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Importance of disulfide bridges in the structure and activity of *Escherichia coli* enterotoxin ST1b

(peptide folding/synthetic peptides/bacterial diarrhea)

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ABSTRACT **A 13-amino acid sequence of the *Escherichia coli* heat-stable enterotoxin ST1b encodes its receptor-binding and diarrheal functions. This sequence includes six cysteines involved in three intramolecular disulfide bridges. To determine the importance of disulfide bridges to the biological activity of ST1b, we synthesized 15 analogues of the tridecapeptide representing all possible replacements of two of the six cysteines by alanines. Only 2 analogues—namely, $A^{6,11}$ST1b-(6–18) and $A^{10,18}$ST1b(6–18)—could inhibit the binding of a radiolabeled analogue of ST1b to rat intestinal cells. The purified peptides were, respectively, 4200 and 130 times less effective as inhibitors than ST1b(6–18), the sequence that includes all six cysteines. In addition, both peptides produce diarrhea when given orally to suckling mice. These analogues share in common only two cysteines (Cys-7 and Cys-15), suggesting that four cysteines, two of which are Cys-7 and Cys-15, are necessary for activity. A pattern of disulfide linkages is proposed where Cys-7 is paired to Cys-15, Cys-6 to Cys-11, and Cys-10 to Cys-18, the preceding disulfide bridges being ranked in descending order of importance in terms of their respective contribution to the activity of the enterotoxin. Using this disulfide bridge arrangement and constraints derived from NMR spectroscopy, we propose a folding pattern for the toxic domain of ST1b.**

The *Escherichia coli* ST1 family of enterotoxins is composed of homologous peptides of <20 amino acids that cause diarrhea in humans and domestic animals. The study of their structure and mode of action may have implications for the prevention of diarrhea in infants in developing countries (1, 2) and in travellers to endemic areas (3). These peptides can be regrouped into two subfamilies, abbreviated ST1a and ST1b, based on amino acid sequence differences (4–7). The receptor binding and enterotoxic properties of the ST1 enterotoxins have been mapped to a highly conserved C-terminal tridecapeptide (8, 9), a sequence also present in the primary structure of the ST1 enterotoxins elaborated by pathogenic strains of *Yersinia enterocolitica* and non 01 *Vibrio cholerae* (10, 11). This sequence includes six cysteines that form three intramolecular disulfide bridges. ST1 toxins initially bind to a surface component located on intestinal cells (8, 12–14). The formation of the ST1–receptor complex activates a membrane-bound guanylate cyclase (15–19). The increase in intracellular cGMP is followed by the secretion of water and electrolytes by the intestinal cell; diarrhea begins within a few hours. The pairing pattern of the three disulfide bridges of ST1 remains speculative (20) and the importance of each of these linkages to the receptor binding and secretory activity of the toxin has not been investigated. This paper addresses these issues through the chemical synthesis of analogues of the active tridecapeptide ST1b(6–18) that lack two of the six cysteines. The results derived from this study lead us to a revised proposal for the disulfide arrangement and for the structure of the toxic domain (21).

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EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification of ST1b(6–18) Analogues. Peptides were synthesized using a peptide synthesizer (model no. 990C; Beckman Instruments) by classical solid-phase methods (22). The crude deprotected peptides were dissolved in distilled water to a concentration of 10 μM. The pH of each solution was adjusted to 8.5 with 10% (vol/vol) aqueous NH_4OH and the disulfide bridges were allowed to air oxidize at room temperature (23). The active analogues with alanine substitutions at positions 6 and 11 [$A^{6,11}$ST1b(6–18)] and at positions 10 and 18 [$A^{10,18}$ST1b(6–18)] were purified by preparative reverse-phase chromatography (gradient: A, 0.1% trifluoroacetic acid in H_2O; B, 35% CH_3CN + 0.1% trifluoroacetic acid in H_2O) and partition chromatography (*n*-BuOH/HAc/H_2O; 4:1:5; vol/vol). The purified analogues were sequenced to confirm their primary structure. The stock solution concentrations were determined by amino acid analysis.

