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Contribution of Individual Disulfide Bonds to Biological Action of *Escherichia coli* Heat-Stable Enterotoxin B

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Heat-stable enterotoxins (STs) of *Escherichia coli* are peptides which alter normal gut physiology by stimulating the loss of water and electrolytes. The action of heat-stable toxin B (STb) is associated with an increase in levels of lumenal 5-hydroxytryptamine and prostaglandin E_2, known mediators of intestinal secretion. In addition, the toxin is responsible for elevation of cytosolic calcium ion levels in cultured cells. STb is a 48-amino-acid basic peptide containing four cysteine residues and two disulfide bonds. Previous work indicates that disulfide bonds are required for intestinal secretory activity, and yet the relative contribution of the two bonds to toxin stability and action is presently unclear. Site-directed mutagenesis was used to alter the cysteine residues of STb to assess the role of the individual disulfide bonds in toxin activity. Our results indicate that loss of a single disulfide bond was sufficient to abolish the intestinal secretory and G protein-coupled calcium ion influx activities associated with STb toxicity. Loss of toxin action was not a function of increased sensitivity of STb mutants to proteolysis, since mutant toxins displayed proteolytic decay rates equivalent to that of wild-type STb. Circular dichroism spectroscopy of mutant STb toxins indicated that single-disulfide-bond elimination did not apparently affect the toxin secondary structure of one mutant, STbC33S,C71S. In contrast, the α-helical content of the other disulfide bond mutant, STbC44S,C59G, was significantly altered, as was that of reduced and alkylated authentic STb. Since both Cys-Cys mutant STbs were completely nontoxic, the absence of biological activity cannot be explained by dramatic secondary structural changes alone; keys to the conformational requirements for STb toxicity undoubtedly reside in the three-dimensional structure of this peptide.

Enterotoxigenic *Escherichia coli* strains are responsible for serious and sometimes fatal diarrheal disease in humans and animals. One class of *E. coli* toxins produced by bacterial strains associated with disease are the heat-stable enterotoxins (STs). *E. coli* STs are peptides which alter normal gut physiology, resulting in intestinal secretion. Two STs (STa and STb) are currently recognized. STa is an 18 (or 19)-amino-acid peptide which exerts its biological effect by elevating levels of intestine mucosal cyclic GMP (8, 14). Elevation of mucosal cyclic GMP levels is the result of activation of the STa receptor-associated guanylyl cyclase activity (26). The STa receptor, guanylyl cyclase C, is a member of a family of receptors possessing an extracellular ligand-binding domain and intracellular domains representing the catalytic guanylyl cyclase and a protein kinase-like regulatory domain (11, 26). The STa-mediated rise in cyclic GMP levels causes a net loss of chloride ions and water from the intestine in vivo (8) and electrogenic secretion of chloride from cultured T_{84} colonic carcinoma cells (19).

The secreted form of STa is an acidic peptide containing six cysteine residues involved in three intramolecular disulfide bonds, all of which are required for full toxicity (5, 12, 27). Despite some variation in the size and amino-terminal sequence of various STa homologs, a 13-amino-acid conserved core sequence bounded by the first and final cysteine residues (Cys-6 and Cys-18 of human STa) is responsible for receptor binding and guanylate cyclase activation identical to that of authentic STa (13, 27).

In contrast to STa, STb is a 48-amino-acid peptide (7, 9, 21) which causes intestinal secretion in the absence of elevated levels of cyclic nucleotides (18, 20, 31). In vitro studies indicate that STb activates a pertussis toxin (PtX)-sensitive GTP-binding regulatory protein (G protein [6, 16]) and, under appropriate conditions, mediates an elevation of the cytosolic calcium ion level (6). In ligated animal intestinal loop assays, STb promotes a dose-dependent release of serotonin (5-hydroxytryptamine [5-HT]) (15, 24) and production of prostaglandin E_2 (PGE_2) (15, 18, 24). Pharmacological blockade studies indicate that intestinal production of PGE_2 and release of 5-HT are involved in the intestinal secretory action of STb (15, 18). Despite these new insights into STb action, the apparent cascade of events leading to intestinal secretion remains unclear.

