  Bull. Chem. Soc. Jpn., 61, 1265—1271 (1988)

Disulfide Linkages in a Heat-Stable Enterotoxin (ST_p) Produced by a Porcine Strain of Enterotoxigenic *Escherichia coli*

Yuji HIDAKA, Hirokazu KUBOTA, Shoko YOSHIMURA, Hideaki ITO,†
Yoshifumi TAKEDA,† and Yasutsugu SHIMONISHI*
Institute for Protein Research, Osaka University, Yamada-oka 3-2, Suita, Osaka 565
†The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108
(Received October 12, 1987)

Heat-stable enterotoxins, produced by various enteric bacteria such as enterotoxigenic *Escherichia coli, Yersinia enterocolitica,* and *Vibrio cholerae* non-01, have common segments consisting of 13 amino acid residues (T. Takao et al., *FEBS Lett.*, **193**, 250 (1985)). These segments have full enterotoxigenic activity and are bound by three intramolecular disulfide bonds. The linkages of these three disulfide bonds in a heat-stable enterotoxin (ST_p) produced by a porcine strain of enterotoxigenic *E. coli* were determined to be

Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys

by stepwise and selective formation of disulfide bonds using different types of removable protecting groups for Cys residues. These disulfide linkages were identical with those of a heat-stable enterotoxin (ST_h) produced by a human strain of enterotoxigenic *E. coli* (Y. Shimonishi et al., *FEBS Lett.*, **215**, 165 (1987)).

Pathogenic enteric bacteria such as enterotoxigenic *Escherichia coli, Yersinia enterocolitica,* and *Vibrio cholerae* non-01 produce heat-stable enterotoxins (STs), which cause acute diarrhea in children in developing countries in the tropics. We and others purified STs to homogeneity from the culture supernatants of these pathogenic enteric bacteria and determined their primary structures,[1-5] as shown in Fig. 1. On the basis of chemical syntheses of various analogues of the STs and measurements of their toxicities, we found that a segment with 13 amino acid residues containing 6 half-cystines (Fig. 1) has the full enterotoxigenic activity of STs.[6] On the other hand, reduction of the three disulfide linkages of the STs resulted in loss of their enterotoxigenic activities. These results indicated that the structural element(s) required for the toxicities of STs is located on the three dimensional structure formed by the three disulfide bonds in the segment with 13 amino acid residues. Recently, we determined that the disulfide bonds of an ST (ST_h) produced by a human strain of enterotoxigenic *E. coli*[7] were as shown in Fig. 7. We also found that these disulfide bonds in ST_h were very important for its toxicity. This observation prompted us to examine whether other STs have the same disulfide linkages as those in ST_h and to investigate the effect of amino acid substitution on the mode of disulfide bond formation or construction of the three dimensional structure and toxicity of ST_h.

In this work, we examined the mode of disulfide bond formation in an ST (ST_p) produced by a porcine strain of enterotoxigenic *E. coli*, in which the primary structure in the common 13 amino acid residues differs from that of ST_h only at position 15. The mode of three disulfide bond formation in ST_p was the same as that in ST_h of a human strain of enterotoxigenic *E. coli*, indicating that other STs produced by enteric bacteria have the same mode of disulfide bond formation.

Experimental

The general and analytical methods used were as described previously.[6] All chemicals used for synthetic experiments were of reagent grade, while those used for analysis were of guaranteed grade and solvents were distilled before use. All amino acids except glycine were of the L-configuration and Boc-amino acid derivatives were purchased from the Peptide Institute Inc. (Minoh, Osaka). A reversed-phase resin (YMC-ODS,s-5) was purchased from Yamamura Chemical Laboratories Co. (Kyoto) and packed into a column of 8×300 mm

Escherichia coli ST_h [a]													N	S	S	N	Y	C	C	E	L	C	C	N	P	A	C	T	G	C	Y
Escherichia coli ST_p [b]													N	T	F	Y	C	C	E	L	C	C	N	P	A	C	A	G	C	Y
Yersinia enterocolitica ST [c]	Q	A	C	D	P	P	S	P	P	A	E	V	S	S	D	W	D	C	C	D	V	C	C	N	P	A	C	A	G	C	
Vibrio cholerae non-01 ST [d]																I	D	C	C	E	I	C	C	N	P	A	C	F	G	C	L	N
Vibrio mimicus ST [e]																I	D	C	C	E	I	C	C	N	P	A	C	F	G	C	L	N

Fig. 1. Amino acid sequences of heat-stable enterotoxins produced by enteric bacteria. Common regions in all the sequences are boxed. Cited from a) Ref. 1, b) Ref. 2, c) Ref. 3, d) Ref. 4, and e) Ref. 5. The single letter notations of amino acid residues are according to *J. Biol. Chem.*, **261**, 1 (1986).

for analytical and preparative purposes in our laboratory. The abbreviations used in this paper are those recommended by the IUPAC-IUB [*J. Biol. Chem.*, **261**, 1 (1986)]. Additional abbreviations are: Acm, acetamidomethyl; HONSu, *N*-hydroxysuccinimide; DMF, *N,N*-dimethylformamide; MBzl, *p*-methylbenzyl; TFA, trifluoroacetic acid; TEA, triethylamine; DMSO, dimethyl sulfoxide.