Radiolabeling of Y^4ST1b(4–18). The two tyrosine residues of the synthetic analogue with a tyrosine substitution at position 4 [Y^4ST1b(4–18)] (1 μg) were iodinated by the lactoperoxidase method using Enzymobeads (BioRad) and 1 mCi of $Na^{125}I$ (1 Ci = 37 GBq) as described (8). Equimolar doses of Y^4ST1b(4–18) and ST1b bind the ST1 enterocyte receptor in a competitive radiobinding assay and induce diarrhea in the suckling mouse bioassay.

Radiobinding Assay. The receptor binding capacity of each analogue was tested with rat intestinal cells. These were collected as described (8) and diluted to a concentration of 10^7 cells per ml in phosphate-buffered saline (PBS) (pH 7.4) containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin (buffer A). Twenty-microliter aliquots of ^{125}I-labeled Y^4ST1b(4–18) [^{125}I-Y^4ST1b(4–18)] (125,000 cpm) were placed in polypropylene tubes (12 × 75 mm) containing 20 μl of an intestinal cell suspension (2 × 10^5 cells), 20 μl of buffer A, and 20 μl of either a known concentration of a synthetic analogue of ST1b to be tested for receptor binding activity or buffer A alone. The reactants were mixed and incubated at 37°C for 30 min. The cells and cell-bound ^{125}I-Y^4ST1b(4–18) were recovered by filtration on

Abbreviations: ST1b, 19-amino acid long heat-stable enterotoxin elaborated by an enterotoxigenic *Escherichia coli* strain of human origin; ST1b(6–18), residues 6–18 of ST1b; $A^{6,11}$ST1b(6–18), synthetic analogue of ST1b(6–18) with alanine substitutions at positions 6 and 11; $A^{10,18}$ST1b(6–18), synthetic analogue of ST1b(6–18) with alanine substitutions at positions 10 and 18; Y^4ST1b(4–18), synthetic analogue of ST1b comprising residues 4–18, with a tyrosine substitution at position 4; ^{125}I-Y^4ST1b(4–18), ^{125}I-labeled Y^4ST1b(4–18).

Whatman GF/A filters. The tubes were washed twice with buffer A containing 0.1% (wt/vol) bovine serum albumin, and the filters were counted. Inhibition curves were constructed by serially diluting peptide solutions in buffer A, with each point representing the average cpm value of triplicate samples.

Suckling Mouse Assay. The enterotoxic potency of active analogues was assessed by determining their capacity to stimulate intestinal secretion using the suckling mouse assay (24, 25). Briefly, peptide stock solutions were serially diluted in PBS containing 0.02% (wt/vol) Evans blue dye. One hundred-microliter aliquots of each solution were directly delivered to the esophagus of 1- to 2-day-old Swiss Webster suckling mice using a 1-ml disposable syringe and a 26-gauge needle fitted with a 1-cm piece of flexible plastic tubing. Three hours later the mice were killed with ether and the weights of their guts and carcasses were determined separately. Each point of the titration curve represents the average gut/carcass weight ratio (G/C) for three mice. A G/C value of 0.09 or greater corresponds to the unambiguous accumulation of fluid in the gut lumen.

RESULTS AND DISCUSSION

Statistical Approach for Assessing Functional Importance and Cysteine Pairing Patterns of Disulfide Bridges. The sequence ST1b(6–18) is depicted in Fig. 1 and encodes the receptor binding and enterotoxic domains of ST1b (8, 9). Mass spectroscopy, ^{13}C NMR (21), and the lack of reactivity with the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) led us to conclude that the six cysteines of ST1b(6–18) form three intramolecular disulfide bridges. Providing that it is equally probable for any cysteine to form a disulfide bridge with any other cysteine of the same peptide, the six cysteines of ST1b(6–18) can adopt 15 possible pairing patterns.§ These are listed in Fig. 1. We were unable to determine which one of these arrangements is correct from analytical studies using biophysical techniques or through identifying the cysteines comprising disulfide bridges in fragments of the toxin produced by FAB mass spectroscopy or enzymatic proteolysis. Consequently, we have used a synthetic strategy to predict the disulfide bridge arrangement of ST1b(6–18).

