Structural Characterization of Functionally Important Regions of the *Escherichia coli* Heat-Stable Enterotoxin STIb†

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ABSTRACT: The biological properties of the *Escherichia coli* enterotoxin STIb (ST_{A-3}, ST_h) reside in a 13 amino acid C-terminal domain, abbreviated STIb(6–18). This tridecapeptide contains six cysteine residues involved in three intramolecular disulfide bridges. The solution structure of STIb(6–18) has been modeled as a series of three consecutive reverse turns [Gariépy et al. (1987) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8907–8911]. Synthetic tridecapeptide analogues of STIb(6–18) with single amino acid substitutions at non-cysteine sites, as well as a truncated decapeptide lacking one of the three disulfide bridges, were prepared in order to examine the relationship between primary sequence and biological activity. The relative affinity of each analogue for intestinal cell receptors only partially correlates with their dose-dependent ability to cause diarrhea in suckling mice, suggesting that subsaturation doses of the enterotoxin with respect to receptor occupancy on intestinal cells may be sufficient to cause diarrhea. Two substitutions in the central-turn region of the molecule, namely, $Asn_{12} \rightarrow$ Ala and $Ala_{14} \rightarrow$ D-Ala, resulted in a large decrease or loss of receptor binding activity as compared to native STIb(6–18), pointing out the functional importance of this region. Analogues containing replacements at other sites showed moderate to slight reductions in biological activity. In particular, residues in the C-terminal region appear to be less important for activity, although their presence remains essential, since a truncated analogue missing the last three amino acids is inactive. Finally, a retrospective analysis of the enterotoxicity data associated with analogues containing D- or L-amino acid replacements supports the view that backbone as well as side chain perturbations in the central-turn region will strongly influence the biological activity of STIb.

STIb,[1] also known as ST_{A-3} and ST_h, is a methanol-soluble 19 amino acid peptide produced by enterotoxigenic strains of *Escherichia coli* (ETEC) and belongs to a class of heat-stable enterotoxins (ST) that collectively are a major cause of diarrheal disease in infants in developing nations, travellers, and domestic livestock (Levine et al., 1977; Sack, 1980). The receptor binding and enterotoxigenic properties of STIb reside in its 13 amino acid C-terminal domain, abbreviated STIb(6–18) (Yoshimura et al., 1985; Gariépy et al., 1987). This sequence contains six cysteine residues which participate in three intramolecular disulfide bridges (Figure 1) (Gariépy et al., 1987; Shimonishi et al., 1987). The disulfide linkages impart a constrained geometry to the molecule and are required for full biological activity of STIb(6–18) (Staples et al., 1980; Greenberg et al., 1983; Gariépy et al., 1987). The enterotoxic domain is homologous to other heat-stable enterotoxins elaborated by *Yersinia enterocolitica* (Takao et al., 1984), *Vibrio cholerae* (Takao et al., 1985), and *Citrobacter freundii* (Guarino et al., 1989). STIb exerts its toxic effects at the level of the mammalian small intestine, where it binds to receptors present on the brush border surface of villus cells (Giannella et al., 1983) and activates a membrane-bound guanylate cyclase. The subsequent elevation of intracellular cGMP results in a net efflux of water and electrolytes from the villus cell into the intestinal human (Hughes et al., 1978; Field et al., 1978). The structure and mechanism of action of the ST family of enterotoxins remain poorly understood and may provide new insights into the control and treatment of ETEC infections.

The importance of STIb as a virulence factor, its small size, and its restricted conformation in solution are important factors favoring the study of its structure and function. The receptor binding and enterotoxic properties of STIb(6–18) and related analogues can be quantified by measuring their ability to inhibit the binding of a radiolabeled analogue of STIb to rat intestinal cells and to cause diarrhea in suckling mice (Gyles, 1979; Gariépy et al., 1987). In this paper, we examine the contributions of the individual amino acids in the sequence to the biological activity of STIb(6–18) through the preparation of synthetic analogues containing single amino acid replacements at non-cysteine positions, as well as a truncated analogue lacking one of the three disulfide bridges.

Experimental Procedures

Preparation of Analogues. Peptides were synthesized by using classical solid-phase methods (Stewart & Young, 1984) with *tert*-butoxycarbonyl-protected amino acids (IAF Biochemicals, Montréal, Canada; Advanced Chemtech, Louisville, KY) coupled to a tBoc-Cys(MBzl)-chloromethyl polystyrene resin (Peninsula Laboratories, Belmont, CA). The completed peptides were cleaved from the resin with anhydrous HF in

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[1] Abbreviations: ED_{50}, enterotoxic dose able to cause a half-maximal increase in fluid accumulation in infant mice; ETEC, enterotoxigenic *E. coli*; G:C, gut-to-carcass ratio; ^{125}I-Y^{4}STIb(4–18), radiolabeled analogue of STIb(4–18) with a tyrosine substitution at position 4; NOE, nuclear Overhauser enhancement; RA, relative binding affinity; RE, relative enterotoxicity; STIa, 18 amino acid long *E. coli* heat-stable enterotoxin having the sequence Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr; STIb, 19 amino acid long *E. coli* heat-stable enterotoxin having the sequence Asn-Ser-Ser-Asn-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr; STIb(6–18), enterotoxic domain of STIb comprising residues 6–18 of the native sequence.

