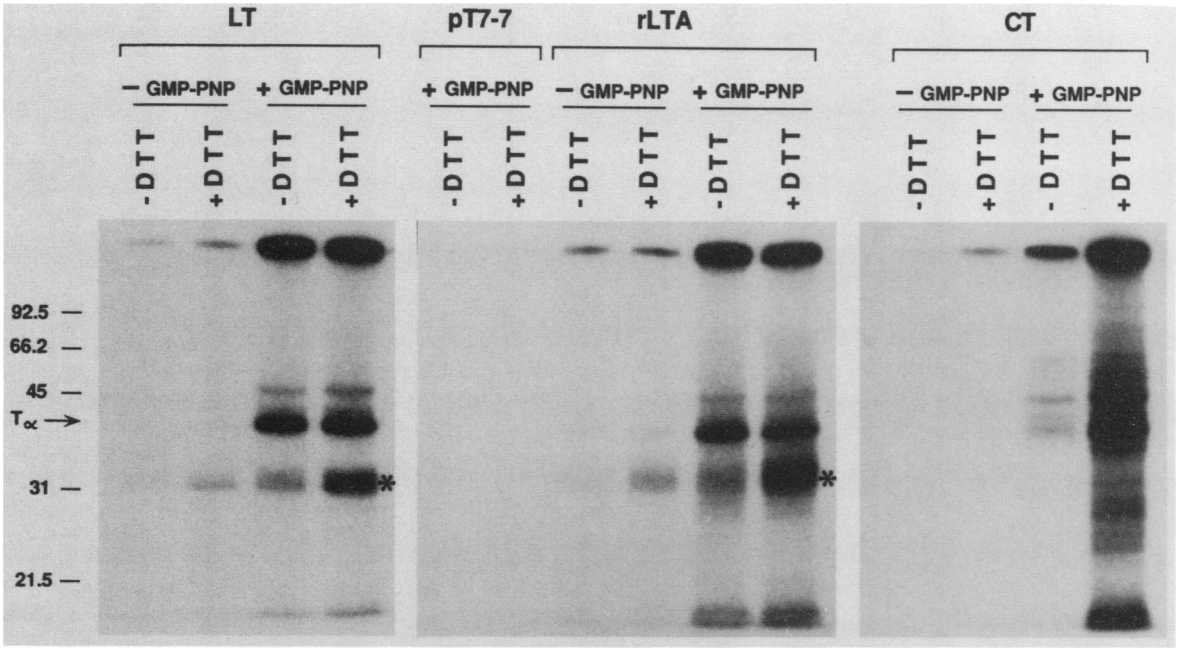

FIG. 3. Effect of DTT and GMP-PNP on ADP-ribosylation of Tα in ROS membranes by LT, rLTA, and CT. ADP-ribosyltransferase activity was assayed as described in Materials and Methods with the indicated omissions and additions. Purified LT and CT were assayed for 2 h at a final concentration of 50 μg/ml. Extracts of cells harboring pT7-7 alone or the rLTA construct were similarly assayed at a final concentration of 100 μg of total protein per ml. Assays were performed with or without added 100 μM GMP-PNP, and samples were preincubated and assayed in the presence or absence of 10 mM DTT. Reaction products were analyzed by SDS-PAGE and autoradiography. The 39,000-M_r band corresponding to Tα is indicated. The positions of the self-ADP-ribosylated forms of the A subunit of authentic LT and the rLTA are denoted with asterisks. The positions of the molecular mass standards are indicated in kilodaltons.

ADP-ribosyltransferase activities of mutant analogs. In contrast to the readily detectable activity associated with rLTA, the substitution or deletion of arginine 7 (rLTA/R7K and rLTA/R7Δ) resulted in a loss of detectable transferase activity (Fig. 4). The activity of these mutant analogs was found to be reduced by a factor of 400 to 500 as determined in several experiments by direct measurement of the amount of radioactivity in the band corresponding to Tα (not shown). The truncated version of rLTA, rLTA/t10, which lacks the first 10 amino acids of the mature protein, also did not exhibit significant transferase activity at any amount tested (not shown). No incorporation was observed with extracts of cells harboring the vector alone, and mixing experiments using rLTA and the various mutant extracts failed to reveal the presence of inhibitory activity in the latter (not shown).

