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Mode of disulfide bond formation of a heat-stable enterotoxin (ST_h) produced by a human strain of enterotoxigenic *Escherichia coli*

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To determine the modes of three disulfide linkages in the heat-stable enterotoxin (ST_h) produced by a human strain of enterotoxigenic *Escherichia coli*, we synthesized ST_h(6–18), which consists of 13 amino acid residues and has the same intramolecular disulfide linkages as native ST_h [(1985) FEBS Lett. 181, 138–142], by stepwise and selective formation of disulfide bonds using different types of removable protecting groups for the Cys residues. Synthesis of the peptide with different modes of disulfide bond formation provided three peptides consistent with standard ST_h(6–18) in their physicochemical and biological properties, thereby indicating that the disulfide bonds in ST_h(6-18) are

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

Thermal stability; Enterotoxin; Disulfide bond; (*E. coli*)

1. INTRODUCTION

Enterotoxigenic *Escherichia coli* elaborates two kinds of heat-stable enterotoxins (STI and STII), which are responsible for acute diarrhea in children in developing countries in the tropics and in domestic animals [1]. STI acts on ST-sensitive cells to stimulate guanylate cyclase and to raise the level of cyclic GMP in the cells [2–5], but the mode of biological action of STII is unknown [6]. STIs have been purified from the culture supernatant of human, bovine and porcine strains of enterotoxigenic *E. coli* and their primary structures with 18 or 19 amino acid residues have been determined [7–9], as shown in fig.1. We named the STIs from human and porcine strains of *E. coli* ST_h and ST_p, respectively. The full enterotoxigenic activity of STI has been found to be expressed by a segment with 13 amino acid residues, which includes 6 half-cystines [10]. Recently, STI-like enterotoxins from *Yersinia enterocolitica* [11,12], *Vibrio cholerae* non-01 [13], and *V. mimicus* (unpublished) have been isolated and sequenced (fig.1). These enterotoxins have common regions with 13 amino acid residues including 6 Cys residues, which are located in the same relative positions and linked intramolecularly by three disulfide bonds. The enterotoxins of *Y. enterocolitica* and *V. cholerae* non-01 have similar biological and physicochemical properties to those of STI of *E. coli*, suggesting that these enterotoxins have the same disulfide bonds and therefore similar tertiary structures.

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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: a, see [7]; b, see [8,9]; c, see [11,12]; d, see [13]; e, unpublished. The single letter notations used are cited from [14].

Detailed information on the conformation of STI is necessary for defining the reactive site(s) on STI for expression of the toxicity of STI, an anti-STI antibody recognition site(s), and the mechanism of the biological system involving STI. On the basis of the chemical syntheses of STI analogues with 13 amino acid residues and various kinds of disulfide bonds and their physicochemical and biological properties, in this paper we propose the mode of disulfide bond formation in STI.

2. MATERIALS AND METHODS

2.1. *Materials and apparatus*

All chemicals and solvents were reagent grade unless otherwise described. CH_3CN was distilled