Rationale for Preparing all Possible Analogues of ST1b(6–18) Lacking Two of the Six Normal Cysteines. The α-conotoxins, a family of 13- to 15-amino acid long neurotoxins from marine snails (26), contain four cysteine residues that form two intramolecular disulfide bridges and share with ST1b(6–18) an identical sequence of six amino acids. This homologous segment specifies a common antigenic determinant (23). The structural and antigenic relatedness of α-conotoxin and ST1b(6–18) suggests the possibility that only two of the three normal disulfide bridges of ST1b are required for receptor binding function and enterotoxicity. If so, then the synthesis of all possible analogues of ST1b(6–18) containing only four of the six normal cysteines (Fig. 2) could yield peptides that retain the biological activities of the original toxin. The four cysteines of each analogue will form two intramolecular disulfide bridges; theoretically, each dicyclic peptide can adopt only three possible disulfide patterns (3 × 1 = 3). The correct arrangement of the three normal disulfide bridges in ST1b(6–18) can be predicted by comparing the sequences and functional activities of each analogue with only two disulfide bridges, providing the following conditions are fulfilled. First, at least two of these analogues must retain function. Second, comparison of the three possible disulfide pairing patterns of each active dicyclic analogue must yield a unique arrangement.

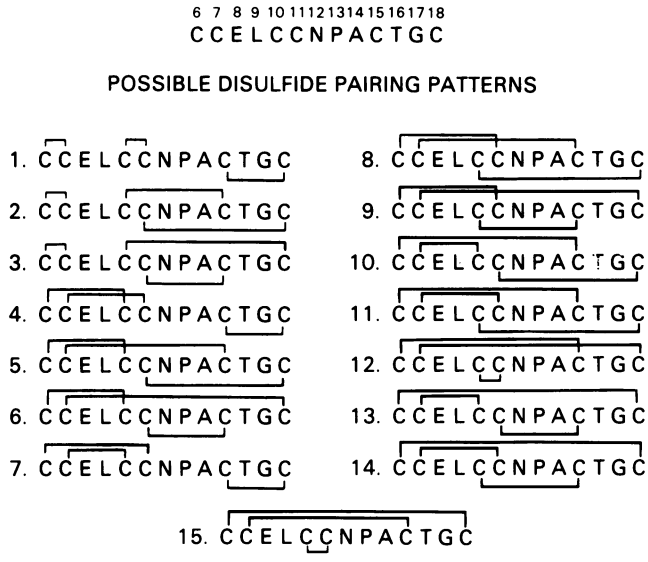

FIG. 1. Possible disulfide pairing patterns of ST1b(6–18). The six cysteines of ST1b(6–18), at positions 6, 7, 10, 11, 15, and 18, can adopt 15 possible disulfide arrangements. Amino acids are identified by the single-letter code.

An important premise of this approach is that only one disulfide pairing pattern is compatible with the active form of ST1b(6–18). If this premise is correct, then all active analogues containing only four cysteines will mimic the remaining disulfide pattern of the natural sequence. The validity of this premise can be analyzed in light of recent proton magnetic resonance studies on ST1b(6–18) and ST1b(6–19) (21). The ^1H NMR spectra of the toxin indicate that the C-terminal part of the molecule (from Asn-12 to Cys-18) adopts a well-defined conformation in solution, whereas the structure of the N-terminal segment (Cys-6 to Cys-11) may not be unique at room temperature. Since all six cysteines (four cysteines in the case of the analogues in this study) are paired and the rate of disulfide interchange is a function of the thiol anion concentration (R-S) (27, 28), then between pH 6 and pH 7.5 the thiol groups (pK_a, ≈8.65) should exist mostly as part of disulfide bridges or as protonated species (R-SH). The rate of formation of intramolecular mixed disulfides is thus expected to be small. For example, the proton spectrum of a 7.4 mM solution of ST1b(6–18) was recorded at pH 6 (21). Assuming that as much as 0.5 mM peptide exists with free thiol groups in solution and that the initial rate constant for