In other experiments not shown, it was determined that extracts containing a recombinant analog that lacks the carboxyl-terminal, 65-amino-acid residues possessed readily detectable, dose-dependent ADP-ribosyltransferase activity. However, this molecule partitioned with the majority of the cellular protein in the soluble fraction of *E. coli* sonic extracts, and only relatively small amounts of this analog were detected in the urea-extracted pellets, making quantitative determinations difficult. Nevertheless, these findings indicated that the regions critical to enzymatic function resided within the first 175 amino-terminal amino acids of the A subunit. In light of this finding, we focused additional attention on the identification of glutamic acid residues in this region that might be analogous to those active-site glutamic acids identified in DT, *Pseudomonas* exotoxin A, and the S1 subunit of PT. Five of the seven glutamic acids among the first 175 residues are conserved among all of the LT variants and include glutamic acids at positions 15, 29, 110, 112, and 159 of the mature A-subunit sequence (44). We chose to alter the glutamic acids at positions 110 and 112 owing to their location in that portion of the A subunit that is similar to those of the active-site glutamic acids of the A fragment of DT (Glu-148) and the S1 subunit of PT (Glu-129). Figure 4 shows that substitution of either glutamic acid 110 or glutamic acid 112 by aspartic acid results in a marked reduction of ADP-ribosyltransferase activity as judged either by labeling of the 39,000-M_r Tα or by evidence of self-ADP-ribosylation. Repeated determinations indicated that the activity of either mutant analog was reduced by a factor of at least 50 to 100, although slightly more activity could be detected in the case of the E110D analog.

Like CT, LT has been shown to catalyze the release of nicotinamide from NAD^+ in the presence of simple guanidino compounds (e.g., agmatine) which serve as ADP-ribose acceptors (39). In both cases, the activity has been shown to be enhanced by the inclusion of low-molecular-weight GTP-binding proteins known as ADP-ribosylation factors (29, 53). As shown in Table 1, preparations containing rLTA possess NAD^+:agmatine ADP-ribosyltransferase activity as judged by a significant increase in the release of [*carbonyl*-^{14}C]nicotinamide from radiolabeled-NAD^+ in the presence of agmatine. The activity associated with rLTA was enhanced by rARF 2. In contrast, and like the results obtained with ROS membranes, the mutant analogs displayed little or no rARF 2-stimulated activity with or without added agmatine.

Limited trypsinolysis of rLTA and mutant analogs. To determine whether introduction of the mutations had detectable effects on conformational integrity, the effect of the mutations on sensitivity to limited trypsin digestion was monitored by using the monoclonal antibodies to the amino-terminal region of similarity and immunoblotting. As shown