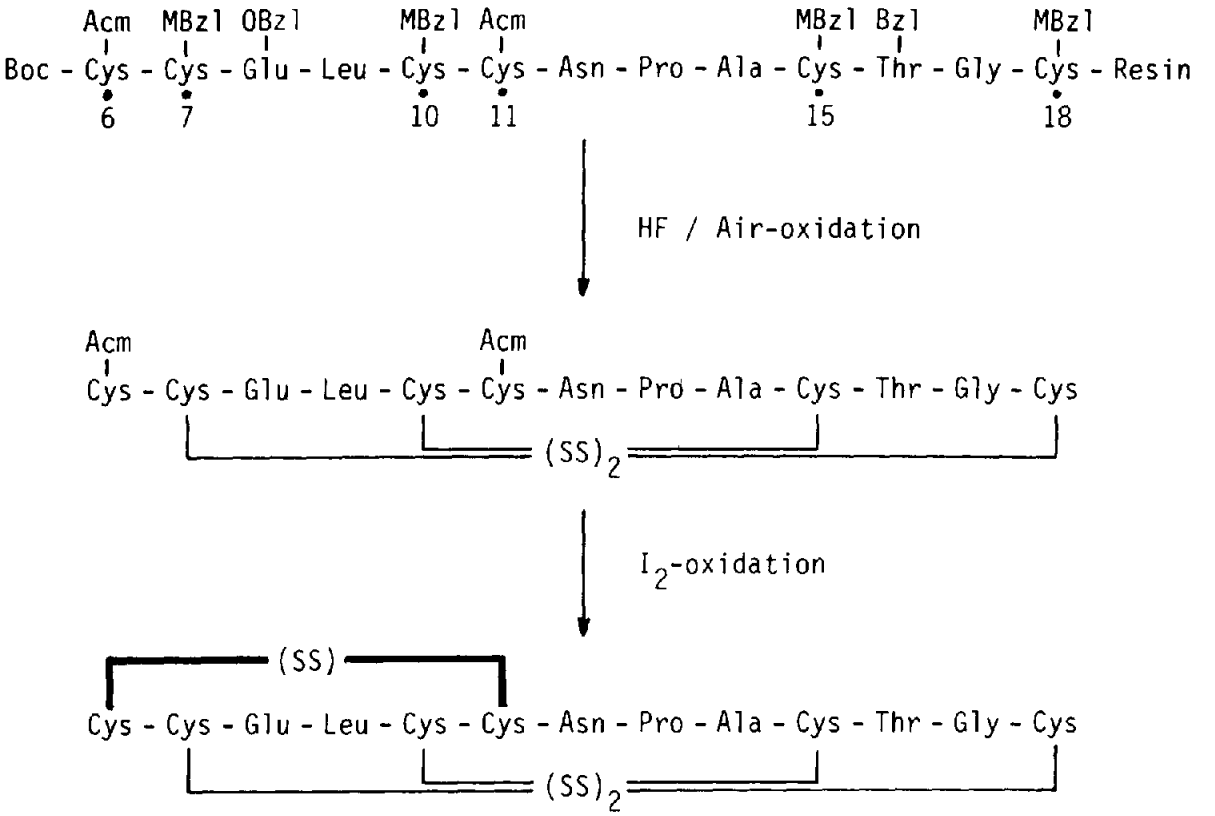

Fig.2. Scheme for synthesis of a shorter analogue, $ST_h(6–18)$, of a heat-stable enterotoxin (ST_h) of *E. coli* by stepwise formation of disulfide bonds.

from phosphorus pentoxide before use for HPLC. The HPLC apparatus consisted of a Hitachi 655 liquid chromatograph equipped with a multiwave ultraviolet monitor, type 655M (Tokyo, Japan). A reversed-phase resin (YMC-ODS, S-5) was purchased from Yamamura Chemical Laboratories (Kyoto, Japan) and packed into a column (4.6 × 250 mm) in our laboratory.

2.2. *Syntheses of peptides*

Peptide synthesis was performed manually by the solid-phase method [15]. The following protecting groups for functional groups were used, *t*-butyloxycarbonyl for -amino groups; 4-methylbenzyl (MBzl) or acetamidomethyl (Acm) for thiol groups of cysteine residues; and benzyl for the -carboxyl of glutamic acid and the hydroxyl group of threonine. The protected peptide resin (70 mg) containing ~0.01 mmol peptide was treated with anisole in anhydrous liquid hydrogen fluoride (HF) [16] at 0°C for 60 min for cleavage of the peptidyl resin linkage and removal of all the protecting groups except Acm groups at given Cys residues. The HF was removed on evaporation in vacuo, and the resulting peptide was extracted into 10% aqueous formic acid (3 ml), washed three times with hexane, and air-oxidized in dilute solution (200 ml) at pH 8.0, until no free thiol group was detectable. The solution was lyophilized and the residual material was purified by HPLC, as described below. The purified peptide with Acm groups at given Cys residues was dissolved at 5×10^{-4} M in MeOH-H_2O (v/v, 4:1), dropped into the same volume of MeOH-1 M HCl (v/v, 4:1)