The primary structure of STb and its intramolecular disulfide bond arrangement (7, 9) indicate that STb forms a disulfide-bonded hairpin-like structure (Fig. 1) with one pair of cysteine residues Cys-44–Cys-59 (codon designations) forming a flexible and charged 16-amino-acid loop. Certain amino acids within the Cys-44–Cys-59 loop are involved in full expression of STb toxicity (7, 10). Residues within the loop structure known to be involved in toxicity are Lys-45 and Lys-46 (mature STb peptide residues Lys-22 and Lys-23) (10) and Arg-52 and Asp-53 (mature STb peptide residues Arg-29 and Asp-30) (7). In addition, lysine residues at peptide positions 18 and 46 appear to be required for expression of wild-type levels of STb toxicity (10). None of the amino acid changes which affected STb biological activity resulted from apparent secondary structural changes in the mutant STbs (10); however, the effect of

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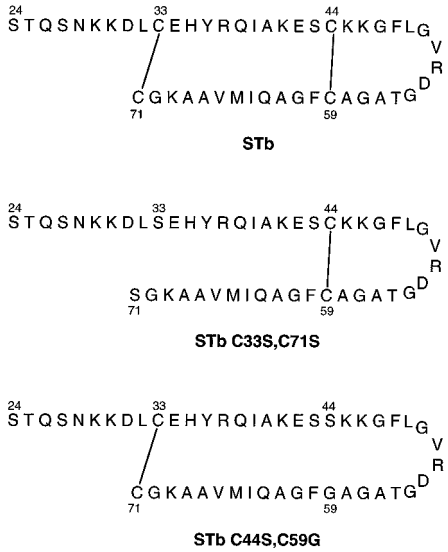

FIG. 1. STb and STb mutants resulting from the elimination of single disulfide bonds. The primary sequence and disulfide bond arrangement of the secreted form of native STb and the two STb mutants constructed in this study are shown. The residue numbering reflects the removal of the first 23 amino acids during secretion as previously reported (21).

these changes on the three-dimensional structure of STb is unknown. The extent to which individual disulfide bonds participate in STb structure and function is presently unclear; however, reduction and alkylation or oxidation of STb results in total loss of activity. In this report, we examined the contribution of the two individual disulfide bonds of STb to activity in both in vitro and in vivo assays.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and media. *E. coli* 1790, a wild-type porcine isolate expressing authentic STb, was previously described and used as a host for pPD21K, an *estB*-encoding vector used for wild-type STb production (6). The following *E. coli* K-12 strains were used. DH5α [F^- ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)U169] was obtained from GIBCO-BRL Life Technologies, Inc., Gaithersburg, Md., XL1-Blue [*recA1 end1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA* λ-*lac*$^-$ (F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10*)] (2) and BMH 71-18 mutS [*thi supE* Δ(*lac-proAB*) (*mutS*::Tn*10*) (F′ *proAB lacI*^{q}*Z*ΔM15)] were obtained from Promega (Madison, Wis.), and MM294 (F^- *endoA1 thi*$^-$ *pro*$^-$ *hsdR*$^-$ *supE44*) was obtained from Barbara Bachman. Plasmid pGEM-7Zf(+), used as a vector during oligonucleotide-directed mutagenesis, was obtained from Promega. The *tac* promoter-containing prokaryotic expression vector pPROK-1 was obtained from Clontech Laboratories, Inc. (Palo Alto, Calif.). Mutant STb constructs subcloned into the *Eco*RI and *Hin*dIII sites of pPROK-1 were pPROK-*estB33-71*, encoding the C-33→S, C-71→S (STbC33S,C71S) mutation and pPROK-*estB44-59*, encoding the STbC44S,C59G mutation. The kanamycin-resistant recombinant M13 phage M13KO7 (30), used as a helper phage in single-stranded DNA template production, was obtained from Promega. Liquid LB medium (10 g of Bacto tryptone, 5 g of Bacto yeast extract, 5 g of NaCl per liter) and LB agar (1.5% Bacto agar per liter of LB medium) were used for routine bacterial culture. After electroporation, transformed bacterial strains were cultured in SOC medium (4). M9-glucose chemically defined medium (22) was used for STb purification. When appropriate, antibiotics were used in liquid and solid media at the following concentrations: carbenicillin, 100 μg ml^{-1}; and kanamycin, 50 μg ml^{-1}.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis of the *estB* gene was conducted with single-stranded phagemid DNA templates prepared from pGEM-based clones of single-Cys STb mutants previously constructed by Dreyfus et al. (7). Oligonucleotides used in mutagenesis of the wild-type *estB* gene and the resulting peptide designations are listed in Table 1. To eliminate disulfide bonds, the four Cys residues in STb were individually changed on the basis of the simplest single-base-pair change to a conserved amino acid residue. Three of the four Cys residues were changed to serines by single-base-pair alteration. The fourth Cys residue (Cys-59) was changed to Gly. In this case, the only possible change to Ser resulted in the introduction of a *Hin*dIII site at that position in the STb gene. Since we use the unique *Hin*dIII site at the 3′ end of the STb gene cassette to move the gene from one vector to another, the TGC→AGC change to a Ser residue was avoided. Oligonucleotide-directed mutagenesis of Cys residues was linked to removal of a unique *Bam*HI restriction site in pGEM-*estB* with the oligonucleotide 5′-AAGCTTGGATCAGGA GAGCTCCCC-3′. For single-stranded template preparation, 5-ml samples of early-log-phase cultures of XL1-Blue (pGEM-*estB*) were infected with M13K07 at a multiplicity of infection of ≥10:1. One hour after phage infection, potassium phosphate buffer (pH 7.0 [20 mM final concentration]) and kanamycin were added, and the culture was allowed to incubate for an additional 16 h at 33°C. After removal of bacteria by centrifugation (5,000 × *g*, 15 min) and recovery of phage by precipitation with polyethylene glycol 8000 (20%), single-stranded DNA template was purified by phenol-chloroform (1:1 [vol/vol]) extraction and ethanol precipitation. After phosphorylation with T4 polynucleotide kinase and ATP (10 mM), oligonucleotides specifying the appropriate cysteine mutant and restriction site removal were annealed to the pGEM-*estB* template, extended, and ligated by the addition of the four deoxynucleotide triphosphates, T4 DNA polymerase (Promega), and T4 DNA ligase (Promega) under standard conditions recommended by the manufacturer. Ligated DNA was precipitated with ethanol, hydrated in 2 μl of H_2O, and used to transform *E. coli* BMH 71-18 mutS by electroporation (4). After 1 h of growth in 1 ml of SOC medium, the culture volume was increased to 5 ml with LB medium, and carbenicillin was added to select for growth of transformants. After overnight growth, plasmid DNA was isolated and digested with *Bam*HI, and 50 ng of digested DNA was used to transform *E. coli* DH5α. After isolation of plasmid DNA from individual transformants, mutations were confirmed by DNA sequence analysis with double-stranded DNA template and the dideoxy chain termination method. Dideoxy nucleotides and Sequenase were obtained from U.S. Biochemical Corp. (Cleveland, Ohio). An *estB*-specific primer, 5′-ATTGCTACAAATGCCTATGCA-3′, was used to prime the DNA sequencing reaction. The mutated *estB* gene cassettes were excised by digestion with *Eco*RI and *Hin*dIII and subcloned into pPROK-1 for expression. Mutations were again confirmed by sequence analysis.