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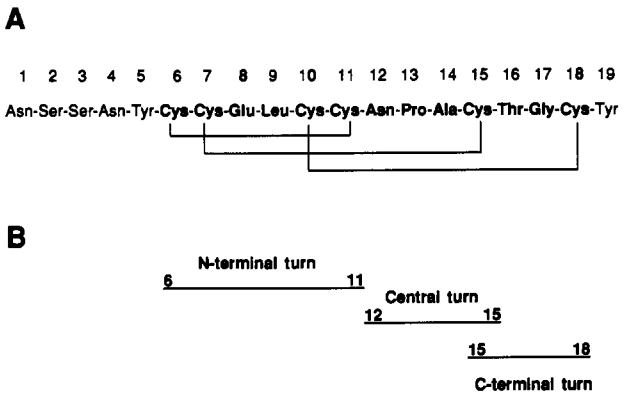

FIGURE 1: (A) Amino acid sequence of the *E. coli* heat-stable enterotoxin STIb (ST_{A-3}). The toxic domain of STIb, comprising residues 6–18 and abbreviated STIb(6–18), is shown in boldface. The six cysteine residues are involved in three disulfide bridges as indicated. Amino acids are identified by their three-letter codes. (B) Turn regions of STIb(6–18) as derived according to Gariépy et al. (1987). The boundaries of each region are indicated by residue numbers.

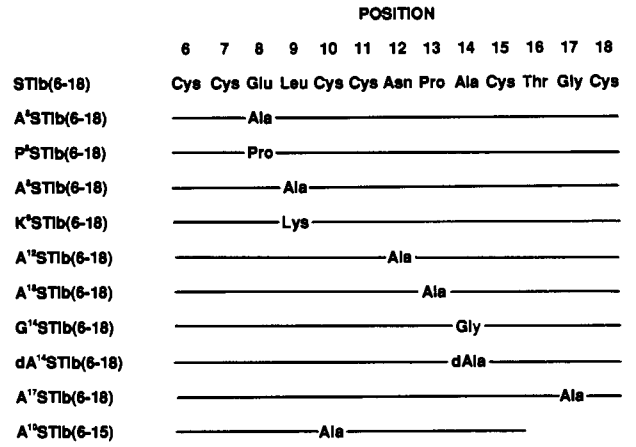

FIGURE 2: Amino acid sequences of all synthetic analogues of STIb(6–18) prepared for this study. The entire sequence of STIb-(6–18) is shown at the top. Solid lines represent regions of each analogue identical in sequence with STIb(6–18). Amino acids are identified by their three-letter codes.

the presence of dimethyl sulfide and anisole, extracted with 50% (v/v) acetic acid in water, and lyophilized. The crude reduced peptides were redissolved in water to a final concentration of 50 μM, adjusted to pH 8.5 with 10% (v/v) aqueous ammonium hydroxide, and air-oxidized for 5 days to allow complete disulfide formation to occur. The oxidized preparations were concentrated by using a preparative reverse-phase C_{18} column, lyophilized, and purified on a semipreparative reverse-phase high-performance liquid chromatography (HPLC) column (μBondapak C_{18} column; Waters, Millipore) as described previously (Gariépy et al., 1987). Active HPLC peaks were identified by measuring their ability to inhibit the binding of ^{125}I-Y^{4}STIb(4–18) to rat intestinal cells (see below). All purified analogues were assayed for composition and homogeneity by amino acid analysis and thin-layer chromatography (TLC) on silica plates. The solvent system used for TLC was 1-butanol/acetic acid/pyridine/water (4:1:1:2).

Preparation of ^{125}I-Y^{4}STIb(4–18), a Radiolabeled Analogue of STIb. One microgram of Y^{4}STIb(4–18), a synthetic analogue consisting of the toxic domain of STIb (residues 6–18) and two additional N-terminal tyrosine residues (Gariépy et al., 1987), was iodinated with 1 mCi of Na^{125}I (Amersham, Oakville, Ontario) using a solid-phase lactoperoxidase method (Enzymobeads; Bio-Rad, Richmond, CA). Following the solid-phase extraction of free iodine (Sep-Pak C_{18} cartridge; Waters, Millipore) and elution from a DEAE ion-exchange column, the radiolabeled peptide was further purified by reverse-phase HPLC on an analytical C_{18} column (μBondapak; Waters, Millipore). The column was equilibrated in 0.1% (v/v) trifluoroacetic acid in water and developed by using a linear gradient of 0.08% (v/v) trifluoroacetic acid in acetonitrile going from 15% to 40% over a period of 40 min. Two radioactive peaks eluting at 27 and 30 min after the start of the gradient were collected. Both peaks corresponded to radioactive species that bound to rat villus cells (see below). Only the ^{125}I-Y^{4}STIb(4–18) which corresponded to the peak eluting at 27 min was used in the radiobinding inhibition assay. The specific activity of the ^{125}I-Y^{4}STIb(4–18) preparation was approximately 400 Ci/mmol.

Inhibition Assay of ^{125}I-Y^{4}STIb(4–18) Binding to Rat Cells by Synthetic Analogues. Villus cells were obtained from small intestines of adult female Sprague-Dawley rats as described elsewhere (Gariépy & Schoolnik, 1986). Aliquots containing 10^5 cpm of ^{125}I-Y^{4}STIb(4–18) were combined with 20 μL of phosphate-buffered saline (PBS: 10 mM NaH_2PO_4/0.15 M NaCl, pH 7.4) containing 5 mM EDTA and 0.02% (w/v) sodium azide, 20 μL of the peptide dilution in PBS, and 5×10^5 rat villus cells in 12 × 75 mm polypropylene test tubes. The samples were incubated for 30 min at 37 °C, treated with 50 μL of 1% (w/v) bovine serum albumin in PBS, and placed on ice for 10 min. The samples were filtered onto Whatman GF/A filters, washed 4 times with PBS, and counted in a γ counter. All experiments were performed in triplicate at each peptide analogue dilution. Inhibition curves were constructed for each analogue and inhibition constants (Table I) calculated from a Scatchard analysis (Segel, 1976) of the displacement data (Figure 3).