General Procedures for Peptide Synthesis in Solution. As a typical procedure the coupling of compound **4** with compound **8** to give compound **9** is described as follows : Compound **4** (1.6 g, 0.75 mmol) was stirred with TFA (10 ml) at room temperature for 30 min and concentrated to a syrup. Meanwhile, compound **8** (0.82 g, 0.85 mmol) was dissolved in DMF (5 ml), cooled below −20 °C, and mixed with 6.72 M HCl (1M=1 mol dm^{-3}) in dioxane (0.88 ml) and isopentyl nitrite (0.13 ml). The solution was stirred at −20 °C for 25 min and mixed with a solution of the above syrup in DMF (10 ml) and TEA (0.94 ml). The mixture was further stirred at 0 °C for 1 day. The precipitate formed in the reaction mixture was separated by filtration and the filtrate was concentrated to a syrup under reduced pressure. The syrup was triturated to a solid in chilled 0.1M HCl. The recrystallization of the solid from EtOH gave **9** (1.40 g).

The deprotection of Z groups, hydrazinolysis of peptide esters, and formation of peptide bonds by an active ester method were carried out as described previously.[6]

Solid-Phase Peptide Synthesis. Peptide synthesis by the solid-phase method[9] was performed as described in a previous paper.[7]

Removal of Protecting Groups by HF and Oxidation by Iodine. The protected peptides (25 μmol each) were treated with anhydrous liquid hydrogen fluoride[10] and air-oxidized as described previously.[6c] The solutions containing the air-oxidized peptides were partially concentrated by lyophilization and purified by HPLC, as described below. The purified peptides with an intramolecular disulfide bond(s) and Acm groups were oxidized by iodine at a peptide-to-iodine molar ratio of 1 : 40 in a mixture of MeOH and 0.1 M HCl as described in a previous paper.[7] The iodine-oxidized peptides were directly purified by HPLC, as described below.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus consisted of a Waters M600 multisolvent delivery system (Milford, MA) connected with a Hitachi UV 655A variable wavelength UV monitor and D-2000 chromato-integrator (Tokyo, Japan). The HPLC column was equilibrated with 10% CH_3CN in 0.05% TFA or 0.01M ammonium acetate (pH 5.7) and after injection of the sample solution, was developed with a linear-gradient of 10—40% CH_3CN with increase in CH_3CN of 0.5 or 1% min^{-1} at a flow rate of 2 ml min^{-1}. Eluates were monitored for absorption at 220 nm.

Amino Acid Analysis and Fast Atom Bombardment (FAB) Mass Spectrometry. The amino acid compositions and molecular weights of purified peptides were determined as described previously.[3]

Biological Assay. Toxicity of synthetic peptides was assayed in suckling mice of 2 days old (1.7±0.1 g) as described previously.[11]

Results and Discussion

Previously,[7] to determine the mode of disulfide bond formation in ST_h (Fig. 7) we adopted a synthetic procedure in which disulfide bonds in ST_h were formed stepwise and selectively using different types of removable protecting groups for Cys residues. We found that this synthetic procedure was suitable for determination of disulfide bond formation in ST_h. Therefore, in the present work we applied this procedure for determination of the positions of disulfide linkages in ST_p.

The three disulfide linkages in ST_h between Cys^6 and Cys^{11}, Cys^7 and Cys^{15}, and Cys^{10} and Cys^{18}, are shown in Fig. 7. These disulfide linkages were expected to be similar in ST_p, because the 13 amino acid sequence including 6 half Cys residues in ST_p differs only at Ala at the fourth position from the C-terminus from that in ST_h and has almost the same biological activity as the corresponding ST_h peptide. Accordingly, we synthesized three kinds of protected linear peptides with the segment from Cys^5 to Cys^{17} of ST_p with two Cys residues at Cys^5 and Cys^{10} or Cys^6 and Cys^{14} or Cys^9 and Cys^{17} protected by an Acm group and the other four Cys residues protected by an MBzl group, which correspond to the sequence from Cys^6 to Cys^{18} in ST_h, as shown in Figs. 2—4. Table 1 summarizes data on protected peptides **1**—**21**. The protected linear peptides thus obtained were treated with liquid