Toxin purification. Wild-type and mutant *estB* constructs were purified as described by Harville and Dreyfus (15). *E. coli* MM294(pPROK-*estB33-71*), MM294(pPROK-*estB44-59*), and MM294(pPD21K) were each grown in 12-liter batch cultures of M9 minimal medium containing glucose (0.02%), carbenicillin (50 μg ml^{-1}), proline (100 μg ml^{-1}), and 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cultures, contained in six 6-liter Erlenmeyer flasks, each containing 2 liters of medium, were grown with aeration (300 rpm) for 18 h at 37°C. After growth, the bacterial cells were removed by ultrafiltration through a 0.1-μm-exclusion limit hollow fiber cartridge attached to a DC10L hollow fiber cartridge system (Amicon, Danvers, Mass.). High-molecular-mass material was removed by filtration through a 100-kDa spiral wound membrane cartridge and then concentrated to approximately 1 liter by passage through a similar cartridge with a 3-kDa exclusion limit (Amicon). The concentrated filtrate was then fractionated by preparative C_4 high-performance liquid chromatography (HPLC) with a Waters DeltaPak C_4 preparative column (25 by 100 mm) (Waters Division, Millipore). Elution of bound material was performed by a methanol-trifluoroacetic acid (0.05% trifluoroacetic acid) step gradient. STb was eluted with 60% methanol-trifluoroacetic acid. Fractions containing STb were dried by rotary evaporation and desalted by G-25 fast protein liquid chromatography (FPLC; Pharmacia LKB Biotechnology, Uppsala, Sweden) in 0.05 M sodium phosphate buffer (pH 6.5). Desalted toxin preparations were then fractionated by

TABLE 1. STb mutations, oligonucleotides, and peptide designations

Target residue and substitution	Oligonucleotide[a]	Designation
Cys-33→Ser	5′-AAAGATCTGT<u>C</u>^{G}TGAACATTAT-3′	STbC33S
Cys-44→Ser[b]	5′-AGGGAAAGTT<u>C</u>^{G}TAAAAAAGGT-3′	STbC44S
Cys-59→Gly	5′-CTGCTGGAGCA<u>G</u>^{T}GCTTTGGCGCCC-3′	STbC59G
Cys-71→Ser[b]	5′-GCAAAAGGAT<u>C</u>^{G}CTAATATATT-3′	STbC71S

[a] Changed bases are underlined. Wild-type bases at mutation sites are shown in superscript.
[b] Mutations were performed as described by Dreyfus et al. (7).