Suckling Mouse Assay. Oral doses (100 μL) of each analogue dilution in PBS containing 0.04% (w/v) Evans blue dye were administered to newborn (2–4-day old) Swiss-Webster suckling mice (Gyles, 1979). After 3 h, the mice were sacrificed and their intestines excised. The guts and remaining carcasses were weighed, and the resulting gut-to-carcass ratio (G:C) was calculated. The G:C determined for each dilution represented the average value recorded from a set of three mice treated with each dilution.

Results

STIb(6–18) Analogues. Peptide analogues of STIb(6–18) were synthesized with alanine substitutions at non-cysteine sites (Figure 2) except at position 14 (alanine), which was replaced by D-alanine and glycine, and at position 16 (threonine). Position 16 is normally occupied by alanine in STIa (STa), an *E. coli* heat-stable enterotoxin with identical diarrheal properties with STIb (Yoshimura et al., 1985). Other analogues were also prepared with substitutions at positions 8 (proline) and 9 (lysine) as well as a truncated form of the enterotoxic domain, abbreviated A^{10}STIb(6–15), missing the three C-terminal residues and one disulfide bridge of STIb-(6–18).

Receptor Binding Activity of STIb(6–18) Analogues. STIb binds to receptors on the apical surface of rat small intestinal villus cells (Giannella et al., 1983; Frantz et al., 1984; Gariépy & Schoolnik, 1986; Kuno et al., 1986). The receptor binding capacities of STIb(6–18) analogues can be quantified with respect to the native sequence according to their ability to inhibit the binding to villus cells of ^{125}I-Y^{4}STIb(4–18), a radiolabeled analogue of STIb (Gariépy et al., 1987). Inhibition curves for eight analogues of STIb(6–18) are shown in Figure 3. Inhibition constants (K_i) calculated for each analogue are listed in Table I. The least active analogue tested in these

Table I: Summary of Biological Properties of STIb(6–18) Analogues[a]

analogue	K_i (M)	relative binding affinity (RA)	ED_{50} (mol)	relative enterotoxicity (RE)	RA:RE
STIb(6–18)	$(4.1 \pm 1.5) \times 10^{-9}$	1.0	5×10^{-12}	1.0	1.0
A^8STIb(6–18)	$(1.1 \pm 1.0) \times 10^{-7}$	0.04	10^{-11}	0.5	0.08
P^8STIb(6–18)	$(2.2 \pm 1.0) \times 10^{-8}$	0.2	10^{-11}	0.5	0.4
A^9STIb(6–18)	$(7.8 \pm 2.0) \times 10^{-8}$	0.05	2×10^{-11}	0.25	0.2
K^9STIb(6–18)	$(5.0 \pm 2.0) \times 10^{-8}$	0.08	3×10^{-11}	0.2	0.4
A^{12}STIb(6–18)		0	ND	ND	
A^{13}STIb(6–18)	$(2.1 \pm 1.0) \times 10^{-8}$	0.2	2×10^{-11}	0.25	0.8
G^{14}STIb(6–18)	$(1.0 \pm 0.5) \times 10^{-7}$	0.04	5×10^{-11}	0.1	0.4
dA^{14}STIb(6–18)	$(3.3 \pm 1.0) \times 10^{-7}$	0.01	3×10^{-10}	0.02	0.5
A^{17}STIb(6–18)	$(2.1 \pm 2.0) \times 10^{-8}$	0.2	5×10^{-12}	1.0	0.2
A^{10}STIb(6–15)		0	ND	ND	

[a] Inhibition constants (K_i) were calculated from a Scatchard analysis of data presented in Figure 3. Binding affinity and enterotoxicity are expressed relative to STIb(6–18). ED_{50}, analogue dose in infant mice resulting in a half-maximal increase in the gut-to-carcass ratio. RA:RE, ratio of the relative binding affinity (from K_i) to the relative toxicity in mice. The analogues A^{12}STIb(6–18) and A^{10}STIb(6–15) showed no inhibition of binding of ^{125}I-Y^4STIb(4–18) to intestinal cells. ND, not determined.

