Vol. 161, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1989 Pages 229-235

A Long-acting Heat-stable Enterotoxin Analog of Enterotoxigenic Escherichia coli with a Single D-Amino Acid

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Received April 12, 1989

SUMMARY: A heat-stable enterotoxin (STp) consisting of 18 amino acid residues including 6 half-cystine residues is produced by a porcine strain of enterotoxigenic Escherichia coli. Analogs of STp with replacements of single residues at each from positions 5 to 17 by the corresponding D-amino acid residue were synthesized by a solid-phase method. Of these analogs, [D-Cys^5]-STp[5-17] showed the same biological properties as STp[5-17]. Moreover, its activity to cause fluid accumulation in suckling mouse lasts more than 24 hours, whereas the activity of STp[5-17] decreases after 6-10 hours. These results indicate that the action of the analog [D-Cys^5]-STp[5-17] is strongly agonistic to that of the native ST.

Enterotoxigenic Escherichia coli produces two kinds of heat-stable enterotoxin, ST_h and STp, that cause acute diarrhoea in humans and domestic animals [1]. Similar heat-stable enterotoxins are produced by several enteric bacteria, such as Yersinia enterocolitica, Vibro cholerae non-01, and Vibro mimicus. Recently, great progress has been made in studies on the molecular characters of ST: Its primary and intramolecular disulfide bond structures [2-7], molecular conformation [8-10], the epitope structure on ST recognized by a monoclonal antibody against ST [11-13] and its binding protein(s) on rat instestinal cell membranes [14-16] have been clarified. However, little is known about the mechanism of recognition of the ST molecule by its binding protein(s) on intestinal cell membranes or the fate of the ST molecule after its binding to the protein(s). For the study on these biological processes it is essential to develop agonists or antagonists of ST and also to determine the structure-function relationship of the molecule.

In this paper, we report the syntheses of ST analogs with single replacements of amino acid residues with D-amino acid residues and toxic activities of these synthetic ST analogs. It was found that an analog with D-Cys at position 5 in STp of a porcine strain of enterotoxigenic E. coli showed the same magnitude of toxicity as native STp and that the activity of the analog to cause fluid accumulation in

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0006-291X/89 $1.50

suckling mouse lasted more than 24 hours whereas the activity of STp[5-17] decreased after 6-10 hours.

MATERIALS AND METHODS

Syntheses of Peptides: Peptides were synthesized manually by the solid-phase method [6, 17]. Briefly, Boc-Cys(MBzl) or Boc-D-Cys(MBzl)-polystyrene (2% divinylbenzene) (0.3 mmol of amino acid per g of resin) was used with the following groups for protection of side-chains : Bzl for glutamic acid and MBzl (4-methyl benzyl) for cysteine. All couplings were carried out with 6-fold excess of Boc-amino acid and DCC, and the coupling of Boc-Asn-OH was repeated twice with 2 eq of 1-hydroxybenzotriazole. After coupling all the amino acid residues, the protected peptide-resin was treated with 10 molar excess of anisole to protecting groups in anhydrous HF at 0°C for 75 min. After removal of HF under reduced pressure, the peptide was extracted with 10% formic acid and washed three times with hexane. The extract was diluted with water to a final peptide concentration of 5×10^{-5}M and the pH of the solution was adjusted to 8.0 by adding aqueous ammonia. This solution was stood at room temperature with occasional stirring until no free mercapto-group was detectable with Ellman's reagent. The peptide was purified by HPLC, as described below.

High-performance Liquid Chromatography (HPLC): The HPLC apparatus consisted of a Waters M600 multisolvent delivery system (Milford, MA) and Hitachi UV 655A variable wavelength UV monitor and D-2000 chromato-integrator (Tokyo, Japan). The HPLC column (4 x 250 mm or 8 x 300 mm) packed with YMC-ODS (s-5) (Yamamura Chemical Laboratories Co., Kyoto, Japan) was equilibrated with 10% CH_3CN in 0.05% trifluoroacetic acid or 0.01M $AcONH_4$ (pH 5.7) and after injection of the peptide solution, it was developed with increase in CH_3CN concentration of 1 %/min at a flow rate of 1 or 2 ml/min. The absorption of the eluate at 220 nm was monitored.