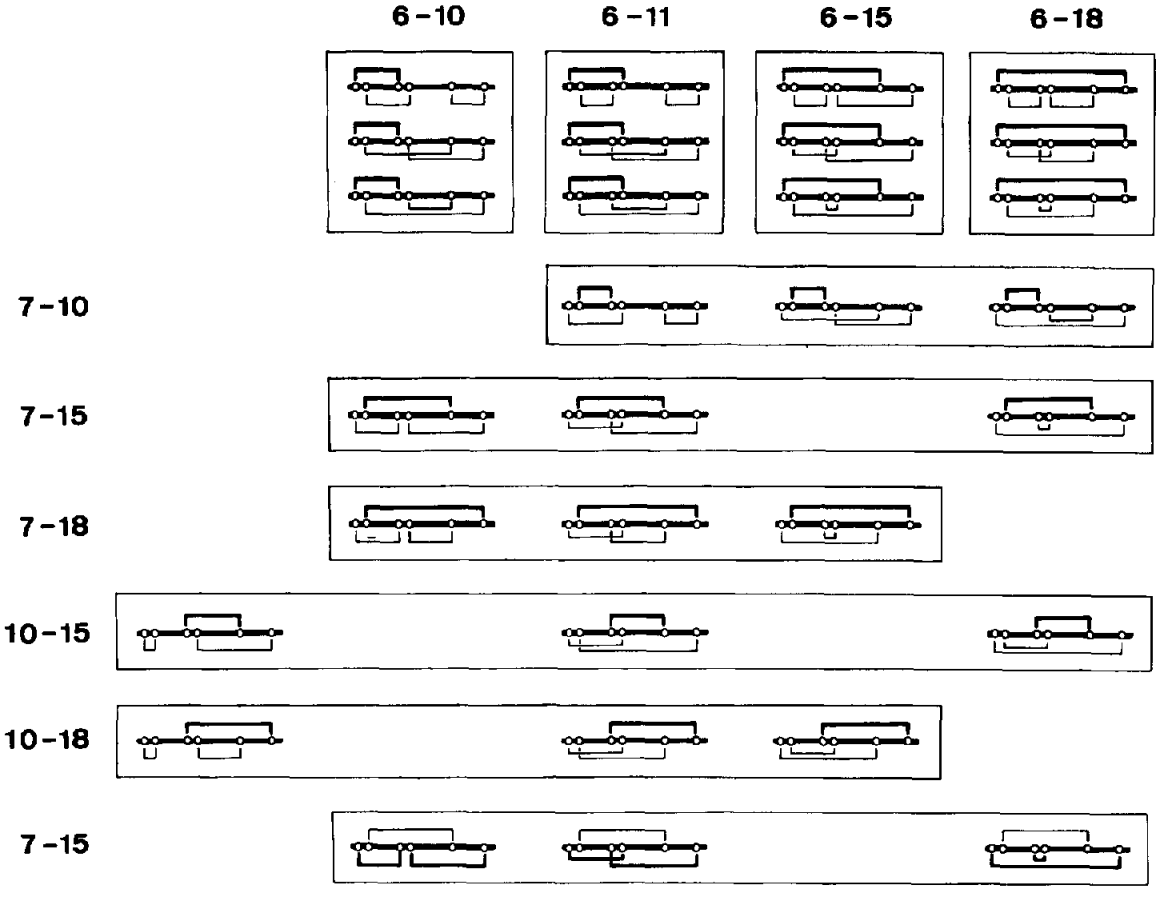

Fig.3. Sets of peptides synthesized by the procedure described in fig.2. Thin lines indicate disulfide bonds formed first, and thick lines those formed finally. Numbers denote positions of Cys residues linked selectively by a disulfide bond.

containing I_2 (40 equivalents of the sample peptide), and stood for 15 min at room temperature [17]. The reaction was stopped by adding L-ascorbic acid and the reaction product was directly purified by HPLC.

2.3. *Purification of synthetic peptides*

The air-oxidized or I_2-oxidized peptide was purified by HPLC on a reversed-phase column. The peptide was eluted with a linear gradient of 10–40% CH_3CN in 0.05% trifluoroacetic acid or 0.01 M ammonium acetate (pH 5.7) with increase of CH_3CN concentration of 0.5%/min or 1%/min at a flow rate of 1 ml/min. Fractions of eluate were monitored for absorbance at 220 nm.

2.4. *Analysis of purified peptides*

The amino acid compositions and molecular masses of purified peptides were examined by amino acid analysis and fast atom bombardment mass spectrometry, respectively, as described [12].

2.5. *Biological assay*

Toxic activity was assayed in suckling mice of 2–4 days old as described [18].