Mono S cation-exchange FPLC (Pharmacia) in a column equilibrated in the same buffer used for desalting STb. Bound material was eluted with a linear NaCl gradient (0 to 1 M). Purity of the STb samples was verified by analytical reverse-phase FPLC with a PepRPC HR 10/10 reverse-phase column (Pharmacia) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tricine buffer (25), followed by staining with Coomassie brilliant blue R (Sigma) and amino-terminal sequence analysis by automated Edman degradation.

Rat intestinal loop assay. The rat intestinal loop assay was performed as described by Whipp (32). After anesthesia with sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, Ill.), the small intestine of female Sprague-Dawley rats (body weight, 125 to 150 mg) was exteriorized and rinsed with 10 to 15 ml of phosphate-buffered saline (PBS) containing 1 mg of soybean trypsin inhibitor (Sigma) ml^{-1}. Toxin was introduced into intestinal loops isolated into 5- to 6-cm lengths by ligation and returned to the abdominal cavity. After a 2-h incubation period, the rats were euthanized by a lethal dose of Nembutal, and the amount of fluid that accumulated in both individual loops and the loop lengths was measured. An average of five loops per rat received control or toxin doses in 100-μl aliquots. Data were reported as microliters of fluid per centimeter of loop length.

Trypsin digestion. Two micrograms of authentic STb and mutant peptides was digested with trypsin under nondenaturing conditions with serially diluted enzyme ranging in concentration from 0.2 to 0.00156 μg per reaction mixture. Digestion was performed at room temperature for 30 min. The reactions were initiated by the addition of toxin and stopped by being heated at 100°C for 15 min. Samples were then analyzed by SDS-PAGE (25). After Coomassie blue staining, the gels were scanned with a Molecular Dynamics Personal Densitometer (Molecular Dynamics) and data were evaluated as the percentage of total toxin remaining at 30 min relative to an identical reaction mixture containing no trypsin.

5-HT and PGE_2 measurements. Rat intestinal fluid which accumulated after treatment with STb or STb mutants was examined for the presence of 5-HT and PGE_2 as previously described (15). 5-HT measurements were made by C_{18} reverse-phase HPLC analysis of acidified gut loop contents and comparison with known standard quantities of 5-HT. PGE_2 measurements were made by radioimmunoassay (Amersham Corp., Arlington Heights, Ill.). Loops lacking any apparent secretion were rinsed with 0.5 ml of PBS, and then the wash fluid was assayed for 5-HT and PGE_2 as described above.

Calcium assay. CMT-93 mouse rectal carcinoma cells (American Type Culture Collection, Rockville, Md.) were grown at 37°C in an atmosphere of 90% air–10% CO_2. Growth medium consisted of Dulbecco's modified Eagle's medium (Sigma) containing 4.5 g of glucose $liter^{-1}$, 10% fetal bovine serum (JRH Scientific, Lenexa, Kans.), penicillin (100 U ml^{-1}), and streptomycin (100 μg ml^{-1}). Cells were grown to 75% confluence and then stripped from culture flasks by treatment with 0.25% trypsin–0.05 mM EDTA. After being washed, 5×10^5 cells were seeded onto sterile no. 00 coverslips, flooded with complete medium, and incubated as described above. STb-mediated calcium ion flux in CMT-93 cells was measured by real-time fluorescence imaging as previously described (6). Simultaneous Ca^{2+} and pH measurements were made with the multi-imaging fluorescence video microscope as described by Morris (23). Coverslips containing CMT-93 cells preloaded with Indo-1,AM and SNARF-1,AM fluorescent dyes (Molecular Probes, Eugene, Oreg.) were held in a thermally controlled microscope-stage perfusion chamber. Cells were treated with 2.5 μg of wild-type or mutant STb at 60 s after the initiation of image recording; at 240 s, $CaCl_2$ was added to a final concentration of 3 mM. The final concentration of STb or STb mutants was 500 nM.