6 10 15 18	
Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys	ST1b(6-18)
[Ala]-[Ala]-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys	A ^{6,7} ST1b(6-18)
[Ala]-Cys-Glu-Leu-[Ala]-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys	A ^{6,10}ST1b(6-18)
[Ala]-Cys-Glu-Leu-Cys-[Ala]-Asn-Pro-Ala-Cys-Thr-Gly-Cys	A ^{6,11}ST1b(6-18)
[Ala]-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-[Ala]-Thr-Gly-Cys	A ^{6,15}ST1b(6-18)
[Ala]-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-[Ala]	A ^{6,18}ST1b(6-18)
Cys-[Ala]-Glu-Leu-[Ala]-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys	A ^{7,10}ST1b(6-18)
Cys-[Ala]-Glu-Leu-Cys-[Ala]-Asn-Pro-Ala-Cys-Thr-Gly-Cys	A ^{7,11}ST1b(6-18)
Cys-[Ala]-Glu-Leu-Cys-Cys-Asn-Pro-Ala-[Ala]-Thr-Gly-Cys	A ^{7,15}ST1b(6-18)
Cys-[Ala]-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-[Ala]	A ^{7,18}ST1b(6-18)
Cys-Cys-Glu-Leu-[Ala]-[Ala]-Asn-Pro-Ala-Cys-Thr-Gly-Cys	A ^{10,11}ST1b(6-18)
Cys-Cys-Glu-Leu-[Ala]-Cys-Asn-Pro-Ala-[Ala]-Thr-Gly-Cys	A ^{10,15}ST1b(6-18)
Cys-Cys-Glu-Leu-[Ala]-Cys-Asn-Pro-Ala-Cys-Thr-Gly-[Ala]	A ^{10,18}ST1b(6-18)
Cys-Cys-Glu-Leu-Cys-[Ala]-Asn-Pro-Ala-[Ala]-Thr-Gly-Cys	A ^{11,15}ST1b(6-18)
Cys-Cys-Glu-Leu-Cys-[Ala]-Asn-Pro-Ala-Cys-Thr-Gly-[Ala]	A ^{11,18}ST1b(6-18)
Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-[Ala]-Thr-Gly-[Ala]	A ^{15,18}ST1b(6-18)

FIG. 2. Amino acid sequences of ST1b(6–18) and the synthetic dicyclic analogues used in the study. The sequences listed below ST1b(6–18) represent all possible substitutions of a pair of cysteines by two alanines (boxed residues). Each dicyclic analogue was tested for receptor binding and secretory activity.

§For n cysteine residues, the number of possible permutations is $(n - 1) \times (n - 3) \times (n - 5) \ldots \times [n - (n - 1)]$.

the formation of mixed disulfides approximates a value of 1.7 $\times$ 10^3 $M^{-1}\cdot min^{-1}$, as observed for the glutathione/*N,N'*-diacetylcystamine system (27), then the thiol anion concentration is 1.12×10^{-6} M and the initial rate of formation of the mixed disulfide is 1.3×10^{-5} $mol\cdot liter^{-1}\cdot min^{-1}$. These values suggest that disulfide interchange occurs slowly on the NMR time scale and should give rise to a defined population of conformers having unique sets of proton resonances in the proton NMR (slow exchange). This prediction is supported by our NMR studies (21), since overlapping resonance patterns were not observed, indicating that ST1b(6–18) probably does not adopt multiple conformations on the NMR time scale. Moreover, if the rate of disulfide interchange were rapid enough to give rise to an averaged population of conformers, a broadening effect (spin–spin relaxation time, t_2) on the width of resonance peaks (29) would have been evident. However, the observed NMR spectrum highlights well-defined resonance patterns for a large proportion of the assigned protons, an indication that disulfide interchange occurs extremely slowly and that only one form of ST1b(6–18), with a unique disulfide geometry predominates. In view of the lack of reactivity of ST1b(6–18) with 5,5'-dithiobis(2-nitrobenzoic acid), a far less likely possibility is that the well-defined resonance patterns are the result of extremely rapid disulfide bridge exchange. Intramolecular disulfide mixing is also a less probable event when the peptide backbone is folded and maintained by other disulfide bridges since disulfide exchange should only occur if the peptide adopts a conformation suitable for the interaction of any possible free thiol group with a disulfide bridge.

