Structure-Activity Relationship of *Escherichia coli* Heat-Stable Enterotoxin: Role of Ala Residue at Position 14 in Toxin-Receptor Interaction[1)]

Shinji YAMASAKI,† Takashi SATO, Yuji HIDAKA, Hiroshi OZAKI, Hideaki ITO,†
Toshiya HIRAYAMA,†† Yoshifumi TAKEDA,† Takashi SUGIMURA,†††
Akira TAI,††† and Yasutsugu SHIMONISHI*

Institute for Protein Research, Osaka University, Suita, Osaka 565
† Faculty of Medicine, Kyoto University, Yoshida-Konoe, Sakyo-ku, Kyoto 606
†† The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108
††† Basic Research Laboratory, Himeji Institute of Technology, 2167 Shosha, Himeji, Hyogo 671-22
(Received March 6, 1990)

A heat-stable enterotoxin (ST_h) produced by a human strain of enterotoxigenic *Escherichia coli* consists of 19 amino residues including 6 half-cystine residues. Analogs of ST_h from positions 6 to 19 with replacements of the Ala residue at position 14 by other amino acid residues were synthesized by a solid-phase method and examined by biological and biochemical methods. This Ala residue was demonstrated to be very important for expression of the toxicity of ST_h and for interaction of the toxin with its receptor protein(s).

Heat-stable enterotoxins produced by enterotoxigenic *Escherichia coli* are peptides with 18 or 19 amino acid residues (named ST_p and ST_h, respectively) that cause acute diarrhea in infants and domestic animals.[2-4)] Similar enterotoxins have been isolated from some pathogenic strains of *Yersinia enterocolitica* and *Vibrio cholerae* non-01.[5,6)] Previous studies have revealed that all the enterotoxigenic properties of these toxins are due to the highly homologous sequence of 13 amino acid residues boxed by dotted lines in Fig. 1,[7)] and that the three disulfide bridges are organized identically in all the toxins.[8)] These findings indicated that the structural element(s) required for generation of the toxicity of these toxins is located on the tertiary structure formed by the segment of this tridecapeptide. Other experiments demonstrated that the initial step of the biological reactions of the toxin is the binding of the toxin to its receptor protein(s) on intestinal epithelial cell membranes.[9)] The formation of a toxin-receptor complex leads to the stimulation of guanylate cyclase, which is bound to or present near the receptor protein(s), followed by elevation of the intracellular concentration of cGMP and change of electrolytes in the cells.[10-12)] However, little is known about the interaction of the toxin with its receptor protein(s) at the molecular level.

Recently, we analyzed the conformation of a tridecapeptide of ST_p (abbreviated as ST_p (5—17)),[13)] consisting of the sequence from Cys^5 to Cys^{17} in ST_p, and demonstrated that this peptide has a right-handed spiral conformation which is constituted from three loop parts from Cys^5 to Cys^{10}, Cys^{10} to Cys^{14}, and Cys^{14}

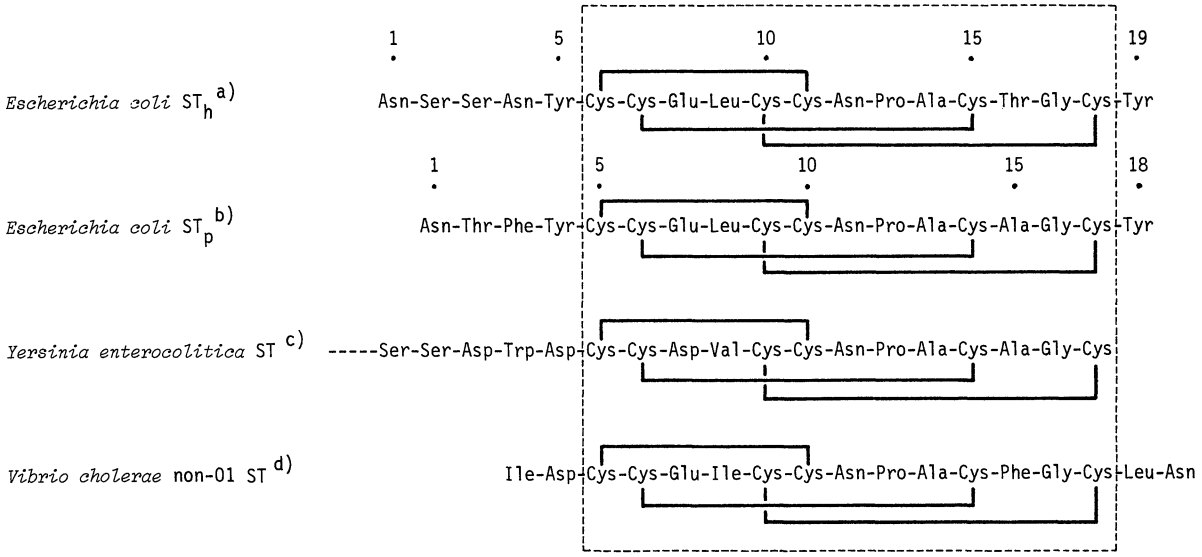

Fig. 1. Amino acid sequences and disulfide pairings of heat-stable enterotoxins produced by various pathogenic bacteria: a) see Refs. 3 and 8; b) see Refs. 4 and 8; c) see Refs. 5 and 8; d) see Refs. 6 and 8.