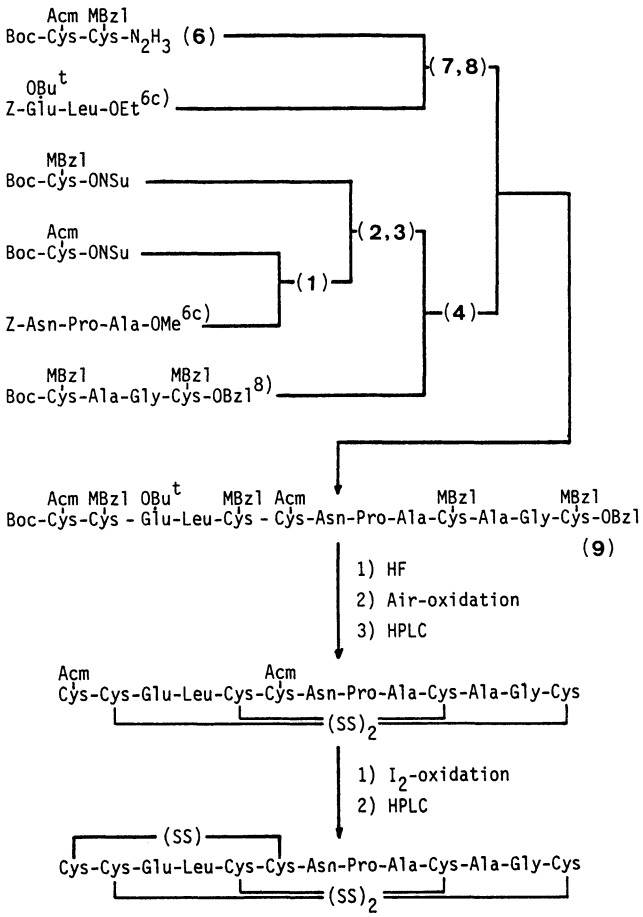

Fig. 2. Scheme for synthesis of a shorter peptide of ST_p linked by a disulfide bond between Cys^5 and Cys^{10}.

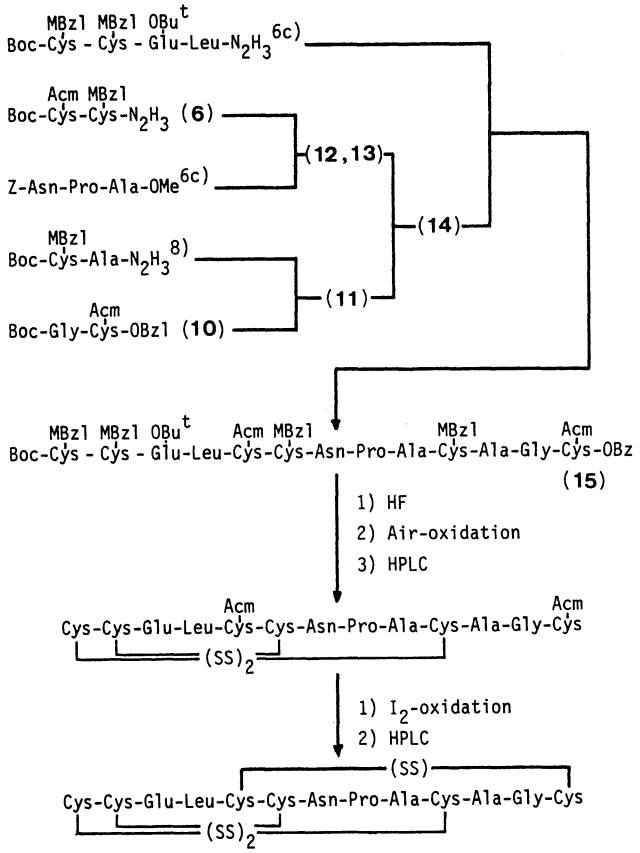

Fig. 3. Scheme for synthesis of a shorter peptide of ST_p linked by a disulfide bond between Cys^9 and Cys^{17}.

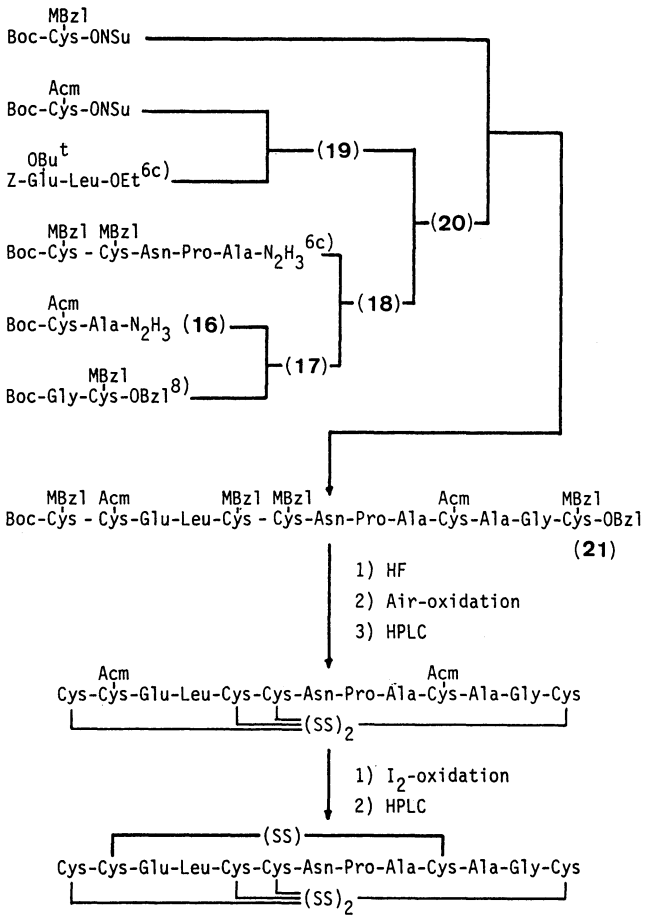

Fig. 4. Scheme for synthesis of a shorter peptide of ST_p linked by a disulfide bond between Cys^6 and Cys^{14}.