Reduction and alkylation of STb. Reduction and alkylation of STb were performed by the following method: 10 μmol of STb (or STb mutants) in 300 μl of 6 M guanidine-HCl–500 mM Tris-HCl–10 mM EDTA (pH 8.6) was incubated with 5.0 mg of dithiothreitol under nitrogen at 37°C for 2 h. Iodoacetamide (13.4 mg in 100 μl of 0.5 N NaOH) was added, and the reaction mixture was incubated in the dark at 25°C for 30 min. The reaction was quenched by the addition of 2-mercaptoethanol (1%). The reaction mixture was then desalted by gel filtration FPLC with a Fast Desalt (G-25) column equilibrated in water containing 0.05% trifluoroacetic acid.

CD. Circular dichroism (CD) spectra were taken of wild-type and mutant STb proteins in both the folded and reduced and alkylated forms with a JASCO J-720 spectrapolarimeter. Five scans were accumulated per spectrum taken at room temperature with a 0.1-cm path length over the wavelength range of 185 to 250 nm. Samples were placed in Milli-Q (Millipore) water (pH 7) and were filtered and degassed prior to CD analysis. Protein concentrations were obtained both prior to and after CD spectral analysis and ranged from 2.6 to 3.6 μM. Secondary structure prediction was made by the matrix multiplication method described by Compton and Johnson (3) and by the k2d program developed by Andrade et al. (1).

Oligonucleotides and protein sequencing. Oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer (Applied Biosystems, Foster City, Calif.). Purified STb and mutant STb peptides were sequenced after reduction and alkylation (described above) with an Applied Biosystems model 477A protein sequencer. Phenylthiohydantoin-derivatized amino acids were separated and identified by an on-line model 120A HPLC (Applied Biosystems). Oligonucleotide synthesis and peptide sequencing were performed in the University of Missouri—Kansas City School of Biological Sciences Molecular Biology Research Core Facility.

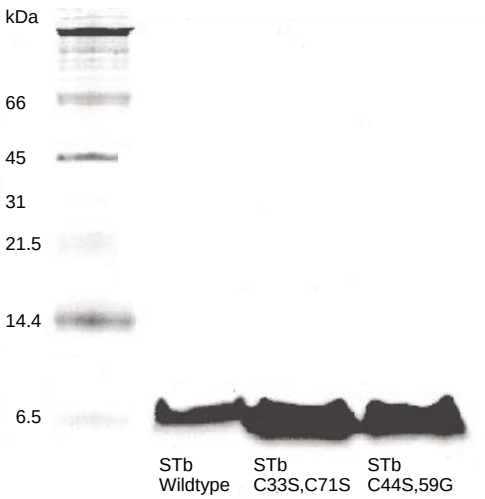

FIG. 2. SDS-PAGE analysis of native and mutant STb peptides. Approximately 1 μg of unreduced STb samples was loaded in SDS-PAGE gel buffer alongside protein standards and electrophoresed as described in Materials and Methods. After electrophoresis, the gel was stained as described in the text and scanned with a Molecular Dynamics personal densitometer.

RESULTS

Genetic construction, expression, and purification of mutant STbs. Construction of cysteine mutant *estB*(s) was performed by oligonucleotide-directed site-specific mutagenesis. The oligonucleotides, residues changed, and toxin nomenclature are shown in Table 1. STb mutant peptides lacking individual disulfide bonds were designated STbC33S,C71S and STbC44S,C59G. DNA sequencing of the desired STb mutants was performed to confirm the correct assignment of the mutations. To assess the biological action of STbs lacking individual disulfide bonds, the toxin constructs were grown in *E. coli* MM294 and the peptides were purified to homogeneity as determined by SDS-PAGE analysis (Fig. 2) and amino acid sequence analysis (not shown). The following yields of the three toxins were obtained: STb, 600 μg liter of $culture^{-1}$; STbC33C,C71S, 200 μg liter of $culture^{-1}$; and STbC44S,C59G, 300 μg liter of $culture^{-1}$.

In vivo biological activity of native STb and STb mutants. Purified STbs were analyzed for secretory action in rat intestinal loops. Compared with authentic STb, which yields reproducible high-level secretion in rat intestine, the two disulfide bond mutant STbs failed to elicit significant secretion (Table 2). In addition to fluid secretion values, mutant STbs also failed to significantly elevate 5-HT and PGE_2 levels in intestinal fluid compared with control values. An increase in the mutant STb dose to 2.5 μg failed to yield measurably higher amounts of secreted fluid, 5-HT, or PGE_2 (not shown). Because STb is partially sensitive to a trypsin-like protease in the gut of experimental animals (32), one explanation for the observed inactivity of the mutant STbs may be an increased sensitivity to proteolysis. Initial attempts to recover native STb from control rat intestinal loops were inconclusive; therefore, we examined the in vitro sensitivity of purified STb, STbC33S,C71S, and STbC44S,C59G to trypsin proteolysis. The results of in vitro proteolysis indicated that all three toxins displayed nearly equivalent sensitivities to degradation by trypsin (Fig. 3). Minor differences in the observed rates of prote-

