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Effect of Alterations of Basic Amino Acid Residues of *Escherichia coli* Heat-Stable Enterotoxin II on Enterotoxicity

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***Escherichia coli* heat-stable enterotoxin II (STII) is composed of 48 amino acid residues. Among these, one histidine, two arginine, and six lysine residues are basic. Isoelectric focusing showed that the isoelectric point of STII is 9.7, indicating that the side chains of some of these basic amino acid residues project outside the molecule. To understand the role that these basic amino acid residues play in toxicity, STII was chemically modified with ethoxyformic anhydride, maleic anhydride, and phenylglyoxal, which alter the side chains of basic amino acid residues in proteins. Maleic anhydride, which modifies the ε amino group, caused a significant loss of enterotoxic activity, but the other two modifiers did not. This indicated that lysine residues play an important role in the expression of the enterotoxic activity of STII and that the contribution of the other basic amino acid residues to the toxicity is relatively low. To confirm this hypothesis, we substituted these nine basic amino acid residues by oligonucleotide-directed site-specific mutagenesis and examined the enterotoxicity of these purified mutant STIIs. The enterotoxic activity was reduced when the lysine residues at positions 18, 22, 23, and 46 were substituted. In particular, the substitution at positions 22 and 23 induced a remarkable reduction. These results demonstrate that the lysine residues at positions 22 and 23 are very important in the expression of the enterotoxic activity of STII.**

Enterotoxigenic *Escherichia coli* strains produce two kinds of heat-stable enterotoxin (STs), which cause intestinal secretion and diarrhea (2, 10). One is termed STI (also referred to as STa), and the other is STII (also referred to as STb). STI is an 18- or 19-amino-acid acidic peptide containing three disulfide bonds and protease-stable peptide (1, 30, 33). STI is active in the suckling mouse assay. The initial step in the action of ST is its interaction with specific high-affinity receptors (12). It has been reported that an STI receptor is guanylate cyclase. The binding of STI to the receptor induces an intracellular increase in cyclic GMP (7, 29). The rise in cyclic GMP presumably results in a rapid increase in net chloride secretion (7, 10). On the other hand, STII was reported to be active only in the weaned-pig ligated intestinal loop assay (3, 17, 34). Because of the lack of a convenient toxin assay, STII was not purified for some time after its detection. Afterwards, STII was reported to be active in the rat intestinal loop assay in the presence of a protease inhibitor (35). Subsequently, we found that STII is also active in the mouse loop test in the presence of protease inhibitor. We purified STII by using this assay and confirmed that the toxin is composed of 48 amino acid residues that contain two disulfide bonds, between Cys-10 and Cys-48 and between Cys-21 and Cys-36 (8). Subsequently, we showed that STII does not increase the levels of cyclic AMP and cyclic GMP, demonstrating that STII induces secretion by a mechanism that is different from that of STI and cholera toxin (13). Recently, Dreyfus et al. suggested that this mechanism involved the increase of intracellular calcium concentration, which is known as one of the mediators of intestinal ion and fluid movement (5). However, the experiments of Dreyfus et al. are insufficient to determine the secondary messengers of STII action.

The analysis of the tertiary structure of STI revealed that STI has a right-hand spiral structure consisting of three β turns fixed by three disulfide linkages (27). The studies on the structure-activity showed that Asn and Ala residues at positions 11 and 13 are important for expression of the toxicity of STp (9, 25, 26, 36). However, the tertiary structure of STII has not been determined, and the functionally important regions of STII have remained unclear. STII is easily degraded by trypsin (8, 34), which catalyzes the hydrolysis of peptide bonds on the C-terminal side of the basic side chains of arginine and lysine (31). This indicates that some side chains of lysine and arginine residues of STII project outside the molecule. These positively charged side chains of amino acid residues of an enzyme can interact with negatively charged groups of substrates. Therefore, basic amino acid residues are often included in the active site of enzymes (16). Furthermore, some basic amino acid residues contribute to maintaining the conformation of a protein throughout interactions with side chains of acidic amino acid residues in the same protein (20). We therefore presumed that the projecting side chains of the basic amino acid residues of STII would play an important role in the expression of enterotoxicity. In this study, we examined the role of the basic amino acid residues of STII in the enterotoxic activity by using chemical modification and oligonucleotide-directed site-specific mutagenesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* HB101 was used as the host strain throughout and was cultured in Luria broth (24). Plasmid pCHL7, which carries the *E. coli* STII gene, was provided by H. W. Moon. The 5.7-kb plasmid was constructed by inserting the 1.3-kb *Hin*dIII-*Hin*dIII fragment of P307 at the *Hin*dIII site of pBR322 (19). We deleted the 500-bp *Pst*I-*Cla*I