anhydrous hydrogen fluoride to remove all the protecting groups except Acm groups and air-oxidized to form intramolecular disulfide bonds between the four Cys residues, which had been protected by MBzl groups. The air-oxidized products were purified by HPLC on a reversed-phase column, as shown in Figs. 5a—5c. On the bases of amino acid and molecular weight analyses of the separated fractions, peptides with two disulfide bonds were concluded to be eluted in the peak fractions shown by asterisks (*) in Figs. 5a—5c. The yields of these peptides, for example those in Fig. 5a, were 14, 20, and 16% in order of their elution on the basis of the protected linear peptide. In this treatment, three kinds of peptides with two intramolecular disulfide bonds should theoretically be obtained. Figure 6 illustrates the HPLC profile of peak *2 in Fig. 5a, which was reduced by dithiothreitol and reoxidized spontaneously in air. Three peptide fractions were obtained similarly to those shown in Fig. 5a except for their peak ratios. However, peptides with a disulfide bond between two vicinal Cys residues were presumably formed with difficulty. In fact, when two disulfide bonds were formed in peptides **15** and **21**, only two of three possible peptides could be isolated by HPLC, as shown in Figs. 5b and 5c. The amino acid compositions, mass values, and biological activities of these peptides with two disulfide bonds are summarized in Table 2.

Then, these monomeric peptides with two disulfide bonds were selectively oxidized by iodine to form a third disulfide linkage at the two Cys residues that had been protected by Acm groups. The peptides with three disulfide linkages were separated and compared by HPLC with standard ST_p peptide, which consists of 13 amino acid residues including 6 Cys residues and has the same disulfide bond formation as native ST_p.[6b] Selective formation of a third disulfide bond between Cys^5 and Cys^{10} or Cys^9 and Cys^{17} in peptides *1 and *5 in Figs. 5a and 5b, respectively, yielded peptides that were identical with standard ST_p peptide on HPLC, as shown by arrows in Figs. 5e and 5f. Peptides *2, *3, and *4 in Figs. 5a and 5b, respectively, did not yield a peptide that was identical with standard ST_p peptide. The results indicated that the disulfide bonds in ST_p were linked between Cys^5 and Cys^{10} and Cys^9 and Cys^{17}, and therefore that the remaining disulfide bond should be between Cys^6 and Cys^{14}. However, when the third disulfide bond was linked between Cys^6

and Cys[14] in two peptides *6 and *7 prepared from peptide **21** (Fig. 5c), no peptide was detected with the same retention time on HPLC as standard ST_p peptide. Figure 5g shows an HPLC profile of the peptide which was prepared by reaction of peptide *7 with iodine. Then, the three disulfide bonds were linked in reverse order; that is, we synthesized a protected linear peptide with two Cys residues at Cys[6] and Cys[14] protected by an MBzl group and the remaining four Cys residues protected by an Acm group by a solid-phase procedure. The first disulfide bond was formed between Cys[6] and Cys[14], and then the second and third disulfide bonds were formed between the remaining four Cys residues, as shown in Figs. 5d and 5h, respectively. When the peak fraction indicated by an asterisk in Fig. 5d was oxidized by iodine, the peak fraction *16 shown by an arrow in Fig. 5h was eluted in the same position as standard ST_p peptide by HPLC. The amino acid compositions and mass values of the peptides in the peak fractions shown by asterisks in Figs. 5e—5h are summarized in Table 3. The peptides eluted by HPLC in the same position as standard ST_p peptide shown in Figs. 5e, 5f, and 5h showed toxicities at doses of 0.8—1.0 ng/mouse, which were almost the same as that of standard ST_p peptide (0.7 ng), as described in Table 3. Thus, we concluded that the three intramolecular disulfide bonds of ST_p were between Cys[5] and Cys[10], Cys[6] and Cys[14], and Cys[9] and Cys[17], as shown in Fig. 7. Other disulfide bond linkages except those described above were not examined, because the positions of the disulfide bonds of ST_p determined above were the same as those of ST_h.[7]

When a third disulfide bond was linked between Cys[5] and Cys[10] and Cys[9] and Cys[17] in peptides *1 and *5 in Figs. 5a and 5b, respectively, they gave two peak fractions on HPLC, as shown in Figs. 5e and 5f, respectively. On HPLC one of them was eluted at the same position as standard ST_p peptide, while the other was eluted earlier. When a third disulfide bond was linked between Cys[6] and Cys[14] in peptide *7 in Fig. 5c, interestingly it gave only a peak fraction with the same retention time as peaks *9 and *11 in Figs. 5e and 5f, respectively. Moreover, these two peak fractions showed different circular dichroism spectra from each other in the range from 190 nm to 260 nm (not shown). These two peptides were not interconvertible, but stable on storage for a long time in a refrigerator. These findings suggested that two topologically stable and different conformers are present in peptides with the same three disulfide linkages and also that only one or both the two conformers are produced dependent upon the order of disulfide bond formation.