Theoretically, only 3 of the 15 synthetic dicyclic analogues shown in Fig. 2 should contain the right pairing pattern for two of the three disulfide linkages, while all of the analogues should contain at least one of the three disulfide linkages observed in the native toxin. Since each oxidized analogue was screened for receptor binding function before purification (as described below), it was assumed that the four cysteines of each analogue would give rise to each of the three possible dicyclic patterns that are dictated by the location of the four cysteines in that analogue. Thus, only a fraction (one-third, assuming a random pairing pattern) of the peptide population of each analogue would be expected to actually contain at least one and at most two, of the three correct disulfide bridges.

In summary, if only two of the three correct disulfide bridges were needed for activity, one would expect to find three active analogues amongst the 15 dicyclic analogues synthesized for this study. If two linkages are needed, but each one contributes differently to the folding and activity of ST1b, one would expect to find less than three active analogues. If only one disulfide linkage is needed for activity, between 6 and 15 of the synthetic analogues should exhibit activity depending on the actual contribution of each disulfide bridge to activity.

Two Analogues Lacking a Pair of Cysteines Exhibit Receptor Binding Function and Enterotoxicity, Indicating That Not All Disulfide Bridges Are Necessary for Activity. The 15 analogues listed in Fig. 2 were prepared by solid-phase peptide synthesis (22); two cysteines were replaced by alanines in each analogue. It should be noted that we chose not to replace two of the four normal cysteines with cysteine derivatives having an acetylamidobenzyl thio protecting group, an approach used by Nishiuchi and Sakakibara (26) to predict the disulfide pairing pattern of conotoxin G1. The acetylamidobenzyl groups were used by these investigators to prevent disulfide bridge formation by the two corresponding cysteine residues. After they allowed the unprotected cysteines to pair, the acetylamidobenzyl groups were removed, permitting the remaining disulfide bridge to form in a selective stepwise manner. However, this approach entails two potential difficulties not encountered in the substitution of cysteine by alanine residues. First, we expected that the removal of any disulfide bridge would decrease the activity of the resulting analogue by a factor of >100. Such a level of activity could falsely result from the partial deprotection of only 1% of thiol side-chain protecting groups during the preparation and use of the protected analogue. Indeed, the acetylamidobenzyl thiol protecting group has been reported to be partly labile to hydrofluoric acid treatment (30). Thus, all the analogues would have had to be purified prior to the initial oxidation step. Second, the formation of unique disulfide linkages through the selective removal of the acetylamidobenzyl protecting group could be nullified by partial disulfide exchange that may occur under the experimental conditions used. This problem arose in the conotoxin studies cited above (26), where disulfide reshuffling and peptide refolding were observed.

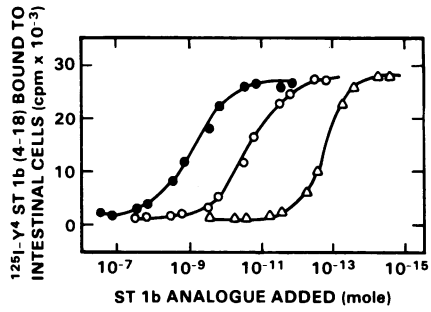

FIG. 3. Inhibition of binding of ^{125}I-Y^{4}ST1b(4–18) to rat intestinal cells by the purified peptides A^{6,11}ST1b(6–18) (●), A^{10,18}ST1b(6–18) (○), and ST1b(6–18) (△). The receptor binding assay is described in *Experimental Procedures*. Each point represents the average of assays performed in triplicate. The standard deviation associated with each point ranges between 50 and 1000 cpm.