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TABLE 1. Plasmids and mutagenic oligonucleotides

Mutant plasmid	Mutagenic oligonucleotide[a]	Replacement of amino acid[b]	Recognition site induced by mutation
pTB252	ATCAAATAATA**T**T**G**ATCTGTGT	Lys-6, Lys-7→Asn, Ile	*Ssp*I
pTB253	CTGTGTGAA***G**TATA*C*AGACAAATA	His-12→Val	*Acc*I
pTB254	GAACATTATC***T**GCA*G*ATAGCC	Arg-14→Leu	*Pst*I
pTB255	AAATAGCC***G**GC*G*AAAGTTGT	Lys-18→Gly	*Cfr*10I
pTB256	GAAAGTTGTC***T**TAA*G*GGTTTTTTA	Lys-22→Leu	*Afl*II
pTB257	AAAAAACC***G**GT*T*TTACAA	Lys-23→Thr	*Cfr*10I
pTB258	AAAGTTGTA**T**TAA*T*GGTTTTTTA	Lys-22, Lys-23→Ile, Asn	*Ase*I
pTB259	GGGGTTA**T**CGATGGTACTG	Arg-29→Ile	*Cla*I
pTB260	GTTGCAGCA***T**CCGGATGCTAA	Lys-46→Ser	*Hpa*II
pTB261	[AAATAGCC***G**GC*G*AAAGTTGT / GTTGCAGCA***T**CCGGATGCTAA]	Lys-18, Lys-46→Gly, Ser	*Cfr*10I, *Hpa*II

[a] Asterisks indicate mismatches with bases of the template plasmid.
[b] Numbers indicate the positions of amino acid residues from the amino-terminal residues of STII composed of 48 amino acid residues.

fragment, which did not contain the STII gene, from plasmid pCHL7. This plasmid, designated pTB251, was used as the wild-type plasmid in this study.

Isoelectrofocusing. The isoelectric point of STII was measured by flat-bed electrofocusing by using an Ultrodex gel and ampholine (pH 9 to 11; Pharmacia LKB, Uppsala, Sweden). Two milligrams of the purified STII was mixed with Ultrodex gel (0.8 g) and ampholine (0.8 ml). The mixture was resolved by electrophoresis after being smeared on a glass plate (14 by 5 cm). Thereafter, 5-mm-wide pieces of the gel were excised and transferred into 1 ml of distilled water. After the gel pieces and water were mixed well, the solution was separated from the gels by centrifugation and the pH of the solution was measured. The STII in the solution was detected by reversed-phase high-performance liquid chromatography (HPLC) with an ODP-50 column (Asahipak, Kawasaki, Japan). After the sample was loaded, the column was developed with a linear gradient of acetonitrile. STII was eluted as a single peak with about 50% acetonitrile, and the amount of STII in the sample was estimated from the height of the peak.

Chemical modification of STII. Purified STIIs were treated with the following three chemical reagents which can modify the side chains of basic amino acid residues in proteins: ethoxyformic anhydride, maleic anhydride, and phenylglyoxal. One hundred micrograms of the purified STII dissolved in 1 ml of appropriate buffer was incubated with these reagents. The concentrations of these reagents in the reaction mixtures are shown in Fig. 1. After the reaction mixture was incubated, it was diluted with distilled water and the enterotoxic activity of the diluent was determined in the mouse intestinal loop assay.

STII was modified with phenylglyoxal by the method of Takahashi (32). The reaction was performed at 25°C for 1 h in 50 mM *N*-ethylmorpholine acetate buffer (pH 8.0). Thus, modified STII was referred to as phenylglyoxal-treated STII.

STII was modified with maleic anhydride by the method of Butler and Hartley (4). The reaction proceeded at 4°C for 40 min in 20 mM phosphate buffer (pH 8.2). It was then terminated with 220 μg of lysine.

STII was modified with ethoxyformic anhydride by the method of Little (21) at 25°C for 20 min in 100 mM phosphate buffer (pH 6.0). The reaction was terminated by adding 310 μg of histidine to the reaction mixture.

Mouse intestinal loop assay. The enterotoxin activity of STII was determined in a mouse intestinal loop assay. Since STII in the mouse intestinal loop is degraded by intestinal proteases, we added aprotinin (Bayer, Leverkusen, Germany), a protease inhibitor, as described previously (13).