While peptide *1 was eluted as a single peak under the conditions for Fig. 5a, it was separated into two broad peak fractions on HPLC under different conditions from that in Fig. 5a, as depicted in Fig. 8. Each fraction showed the same profile on repeated HPLC as that before separation (not shown). Peptides in the

Table 1. Yields, Melting Points,[a] Solvents for Recrystallization, Optical Rotations, and Analytical Data of Intermediates

Compound	Yield	Mp	Solvent for recrystallization	$[\alpha]_D^{25}$ [b]	Found (%)			Calcd (%)		
	%	θ_m/°C		degree	C	H	N	C	H	N
1	91.8	79—80	$CHCl_3$-hexane	−46.5						
2[c]	67.0	148—149	AcOEt-EtOH-ether	−70.5	52.65	6.77	12.27	52.82	6.71	12.32
3[d]	97.7	92—94	EtOH-ether	−64.3	50.21	6.87	15.53	50.17	6.80	15.49
4	93.1	169—171	EtOH-ether	−67.8	56.79	6.57	11.01	57.01	6.54	10.76
5[e]	94.1	126—127	AcOEt-hexane	−36.4	54.18	7.00	8.29	53.79	6.87	8.18
6	91.6	168—169	MeOH-ether	−23.8	51.13	6.86	13.52	51.45	6.87	13.64
7[f]	94.2	128—129.5	AcOEt-ether-hexane	−25.9	56.53	7.79	8.40	56.71	7.69	8.48
8[g]	94.7	148—149	MeOH-ether	−15.7	54.59	7.59	12.12	54.73	7.57	12.08
9	89.2	211—212	EtOH	−52.0[h]	57.14	6.99	10.42	57.35	6.74	10.70
10	88.1	94—95	AcOEt-hexane	−21.1	54.78	6.64	9.61	54.66	6.65	9.56
11	96.3	161—162	EtOH-ether	−18.1	56.49	6.61	9.80	56.89	6.60	9.76
12[i]	70.5	152—153	EtOH-$CHCl_3$-ether	−59.0	52.64	6.82	12.21	52.82	6.71	12.32
13[j]	90.5	105—106	EtOH-ether	−43.8	49.97	6.85	16.00	50.17	6.80	15.49
14	83.3	177—178	EtOH	−60.2	54.25	6.43	12.10	54.77	6.42	12.17
15	83.8	225 (decomp)	EtOH	−64.2[h]	57.02	6.77	10.67	57.35	6.74	10.70
16	62.7		MeOH-ether	−6.1	44.13	7.25	18.50	44.55	7.21	18.56
17	89.4	148—149	AcOEt-hexane	−23.1	56.72	6.63	9.69	56.89	6.60	9.76
18	92.2	170—172	EtOH	−64.1	56.68	6.48	10.81	57.01	6.54	10.76
19	82.8	163—164.5	MeOH-ether	−16.6	49.56	7.83	16.06	49.04	8.22	15.40
20	84.1	202—203	EtOH-AcOEt	−47.7[k]	56.21	6.82	11.26	56.10	6.83	11.03
21	84.5	197—198	EtOH	−59.6[h]	55.80	6.46	11.32	56.07	6.57	10.90

a) Melting points were uncorrected. b) c=1.0 in DMF. c) Boc-Cys(MBzl)-Cys(Acm)-Asn-Pro-Ala-OMe. d) Boc-Cys(MBzl)-Cys(Acm)-Asn-Pro-Ala-N_2H_3. e) Boc-Cys(Acm)-Cys(MBzl)-OMe. f) Boc-Cys(Acm)-Cys(MBzl)-Glu(OBu^t)-Leu-OEt. g) Boc-Cys(Acm)-Cys(MBzl)-Glu(OBu^t)-Leu-N_2H_3. h) c=0.5 in DMSO. i) Boc-Cys(Acm)-Cys(MBzl)-Asn-Pro-Ala-OMe. j) Boc-Cys(Acm)-Cys(MBzl)-Asn-Pro-Ala-N_2H_3. k) c=1.0 in DMSO.