Analyses of Purified Peptides: The amino acid compositions and molecular weights of purified peptides were examined by amino acid analysis and fast atom bombardment mass spectrometry, respectively, as described previously [18].

Toxic Activity: The toxicity of synthetic peptides was assayed in suckling mouse of 2 days old (1.7 ± 0.1 g) as described previously [19]. An aliquot of 0.1 ml of each sample was administered orally. Three hours after the administration, the animal was sacrificed. Fluid accumulation (FA) ratio was calculated as the ratio of the weight of the entire intestine to that of the rest of the body. One mouse unit (MU) was defined as a minimum effective dose that gives FA ratio more than 0.09.

Receptor Binding Assay: The binding of synthetic peptides to the receptor protein(s) was assayed using brush-border membranes isolated from male Sprague-Dawley rats (8 weeks old, 200-250 g), as described [16]. 5-Azido-2-nitrobenzoyl(ANB)-STh[5-19] and ^{125}I-ANB-STh[5-19] were synthesized as described [16].

RESULTS AND DISCUSSION

Figure 1 shows the primary and disulfide bond structures of STs produced by enteric bacteria [2-7]. The activities of these STs to cause fluid accumulation in suckling mouse are located on the spatial structure formed by 13 amino acid residues including 6 half-cystine residues that are circled by a dotted line [20]. For developing agonists or antagonists of STs or elucidating the stereospecific requirements of the amino acid residues for their receptor binding, we synthesized analogs of STp, which consisted of these 13 amino acid residues with a single replacements of each amino acid residues with their corresponding D-isomer except

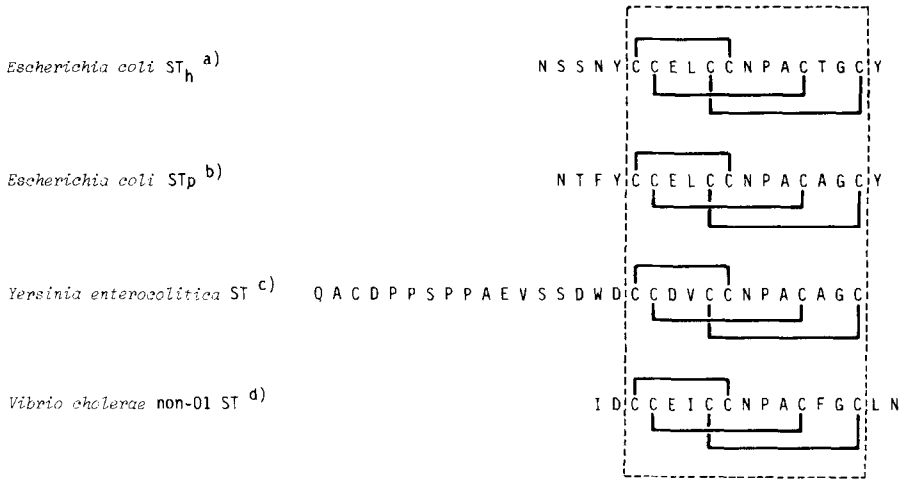

Fig. 1. Primary and disulfide bond structures of ST (ST_p) isolated from a) strain SK-1 [2,6] and b) strain 18D [3,7] of enterotoxigenic E. coli, c) Y. enterocolitica [4], and d) V. cholerae non-01 [5].

for Gly at position 16. The analogs were prepared by a solid-phase method. The peptides liberated from a polymer-support were air-oxidized in dilute aqueous solution to link the three intramolecular disulfide bonds and were purified by HPLC. The purified products were analyzed by amino acid and mass spectrometric analyses.