3. RESULTS AND DISCUSSION

Reduction of the disulfide bonds in STI results in loss of its toxic activity, indicating that the tertiary structure formed by the disulfide bonds is necessary for expression of this toxicity. We previously found that the sequences of both ST_h and ST_p consisting of 13 amino acids and including 6 half-cystine residues have the same toxic activities as native toxins [10]. These findings suggested that the structural information for the biological properties of STI is involved in the segments formed from these 13 amino acid residues.

Two general procedures for determination of disulfide bridges in peptides and proteins (partial acid or enzymatic hydrolysis of peptides and proteins and selective reduction and alkylation of disulfide bonds in peptides and proteins) were applied for determination of the disulfide bonds of ST_h, but without success. Therefore, in this work, we adopted a stepwise synthetic procedure to form disulfide bonds using selectively removable pro-

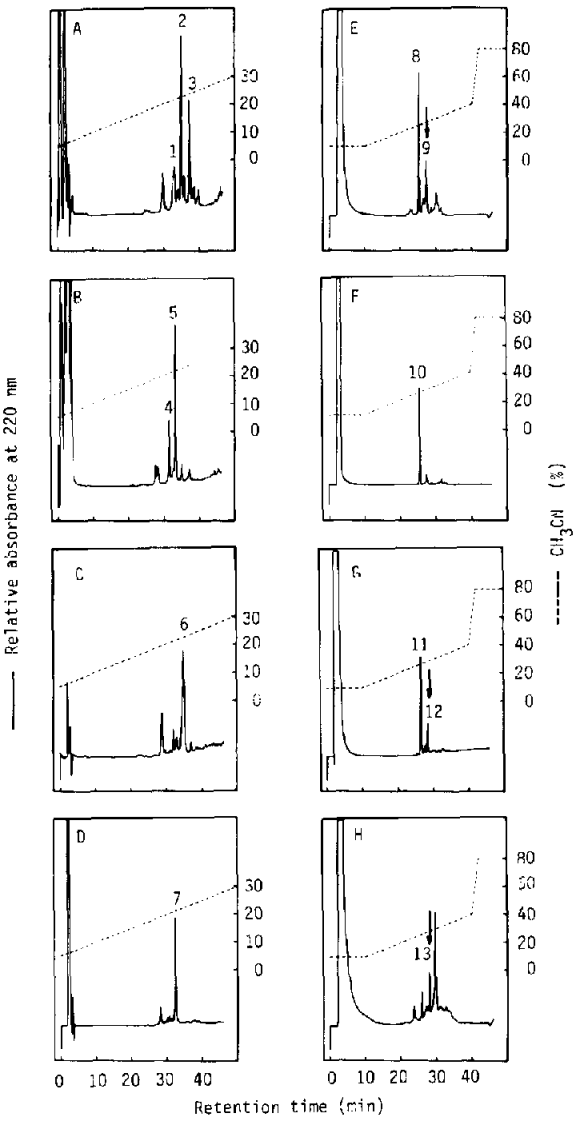

Fig.4. Reversed-phase HPLC of (A) a deprotected and air-oxidized solution of peptides with two disulfide bonds at position 7, 10, 15, and 18; (B) a deprotected and air-oxidized solution of peptides with two disulfide bonds at positions 6, 10, 11, and 18; (C) a deprotected and air-oxidized solution of peptides with two disulfide bonds at positions 6, 7, 11, and 15; (D) a deprotected and air-oxidized solution of peptides with a disulfide bond at positions 7 and 15; (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 6 in C; (H) an I_2-oxidized solution of peak 7 in D.

Table 1

Amino acid compositions[a], mass values and toxic activities of synthetic $ST_h(6-18)$[b]

	Peak 8	Peak 9	Peak 10	Peak 11	Peak 12	Peak 13	Theoretical value
Asp	1.00	1.03	1.03	1.00	1.00	1.11	1
Thr	1.01	0.99	1.01	1.03	1.02	1.03	1
Glu	1.03	1.02	1.05	1.02	1.02	1.06	1
Gly	0.96	0.99	1.04	1.00	1.05	1.05	1
Ala	1	1	1	1	1	1	1
Cys	4.74	4.39	5.37	5.13	4.96	5.26	6
Leu	1.11	0.97	1.00	1.10	1.02	1.04	1
Pro	0.98	1.09	1.01	0.98	0.92	0.96	1
$[M+H]^+$	1313.1	1313.2	1313.2	1313.3	1313.2	1313.1	1313.4
MED[c]	9	0.7	12	10	0.9	0.3	0.6[d]