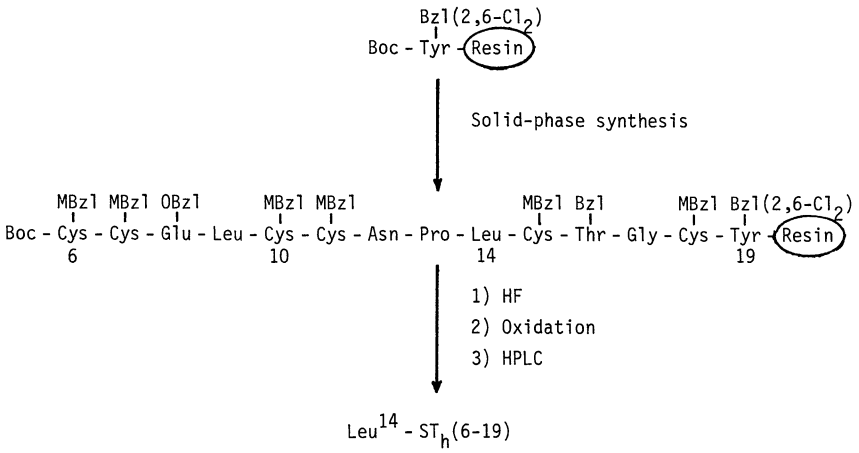

Fig. 2. Scheme for synthesis of Leu^{14}-ST$_h$(6—19).

to Cys^{17}. This finding has made it possible to investigate the relation between the conformation of the toxin and its toxicity and to determine the effect of amino acid replacements on the receptor binding ability of the toxin, in other words, to reside the amino acid residue(s) concerned in interaction of the toxin with its receptor protein(s).

In this paper we describe the synthesis of various analogs of ST$_h$, which consisted of the sequence from Cys^6 to Tyr^{19} in ST$_h$ but with replacements of Ala at position 14 by other amino acid residues, and the effects of replacement of this Ala residue on the biological properties of ST$_h$. This replacement was chosen to determine the effect of a replacement in the second loop from the N-terminus of ST$_h$ on its conformational and biological properties. Other reasons of this choice were based on the following previous findings: 1) The sequence from Cys^{11} to Cys^{15} is conserved in all the enterotoxins.[6] 2) The sequence from Cys^6 to Cys^{15} is the shortest peptide of ST$_h$ showing enterotoxigenic activity.[14] 3) The Pro residue seems important for formation of loop structure from Cys^{10} to Cys^{14}, because this residue is often located in β-turn structures in proteins.[15] Using ^{1}H NMR spectroscopy, we also examined the conformation of the Leu^{13}-analog of ST$_p$(5—17) (abbreviated as Leu^{13}-ST$_p$(5—17)), in which Ala at position 13 of ST$_p$ was replaced by Leu.

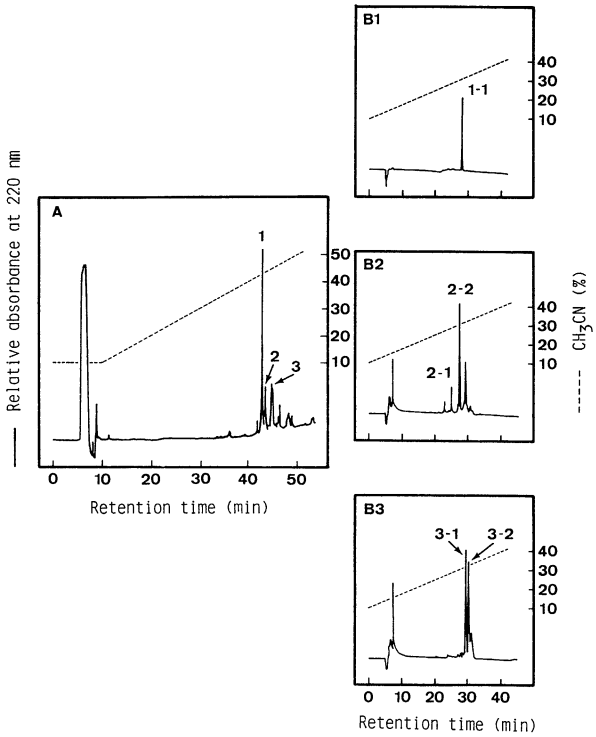

Fig. 3. HPLC profiles of Leu^{14}-ST$_h$(6—19): A) Profile of the crude peptide on elution with an increasing concentration of CH_3CN in 0.05% TFA, B1—B3) Profiles on rechromatography of peaks 1—3 in A), respectively, on elution with an increasing concentration of CH_3CN in 0.01 M ammonium acetate (pH 5.7).

Results and Discussion

Synthesis of Analogs of ST$_h$(6—19). The analogs of ST$_h$(6—19) were synthesized by a stepwise strategy of the solid-phase method.[8,16] As an example the scheme for synthesis of the Leu^{14}-analog of ST$_h$(6—19)(Leu^{14}-ST$_h$(6—19)) is shown in Fig. 2. The C-terminal Tyr at position 19 was used as a starting amino acid residue, although this Tyr was not essential for expression of the toxicity of ST$_h$.[7] The protected peptide was treated with anhydrous liquid hydrogen fluoride to liberate it from the resin and remove all protecting groups, and the resulting free linear peptide was spontaneously oxidized by air to form disulfide linkages. The crude peptide was purified by high-performance liquid chromatography (HPLC) and one major and several minor peak fractions were obtained, as shown in Fig. 3A. All the peak fractions obtained were isolated and examined by amino acid and FAB mass spectrometric

Table 1. Amino Acid Compositions,[a] Mass Values[b] and Toxic Activities[c] of Leu^{14}-$ST_h(6—19)$