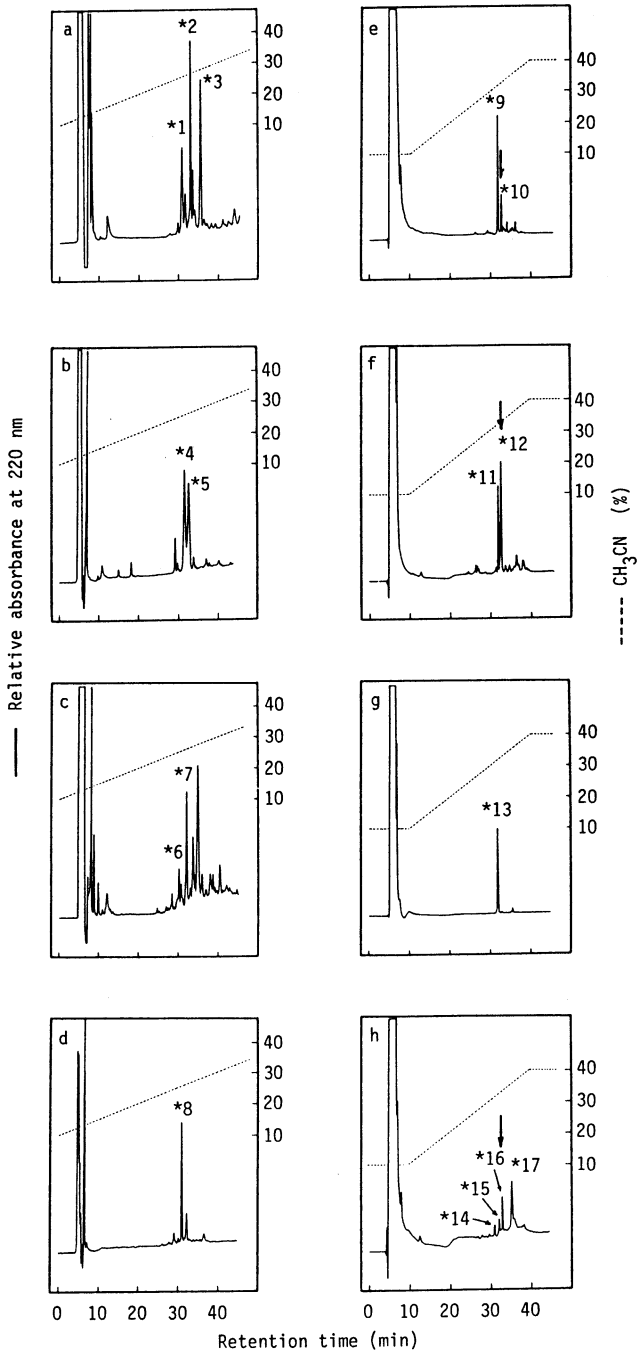

Fig. 5. HPLC profiles of: a) a deprotected and air-oxidized solution of peptides with two disulfide bonds at four Cys residues other than Cys^5 and Cys^{10} (Fig. 2), b) a deprotected and air-oxidized solution of peptides with two disulfide bonds at four Cys residues other than Cys^9 and Cys^{17} (Fig. 3.), c) a deprotected and air-oxidized solution of peptides with two disulfide bonds at four Cys residues other than Cys^6 and Cys^{14} (Fig. 4), d) a deprotected and air-oxidized solution of a peptide with a disulfide bond between Cys^6 and Cys^{14}, e) an I_2-oxidized solution of peak *1 in a), f) an I_2-oxidized solution of peak *5 in b), g) an I_2-oxidized solution of peak *7 in c), and h) an I_2-oxidized solution of peak *8 in d). Peaks *10, *12, and *16 shown by arrows were eluted at the same retention time as standard ST_p peptide.

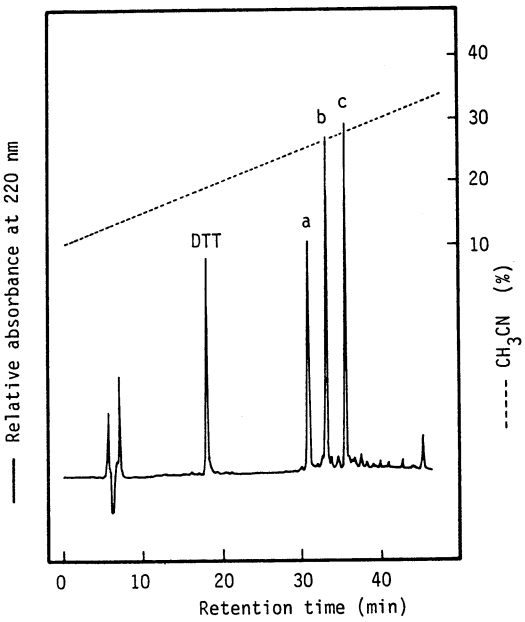

Fig. 6. HPLC profile of peptide *2 in Fig. 5a, which was reduced and reoxidized by air. Peaks a, b, and c had the same retention times as those of peaks *1, *2, and *3 in Fig. 5a, respectively.

two peak fractions 1 and 2 shown in Fig. 8 were oxidized by iodine immediately after their elution from an HPLC column. The peptide in the first fraction gave two peaks like that shown in Fig. 9a, one of which was identical with standard ST_p peptide, whereas the peptide in the second fraction gave only a peptide that was not identical with standard ST_p peptide, as shown in Figs. 9b and 9c, respectively. These findings suggested that peptide *1 consisted of two components, which were rapidly interconvertible and therefore were in equilibrium. These peptides are presumed to be two conformers with cis and trans configurations around the peptide bond between Asn^{11} and Pro^{12}, one of them corresponding to a peptide in the first fraction, and the other to a peptide in the second fraction in Fig. 8. One of the two conformers yielded two peptides, and the other only one peptide, when they were oxidized by iodine. Since peptide *5 gave two peak fractions on treatment with iodine (Fig. 5f), it was considered to consist of two components like peptide *1. However, peptide *5 could not be separated by HPLC under the same conditions as for peptide *1 in Fig. 8. The two conformers in peptide *1 might have dissimilar physicochemical properties and those in peptide *5 similar properties, or they might be rapidly equilibrated, because on reversed-phase HPLC peptide *1 could be separated but peptide *5 could not be separated.