TABLE 2. In vivo action of native and mutant STbs

Toxin[a]	Amt of secretion[b]	Amt of[c]:	
		PGE_2	5-HT
PBS	20 ± 10	0.52 ± 0.4	0.33 ± 4
STb	135 ± 25	14 ± 2	6.6 ± 1
STbC33S,C71S	35 ± 8	0.66 ± 0.4	0.8 ± 4
STbC44S,C59G	28 ± 10	0.5 ± 0.5	0.3 ± 0.2

[a] Toxin doses were 1 μg per loop, administered in PBS containing soybean trypsin inhibitor (1 mg ml^{-1}).

[b] Data are the mean microliters of fluid per centimeter of loop length ± standard error from five to six loops. Loop contents were analyzed 90 min after introduction of toxin.

[c] Data are mean picograms of PGE_2 or 5-HT per centimeter of loop length ± standard error determined from loop fluids indicated under the "Amt of secretion" heading. The data are taken from six loops.

olysis were therefore insufficient to account for the in vivo toxicities elaborated by the STbs.

STb-mediated calcium ion flux in cultured intestinal epithelial cells. Previous work in our laboratory indicated that STb activates a PtX-sensitive G protein in cultured cells and that activation can be assessed by an influx of calcium ions under the appropriate experimental conditions (6). Calcium ion flux in response to STb is dose dependent and requires a biologically active form of STb, since reduced and alkylated STb is inactive in this in vitro toxin assay. The contribution of individual disulfide bonds to the ability of STb to activate the PtX-sensitive G protein response in cultured CMT-93 mouse rectal carcinoma cells was assessed and is described in Materials and Methods. Under the experimental conditions, authentic STb (500 nM) induced a rapid and dramatic increase in cytosolic calcium levels (Fig. 4A). As previously reported (6), the response was independent of changes in intracellular pH (Fig. 4A) and was sensitive to PtX treatment (not shown). In contrast, equivalent doses (500 nM) of C33S,C71S and C44S,C59G STb failed to induce calcium ion flux under identical experimental conditions (Fig. 4B and C, respectively).

CD spectroscopy of STb and the STb disulfide bond mutants. The apparent loss of biological activity after selective disulfide bond disruption of STb may be the result of significant alteration in secondary structure. To assess the impact of selective disulfide bond removal on apparent secondary structure, we examined authentic and mutant STbs by CD spectroscopy in the far-UV range. Nonreduced wild-type STb yielded a

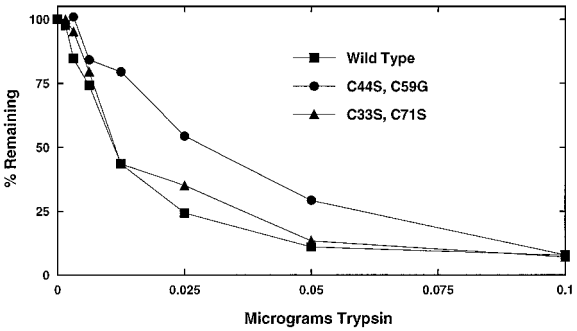

FIG. 3. Protease sensitivity of authentic and mutant STb peptides. Authentic STb and mutant peptides were subjected to proteolysis with serially diluted trypsin as described in the text. After protease digestion, samples were separated by SDS-PAGE and stained as described in the text. Stained gels were scanned, and the data from three scannings were averaged and analyzed with Delta Graph Pro software (Delta Point, Inc.).