	1-1	2-1	2-2	3-1	3-2	Theoretical value
Asp	1.06	1.14	1.07	1.06	1.06	1
Thr	1.01	1.02	1.04	1.03	1.00	1
Glu	1.06	1.19	1.01	0.97	1.04	1
Pro	0.98	0.97	1.08	1.02	1.03	1
Gly	1.03	1.40	1.01	1.02	1.04	1
Cys	5.32	4.52	4.72	4.39	4.47	6
Leu	2	2	2	2	2	2
Tyr	0.96	0.84	0.86	0.76	0.97	1
$[M+H]^+$	1518.9	1518.2	1518.2	1518.2	1518.1	1518.5
MED (ng)	>10000	>4000	>10000	>20000	>20000	
(pmol)	>6600	>2600	>6600	>13000	>13000	

a) Values were calculated as mol/mol of Leu. b) $[M+H]^+$, mass value of quasi-molecular ion. c) MED: Minimum effective dose.

Table 2. Amino Acid Compositions[a] and Mass Values[b] of Various Analogs of $ST_h(6—19)$

	Gly^{14}	Ser^{14}	Asp^{14}	Glu^{14}	Gln^{14}	Val^{14}	Ile^{14}	Leu^{14}	Phe^{14}	Lys^{14}	Arg^{14}
Asp	1.02(1)	1.06(1)	2.32(2)	1.06(1)	1.12(1)	1.06(1)	1.32(1)	1.06(1)	1.05(1)	1.05(1)	1.03(1)
Thr	0.97(1)	1.02(1)	0.98(1)	0.97(1)	1.02(1)	1.01(1)	0.99(1)	1.01(1)	1.00(1)	1.00(1)	0.98(1)
Ser	—	0.97(1)	—	—	—	—	—	—	—	—	—
Glu	0.98(1)	1.05(1)	1.04(1)	1.87(2)	2.05(2)	1.05(1)	1.04(1)	1.06(1)	1.03(1)	1.05(1)	1.02(1)
Pro	0.92(1)	1.05(1)	1.02(1)	0.94(1)	0.84(1)	0.87(1)	1.02(1)	0.98(1)	1.12(1)	0.97(1)	0.89(1)
Gly	1.99(2)	1.17(1)	1.02(1)	1.12(1)	1.02(1)	1.04(1)	1.04(1)	1.03(1)	1.05(1)	1.01(1)	1.02(1)
Ala	—	—	—	—	—	—	—	—	—	—	—
Cys	4.81(6)	5.94(6)	3.68(6)	4.41(6)	5.81(6)	5.36(6)	4.21(6)	5.32(6)	5.62(6)	5.05(6)	5.51(6)
Val	—	—	—	—	—	0.98(1)	—	—	—	—	—
Leu	1(1)	1(1)	1(1)	1(1)	1(1)	1(1)	1(1)	2(2)	1(1)	1(1)	1(1)
Ile	—	—	—	—	—	—	0.91(1)	—	—	—	—
Tyr	0.93(1)	1.00(1)	0.95(1)	0.90(1)	0.86(1)	0.95(1)	1.02(1)	0.96(1)	0.93(1)	0.95(1)	0.90(1)
Phe	—	—	—	—	—	—	—	—	1.06(1)	—	—
Lys	—	—	—	—	—	—	—	—	—	1.00(1)	—
Arg	—	—	—	—	—	—	—	—	—	—	0.96(1)
$[M+H]^+$	1462.5	1492.0	1520.5	1534.4	1533.2	1504.4	1518.2	1518.9	1552.4	1533.7	1561.5
	(1462.4)	(1492.4)	(1520.4)	(1534.4)	(1533.4)	(1504.4)	(1518.5)	(1518.5)	(1552.4)	(1533.5)	(1561.5)

a) Values were calculated as mol/mol of Leu. Numbers in parentheses indicates theoretical values. b) $[M+H]^+$, mass value of quasi-molecular ion. Numbers in parentheses indicate theoretical values.

analyses. The peak fractions (peaks 1, 2, and 3 in Fig. 3A), which had amino acid compositions and mass values compatible with the theoretical values, were collected. These peak fractions were further chromatographed under different conditions from those in Fig. 3A, as shown in Fig. 3B1—3B3. The main fraction (peak 1) gave only one peak (1-1 in Fig. 3B1), while the minor peak fractions 2 and 3 were each separated into several fractions. Of these peak fractions peaks 1-1, 2-1, 2-2, 3-1, and 3-2 were confirmed to be single by HPLC under the same conditions as those for Fig. 3A, and found to show identical analytical data to the theoretical values (Table 1).

Other peptides were synthesized and purified in similar ways to those shown in Figs. 2 and 3. Almost all the analogs of $ST_h(6—19)$ gave one major peak and several minor peaks on HPLC with similar amino acid compositions and mass values to the theoretical values, like Leu^{14}-$ST_h(6—19)$ shown in Fig. 3A. Analytical data on the major peak fractions of these analogs are summarized in Table 2.