Mice weighing 30 to 35 g were anesthetized with sodium pentobarbital, and the intestines were exteriorized through a midline incision. The intestinal lumen was rinsed three times with saline containing 100 U of aprotinin per ml. After the rinses were performed, a series of ligated intestinal segments (loops), about 4 cm long and separated by a 0.5- to 1-cm interloop, were created. The most-proximal loop was placed about 4 cm distal to the ligament of Treitz. One or two loops were created per intestine. Each loop was injected with 0.2 ml of toxin solution containing 500 U of aprotinin. Three hours after the injection, the mice were killed and the sample activities were measured and expressed as the ratio of the weight of the loop (in grams) to its length (in centimeters). A ratio above 0.20 was regarded as a positive response.

Oligonucleotide-directed mutagenesis and DNA sequencing. Table 1 shows the mutagenic oligonucleotides and the mutant plasmids obtained in this experiment. Mutant plasmids pTB252, pTB253, pTB254, pTB255, pTB256, pTB257, pTB258, pTB259, pTB260, and pTB261 were constructed to produce the following mutant STIIs: STII(K6N, K7I), STII(H12V), STII(R14L), STII(K18G), STII(K22L), STII(K23T), STII(K22I, K23N), STII(R29I), STII(K46S), and STII(K18G, K46S), respectively. To promptly screen the mutated plasmids, the sequences of the mutagenic oligonucleotides were designed to introduce a recognition site for a restriction endonuclease into the mutant STII gene (Table 1). Oligonucleotide-directed mutagenesis was performed with plasmid pTB251 by using the plasmid method described by Inouye and Inouye (15). Plasmid pTB261 which encodes the gene for STII(K18G, K46S) was constructed from plasmid pTB260 by using the oligonucleotide designed to induce the Lys-18→Gly mutation.

DNA sequences were determined by dideoxy chain termination from a double-stranded template with a model 373A DNA sequencing system (Applied Biosystems, Foster City, Calif.). Reactions proceeded as described in the instructions supplied with the Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems). The sequences of the primers used for the sequencing were 5′-TTTAACTGTGATAAACTACC-3′ (sense) and 5′-TTCTCATGTTTGACAGCTTA 3′ (antisense).

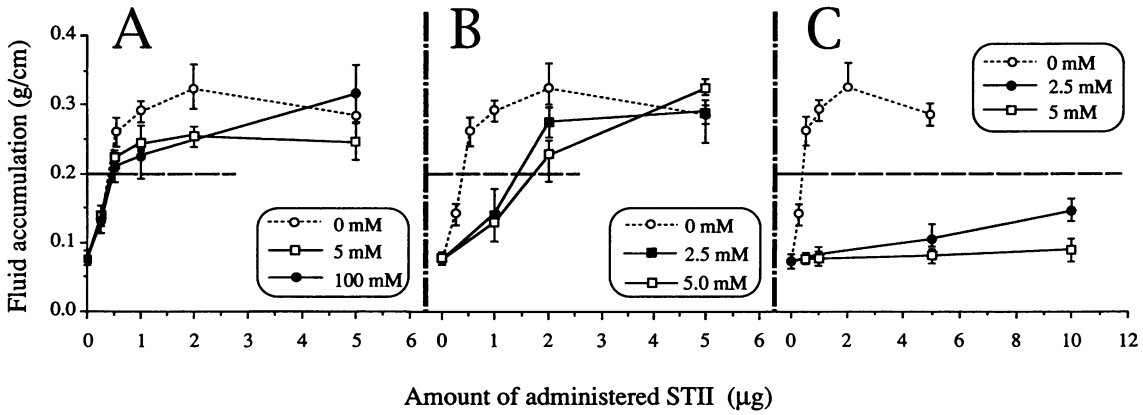

FIG. 1. Enterotoxic activity of chemically modified STII. One hundred micrograms of purified STII was treated with phenylglyoxal (A), ethoxyformic anhydride (B), and maleic anhydride (C) as described in Materials and Methods. The concentrations of the reagents used for the treatment are shown on the figure. The volume of the reaction mixture was 1 ml. After modification, the reaction mixture was diluted with distilled water and the enterotoxic activity of the diluent was determined in the mouse intestinal loop assay of STII. Values represent means ± standard errors for five determinations.

Purification of STII. Native and mutant STIIs were purified from the culture supernatants of *E. coli* HB101 harboring the appropriate plasmid by using methods described previously (8). Briefly, the cells were grown in Luria broth containing ampicillin (50 μg/ml) at 37°C overnight with shaking. The supernatant was separated from the cells by centrifugation. STII was precipitated from the supernatant with ammonium sulfate (40 g/100 ml). The resulting precipitate was recovered by centrifugation and then dissolved in distilled water. The crude preparation of STII was further purified by successive column chromatography on DEAE-Sephadex A-25, SP-Sephadex C-50, and Pep (Pharmacia LKB).