[a] Values are those in acid hydrolysates (110°C, 24 h) of synthetic peptides and are shown as mol/mol of Ala

[b] Peak 8, 9, etc. correspond to those in fig.4

[c] MED, minimum effective dose

[d] This value is cited from [10]

tecting groups at Cys residues, as shown in fig.2, and selectively synthesized peptides with nine sets of disulfide bonds: that is, between 6 and 10, 6 and 11, 6 and 15, 6 and 18, 7 and 10, 7 and 15, 7 and 18, 10 and 15, and 10 and 18, as shown in fig.3. Of the peptides with these sets of disulfide bonds, the three in which two disulfide bonds were first formed between four Cys residues except Cys-6 and Cys-11 or Cys-10 and Cys-18 and the remaining one disulfide bond then between Cys-6 and Cys-11 or Cys-10 and Cys-18, respectively, or one disulfide bond was first formed between Cys-7 and Cys-15 and the remaining two disulfide bonds then between the remaining four Cys residues, were identical on HPLC with standard $ST_h(6-18)$, as shown in fig.4. Other peptides did not have the same retention time as standard $ST_h(6-18)$ on HPLC.

The amino acid compositions and mass values of the peptides obtained were identical with the theoretical values for $ST_h(6-18)$ within the limits of experimental error, as summarized in table 1. Moreover, the toxicities, that is the minimum effective doses, of these peptides were 0.3–1.0 ng, which were almost the same as that (0.6 ng) of standard $ST_h(6-18)$ [10]. These activities were

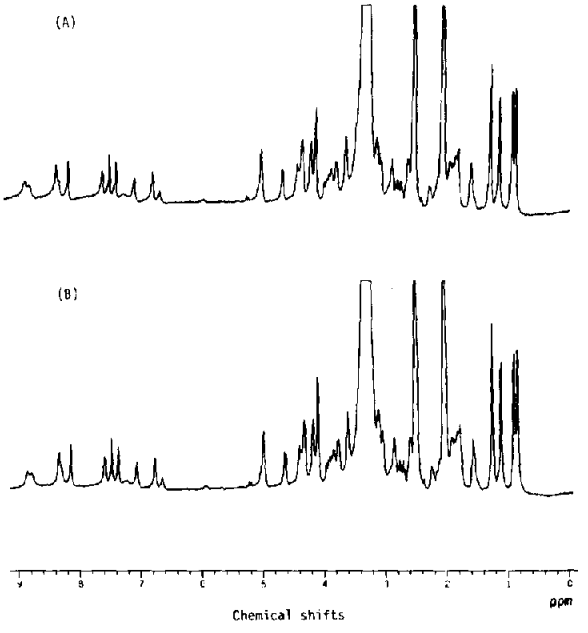

Fig.5. 500 MHz ^{1}H-NMR spectra in a mixture of CD_3CN and DMSO-d_6 (v/v, 15:85). (A) A peptide linked between Cys-6 and Cys-11 as a third disulfide bond and (B) standard $ST_h(6-18)$.

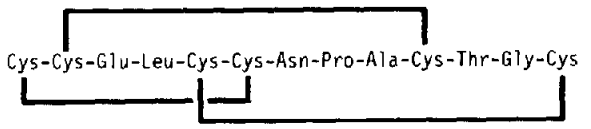

Fig.6. Mode of three disulfide linkages of $ST_h(6–18)$.

neutralized by anti-native ST_h polyclonal antibodies. Furthermore, the ^{1}H-NMR spectra of these peptides were identical with that of standard $ST_h(6–18)$, as shown in fig.5.

Thus, we conclude that the disulfide linkages in $ST_h(6–18)$ are formed between Cys-6 and Cys-11, Cys-7 and Cys-15, and Cys-10 and Cys-18, as shown in fig.6. We recently found that $ST_p(5–17)$, which has Ala in place of Thr in $ST_h(6–18)$, has the same disulfide bonds as $ST_h(6–18)$ (not shown). This indicates that *E. coli*-ST_h and ST_p have the same disulfide bonds as those shown in fig.6.

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