Houghten et al.[12] proposed the mode of disulfide bond formation of ST_p from an experiment on the reoxidation kinetics of ST_p, which they synthesized according to the amino acid sequence proposed previously,[13] although the amino acid sequence was later revised to the sequence shown in Fig. 1.[2] The pro-

Table 2. Amino Acid Composition,[a] Mass Values,[b] and Toxic Activities[c] of Synthetic Peptides with One or Two Disulfide Bonds[d]

	*1	*2	*3	*4	*5	*6	*7	Theoretical value	*8	Theoretical value
Asp	0.99	1.01	1.02	1.17	1.03	1.15	1.12	1	1.07	1
Glu	0.98	0.98	1.01	1.01	1.00	1.07	1.01	1	1.02	1
Gly	0.94	0.98	0.98	1.01	0.97	1.13	1.05	1	1.02	1
Ala	2	2	2	2	2	2	2	2	2	2
1/2 Cys	5.11	3.99	6.47	5.18	5.65	6.19	6.61	6	4.34	6
Leu	0.95	0.96	0.98	1.02	1.01	1.06	1.04	1	1.01	1
Pro	1.17	0.99	1.12	1.10	1.00	1.24	1.19	1	1.14	1
$[M+H]^{+}$ [b]	1427.3	1427.1	1427.1	1427.3	1427.1	1427.0	1427.0	1427.4	1572.0	1571.5
MED/ng[c]	290	840	$\simeq 10\times10^3$	400	790	620	$>10\times10^3$		$>10\times10^3$	

a) Values are those in acid hydrolysates (110 °C, 24 h) of synthetic peptides and are shown in mol/mol of Ala. b) $[M+H]^{+}$, mass values of quasi-molecular ion. c) MED, minimum effective dose. d) *1, *2, etc. correspond to those in Fig. 5.

Table 3. Amino Acid Composition,[a] Mass Values,[b] and Toxic Activities[c] of Synthetic $ST_p(5—17)$[d]

	*9	*10	*11	*12	*13	*14	*15	*16	*17	Theoretical value
Asp	1.19	1.02	1.05	1.00	1.02	1.34	1.10	1.30	1.05	1
Glu	1.12	0.97	1.26	0.98	1.08	1.26	1.07	1.00	1.03	1
Gly	1.19	0.99	1.14	0.87	1.02	1.47	1.03	0.97	1.03	1
Ala	2	2	2	2	2	2	2	2	2	2
1/2 Cys	2.95	4.86	4.56	4.78	4.87	5.13	4.41	5.65	5.08	6
Leu	1.04	1.07	1.04	1.02	1.01	1.12	1.06	1.01	1.01	1
Pro	1.17	0.99	1.18	1.10	1.03	1.18	1.04	1.00	1.03	1
$[M+H]^{+}$[b]	1282.9	1283.0	1283.2	1283.0	1283.1	1282.8	1282.8	1283.0	1282.8	1283.3
MED/ng[c]	14	1.0	11	0.8	15	138	14	0.8	61	0.7

a) Values are those in acid hydrolysates (110 °C, 24 h) of synthetic peptides and are shown as mol/mol of Ala. b) $[M+H]^{+}$, mass values of quasi-molecular ion. c) MED, minimum effective dose per mouse. d) *9, *10, etc. correspond to those in Fig. 5.

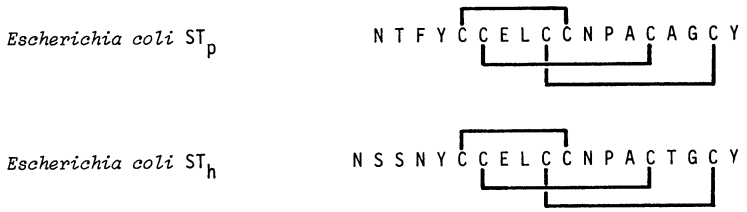

Fig. 7. Modes of three disulfide linkages of heat-stable enterotoxins (ST_p and ST_h[7]) of porcine and human strains, respectively, of enterotoxigenic *E. coli*.

posed disulfide bonds were between Cys^5 and Cys^{14}, Cys^6 and Cys^{10}, and Cys^9 and Cys^{17}. This disulfide bond formation is not consistent with that determined in this work. That is, the partners of Cys^5 and Cys^6 in disulfide bond formation are reversed. These mismatched pairings of disulfide linkages might be because they used a peptide with a different amino acid sequence from that shown in Fig. 1. If they had used a peptide with the correct amino acid sequence, they might have obtained the same results as ours.

Recently, we determined the mode of disulfide bond formation of ST_h produced by a human strain of enterotoxigenic *E. coli*, as shown in Fig. 7, and also of ST produced by *Vibrio-mimicus*.[5] The positions of the disulfide linkages were identical with those of ST_p examined in this work. These results imply that the three dimensional structure in the common 13 amino acid region as shown in Fig. 1 is very similar in all ST's examined to date. Further elucidation of this spatial structure should be useful in understanding the structure-activity relation of ST and its mechanism of binding to receptor proteins on the membranes of

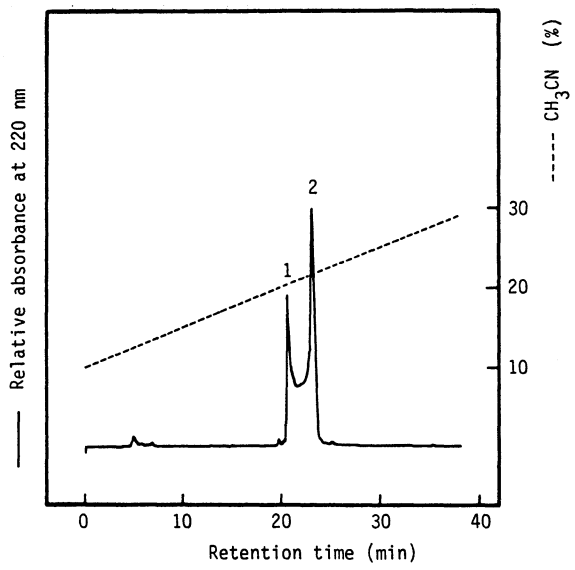

Fig. 8. HPLC profile of peptide *1 in Fig. 5a eluted with a linear-gradient of 10—30% CH_3CN concentration with increase in CH_3CN of 0.5% min^{-1} in 0.01 M ammonium acetate (pH 5.7).