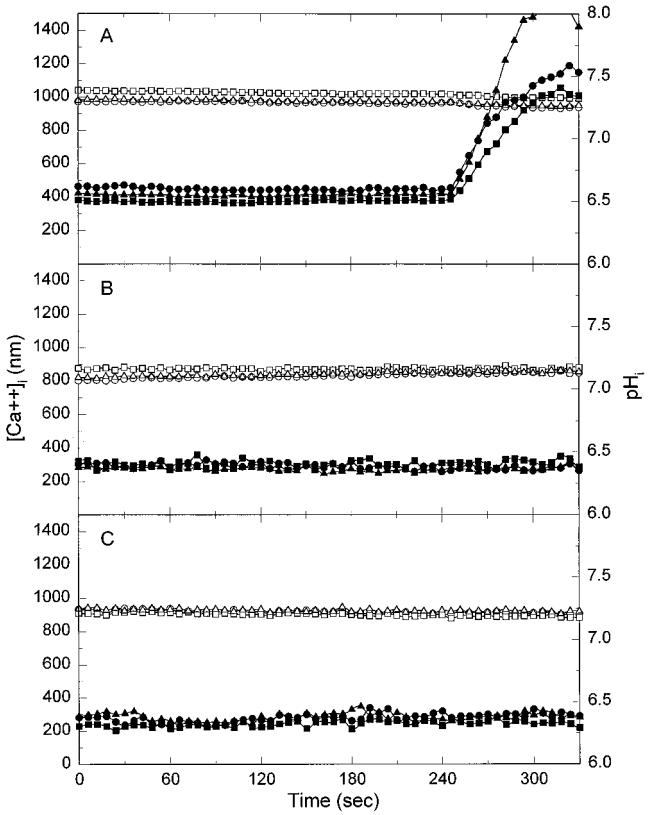

FIG. 4. Calcium ion flux mediated by STb wild-type and mutant peptides. A calcium ion influx assay was performed with mouse CMT-93 rectal carcinoma cells in culture as described in the text. Changes in internal calcium ion concentration ($[Ca^{2+}]_i$) are represented by solid symbols; open symbols represent internal pH (pH_i). Each pair of solid and open symbols represents calcium and pH measurements from a single cell. At least six individual cells were simultaneously analyzed; the data presented are representative of three individual cells per toxin sample. (A) Native STb. (B) STbC33S,C71S. (C) STbC44S,C59G.

far-UV CD spectrum consistent with significant α-helical content as evidenced by characteristic [Θ] minima at approximately 208 and 220 nm (Fig. 5A). In contrast, reduced and alkylated STb exhibits primarily a random coil structure with a calculated reduction in apparent α-helical content exceeding 70% of that of authentic STb (Fig. 5A). Unlike reduced STb, STbC33S,C71S yielded a CD spectrum that was nearly superimposable over that of native STb (Fig. 5B). In fact, calculation of secondary structure indicated that STbC33S,C71S had slightly more α-helical content than authentic STb. In contrast to these data, the CD spectrum of STbC44S,C59G was flattened considerably in the range of 200 to 220 nm (Fig. 5B) and, on the basis of secondary structure prediction, retained only 79% of the α-helical content of authentic STb or STb C33S,C71S with a concomitant increase in random coil.

DISCUSSION

Native STb contains two disulfide bonds in the arrangement shown in Fig. 1. Reduction and alkylation (7) or prolonged incubation at elevated pH (9), conditions that promote disulfide bond disruption, results in the loss of STb biological activity. Here, we wished to examine the relative contribution of individual disulfide bonds to the biological and physical properties of STb, including intestinal secretory activity, in vitro calcium ion flux, and peptide secondary structure. Our results

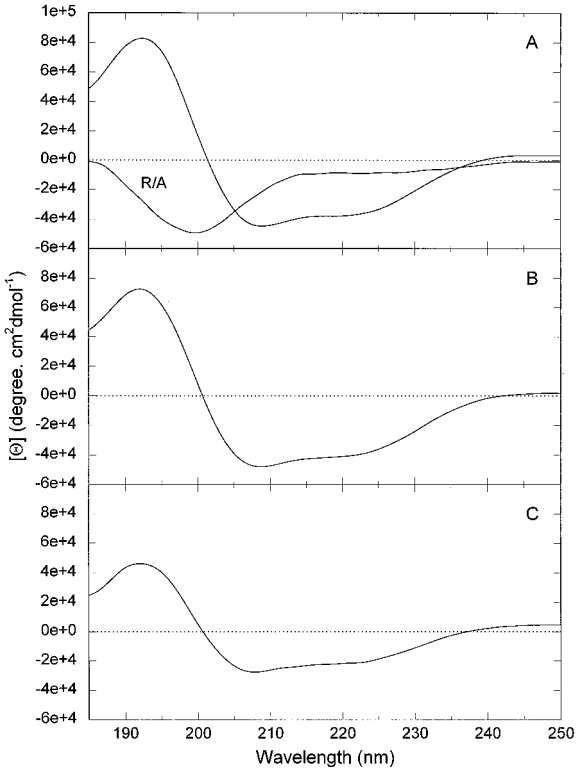

FIG. 5. CD spectroscopy of STb peptides. Five scans of each toxin preparation were performed and were used to generate the spectra shown. The samples were scanned with a 0.1-cm path length with a range from 185 to 250 nm as described in Materials and Methods. The CD data were collected with Sigma Scan (Jandel, Inc.) and converted to graphic form with SigmaPlot (Jandel, Inc.). (A) Native STb. R/A, reduced and alkylated native STb. (B) STbC33S,C71S. (C) STbC44S,C59G.