The activities of the synthetic analogs to cause fluid accumulation in suckling mouse are summarized in Table 1. The analogs [D-Cys^5]-, [D-Cys^9]-, and [D-Cys^{17}]-STp[5-17] (No. 2, 6, and 13 in Table 1, respectively) had almost the same activities as STp[1-18] (Ref. 20) and STp[5-17] (No. 1 in Table 1), and these toxic activities were completely neutralized by an anti-ST_h monoclonal antibody that neutralizes the toxic activities of synthetic STp and ST_h [21]. These peptides were also found by the procedure described previously [6] to have the same structure of disulfide bonds as that in STp. The analogs [D-Pro^{12}]- and [D-Ala^{15}]-STp[5-17] (No. 9 and 12 in Table 1) had only one-twentieth and one-tenth, respectively, of the activity of STp[5-17]. These results indicate that the conformation of STp[5-17] is not influenced by substitutions of amino acid residues at these positions with the corresponding D-enantiomers or that the conformational change of STp[5-17] caused by such substitutions, if any, does not affect the biological activity. Similarly, analogs of ST_h of E. coli and STs of Y. enterocolitica and V. cholerae non-01 (No. 14-20 in Table 1), in which the same amino acid residues were replaced by either D-Cys or β-mercaptopropionic acid, had almost the same activities as those of the corresponding native STs. On the other hand, the toxic activities of analogs in which residues at other positions were replaced by the corresponding D-amino acid residues were very weak. These results suggest that amino acid residues at these other positions are involved in the binding of the STs to the receptor protein(s), or that the chirality of amino acid residues at these positions is important for the

Table 1. Analogs of heat-stable enterotoxin (ST_p) in which single amino acids at each position were replaced by their corresponding D-amino acid residues

No.	5 Cys	6 Cys	Glu	Leu	9 Cys	10 Cys	Asn	Pro	Ala	14 Cys	Ala	Gly	17 Cys	MED*(ng)
1**	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6
2	D-Cys													0.6
3		D-Cys												$5.2x10^3$
4			D-Glu											$1.4x10^3$
5				D-Leu										$7.1x10^3$
6					D-Cys									1.2
7						D-Cys								$3.6x10^3$
8							D-Asn							$11x10^3$
9								D-Pro						20
10									D-Ala					760
11										D-Cys				470
12											D-Ala			5.8
13													D-Cys	1.7
14	D-Cys										Thr			0.4
15	D-Cys			Ile							Phe			0.6
16	D-Cys		Asp	Val										1.1
17	β-Mpr***													0.3
18	β-Mpr										Thr			0.6
19	β-Mpr			Ile							Phe			0.7
20	β-Mpr		Asp	Val										3.7

*, minimum effective dose

**, all L-amino acids

***, β-mercaptopropionic acid

folding of the peptide chain and hence for the construction of the spatial structure necessary for its binding to the receptor protein(s).

Next, we examined the bindings of the synthetic analogs to the receptor protein(s) on rat intestinal cell membranes using a radio-iodinated photo-affinity-labeled ST analog, ^{125}I-ANB-ST_h[5-19], which was synthesized as described previously [16]. The binding of ^{125}I-ANB-ST_h[5-19] to the receptor protein(s) on the cell membranes was competitively inhibited by an analog [D-Cys^5]-STp[5-17] at the same

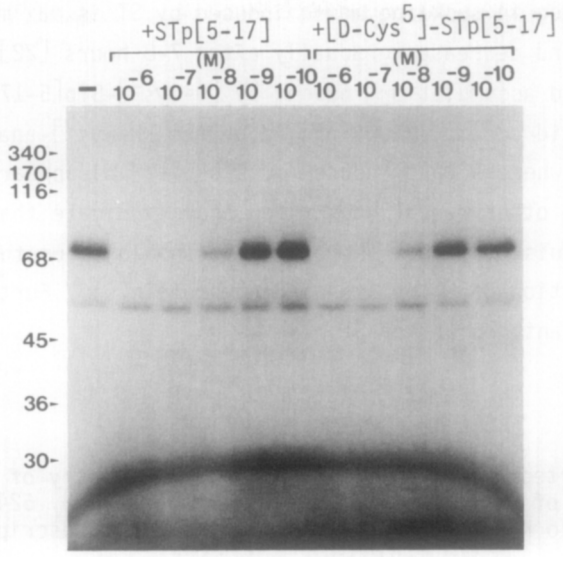

Fig. 2. SDS polyacrylamide gel electrophoresis of the binding protein(s) from rat intestinal cell membranes labeled with ANB-ST_h[5-19].