Biological and Biochemical Properties of Synthetic Peptides. In the suckling mice assay,[17] peak fractions 1-1, 2-1, 2-2, 3-1, and 3-2 in Fig. 3B, did not show toxicity at doses of less than 4,000 ng (Table 1), while $ST_h(6—19)$ showed toxicity at a dose of 0.6 ng.[7] These results indicated that Leu^{14}-$ST_h(6\text{-}19)$ is inactive. The reactivities of these fractions with a monoclonal antibody against ST_h were examined by a competitive enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 4, the major fraction (peak 1-1 in Fig. 3B) reacted with the monoclonal antibody similarly to $ST_h(6—19)$, whereas the minor fractions 2-2 and 3-1 reacted only weakly with the antibody and 2-1 and 3-2 showed no reactivity. Thus the major fraction (peak 1-1) had the same epitope as that in $ST_h(6—19)$, in

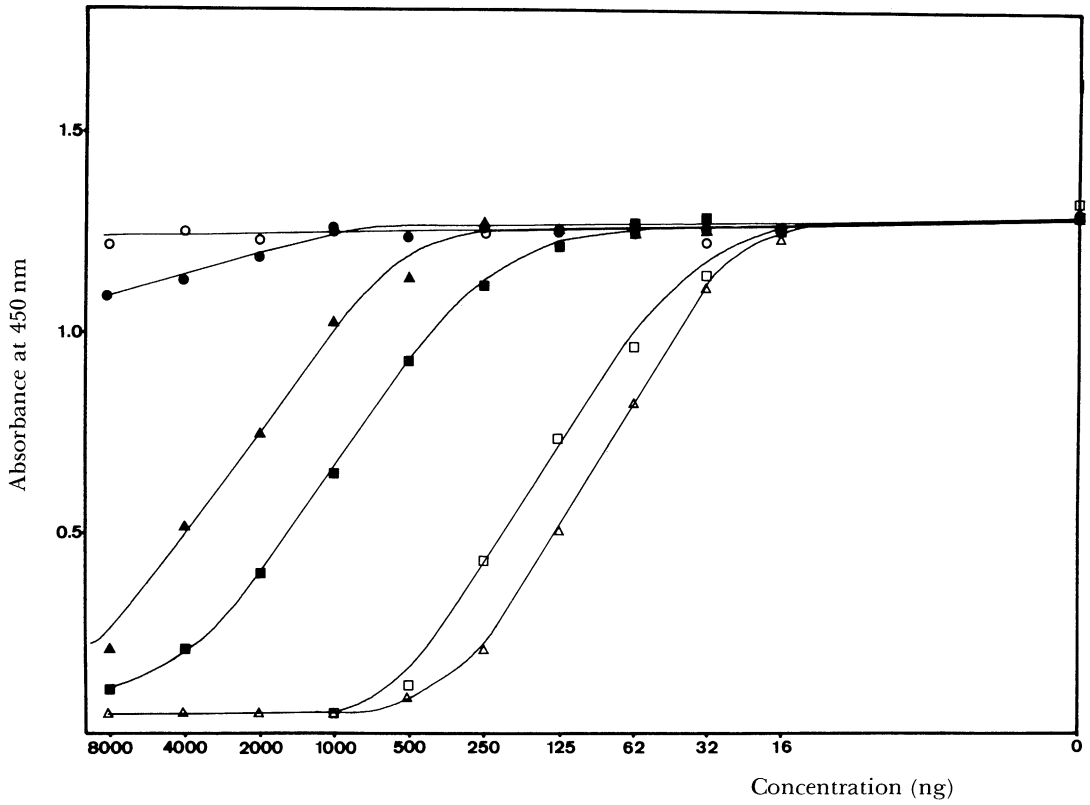

Fig. 4. Reactivity of peak fractions in Fig. 3B with monoclonal antibody ST_h53-4 in competitive ELISA: ST_h(6—19) (□), peak 1-1 (△), peak 2-1 (○), peak 2-2 (■), peak 3-1 (▲), and peak 3-2 (●).

Table 3. Toxicities of Various Analogs of ST_h(6—19)

Amino acid sequence														MED/pmol
6								14					19	
Cys-	Cys-	Glu-	Leu-	Cys-	Cys-	Asn-	Pro-	Ala-	Cys-	Thr-	Gly-	Cys-	Tyr	0.4
—	—	—	—	—	—	—	—	Gly	—	—	—	—	—	10
—	—	—	—	—	—	—	—	Ser	—	—	—	—	—	14
—	—	—	—	—	—	—	—	Asp	—	—	—	—	—	506
—	—	—	—	—	—	—	—	Glu	—	—	—	—	—	540
—	—	—	—	—	—	—	—	Gln	—	—	—	—	—	2090
—	—	—	—	—	—	—	—	Val	—	—	—	—	—	>6600
—	—	—	—	—	—	—	—	Ile	—	—	—	—	—	>1300
—	—	—	—	—	—	—	—	Leu	—	—	—	—	—	>6600
—	—	—	—	—	—	—	—	Phe	—	—	—	—	—	>6400
—	—	—	—	—	—	—	—	Lys	—	—	—	—	—	>6400
—	—	—	—	—	—	—	—	Arg	—	—	—	—	—	>6500
—	—	—	—	—	—	—	—	Leu	—	Ala	—	—	—	>6400

MED: minimum effective dose.

other words, the same surface structure to interact with the monoclonal antibody as that of ST_h(6—19).

Table 3 shows the toxicities of other analogs of ST_h(6—19). Gly^{14}- and Ser^{14}-ST_h(6—19) had about one-twentieth and one-thirtieth, respectively, of the toxicity of ST_h(6—19), whereas the other peptides were almost inactive. These results indicated that with increase in the size of the side chain of the amino acid residue at position 14, the peptides become less active, and that replacement of the amino acid residue at this position by an amino acid residue with a bulky side chain results in marked reduction of toxicity. The Gly^{14}-analog with a smaller side chain than that of the original peptide was less active than ST_h(6—19), the peptide with the native sequence. This implies that the size of the side chain at the amino acid residue at this position is an important factor for expression of the toxicity of ST_h. The initial step of the biochemical

reactions of STh is its binding to the receptor protein(s): the formation of the STh-receptor protein complex.[10] The present results strongly suggest that Ala at position 14 takes part in the interaction of STh with its receptor protein(s) on the target cell and that the methyl group may be a suitable size for the binding of STh to its receptor protein(s).