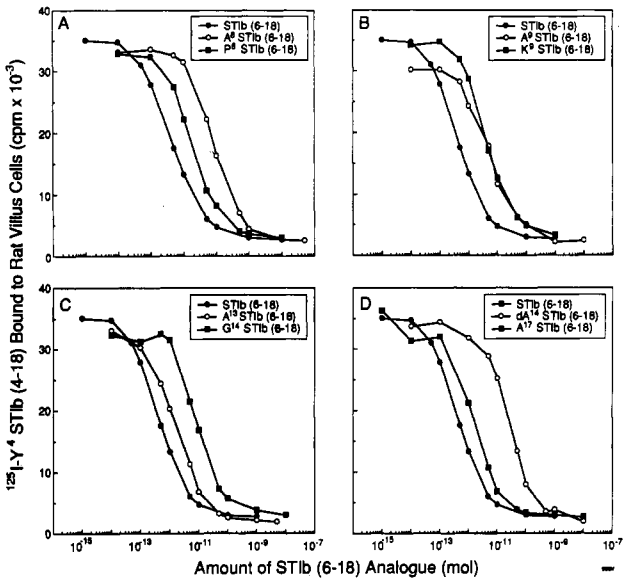

FIGURE 3: Radiobinding inhibition curves for STIb(6–18) and analogues containing single amino acid substitutions as indicated. Each analogue was assayed for its ability to inhibit the binding to rat small intestinal cells of ^{125}I-Y^4STIb(4–18), as described under Experimental Procedures. The curve for STIb(6–18) is reproduced in all four panels for comparison. Each data point represents the average of experiments performed in triplicate at each analogue dilution. The standard deviation associated with all points was typically less than ±6% of the total average counts per minute.

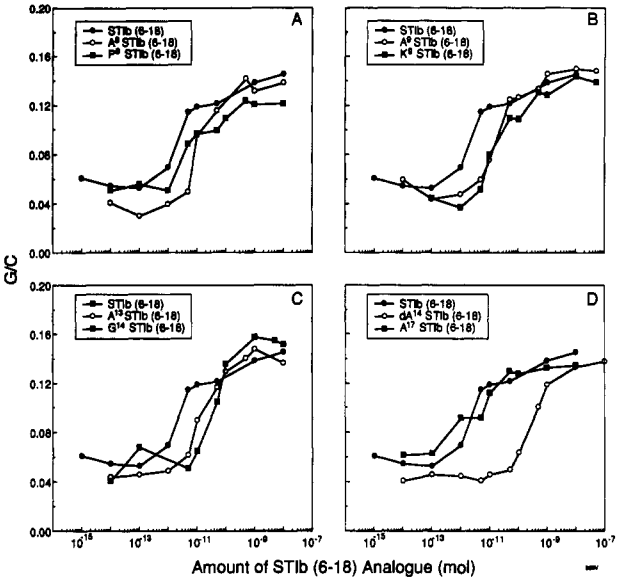

FIGURE 4: Dose–response curves for the administration of STIb(6–18) analogue dilutions to newborn suckling mice. A quantitative assessment of the diarrheal response in mice is reported in terms of the gut-to-carcass ratio (G:C), which was calculated as described under Experimental Procedures. The curve for STIb(6–18) is reproduced in all four panels for comparison. Each data point represents the average G:C for three animals at each analogue dilution.

experiments was dA^{14}STIb(6–18), which was approximately 100-fold weaker as an inhibitor than STIb(6–18) (Figure 3D, Table I). The remaining analogues were between 5- and 25-fold less active than STIb(6–18) (Figure 3, Table I). An inhibition constant of $(4.1 \pm 1.5) \times 10^{-9}$ M was calculated for STIb(6–18) (Table I), in agreement with the value of $(5.2 \times 1.5) \times 10^{-9}$ M derived by Waldman and O'Hanley for native STIa (Waldman & O'Hanley, 1989).

Two synthetic analogues, namely, A^{12}STIb(6–18) and A^{10}STIb(6–15), showed no receptor binding activity. Oxidized preparations of these analogues could not inhibit the binding of a radiolabeled analogue of STIb to intestinal cells even at peptide concentrations in excess of 50 μg. No active HPLC peaks could be identified in either chromatogram.

Enterotoxic Activity of STIb(6–18) Analogues in Infant Mice. All analogues that showed receptor binding activity (Figure 3) were assayed for their ability to cause intestinal fluid accumulation in suckling mice (Gyles, 1979). Dose–response curves for the eight active analogues are shown in Figure 4. The amount of each analogue required to achieve a half-maximal diarrheal response is listed in Table I and typically corresponded to a G:C value of about 0.090. Also summarized in Table I is the ratio of the relative K_i value (RA) to the relative half-maximal dose in mice (RE) for each analogue with respect to STIb(6–18) (RA:RE ratio). If the binding of STIb to its receptor triggered a proportional cellular response leading to diarrhea, one would expect the binding affinity of analogues for the STI receptor to parallel the observed diarrheal response, giving rise to an RA:RE value close to unity. In the case of most analogues in this study, the RA:RE parameter ranged in value between 0.2 and 0.8, indicating that, in general, reductions in the binding capacity of the analogues for the STIb receptor relative to that of STIb(6–18) were correlated with their observed enterotoxicity. The largest deviation between receptor binding and enterotoxicity was in the case of A^8STIb(6–18), which had an RA:RE ratio of 0.08. In other words, this analogue was 12.5 times more active in the suckling mouse assay as would be expected from the binding assay data. The least active analogue in the suckling mouse assay was dA^{14}STIb(6–18), which was approximately 60-fold less active than STIb(6–18), a result which correlated with the 100-fold reduction in binding affinity. The remaining analogues were between 1- and 10-fold less active than STIb(6–18) in this assay (Table I).