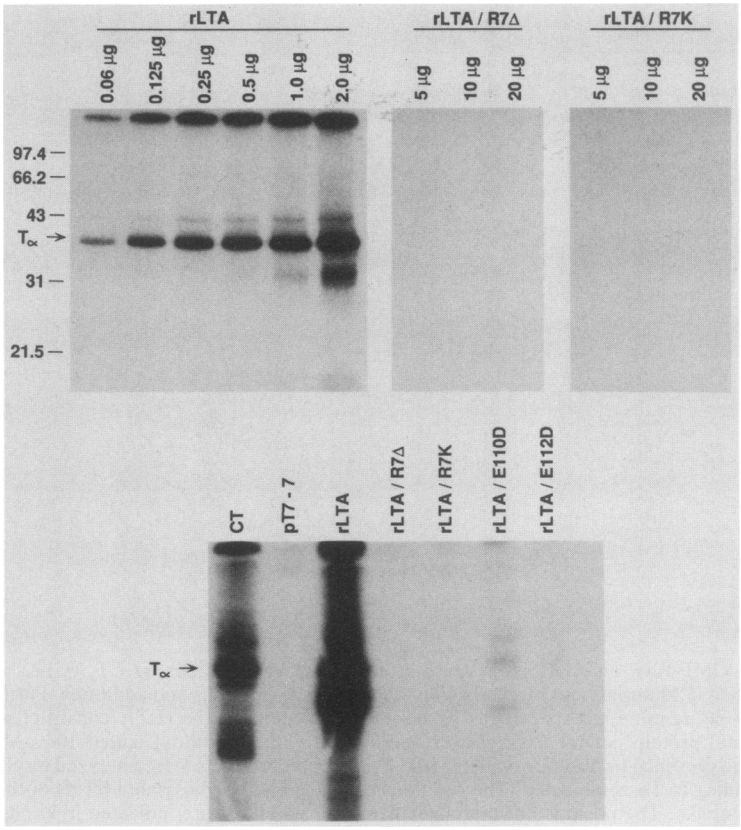

FIG. 4. ADP-ribosyltransferase activities of rLTA and mutant analogs. Dialyzed urea extracts containing the indicated recombinant proteins were assayed for ADP-ribosyltransferase activity for 2 h as described in Materials and Methods. For the upper panels showing reactions using rLTA, rLTA/R7K, and rLTA/R7Δ, the total amount of sample protein used per assay is indicated above each lane. The gel containing the rLTA reaction products was exposed to X-ray film for 2 h. The remaining autoradiographs (rLTA/R7K and rLTA/R7Δ) represent 2-h exposures. In the bottom panel, aliquots containing 15 to 20 μg (total protein) of the rLTA/E110D and rLTA/E112D and the other indicated extracts were assayed. The CT reaction was included for reference purposes. The autoradiograph shown represents a 17-h exposure. Molecular mass standards are indicated in kilodaltons. The position of Tα is also indicated.

in Figure 5, incubation of rLTA with increasing amounts of trypsin resulted in the appearance of a stable fragment, derived from the amino terminus, with an electrophoretic mobility corresponding to an M_r of approximately 23,000. Trypsinolysis of authentic LT also results in the appearance of a fragment of similar size; this fragment has been termed the A1 fragment (19). Both rLTA/E110D and rLTA/E112D yielded similar, if not identical, cleavage patterns. However, trypsinolysis of rLTA/R7K resulted in the simultaneous disappearance of both the intact subunit and the fragment corresponding to an M_r of 23,000. A similar result was obtained with rLTA/R7Δ (not shown). The latter results indicate that substitutions at position 7, while profoundly affecting transferase activity, also appear to affect the overall conformation of the amino-terminal portion of the subunit as judged by increased trypsin sensitivity. This alteration does not appear to have occurred in the mutant analogs rLTA/E110D and rLTA/E112D.

TABLE 1. NAD^+:agmatine ADP-ribosyltransferase activity of rLTA and mutant analogs

Recombinant subunit	Enzyme activity (pmol of nicotinamide released per min)[a]			
	−Agmatine		+Agmatine	
	−ARF	+ARF	−ARF	+ARF
pT7-7 (control)	1.16 ± 0.04	1.23 ± 0.03	1.63 ± 0.07	1.67 ± 0.01
rLTA	1.03 ± 0.04	1.31 ± 0.05	4.59 ± 0.02	7.91 ± 0.15
rLTA/R7Δ	0.79 ± 0.06	0.96 ± 0.04	1.08 ± 0.03	1.36 ± 0.06
rLRA/R7K	0.74 ± 0.03	0.85 ± 0.04	1.04 ± 0.04	1.20 ± 0.05
rLTA/E110D	1.11 ± 0.02	1.59 ± 0.04	1.56 ± 0.06	2.04 ± 0.11
rLTA/E112D	1.38 ± 0.02	1.64 ± 0.06	1.72 ± 0.05	2.13 ± 0.07

[a] The assays were performed as described in Materials and Methods with and without added agmatine or rARF 2 as indicated. Values represent the means of triplicate determinations ± standard deviations.

DISCUSSION

The limited regions of similarity shared by the S1 subunit of PT and active A subunits of LT and CT prompted initial experiments designed to determine whether these regions might be important to the enzymatic function of PT (5, 11). The results indicated that the amino-terminal region of S1, delineated by residues 8 through 15, and arginine 9 in particular, was important to the enzymatic activity of S1 as judged by the dramatic reductions in ADP-ribosyltransferase activity that accompanied either deletion of the region or specific alterations at position 9. An obvious prediction that resulted from this work was that, if the region has genuine