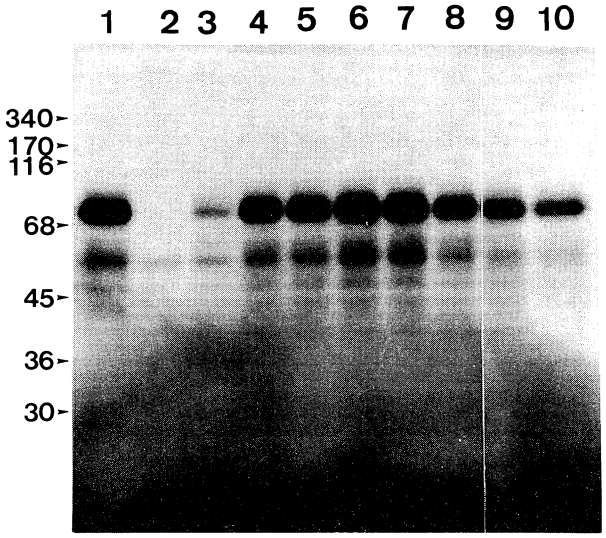

Fig. 5. Radioautogram of polyacrylamide gel after electrophoresis of the membrane proteins isolated from rat intestine and cross-linked with radio-iodinated ^{125}I-5-azido-2-nitrobenzoyl-STh(5—19): (lane 1) in the absence of STh(6—19), (lane 2) in the presence of STh(6—19), (lanes 3—10) in the presence of Gly^{14}-STh(6—19), Glu^{14}-STh(6—19), Gln^{14}-STh(6—19), Phe^{14}-STh(6—19), Arg^{14}-STh(6—19), Val^{14}-STh(6—19), Lys^{14}-STh(6—19), and Leu^{14}-STh(6—19), respectively. Numbers on the left side indicate the positions of standard markers of molecular weight ($\times 10^3$).

Recently, Hirayama *et al.*[12] identified a protein of 70 kDa from rat intestinal epithelial cell membranes that binds specifically to STh and is labeled with a photoaffinity-labeled and radio-iodinated analog of STh (^{125}I-5-azido-2-nitrobenzoyl-STh(5—19)). We examined the competitive binding of synthetic analogs of STh(6—19) with this labeled analog to the 70 kDa protein. As shown in Fig. 5, the binding of the labeled analog to this protein was completely inhibited by STh(6—19) (lane 2 in Fig. 5) and partly by the Gly^{14}-analog of STh(6—19) (lane 3), which showed about one-twentieth of the activity of the original peptide. But at the same molar level as that of STh(6-19) other peptides did not inhibit the binding of the labeled compound to the 70 kDa protein (lanes 4 to 10). Although the competitive binding of the analogs of STh(6—19) to 70 kDa protein was not examined quantitatively in this experiment, the results clearly demonstrate that the binding abilities of synthetic peptides to the 70 kDa protein on the target cells are correlated with their toxic activities.

Conformational Studies. As described above, Leu^{14}-STh(6—19) was suggested to have a similar conformation to STh(6—19). For confirmation of this suggestion and elucidation of the role of the amino acid residue at position 14 in STh(6—19) in its spatial structure-function relationship, we examined the conformation of Leu^{13}-STp(5—17). This peptide was chosen instead of Leu^{14}-STh(6—19) for the following reasons: 1) STp(5—17) has almost the same chemical and biological properties as those of STh(6—18)[7] and hence its conformation is assumed to be almost the