The synthesized analogues depicted in Fig. 2 were air-oxidized immediately after their cleavage from the resin and tested in a radiobinding assay to determine whether they could competitively inhibit the binding of radiolabeled Y^4-ST1b(4–18) to rat intestinal cells. Each synthetic analogue (Fig. 2) was tested in a radiobinding assay at doses of 500 ng and 50 μg, which represent inhibition doses 900 and 90,000 times greater than for ST1b(6–18), the analogue that includes all three disulfide bridges. Only two crude analogues, abbreviated A^{6,11}ST1b(6–18) and A^{10,18}ST1b(6–18), showed significant inhibition of ^{125}I-Y^{4}ST1b(4–18) binding to intestinal cells at a dose of 50 μg (results not shown). These two active analogues were purified and their amino acid sequences were determined and shown to agree with the syntheses performed. Inhibition curves were constructed to determine the amount of each purified synthetic peptide that inhibited 50% (IC_{50}) of the specific binding of ^{125}I-Y^{4}ST1b(4–18) to rat

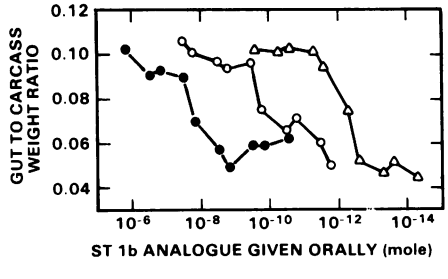

FIG. 4. Diarrheal response curves for the purified peptides A^{6,11}ST1b(6–18) (●), A^{10,18}ST1b(6–18) (○), and ST1b(6–18) (△). A gut/carcass ratio value of 0.09 or above is correlated with a strong episode of diarrhea in all mice tested. Each point represents the average ratio value derived from weight measurements performed on three suckling mice.

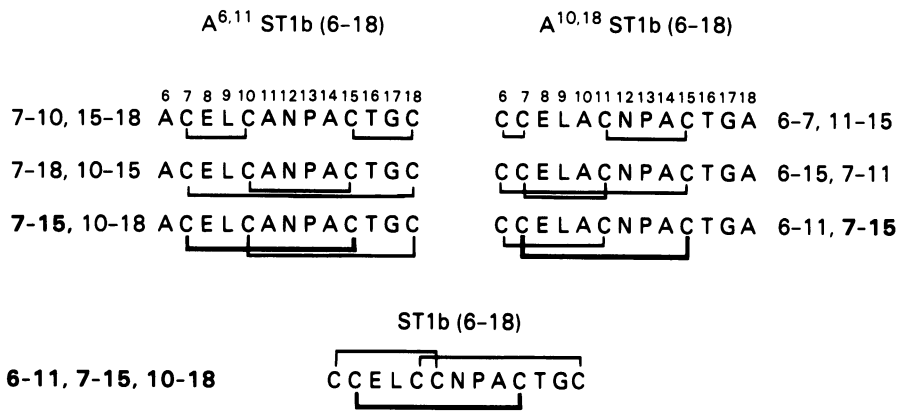

FIG. 5. Possible disulfide pairing patterns of the two active dicyclic analogues $A^{6,11}$ST1b(6–18) and $A^{10,18}$ST1b(6–18). The four cysteines of each analogue can adopt three possible disulfide arrangements. The disulfide bridge Cys-7 to Cys-15 (indicated in boldface) is common to both patterns, leaving Cys-10 to pair with Cys-18 in $A^{6,11}$ST1b(6–18) and Cys-6 to pair with Cys-11 in $A^{10,18}$ST1b(6–18). The proposed disulfide bridge arrangement of ST1b(6–18) is indicated. Amino acids are identified by the single-letter code.

intestinal cells (Fig. 3). Analogues $A^{6,11}$ST1b(6–18) and $A^{10,18}$ST1b(6–18) were 4200 and 130 times less active, respectively, than ST1b(6–18) based on their IC_{50} values. Both analogues also elicited strong diarrheal responses based on significant increases in the gut/carcass weight ratio, when given orally to suckling mice (Fig. 4). The minimal doses resulting in diarrhea were 1.1×10^{-12} mol for ST1b(6–18), 2.1×10^{-10} mol for $A^{10,18}$ST1b(6–18), and 1.8×10^{-8} mol for $A^{6,11}$ST1b(6–18).