Discussion

Design Strategy for STIb(6–18) Analogues. The aim of this study was to analyze analogues of STIb(6–18) with single amino acid replacements at non-cysteine positions, along with a truncated analogue lacking two cysteines, in order to further identify functional groups and structural features of this tridecapeptide relevant to its biological activity. Staples et al. (1980), Greenberg et al. (1983), and Gariépy et al. (1987) have shown that the receptor binding and enterotoxic properties of STIb(6–18) are critically dependent on the intact nature of the three disulfide bridges (Figure 1A). Furthermore, Gariépy et al. (1987) suggested that there is a hierarchy of importance of the disulfide linkages, the bond between Cys-7 and Cys-15 being the most important, followed by that between Cys-6 and Cys-11, with the disulfide bridge involving Cys-10 and Cys-18 being the least important in terms of its contribution to the biological activity of STIb(6–18) (Gariépy et al., 1987). A set of possible structures for STIb(6–18) were derived from a database of NMR constraints (NOEs, dihedral angles, and amide proton exchange rates; Gariépy et al., 1986) and the correct pairing pattern of cysteine residues in STIb(6–18) (Gariépy et al., 1987; Shimonishi et al., 1987) suggesting the existence of a dominant pattern of secondary structure within the molecule, namely, three consecutive reverse-turn regions spanning the entire sequence of the tridecapeptide (Figure 1B). We therefore postulated that both the binding and diarrheal properties of this peptide were dependent on the conformation and integrity of these three putative turns. Additional support for this postulate comes from the work of Kobota et al. (1989), who demonstrated recently that synthetic analogues of STIa with D-amino acid replacements resulted in most cases in greatly reduced enterotoxicity in mice. D-Amino acids introduce large changes in the allowed range of dihedral angles taken by residues along the peptide backbone, and will consequently induce different types of reverse turns in peptides (Rose et al., 1985). In order to fully characterize the importance of each position along the sequence of STIb(6–18), it is crucial to compare the effects of substitutions with L-amino acids having different side chains from those present in the native structure. In particular, a reduction of the side chain to a single methyl group as generated by an alanine substitution would permit the evaluation of individual side chain contribution to the stability of the receptor–toxin complex while producing a minimal perturbation in backbone flexibility or orientation. The amino acid replacements attempted could potentially affect the binding and toxicity of STIb(6–18) either through the loss of a side chain critical for receptor binding or by the disruption of its tertiary structure.

Correlation between Receptor Binding Affinity and Enterotoxicity. Several analogues of STIa or STIb have been generated by chemical synthesis and mutagenesis (Yoshimura et al., 1985; Gariépy et al., 1987; Shimonishi et al., 1987; Okamoto et al., 1988; Waldman & O'Hanley, 1989; Kubota et al., 1989). However, in most cases the evaluation of their structural and functional integrity has been limited to the suckling mouse assay. It is important to establish for each analogue the correlation between its capacity to inhibit the binding of radiolabeled Y^4STIb(4–18) to intestinal cell receptors and its potency in causing diarrhea in infant mice. This could provide clues as to the complexity of the cellular events giving rise to the secretory response following the binding of the enterotoxin to its membrane receptor. In addition, one would like to determine whether a given analogue can act as a therapeutic inhibitor able to counter the action of this class of toxins. The ratio of the relative binding affinity of a given analogue [with respect to STIb(6–18)] to its relative enterotoxicity to mice (RA:RE; Table I) provides a qualitative estimate of such a property. Analogues having an RA:RE value significantly greater than unity would represent potential antagonists, meaning that their tight binding to the receptor does not generate a proportional fluid secretion and, thus, that they could be used to inhibit the binding of the natural enterotoxin. Most analogues tested had relative binding-to-toxicity ratios between 0.2 and 0.8, indicating that the ability of STIb(6–18) analogues to cause diarrhea is closely but not linearly related to their affinity for the STIb receptor. The lowest ratio observed was 0.08, for A^8STIb(6–18), which was approximately 25-fold less active in the binding assay compared to STIb(6–18), but only 2-fold less enterotoxic to mice. Thus, it is possible for an analogue of STIb(6–18) with moderately weaker binding affinity to nonetheless be capable of inducing a potent diarrheal response in mice. Since the observed RA:RE ratios for all analogues prepared are lower than unity, it suggests that the subsequent intracellular signal(s) engendered in response to the receptor binding event may generate a nonlinear response (a threshold activation of the guanylate cyclase for example). Mechanistically, subsaturating doses of STIb analogues with respect to receptor occupancy on the surface of intestinal cells may still trigger an intracellular response leading to the macroscopic manifestation of diarrhea.

Side Chain versus Backbone Alterations. D-Amino acid replacements for both the cysteine and non-cysteine residues of STIa(5–17) resulted in several orders of magnitude decreases in enterotoxicity, particularly for substitutions at positions 6–8 (Cys-Glu-Leu), and at positions 10–14 (Cys-Asn-Pro-Ala-Cys) of STIa(5–17) (Kubota et al., 1989). The relative enterotoxicity of D-amino acid containing analogues ranged from 0.005% to 3% of that of STIa(5–17) for substitutions in these two regions (Kubota et al., 1989). In the present study, analogues with side chain replacements involving L-amino acids were found to display enterotoxicity values between 10 and 100% of the value observed for STIb(6–18) (Table I), suggesting that side chain replacements (L-amino acids) are less perturbing than backbone ones (D-amino acids) in terms of binding affinity and diarrhea. For example, A^9STIb(6–18), which contains a leucine to alanine substitution at position 9, was 20-fold less active than STIb(6–18) in the binding assay and 4-fold less potent in the suckling mouse assay (Table I). By contrast, an STIa analogue with a D-leucine replacement at this position was more than a thousandfold less enterotoxic to mice than the native sequence (Kubota et al., 1989). In the present study, only one analogue was prepared that contained a D-amino acid replacement. This peptide, abbreviated dA^{14}STIb(6–18), was the weakest of all the active analogues tested in both assays, showing a 100-fold reduction in binding affinity and a 60-fold reduction in enterotoxicity relative to STIb(6–18) (Figure 3D, Figure 4D, Table I). An analogue with a glycine replacement at this site, G^{14}STIb(6–18), had 25-fold and 10-fold reductions, respectively, in receptor binding and diarrheal activity (Table I), suggesting that the increase in backbone flexibility is also partly detrimental toward activity.