molar concentration as that of STp[5-17] (Fig. 2). The binding of ^{125}I-ANB-ST_h[5-19] to the receptor protein(s) was also inhibited completely by analogs [D-Cys^9]- and [D-Cys^{17}]-STp[5-17] at the same molar concentration as that of STp[5-17] (data not shown). On the contrary, the analogs with low toxic activity inhibited the binding of ^{125}I-ANB-ST_h[5-19] to the protein(s) only at higher concentrations, or incompletely (data not shown). These results indicate that toxic analogs [D-Cys^5]-, [D-Cys^9]-, and [D-Cys^{17}]-STp[5-17] can bind to the receptor protein(s) similarly to STp and hence imply that these toxic peptides have similar spatial structures to the structure of STp[5-17] that is essential for its binding to the receptor protein(s).

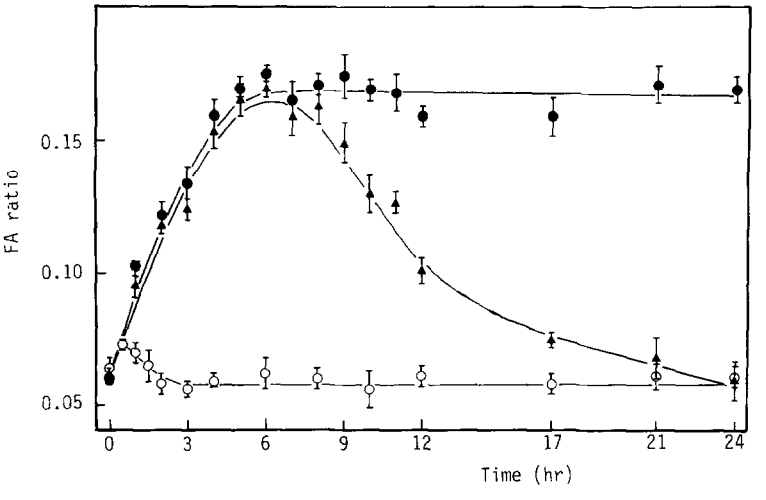

Fig. 3. Time course of fluid accumulation caused by ST_p[5-17] (▲), [D- Cys^5]-ST_p[5-17] in 0.1 ml of PBS or 0.1 ml of PBS alone (●), and phosphate buffered saline (PBS) (○) in suckling mouse. Either 3 MU of each ST was administered to suckling mouse. Values are means of three determinations.

Fluid accumulation in suckling mouse induced by ST is maximal 3-5 hours after its administration and decreases gradually after 7-8 hours [22]. We compared the time courses of fluid accumulation induced by [D-Cys^5]-STp[5-17] and STp[5-17]. As shown in Fig. 3, fluid accumulation induced by the [D-Cys^5]-analog persisted for more than 24 hours, whereas that induced by STp[5-17] disappeared after 9-10 hours. This finding and the other results described above indicate that [D-Cys^5]-STp[5-17] acts as a strong agonist of STp. Therefore, it should be useful in studies on the mechanism of recognition of ST by its binding protein(s). Further work is in progress to develop antagonists of ST.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid from the Ministry of Education, and Science and Culture of Japan (Scientific Research Grant No. 62470022). We are indebted to Mrs. Aiko Kobatake for preparation of the manuscript.