Analysis of Possible Cysteine Linkages for the Two Active Analogues Suggests a Unique Disulfide Pairing Pattern. The four cysteines of each active analogue can form three possible disulfide bridge patterns (Fig. 5). Only a disulfide bridge between Cys-7 and Cys-15 is common to both sets of patterns. All other cysteine pairs occur in only one of these two analogues or in inactive analogues. If the analogues $A^{6,11}$ST1b(6–18) and $A^{10,18}$ST1b(6–18) are active because they adopt the same disulfide arrangement as ST1b(6–18), then not only are Cys-7 and Cys-15 paired, but Cys-10 and Cys-18 must be disulfide linked in $A^{6,11}$ST1b(6–18) and Cys-6 must be disulfide linked to Cys-11 in $A^{10,18}$ST1b(6–18) to complete the pairing pattern (Fig. 5). Thus, according to this analysis the predicted disulfide bridge pattern for ST1b(6–18) is Cys-7 to Cys-15, Cys-6 to Cys-11, and Cys-10 to Cys-18. Besides the two analogues that are active, four inactive analogues also contain Cys-7 and Cys-15, implying that these two cysteines (as a single disulfide bridge) are not sufficient for activity.

Contribution of Each Disulfide Bridge to Activity Is Reflected by Potency of Active Analogues $A^{6,11}$ST1b(6–18) and $A^{10,18}$-ST1b(6–18). In comparing the performance of $A^{6,11}$ST1b(6–18) and $A^{10,18}$ST1b(6–18) to ST1b(6–18) in the radiobinding assay, the removal of cysteines 6 and 11 resulted in a 32-times greater decrease in activity than the removal of cysteines 10 and 18 (ratio of IC_{50} values, 4200:130). Assuming that the deduced pairing pattern is correct, the disulfide bridge composed of Cys-6 and Cys-11 can be considered more important with respect to maximal binding activity than the Cys-10/Cys-18 linkage. The remaining disulfide linkage involving Cys-7 and Cys-15 is the most crucial since all analogues lacking either one or both of these cysteines are inactive.

Reevaluation of Structure of Toxic Domain. Based on the differential rate of oxidation of the six cysteines of $Y^{11}N^{18}$-ST1a(1–18), Houghten *et al.* (20)¶ have suggested the following pairing pattern for the three disulfide bridges of ST1a: Cys-5 to Cys-14, Cys-6 to Cys-10, and Cys-9 to Cys-17. This tentative assignment corresponds to the following linkages for ST1b; Cys-6 to Cys-15, Cys-7 to Cys-11, and Cys-10 to Cys-18 (Fig. 1, pattern 11). In a recent structural study of