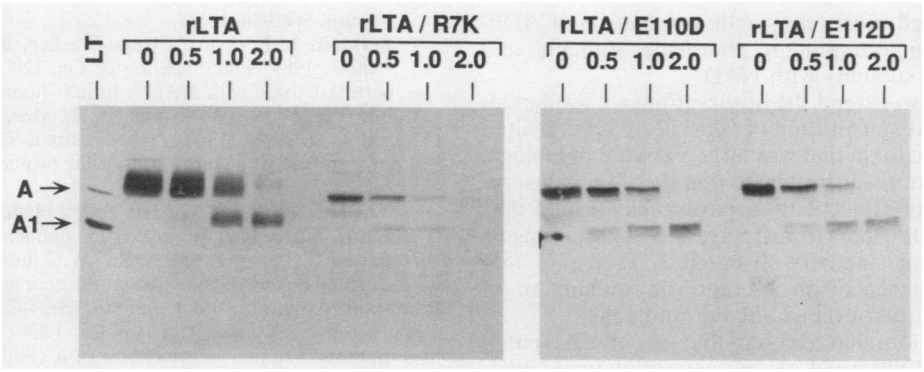

FIG. 5. Trypsin sensitivity of rLTA and mutant analogs. Extracts (1-mg/ml total protein concentration) were incubated for 0.5 h at 30°C with the final concentrations (0 to 2 μg/ml) of trypsin indicated above each lane. The reactions were terminated by the addition of soybean trypsin inhibitor to a final concentration of 100 μg/ml. The reaction mixtures were then diluted with an equal volume of double-strength electrophoresis sample buffer, heated to 95°C for 5 min, and then analyzed by SDS-PAGE in 12.5% minigels. Authentic purified LT (LT lane) was incubated with 1.0 μg of trypsin per ml under the same conditions. The positions of the intact A subunit and the A1 tryptic fragment of LT are indicated.

mechanistic importance in ADP-ribosylation, then similar alterations in either LT or CT should exert comparable effects.

Prior to investigating the effects of mutations, we examined the characteristics of the enzymatic activity of the recombinant A subunit, rLTA, as compared with that of authentic LT. As noted, the two enzymes appeared to be virtually identical as judged by several criteria. These findings indicated that the recombinants provided a legitimate and reliable system with which to interpret the effects of the mutations on LT activity. The self-ADP-ribosylation by rLTA deserves comment. In contrast to the authentic LT A subunit, rLTA is synthesized without a signal peptide and consequently is not exported. This feature likely results in unusually high levels of cell-associated A subunit, which may explain the occurrence of self-ADP-ribosylation during synthesis. The significance of self-ADP-ribosylation and its relationship to the activity of LT or CT are not well understood, but Moss et al. (41) have reported that ADP-ribosylation of the A1 subunit of CT results in enhanced ADP-ribosyltransferase activity. However, it is unlikely that self-ADP-ribosylation represents a requisite step in enzymatic or toxic activities of LT or CT (43).

Of greater interest to the present study is the finding that substitution or deletion of Arg-7 in rLTA results in the loss of detectable ADP-ribosyltransferase activity. However, the effects of these mutations can be distinguished from those observed with the analogous Arg-9 mutations in the S1 subunit of PT in one important respect. In the case of substitution of Arg-9 by lysine in S1, no evidence of conformational alteration, as judged by retention of reactivity with a monoclonal antibody which recognizes a conformation-dependent epitope, was detected (46). Indeed, an Arg-9 to Lys mutant analog of S1 has been incorporated into an enzymatically inactive recombinant holotoxin without any apparent effect on other nonenzymatic biologic properties (47). As reported here, substitution or deletion of Arg-7 in rLTA results in enhanced tryptic sensitivity when compared with the wild-type recombinant subunit and authentic LT. While it might be argued that the increased sensitivity of the 23,000-M_r mutant fragment appears secondarily to the initial cleavage (presumably at Arg-192 within the region subtended by the disulfide bond), these results pose a difficulty concerning interpretation of the specificity of the effects of these mutations. Indeed, a recent comparative X-ray crystallographic study of a mutant form of staphylococcal nuclease suggests that significant nonlocal conformational changes may accompany seemingly conservative mutations in the active sites of enzymes and that independent structural data must be used to support assertions concerning the specificity and the locality of the effects imparted by mutagenic substitutions (36). While the current results may reflect an important enzymatic role for Arg-7 of the LT A subunit, they cannot be used without reservation to support a proposed specific role of these arginine residues in the enzymatic mechanisms of the A subunits of LT, CT, and PT, given the apparent effect of the amino-terminal substitutions on conformation. However, in support of functional conservation of Arg-7 and its apparent homologs in PT and CT is the recent finding that substitution of Arg-7 by Lys also results in the abolition of ADP-ribosyltransferase activity of the A subunit of CT (6). It is not known whether this mutation in CT induces a conformational alteration in the A subunit similar to that observed here.