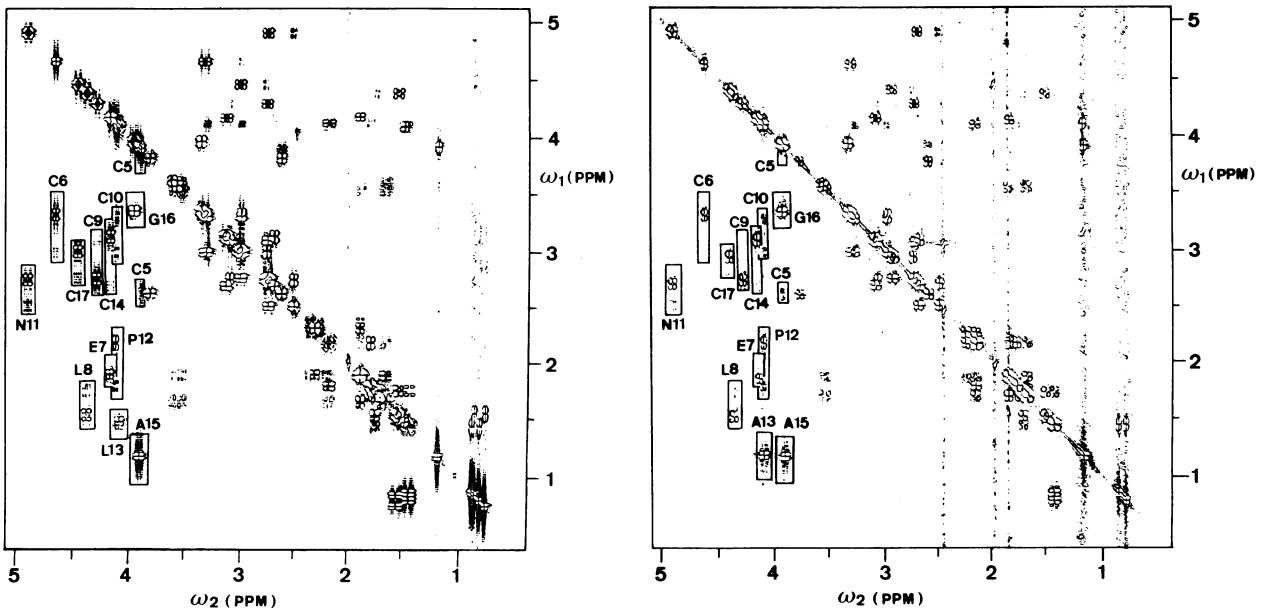

Fig. 6. DQF-COSY spectra of Leu^{13}-STp(5—17) (left) and STp(5—17) (right) in the aliphatic regions showing the CαH–CβH ^{1}H cross peaks.

same as that of ST_h(6—18), 2) the C-terminal Tyr residue at position 19 in ST_h is not important for toxicity,[7] and 3) the conformation of ST_p(5—17) has been analyzed in detail by NMR spectroscopy,[13] while the conformation of ST_h(6—18) is yet obscure. Leu^{13}-ST_p(5—17) was synthesized and purified in a similar manner to that described above.

We measured the DQF-COSY and NOESY spectra of Leu^{13}-ST_p(5—17) and compared them with those of ST_p(5—17). The assignment of the spin system of constituent amino acids of Leu^{13}-ST_p(5—17) and their sequential resonances was attained by the established procedures described in Refs. 18 and 19, respectively. The chemical shifts of the protons of Leu^{13}-ST_p(5—17) were almost the same as those of ST_p(5—17) except for the C_β-protons of Leu^{13}, as shown in Fig. 6. The NOEs were observed with similar relative intensities between the same protons in both Leu^{13}-ST_p(5—17) and ST_p(5—17) and these are summarized in Fig. 7.

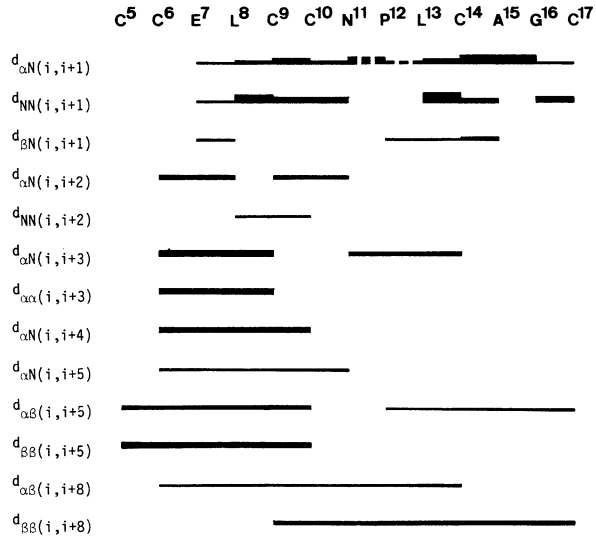

Fig. 7. Summary of NOEs observed in Leu^{13}-ST_p(5—17): The intensities of NOEs are indicated by line thicknesses, and NOEs involving $C_\delta H$ protons of Pro^{12} by dotted lines.

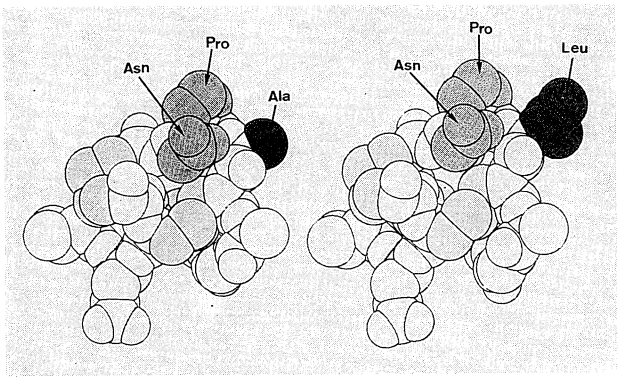

Fig. 8. Space-filling models of Leu^{13}-ST_p(5—17) (right) and ST_p(5—17) (left).

Moreover, the temperature dependency of the chemical shifts showed that the NH protons of Cys^9, Cys^{14}, and Cys^{17} in Leu^{13}-ST_p(5—17) took part in hydrogen bondings similarly to those of ST_p(5—17), which contributed to stabilization of its tertiary structure.[13] These results indicate that the back bone structure of Leu^{13}-ST_p(5—17) is quite similar to that of ST_p(5—17); that is, the back bone structure was not influenced by replacement of Ala at position 13 (corresponding to Ala^{14} in ST_h) by Leu, and the overall folding was similar in the two peptides. The stability of this conformation may be maintained by the three intramolecular disulfide linkages. This finding suggests that the replacement of this residue by the amino acid residues listed in Table 3 did not affect the conformation of ST_p(5—17).