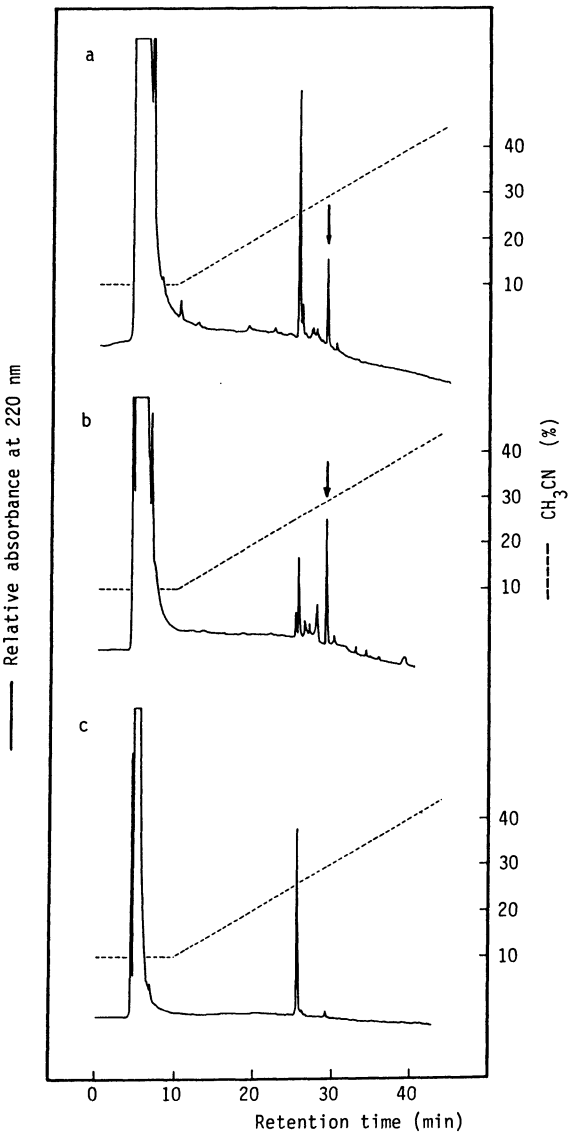

Fig. 9. HPLC profile of peak fractions eluted in Fig. 8 and oxidized by iodine: a) a mixture of peak fractions 1 and 2, b) peak fraction 1, and c) peak fraction 2. Peaks shown by arrows had the same retention times as standard ST_p peptide.

intestinal cells.

This work was supported in part by a Scientific Research Grant (No. 62470022 to Y. S) from the Ministry of Education, Science and Culture and in part by a grant from the Naito Foundation. We are indebted to Mrs. Noriko Mizoguchi for amino acid analysis and also to Mrs. Aiko Kobatake for elemental analysis and preparation of the manuscript.

References

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

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

3) T. Takao, N. Tominaga, Y. Shimonishi, S. Hara, T. Inoue, and A. Miyama, *Biochem. Biophys. Res. Commun.*, **125**, 845 (1984); T. Takao, N. Tominaga, S. Yoshimura, Y. Shimonishi, S. Hara, T. Inoue, and A. Miyama, *Eur. J. Biochem.*, **152**, 199 (1985).

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

5) T. Takao, Y. Hidaka, M. Kobayashi, O. Nishimura, M. Arita, T. Takeda, H. Imaishi, T. Honda, T. Miwatani, and Y. Shimonishi, the manuscript in preparation.

6) a) S. Yoshimura, T. Takao, H. Ikemura, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Bull. Chem. Soc. Jpn.*, **57**, 1381 (1984); b) S. Yoshimura, H. Ikemura, H. Watanabe, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, T. Miwatani, and Y. Takeda, *FEBS. Lett.*, **181**, 138 (1985); c) H. Ikemura, S. Yoshimura, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Bull. Chem. Soc. Jpn.*, **57**, 2550 (1984).

7) Y. Shimonishi, Y. Hidaka, M. Hane, M. Koizumi, S. Aimoto, T. Takeda, T. Miwatani, and Y. Takeda, *FEBS Lett.*, **215**, 165 (1987).

8) S. Yoshimura and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, **58**, 2805 (1985).

9) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).

10) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).

11) Y. Takeda, T. Takeda, T. Yano, K. Yamamoto, and T. Miwatani, *Infect. Immun.*, **25**, 978 (1979).

12) R. A. Houghten, J. M. Ostresh, and F. A. Klipstein, *Eur. J. Biochem.*, **145**, 157 (1984).

13) S. K. Chan and R. A. Giannella, *J. Biol. Chem.*, **256**, 7744 (1981).