indicated that both disulfide bonds are absolutely required for the intestinal secretory action of STb. The observed loss of toxicity after individual disulfide bond removal was not the result of apparent increased sensitivity of mutant STbs to proteolytic degradation. In addition, unlike native STb, both disulfide bond mutants were unable to effect PtX-sensitive G protein activation, as evidenced by STb-induced calcium ion entry into cultured cells. The relationship between G protein activation, calcium ion entry, and intestinal secretion is presently unclear. The finding that specific disulfide bond disruption results in loss of calcium entry, the monitor for G protein activation, however, does support the notion that G protein coupling and possibly calcium ion entry are events that are absolutely required for elaboration of STb intestinal secretory action.

Previous work by our laboratory attempted to evaluate the role of Cys residues and disulfide bonds in STb intestinal secretory activity. STb molecules bearing the single-Cys mutations C-71→S and C-44→S, however, apparently failed to translocate across the outer membrane of the *E. coli* host (7). While expression of the constructs from sucrose-stabilized EDTA and lysozyme-generated spheroplasts did allow detection of the STb Cys mutants (7), the production and purification of a sufficient quantity of the toxins for biological assay would have been hampered by this regimen. We hypothesized that the free sulfhydryl group on the unpaired Cys residue of the C-44→S and C-71→S STbs was promoting either inappropriate intermolecular disulfide bonding or intramolecular disulfide bond formation in the *E. coli* host periplasm; the result of either scenario would be inefficient or impossible outer membrane translocation. We have also observed lower yields of extracellular STb generated from T7 promoter-polymerase expression systems than those generated with either *tac* or *lac* promoters (see reference 29 and unpublished results), presumably because of inefficient outer membrane translocation caused by the rapid accumulation of periplasmic toxin. Here, we were able to overcome the problems previously encountered by constructing disulfide bond pair double Cys mutants, thus preventing inappropriate intramolecular disulfide bond formation, and expression from a *tac* promoter vector to better control the yield of extracellular toxin.

In vivo studies of STb action have indicated that release of 5-HT is associated with STb action in the gut (15, 24) and that 5-HT release is in part responsible for intestinal secretion (15). In addition, STb causes a dose-dependent rise in lumenal PGE_2; blocking PGE_2 formation by treating experimental animals with indomethacin blocks STb-mediated intestinal secretion (15, 18). The inability of the disulfide bond mutants to elicit 5-HT release and PGE_2 formation indicates again that these two intestinal secretagogs are involved in the mechanism of STb biological action. As a model of 5-HT release in vivo, we have examined the effect of STb on 5-HT secretion from the rat basophilic leukemia cell line RBL 2H3 (17, 33). In this model, STb causes dose-dependent release of 5-HT through a PtX-sensitive pathway. Furthermore, reduced and alkylated STb and the disulfide bond STb mutants described in this report fail to induce 5-HT release (see reference 17 and unpublished results), thus indicating again that native STb is required for the apparent activation of heterotrimeric G protein and subsequent coupling to 5-HT release in vitro and, presumably, in vivo.

Reduction and alkylation of STb caused a dramatic change in apparent secondary structure on the basis of the observed far-UV CD spectrum; thus, maintenance of a native conformation is dependent upon the two disulfide bonds. These data are in agreement with those from recent structural studies which indicate STb is converted to nearly total random coil structure after reduction and alkylation (28). Our structural studies indicate that STb is helical between residues 33 and 45 and 61 and 67. Elimination of both disulfide bonds apparently eliminates all α-helical content on the basis of CD measurements. Removal of the Cys-44–Cys-59 disulfide bond, as reported here, also significantly reduced the molar ellipticity between 200 and 220 nm, indicating a reduction in the α-helical content of this mutant STb. An unexpected finding was the nearly identical CD spectrum of the STbC33S,C71S mutant compared with that of native STb. Despite the preservation of secondary structure, the loss of activity of STb C33S,C71S indicates that preservation of secondary structure alone is not necessarily a valid indicator of STb toxicity. The findings presented here indicate that STbC33S,C71S represents another potentially useful model peptide like those previously described (7, 10) for structure and function analysis of STb action. The usefulness of all of these mutants in determining structure and function relationships, however, awaits determination of their solution structures and comparison with that of native STb (28).

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