An exception to this pattern of results was A^{12}STIb(6–18), which contains an asparagine to alanine substitution at position 12. This analogue showed no receptor binding activity. Okamoto et al. (1988) have reported that L-lysine and L-arginine replacements at this position resulted in inactive analogues while a D-asparagine substitution yielded a weak but active analogue (Kubota et al., 1989). This site does tolerate sub-

stituents such as aspartic acid, histidine, glutamine, and tyrosine with the potency of these analogues ranging in value between 2 and 12% of that expected for the natural sequence (Okamoto et al., 1988). In summary, amino acid replacements that alter the orientation of the peptide backbone (D-amino acid substitutions, for example) have a much greater impact upon the biological activity of STIb(6–18) than do replacements which only alter the nature of the side chain (L-amino acid substitutions).

Effects of Amino Acid Replacements: N-Terminal-Turn Region. The peptide backbone of the six first residues of STIb(6–18) probably adopts a turn conformation with four of these residues being cysteines involved in three disulfide linkages and cysteines-6 and -11 pairing to form a cyclic hexapeptide subdomain (Figure 1). On the basis of proton magnetic resonance experiments (^{1}H NMR), this region undergoes a temperature-dependent conformational change (Gariépy et al., 1986). Thus, the N-terminal region may represent a flexible part of the molecule. To investigate the impact of restricting the peptide backbone in this region, a glutamic acid to proline substitution was introduced at position 8. Marginal decreases in potency and binding affinity were observed (Table I) which contrast dramatically with the large decrease in enterotoxicity observed for the D-glutamic acid analogue (Kubota et al., 1989). The analogues P^{8}STIb(6–18) and A^{8}STIb(6–18) also confirmed that the removal of the side chain negative charge at position 8 had a small effect on activity (Table I). Thus, the stability of the complex between STIb and its receptor is not strongly influenced by the absence of a negative charge or the truncation of the side chain. The nature of the backbone folding at this position is important, since large perturbations in the allowed values in dihedral angles result in significant losses in activity (Kubota et al., 1989).

Leucine at position 9 represents the other non-cysteine residue within this turn. Removal of the hydrophobic side chain as exemplified by an alanine substitution yielded a moderately less active analogue with 8% of the binding affinity and 20% of the enterotoxicity normally observed for the natural sequence (Table I). Substitution with other hydrophobic residues (isoleucine and valine) at position 9 in analogues with aspartic acid at position 8 resulted in analogues with comparable ability to cause diarrhea in mice (Kubota et al., 1989). Additionally, a lysine substitution was attempted at position 9 in order to locate a positive electrostatic charge adjacent to the negative charge of glutamic acid (position 8) and to change the polarity of the side chain at this site. In relation to STIb(6–18), the analogue K^{9}STIb(6–18) displayed a 12.5-fold decrease in binding affinity and a 5-fold reduction in enterotoxicity as monitored in the suckling mouse assay (Table I). Since the D-leucine-containing analogue is several orders of magnitude less enterotoxic than the native sequence (Kubota et al., 1989), one can conclude that as in the case of position 8, the modification or removal of the side chain is less critical in terms of activity than a perturbation affecting the geometry of the peptide backbone at that position.

Central-Turn Region. In our model, a second chain reversal in STIb(6–18) occurs between residues 12 and 15. The turn is stabilized at both ends by the essential disulfide bridge between cysteines-7 and -15 and by the disulfide linkage involving cysteine-11 (Gariépy et al., 1987) (Figure 1). The tetrapeptide Asn-Pro-Ala-Cys has a high probability of existing in a β-turn conformation based on the secondary structure algorithm developed by Chou and Fasman (1978). Amino acid substitutions at all three non-cysteine positions were attempted. Replacement of asparagine-12 with alanine resulted in an analogue, A^{12}STIb(6–18), which showed no activity in the radiobinding inhibition assay although the amino acid composition of the analogue was verified. Other groups have reported the lack of enterotoxic activity in mice of analogues containing lysine and arginine substitutions at this site (Okamoto et al., 1988). The tolerance for other side chain substituents may indicate the need for a hydrogen bond acceptor group (His, Gln), but the reduced activity observed for the glutamine analogue suggests that another factor (side chain length, for example) may favor asparagine at this site. Since the next residue in the sequence is normally a proline, the range of backbone dihedral angles for the asparagine is restricted (Cantor & Schimmel, 1980). This fact may explain why the D-asparagine-containing analogue was 4 orders of magnitude less active than the native sequence (Kubota et al., 1989).