Circular dichroism. The circular dichroic (CD) spectra of STII were recorded with a CD spectrophotometer (model J-500C; Japan Spectroscopic Co., Ltd., Tokyo). Purified STII was dissolved in distilled water at a concentration of 50 μg/ml, and the CD spectra were recorded under the following conditions: scanning speed, 50 nm/min; slit size, 160 μm; temperature, 20°C.

Amino acid sequence and protein assay. The N-terminal amino acid sequence was determined with a protein sequencer (model 473A; Applied Biosystems). The protein content was determined by the method of Lowry et al. (22), using bovine serum albumin as a standard.

RESULTS

Isoelectric point of STII. The isoelectric point of purified native STII was about 9.7 when determined on the basis of its mobility in a pH gradient Ultrodex gel. The STII sequence revealed that there are nine basic amino acid residues consisting of six lysine residues, two arginine residues, and one histidine residue (8). The isoelectric point of STII shows that the side chains of some of these basic amino acid residues project outside the molecule.

Chemical modification of STII. To understand the role of these basic amino residues in the enterotoxicity of STII, we chemically modified the basic amino acid residues of STII with phenylglyoxal, maleic anhydride, and ethoxyformic anhydride.

Phenylglyoxal modifies arginine residues (32). The enterotoxic activity of the phenylglyoxal-treated STII was determined and compared with that of the control STII (STII incubated in the absence of the chemicals) (Fig. 1A). There was a negligible difference in the enterotoxicities of the treated and control STIIs. This indicated that arginine residues are not important for the expression of the enterotoxic STII activity.

Ethoxyformic anhydride selectively modifies histidine residues in proteins (21). To clarify the contribution of these residues to the enterotoxic activity of STII, we modified the purified STIIs with ethoxyformic anhydride (2.5 and 5.0 mM). The enterotoxic activity of the modified STII is shown in Fig. 1B. There was no significant difference in the enterotoxicities of the two STIIs modified with 2.5 and 5.0 mM ethoxyformic anhydride. The minimal amount of both modified STIIs to induce a positive response was 2 μg. On the other hand, the control STII induced a positive response at a dose of 0.5 μg. This showed that the modification of STII with ethoxyformic anhydride caused a fourfold reduction (the reduction rate was estimated from a comparison of the minimal effective dose of modified STII with that of the control STII).

Judging from the specificity of ethoxyformic anhydride in modification, the amino acid residue which is responsible for the fourfold reduction was considered to be histidine. However, this cannot be definitely concluded because chemical modification is often accompanied by a side reaction. Ethoxyformic anhydride also binds to α amino groups other than the imidazole group of histidine residues in proteins at low pH. When bound to the imidazole group of histidine, it can be removed from the protein with hydroxylamine at pH 7.0 (23). We therefore treated the ethoxyformic anhydride-modified STII with hydroxylamine at pH 7.0 (10 mM) and examined the enterotoxicity of the modified STII. The enterotoxicity of the ethoxyformic anhydride-modified STII was increased to the level of the control STII (data not shown). This result indicates that the fourfold reduction of the enterotoxic activity of STII caused by ethoxyformic anhydride should be attributed to the modification of the imidazole group of histidine residues in STII.

Maleic anhydride can acylate both the α and ε amino groups of histidine and lysine residues, respectively (4). The purified STIIs were modified with either 2.5 mM or 5.0 mM maleic anhydride as described in Materials and Methods. The enterotoxic activities of these modified STIIs are shown in Fig. 1C. A positive response was not observed in any mouse intestinal loop administered up to 10 μg of maleyl STII. This result