Figure 8 illustrates the molecular model of Leu^{13}-ST_p(5—17), which was drawn up by replacing the methyl group of Ala^{13} in ST_p(5—17) with an isobutyl group, and also that of ST_p(5—17). The model of ST_p-(5—17) shows that side chains of the sequence Asn^{11}–Pro^{12}–Ala^{13} are oriented in the same direction. The replacement of the methyl group of the Ala residue by a bulky side chain such as an isobutyl group may result in changes in the surface structure of this region and the bulky side chain may intrude upon the contact of the ST molecule with its receptor protein(s). This inference may confer to ST_h(6—19). Gly^{14}- and Ser^{14}-ST_h(6—19) with side chains of more or less similar size to that of the native toxin may still come into contact with the receptor protein(s) and hence retain toxicity. Presumably, other amino acid residues besides Ala^{14} in ST_h take part in the interaction of the toxin with its receptor protein(s). Further detailed analyses of the conformation-activity relationship should provide insights into the molecular basis of the toxin-receptor interaction. The results should also be useful in understanding the molecular basis of the peptide-to-protein binding interaction.

Experimental

The general and analytical methods used were described in the preceding papers.[7,14] All chemicals used for preparative experiments were of reagent grade, those used for analyses were of guaranteed grade, and solvents were distilled before use. All amino acids used were of the L-configuration except glycine. Boc-amino acids were purchased from the Peptide Institute Inc. (Minoh, Osaka). Solvent for NMR experiments (DMSO-d_6 (99.96%), CH_3CN-d_3 (99.9%), and D_2O (99.95%)) were obtained from CEA (Commissarriat a L'Energie Atomique, France). The abbreviations used in this paper are those recommended by the IUPAC-IUB [*J. Biol. Chem.*, **261**, 1 (1986)]. Additional abbreviations are: MBzl, 4-methylbenzyl; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; FAB, fast atom bombardment; DQF, double quantum-filtered; COSY, two dimensional correlated spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, two dimensional NOE spectroscopy; ELISA,

enzyme-linked immunosorbent assay.

Synthesis of Peptides. Peptides were synthesized manually by the solid-phase method.[8,16] Boc-Tyr(Bzl(2,6-Cl_2))-polystyrene (2% divinylbenzene) (0.2—0.3 mmol of amino acid per 1 g of resin) was elongated one amino acid at a time in order by coupling Boc-amino acids with the aid of DCC. The following protecting groups for functional groups of side-chains were used: Bzl for serine, threonine, aspartic acid, and glutamic acid, MBzl for cysteine, 2-chlorobenzyloxycarbonyl for lysine, and *p*-toluenesulfonyl for arginine. After coupling all the amino acids, the protected peptide-resin was treated with the same amount of anisole in anhydrous hydrogen fluoride at 0 °C for 1 h. The hydrogen fluoride was removed under reduced pressure and the residue was diluted with water to a peptide concentration of 5×10^{-5}M (1M=1 mol dm^{-3}) and adjusted to pH 8.0. The solution was kept at room temperature with occasional stirring until no free mercapto groups were detectable. The resulting peptide was purified by high-performance liquid chromatography, as described below.

High-Performance Liquid Chromatography (HPLC). The synthetic peptides were purified on a reversed-phase column (YMC-ODS, 8×250 mm, Yamamura Chemical Industries Ltd., Kyoto) using an HPLC delivery system consisting of a Waters M600 pump (Milford, MA) and Hitachi 655A variable wavelength UV monitor and D-2000 chromatointegrator (Tokyo). The peptides were eluted with a linear gradient of increasing concentration of CH_3CN in 0.05% TFA or 0.01 M ammonium acetate (pH 5.7). The absorbance of the eluate at 220 nm was monitored.

Amino Acid and Mass Analyses. The purified peptides were hydrolyzed for 24 h at 110 °C in constant boiling hydrochloric acid and the amino acid compositions of the hydrolyzates were analyzed in a Hitachi L-8500 amino acid analyzer. The mass values were examined by FAB mass spectrometry, as described in Ref. 20.

Biological Assays. Toxic activities of synthetic peptides were examined in suckling mice (1.7±0.1 g) of 2—3 days old as described previously.[17] Assay of binding of synthetic peptides to the receptor protein(s) was carried out using brush-border membranes isolated from male Sprague-Dawley rats (8 weeks old, 200—250 g), as described in Ref. 12. 5-Azido-2-nitrobenzoyl-ST_h(5—19) and ^{125}I-5-azido-2-nitrobenzoyl-ST_h(5—19) were synthesized as described previously.[12] In competition experiments, the membranes (200 μg of proteins) were incubated with ^{125}I-5-azido-2-nitrobenzoyl-ST_h(5—19) (3×10^{-6} M) in the presence of ST_h(5—19) or its analogs (10^{-6} M). After photolysis at 4 °C by exposure to light at 254 nm, the photoaffinity-labeled proteins were solubilized in the presence of 0.1% Lubrol PX and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis using 10% separating gel.[21]

Enzyme-Linked Immunosorbent Assay (ELISA). Competitive ELISA was performed using monoclonal anti-ST_h antibody which recognizes at least Leu^9 in ST_h.[22] ST_h(1—19) (1 μg ml^{-1} of phosphate-buffered saline (PBS)) (0.1 ml) was added to each well of a Falcon microtest III flexible assay plate (Becton Dickinson and Co., Oxnard, Ca., USA). The plate was incubated at 37 °C for 2 h and washed three times with PBS. PBS (0.15 ml) containing 1% bovine serum albumin (BSA) was added to each well and incubated at 37 °C for 1 h. After washing the wells three times with PBS containing 0.05% Tween 20 (PBS T), 0.05 ml of sample and 0.05 ml monoclonal antibody solution appropriately diluted with PBS T were mixed and added to each well. After incubation at 37 °C for 1 h, the wells were washed three times with PBS T. PBS T solution (0.1 ml) of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Zymed Laboratories, Inc., San Francisco, Ca., USA) was added to each well and incubated at 37 °C for 1 h. After washing three times with PBS T, 0.1 ml of 0.1% *o*-phenylenediamine (Sigma) in 100 mM citrate buffer (pH 4.5) containing 0.015% hydrogen peroxide was added, and after incubation at room temperature, the optical density at 450 nm was measured with a spectrophotometer.