Proline substitution with alanine at position 13 resulted in a partial loss of activity (ED_{50}, 2×10^{-11} mol) and binding affinity (K_i, 2.1×10^{-8} M). However, these values are far greater than the ones recently reported for a glycine ($K_i \sim 10^{-6}$ M; ED_{50}, 9.5×10^{-9} mol; Waldman & O'Hanely, 1989) or a D-proline substitution (Kubota et al., 1989). The restrictive nature of the proline side chain on the local conformation of the peptide backbone does not appear to be essential for activity [as demonstrated by A^{13}STIb(6–18)]. However, an increase in backbone flexibility (glycine) or a change in the allowed orientation of the side chain and peptide backbone (D-proline) has a dramatic effect on the activity and thus the proper folding of the toxic domain. In this study, alanine at position 14 was replaced by D-alanine and glycine. In both cases, a large drop in both receptor binding and enterotoxicity was observed (Table I), suggesting that the conformation of the peptide backbone is critical at that position as in the case of proline-13.

In summary, perturbations in the backbone conformation of the central-turn region are far more detrimental than side chain substitutions although decreases in activity have been observed for all substitutions made in this region. It is possible that the formation of secondary structure within this region represents an important event in the proper folding and/or disulfide pairing of STIb(6–18) since the orientation of two functionally important cysteines (cysteines-11 and -15) flanking both sides of this turn would influence the preferential pairing of all three disulfide bridges.

C-Terminal-Turn Region. The remaining two non-cysteine residues (positions 16 and 17) fall within a third turn region of STIb(6–18) predicted by our model (Gariépy et al., 1987). No alanine substitution was attempted at position 16, because this site is normally occupied by alanine in STIa, an *E. coli* heat-stable enterotoxin with an otherwise identical tridecapeptide sequence with STIb(6–18), and similar diarrheal properties (Yoshimura et al., 1985). Position 16 in STIa appears to be relatively insensitive to amino acid substitutions with an analogue containing a D-alanine replacement at this site showing only a 10-fold reduction in diarrheal activity in mice (Kubota et al., 1989). Naturally occurring mutations at that site include alanine, phenylalanine, and threonine, all of which are similarly active. The amino acid degeneracy at this position is thus large. Replacement of glycine-17 by alanine had no effect on the enterotoxigenic activity of the analogue A^{17}STIb(6–18) as compared with STIb(6–18), and only a small decrease (a 5-fold reduction) in receptor binding affinity was observed (Table I). This region of the molecule is stabilized by a disulfide bridge (Cys-10 to Cys-18) which has been found to be the least important disulfide of STIb-

Table II: Loss of Enterotoxicity Resulting from Amino Acid Substitutions along the Sequence of STIb(6–18)[a]

position	6	7	8	9	10	11	12	13	14	15	16	17	18
wild-type amino acid	Cys	Cys	Glu	Leu	Cys	Cys	Asn	Pro	Ala	Cys	Thr	Gly	Cys
backbone substitutions (D-amino acids)	0	**4.0**	**3.4**	**4.1**, 0.3	0.3	**3.7**	**4.3**	1.5	1.7	**2.9**	1.0	0 (Ala)	0.4
side chain substitutions (alanine)	**4.2**	i	0.3	0.6, 0.7 (Lys)	**2.3**	**4.2**	i, 0.9 (His)	0.6	1.0	i	0	0	**2.3**

[a] Wild-type amino acids are identified by their three-letter codes. Each number represents the negative logarithm of the relative loss of enterotoxicity observed from a substitution at that position. Numbers associated with backbone effects reflect equivalent D-amino acid substitutions except when indicated in parentheses. Numbers associated with side chain effects represent alanine substitutions except when indicated in parentheses. The letter i denotes an inactive analogue. Letters in parentheses represent the following substitutions: Ala, alanine; His, histidine; Lys, lysine; Pro, proline. Boldface denotes positions particularly sensitive to structural perturbations (>100-fold loss in enterotoxicity). Data compiled from Yoshimura et al. (1985), Gariépy et al. (1987), Okamoto et al. (1988), Kubota et al. (1989), and Table I.

(6–18) in terms of its requirement for the maintenance of biological activity (Gariépy et al., 1987). However, the presence of the three C-terminal residues is required in order for the enterotoxin to maintain its receptor binding activity, since the truncated decapeptide, A^{10}STIb(6–15), lacks activity in the binding assay.

STIb(6–18) Structure and Activity: A Current View. The impact of substitutions made to date to the sequence of STI enterotoxins (Yoshimura et al., 1985; Gariepy et al., 1987; Kubota et al., 1989; Okamoto et al., 1988) is presented in Table II as a table of relative enterotoxicity observed at each position along the primary sequence of STIb(6–18). Substitutions at each amino acid position are classified as predominantly resulting either in a peptide backbone or in a side chain perturbation. Numerical values listed represent the negative logarithm of the relative loss in enterotoxicity resulting from an amino acid replacement at a particular position. A value of 0 implies an identical potency with the natural toxin while numbers above 2 indicate a greater than 100-fold decrease in activity. Since small differences exist in the STI sequences examined or the approaches used by investigators to determine the enterotoxicity of their analogues, we report values representing the relative potency of these analogues in relation to the natural sequence within each experimental data set. This qualitative representation of the data (Table II) clearly points out that the backbone geometry of the first two turns of STIb(6–18) (Figure 1B) as well as the identity of side chains at positions 6, 7, 10, 11, 12, 15, and 18 represents the most sensitive areas of the sequence (boldface numbers in Table II). Of these seven critical side chains, six are cysteines with asparagine at position 12 being the lone noncysteine residue that strongly influences the activity of STIb(6–18).