Both rLTA/E110D and rLTA/E112D also possessed severely reduced ADP-ribosyltransferase activity (<1% of wild type), but exhibited a pattern of tryptic fragmentation that was indistiguishable from that of the wild-type enzyme, rLTA. It might have been anticipated that replacement of at least one of these residues would have little or perhaps no effect on enzymatic activity if the effects of the substitutions were entirely specific to the active site. One explanation for this finding is that, if one of these two glutamic acids represents a true active-site residue, then substitution of the other might have a significant steric effect on the function of the other, owing to their proximity. Alternatively, we cannot formally exclude the possibility that these analogs possess conformational (folding) changes that occur either spontaneously or during the denaturation/renaturation steps involved in the urea extraction protocol and that are not detected by our assays of relative trypsin sensitivity. Nevertheless, the general position of Glu-110 and Glu-112 in the LT A subunit resembles that of the critical glutamic acids in the enzymatically active portions of DT and PT and enhances the theoretical likelihood that one of these two glutamic acid residues represents the catalytically important analog of the other ADP-ribosylating toxins. To help identify which of these glutamic acid residues (110 or 112), if either, is

functionally involved in the enzymatic mechanism of ADP-ribosylation, we are attempting to photolabel authentic and recombinant LT A subunits with NAD^+.

These findings also extend the observations of Tsuji et al. (56), who found that substitution of Glu-112 by Lys resulted in the formation of a toxin that was largely devoid of biologic activity. The current results indicate that the more conservative substitution of Glu-112 by aspartic acid results in a dramatic loss of ADP-ribosyltransferase activity and suggest that the loss of biologic activity observed by Tsuji et al. (56) was not a consequence of an alteration in stability or of defective translocation of the A subunit into cells.

Wozniak et al. (58) have noted that a defined spatial relationship can be observed among certain glutamic acid, histidine, and tryptophan residues in at least five bacterial ADP-ribosylating toxins and that this triad may represent a functionally conserved feature. The proposed conserved relationship yields the following pattern: **His**/126 amino acids/**Glu**/4 amino acids/**Trp**. Glu-148 of DT and Glu-553 of *Pseudomonas* exotoxin A, both identified as catalytically important residues, were used to define this relationship. This observation led to the proposal that Glu-210 of the S1 subunit of PT and Asp-170 of the LT A subunit represented analogous residues flanked by a conserved histidine and tryptophan. More recently, Lax et al. (28) have noted the presence of such a His-Glu-Trp motif in the deduced amino acid sequence of a mitogenic ADP-ribosylating toxin from *Pasteurella multocida*. However, these authors also note that the same motif can be found in other proteins not thought to have any ADP-ribosylating activity. Previous results from our laboratory and others (3, 33) have shown that Glu-210 of the S1 subunit of PT is not essential for transferase activity and that Glu-129 appears to represent the functional equivalent of Glu-148 in DT and Glu-553 in *Pseudomonas* exotoxin A. The current results, although not directly addressing the functional significance of Asp-170, suggest that either Glu-110 or Glu-112 is more likely to represent such an essential acidic residue in LT.

In summary, the results of this and other studies support the contention that an enzymatically inactive and nontoxic form of LT, and perhaps CT, can be constructed through the use of site-specific mutations. However, further studies will be required to define formally the roles of Arg-7, Glu-110, and Glu-112 in the enzymatic mechanisms of both LT and CT.

ACKNOWLEDGMENTS

We thank R. Belland, W. Simpson, and N. Harford for critical review of the manuscript. We gratefully acknowledge the assistance of M. G. Peacock and R. J. Messer in the manipulation of expression vectors and in nucleotide sequence analyses. We thank S. G. Smith for assistance with sample preparation and enzyme assays, S. Perryman for synthesis of the oligonucleotides, the staff of the Hybridoma Facility of the Laboratory of Cellular Physiology, CBER, Food and Drug Administration, for providing ascites fluid containing monoclonal antibodies, and R. Evans and G. Hettrick for preparation of the graphics.

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