NMR Spectroscopy. The sample peptide was dissolved in a mixture (0.5 ml) of DMSO-d_6 and CH_3CN-d_3 (v/v, 80/20) at a peptide concentration of 8—12 mM. ^{1}H NMR spectra were recorded with a JEOL GX-400s spectrometer with a proton probe maintained thermostatically at 10 °C. Chemical shifts were measured relative to the methyl resonance of tetramethylsilane used as an internal standard. The DQF- COSY and NOESY spectra were obtained in a phase sensitive mode,[23] using quadrature detection in both dimensions. Other experimental conditions were as described previously.[13]

This work was supported in part by a Grant-in Aid from the Ministry of Education, Science and Culture. We are indebted to Miss Chieko Katsu for preparation of the Manuscript.

References

1) Presented in part at the 24th Joint Conference of the U. S. -Japan Cooperative Medical Science Program—Cholera and Related Diarrheal Diseases Panel—, Tokyo, November 13—16, 1988.

2) H. W. Smith and C. L. Gyles, *J. Med. Microbiol.*, **3**, 387 (1970).

3) S. Aimoto, T. Takao, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Eur. J. Biochem.*, **129**, 257 (1982).

4) T. Takao, T. Hitouji, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *FEBS Lett.*, **152**, 1 (1983); M. R. Thompson and R. A. Giannella, *Infect. Immun.*, **47**, 834 (1985).

5) T. Takao, N. Tominaga, Y. Shimonishi, S. Hara, T. Inoue, and A. Miyama, *Biochem. Biophys. Res. Commun.*, **125**, 845 (1984).

6) T. Takao, Y. Shimonishi, M. Kobayashi, O. Nishimura, M. Arita, T. Takeda, T. Honda, and T. Miwatani, *FEBS Lett.*, **193**, 250 (1985).

7) S. Yoshimura, H. Ikemura, H. Watanabe, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, T. Miwatani, and Y. Takeda, *FEBS Lett.*, **181**, 138 (1985).

8) Y. Shimonishi, Y. Hidaka, M. Koizumi, M. Hane, S. Aimoto, T. Takeda, T. Miwatani, and Y. Takeda, *FEBS Lett.*, **215**, 165 (1987); Y. Hidaka, H. Kubota, S. Yoshimura, H. Ito, Y. Takeda, and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, **61**, 1265 (1988); Y. Hidaka, H. Kubota, H. Ito, Y. Takeda, and Y. Shimonishi, "Peptide Chemistry 1987," ed by T. Shiba and S. Sakakibara, Protein Research Foundation, Osaka (1988), p. 159.

9) J. Gariépy and G. K. Schoolnik, *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 483 (1986).
10) S. A. Waldman, T. Kuno, Y. Kamisaki, L. Y. Chang, J. Gariépy, P. O'Hanley, G. K. Schoolnik, and F. Murad, *Infect. Immun.*, **51**, 320 (1986).
11) J. Gariépy, A. K. Judd, and G. K. Schoolnik, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 8907 (1987).
12) T. Hirayama, Y. Oku, Y. Takeda, N. Iwata, S. Aimoto, and Y. Shimonishi, "Advances in Cholera and Related Diarrheas," ed by R. B. Sack and Y. Zinnaka, **7**, 105 (1990).
13) H. Ozaki, H. Kubota, Y. Hidaka, T. Ohkubo, H. Tamaoki, Y. Kobayashi, Y. Kyogoku, T. Sugimura, A. Tai, and Y. Shimonishi, "Peptide Chemistry 1988," ed by M. Ueki, Protein Research Foundation, Osaka (1989), p. 179.
14) S. Yamasaki, Y. Hidaka, H. Ito, Y. Takeda, and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, **61**, 1701 (1988).
15) J. S. Richardson, "Adv. Protein Chemistry," ed by C. B. Anfinsen, J. T. Edsall, and F. M. Richards, Academic Press, Inc., New York, Vol. 34, p. 167 (1981).
16) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
17) Y. Takeda, T. Takeda, Y. Yano, K. Yamamoto, and T. Miwatani, *Infect. Immun.*, **25**, 978 (1979).
18) G. M. Clore and A. M. Gronenborn, *Protein Eng.*, **1**, 275 (1987).
19) K. Wüthrich, G. Wider, G. Wagner, and W. Braun, *J. Mol. Biol.*, **155**, 311 (1982).
20) T. Takao, T. Hitouji, Y. Shimonishi, T. Tanabe, S. Inouye, and M. Inouye, *J. Biol. Chem.*, **259**, 6105 (1984).
21) U. K. Laemmli, *Nature*, **227**, 680 (1970).
22) T. Takeda, K. Suzuki, Y. Yokoo, S. Yamasaki, H. Ito, Y. Takeda, and Y. Shimonishi, "Advances in Cholera and Related Diarrheas," ed by R. B. Sack and Y. Zinnaka, **7**, 183 (1990).
23) D. J. States, R. A. Haberkorn, and D. J. Reuben, *J. Magn. Reson.*, **48**, 286 (1982).