In view of the complexity of the tridecapeptide, one would like to assess areas of the molecule that may allow alterations leading to a simpler structure. A visual analysis of Table II indicates that cysteines-10 and -18 normally paired as a disulfide bridge may tolerate backbone changes without a large loss in activity. Observed reductions in potency associated with the replacement of non-cysteine residues at positions 16 and 17 are also small. Thus, a truncation of the C-terminal region of the molecule in conjunction with the maintenance of a covalent bond between the side chain of residue 10 and the C-terminal end of the molecule may afford a shorter but active analogue. The design of shorter analogues along this route is supported by the fact that $A^{10,18}$STIb(6–18) lacking this disulfide bond has been shown to be active (Gariépy et al., 1987) while the truncated analogue A^{10}STIb(6–15) is not (Table I). Another interesting point arising from the data in Table II is the dominant impact of D-amino acids at positions 8 and 9, resulting in large decreases in activity as opposed to side chain truncations or replacements. These results suggest that the folding of this region is the relevant factor in generating an active STIb(6–18) sequence and that its effect may be distal to that site. Hypothetically, the N-terminal cyclic region comprising residues 6–11 (stabilized by a disulfide bridge between cysteines-6 and -11) may allow for the proper orientation of residues located in the central turn of the molecule (residues 12–15), this latter turn constituting the contact site with the receptor.

Loss of enterotoxicity can also be interpreted in terms of substitutions that affect important interactions between the enterotoxin and its receptor binding site, rather than the overall folded conformation of the peptide. In the present study, alanine replacements at non-cysteine positions generally result in a partial loss of potency (Table I). Okamoto et al. (1988) have argued that their analogues which contain substitutions at position 12 have the same folded conformation as STI since they were recognized by monoclonal and polyclonal antisera to the native toxin (Okamoto et al., 1988). However, the specificity and quantitative affinity of such probes for each analogue have not been analyzed. Moreover, it is well established that antibodies can react with epitopes within different structural contexts in polypeptides (Dyson et al., 1988). Finally, Svennerholm et al. (1988) reported that biologically inactive fragments of STIb as short as six amino acids were reactive with anti-STIb antibodies. These findings support our view that these immunological probes are probably unable to differentiate between active, folded, or denatured forms of small peptides such as the heat-stable enterotoxins.

In summary, the receptor binding affinity of STIb(6–18) analogues correlates with their ability to cause diarrhea. This relationship, however, may not be linear, suggesting the activation of cellular mechanisms at subsaturating doses of the cell surface receptors. It is likely that proper turn formation in the N-terminal and central regions of STIb(6–18) is necessary for generating a biologically active conformation. The C-terminal region of the molecule although important for activity will tolerate backbone perturbations and side chain modifications.

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Synthesis of 25-Hydroxyvitamin D_3 3β-3′-[*N*-(4-Azido-2-nitrophenyl)amino]propyl Ether, A Second-Generation Photoaffinity Analogue of 25-Hydroxyvitamin D_3: Photoaffinity Labeling of Rat Serum Vitamin D Binding Protein†

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ABSTRACT: Vulnerability of 25-hydroxy-[26,27-^{3}H]vitamin D_3 3β-*N*-(4-azido-2-nitrophenyl)glycinate, a photoaffinity analogue of 25-hydroxyvitamin D_3 (25-OH-D_3) (Ray et al., 1986) toward standard conditions of carboxymethylatioin promoted us to synthesize 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitrophenyl)amino]propyl ether (25-ANE), a hydrolytically stable photoaffinity analogue of 25-OH-D_3, and 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitro-[3,5-^{3}H]phenyl)amino]propyl ether (^{3}H-25-ANE), the radiolabeled counterpart of 25-ANE. Competitive binding assays of 25-OH-D_3 and 25-ANE with rat serum demonstrated that 25-ANE competes for the 25-OH-D_3 binding site in rat serum vitamin D binding protein (rDBP). On the other hand, UV exposure of a sample of purified rat DBP (rDBP), preincubated in the dark with ^{3}H-25-ANE, covalently labeled the protein. However, very little covalent labeling was observed in the absence of UV light or in the presence of a large excess of 25-OH-D_3. These results provide strong evidence for the covalent labeling of the 25-OH-D_3 binding site in rDBP by ^{3}H-25-ANE.

It is well established that vitamin D binding protein (DBP), a major serum constituent, transports vitamin D and its metabolites to target organs and tissues leading to the observed calciotropic properties of the vitamin D hormone (DeLuca, 1979; Cooke & Haddad, 1989). DBP binds to metabolites of vitamin D with high affinities, and one of the highest affinities is toward 25-hydroxyvitamin D_3 (25-OH-D_3) (Francheschi et al., 1981; Daiger et al., 1975; Haddad & Walgate, 1976).

Recently other functions of DBP have been recognized. DBP binds and sequesters monomers of actin with high affinity (Van Baelen et al., 1980; Haddad, 1982). DBP has also been found to be associated with unsaturated fatty acids (Williams et al., 1988; D. Z. Xiang and R. Bouillon, unpublished results) as well as with various cell types including B- and T-lymphocytes and the cytotrophoblasts of placenta (Petrini et al., 1983, 1985). The physiologic significance of the observed multifunctional properties of DBP remains unknown. On the other hand, the primary amino acid structures of human and rat serum DBPs have been determined recently (Yang et al., 1985; Cooke & David, 1985; Schoentgen et al., 1986; Cooke, 1986).

Photoaffinity labeling has been a very important biochemical tool for probing ligand-binding sites of steroid hormone binding

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