REFERENCES

1. Smith, H. W., and Gyles, C. L. (1970) J. Med. Microbiol. 3, 387-401.
2. Aimoto, S., Takao, T., Shimonishi, Y., Hara, S., Takeda, T., Takeda, Y., and Miwatani, T. (1982) Eur. J. Biochem. 129, 257-263.
3. Takao, T., Hitouji, T., Aimoto, S., Shimonishi, Y., Hara, S., Takeda, T., Takeda, Y., and Miwatani, T. (1983) FEBS Lett. 152, 1-5; Thompson, M. R., and Giannella, R. A. (1985) Infect. Immun. 47, 834-836.
4. Takao, T., Tominaga, N., Shimonishi, Y., Hara, S., Inoue, T., and Miyama, A. (1984) Biochem. Biophys. Res. Commun. 125, 845-851.
5. Takao, T., Shimonishi, Y., Kobayashi, M., Nishimura, O., Arita, M., Takeda, T., Honda, T., and Miwatani, T. (1985) FEBS Lett. 193, 250-254.
6. Shimonishi, Y., Hidaka, Y., Koizumi, M., Hane, M., Aimoto, S., Takeda, T., Miwatani, T., and Takeda, Y. (1987) FEBS Lett. 215, 165-170.
7. Hidaka, Y., Kubota, H., Yoshimura, S., Ito, H., Takeda, Y., and Shimonishi, Y. (1988) Bull. Chem. Soc. Jpn. 61, 1265-1271.
8. Ohkubo, T., Kobayashi, Y., Shimonishi, Y., Kyogoku, Y., Braun, W., and Go, N. (1986) Biopolymers 25, S123-S134.
9. Gariepy, J., Lane, A., Frayman, F., Wilbur, D., Robien, W., Schoolnik, G., and Jardetzky, O. (1986) Biochemistry 25, 7854-7866.
10. Yamasaki, S., Hidaka, Y., Ito, H., Takeda, Y., and Shimonishi, Y. (1988) Bull. Chem. Soc. Jpn. 61, 1701-1706.
11. Brandwein, H., Deutsch, A., Thompson, M., and Giannella, R. (1985) Infect. Immun. 47, 242-246.
12. Hemelhof, W., Retore, P., De Mol, P., Butzler, J. P., Takeda, T., Miwatani, T., and Takeda, Y. (1984) Lancet i, 1011-1012.
13. Svennerholm, A. M., Wikstrom, M., Lindblad, M., and Holmgren, J. (1986) J. Clin. Microbiol. 24, 585-590.
14. Waldman, S. A., Kuno, T., Kamisaki, Y., Chang, L. Y., Gariepy, J., O'Hanley, P., Schoolnik, G. K., and Murad, F. (1986) Infect. immun. 51, 320-326.
15. Gariepy, J., Judd, A. K., and Schoolnik, G. K. (1987) Proc. Natl. Acad. Sci., USA. 84, 8907-8911.
16. Hirayama, T., Oku, Y., Takeda, Y., Iwata, N., Aimoto, S., and Shimonishi, Y. (1987) Abstract of the 23rd Joint Conference on Cholera -The U.S.-Japan Cooperative Medical Science Program-, pp.102-103, Williamsburg, Virginia, Nov.10-12, 1987.
17. Merrifield, R. B. (1963) J. Am. Chem. Soc. 85, 2149-2154.
18. Takao, T., Tominaga, N., Yoshimura, S., Shimonishi, Y., Hara, S., Inoue, T., and Miyama, A. (1985) Eur. J. Biochem. 152, 199-206.

19. Takeda, Y., Takeda, T., Yano, T., Yamamoto, K., and Miwatani, T. (1979) Infect. Immun. 25, 978-985.
20. Yoshimura, S., Ikemura, H., Watanabe, H., Aimoto, S., Shimonishi, Y., Hara, S., Takeda, T., Miwatani, T., and Takeda, Y. (1985) FEBS Lett. 181, 138-142.
21. Takeda, T., Yokoo, Y., Yamasaki, S., Ito, H., Takeda, Y., and Shimonishi, Y. (1987) Abstract of the 23rd Joint Conference on Cholera -The U.S.-Japan Cooperative Medical Science Program-, pp.50-51, Williamsburg, Virginia, Nov.10-12, 1987.
22. Aimoto, S., Watanabe, H., Ikemura, H., Shimonishi, Y., Takeda, T., Takeda, Y., and Miwatani, T. (1983) Biochem. Biophys. Res. Commun. 112, 320-326.