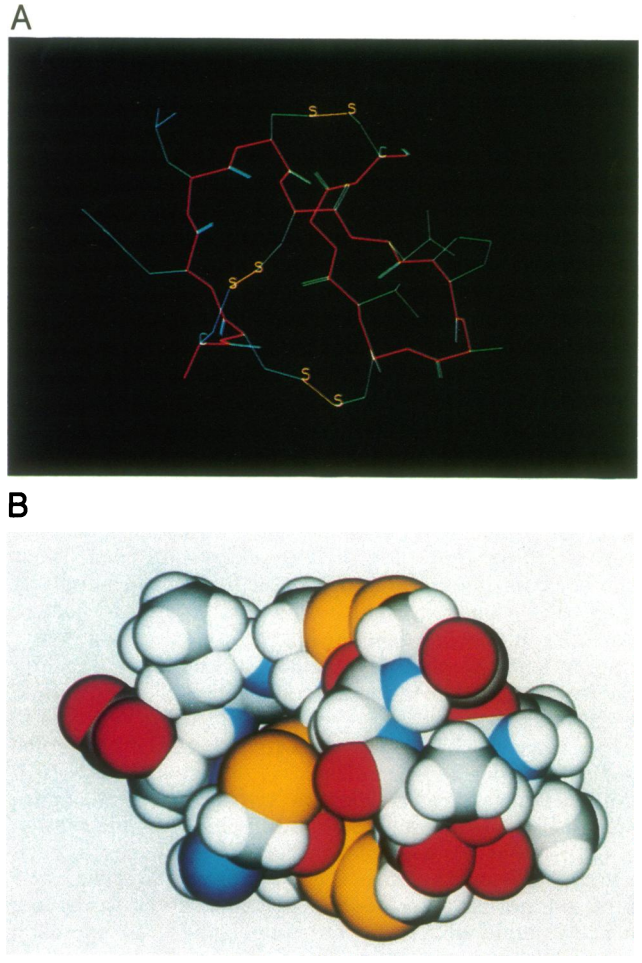

FIG. 6. Possible folding pattern for the peptide ST1b(6–18). A set of atomic coordinates was derived as described in the text and the resulting structure was displayed on an Evans and Sutherland graphics system using the program MOGLI. (*A*) Wire representation of ST1b(6–18). The hydrogen atoms have been omitted for clarity. Red, peptide backbone; blue and green, side chains and carbonyl bonds; yellow, disulfide bonds. (*B*) Space filling representation of *A*.

¶The original sequence of ST1a has recently been revised and corrected as outlined in ref. 7.

the toxic domain of ST1b using NMR spectroscopy, we included the disulfide linkage pattern proposed by Houghten *et al.* (20)¶ as a major geometric constraint to analyze the folding patterns adopted by the peptide chain in solution. This constraint was particularly important in light of the fact that the N-terminal region of ST1b(6–18) is underdetermined in terms of experimentally derived NMR constraints. Our pattern of disulfide linkages differs from the proposal of Houghten *et al.* (20)¶ in that we found Cys-6 paired with Cys-11 and Cys-7 paired with Cys-15 (Fig. 1, pattern 8). This newly deduced pattern was used in conjunction with the constraint data base derived earlier (21) and the interdependent constraint-satisfaction algorithm of Frayman (program PROTO; see refs. 21 and 31) to derive possible structures for the peptide ST1b(6–18). One possible folding pattern for the peptide consistent with the ensemble of constraints is presented in Fig. 6. This representation entails only one major constraint violation: a calculated distance of 4.68 Å separating the two α-carbon protons of Asn-12 and Gly-17. We experimentally observed a strong nuclear Overhauser enhancement signal for these protons in the spectrum of ST1b(6–18) and would have expected this distance to be <4 Å. Fig. 6 outlines features common to all the calculated structures: a folded peptide backbone consisting of three turns and stabilized by the three disulfide bridges with the position of the backbone region between Asn-12 and Cys-18 being more defined than the N-terminal region. Although the positions of all side-chain atoms remain unknown, the examination of structures that satisfy the NMR constraints in conjunction with the information obtained from this study provide a basis for the design of other analogues.

In conclusion, this study has examined the importance of disulfide bridges in the biological activity of ST1b through the synthesis of 15 analogues of the active tridecapeptide ST1b-(6–18) that lack all possible pairs of cysteines. Two analogues, $A^{6,11}$ST1b(6–18) and $A^{10,18}$ST1b(6–18) inhibited the binding of a radiolabeled analogue of ST1b to rat intestinal cells and caused diarrhea in suckling mice. Analysis of the primary sequence of the two active analogues indicates that four cysteines, including Cys-7 and Cys-15, are necessary for activity and led to a disulfide pairing pattern linking Cys-7 to Cys-15, Cys-6 to Cys-11, and Cys-10 to Cys-18. The contribution of each disulfide bridge to potency is not equivalent: Cys-7 and Cys-15 is the most crucial pair for activity, followed by Cys-6 and Cys-11. The least important linkage, Cys-10 and Cys-18, still contributes significantly to maximal potency.

Note Added in Proof. After completion of this work, Shimonishi *et al.* (32), using the acetylamidomethyl protecting group for the stepwise and selective formation of disulfide bonds, proposed the same disulfide pairing pattern as reported here.

We are grateful to Dr. Felix Frayman for deriving new sets of possible atomic coordinates for ST1b(6–18) using the Program PROTO. In addition, we thank Drs. Oleg Jardetzky and Andrew Lane of the Stanford University Magnetic Resonance Laboratory for useful and stimulating discussions. J.G. is a Scholar of the Medical Research Council of Canada.

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