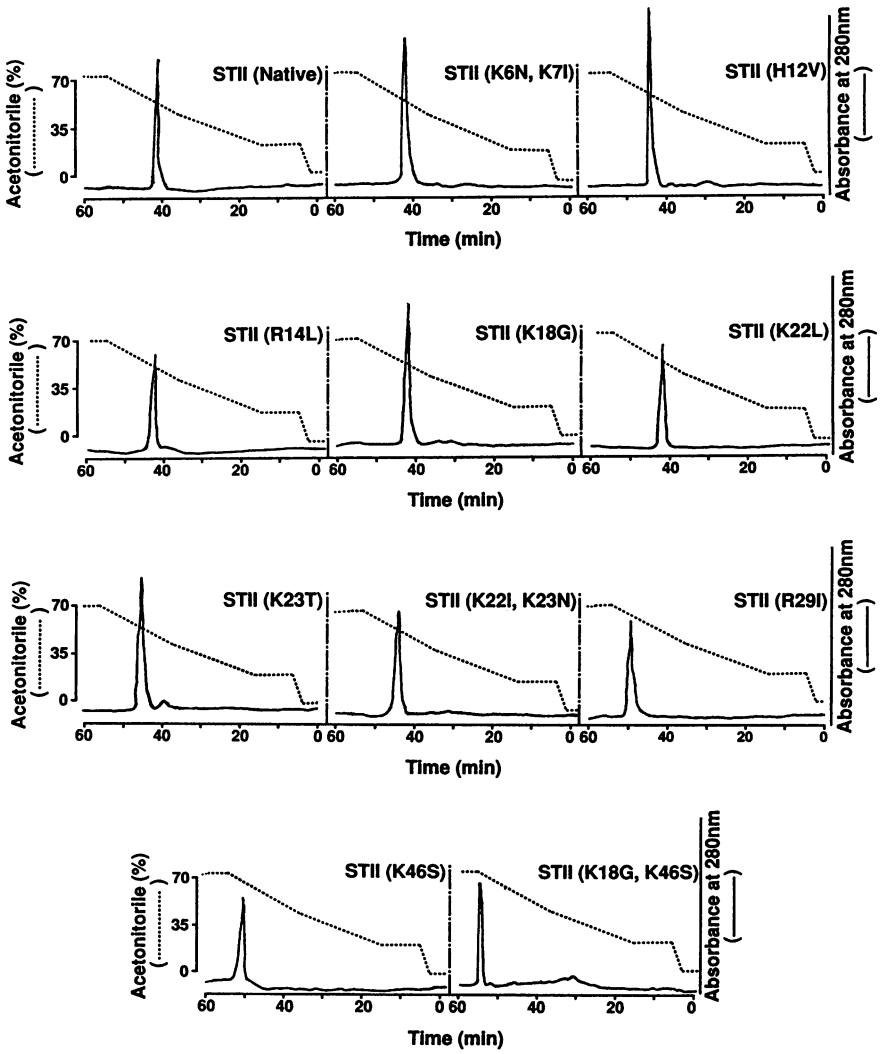

FIG. 2. Profiles for reversed-phase HPLC with an ODP-50 column of purified STII. Toxins were eluted with a gradient (------) of acetonitrile in 0.05% trifluoroacetic acid.

indicates that lysine or/and histidine residues are important for the expression of the enterotoxic activity of STII.

As described above, the severe loss of enterotoxic activity induced by maleic anhydride is thought to be ascribed to the modifications of side chains of lysine and/or histidine. However, the ethoxyformic anhydride modification of the histidine side chains caused only a fourfold reduction of the enterotoxic activity (Fig. 1B). Therefore, the severe loss of enterotoxicity induced by maleic anhydride was probably induced by modification of the lysine residues.

Preparation of mutant STII. Mutant STII plasmids were obtained by oligonucleotide-directed mutagenesis as described in Materials and Methods. Transformants carrying the mutated plasmids were screened by colony hybridization with the same synthetic oligonucleotide that was used as the mutagen. Plasmids of putative mutants that were identified by hybridization were isolated and digested with the appropriate restriction endonuclease shown in Table 1. Plasmids possessing restriction sites at desired positions were selected and sequenced. The correct mutations were confirmed in every plasmid. This means that the desired mutations were introduced into the predicted positions, and the strains harboring these plasmids produced the desired mutant STIIs.

All mutant STIIs were purified from culture supernatants of these strains. The purified mutant STIIs were examined by reversed-phase HPLC with an ODP-50 column. The elution was performed with a 0 to 70% acetonitrile gradient in 0.05% trifluoroacetic acid. As shown in Fig. 2, every examined mutant STII was eluted as a single peak, indicating that these mutant STIIs were purified to homogeneity. The concentrations of acetonitrile at which these STIIs were eluted were between 50 and 68%.

Properties of mutant STII. It has been demonstrated that native STII is synthesized as a 71-amino-acid-residue precursor. The precursor is cleaved between amino acid residues 23 and 24 by signal peptidase, and the resulting mature native STII region composed of 48 amino acid residues is secreted into the culture medium (8, 18, 19, 28).

To clarify whether or not these mutant STIIs were cleaved at the same site as that of native STII by signal peptidase, their amino acid sequences were determined. All mutant STIIs were cleaved at the same site as that of native STII. Furthermore,

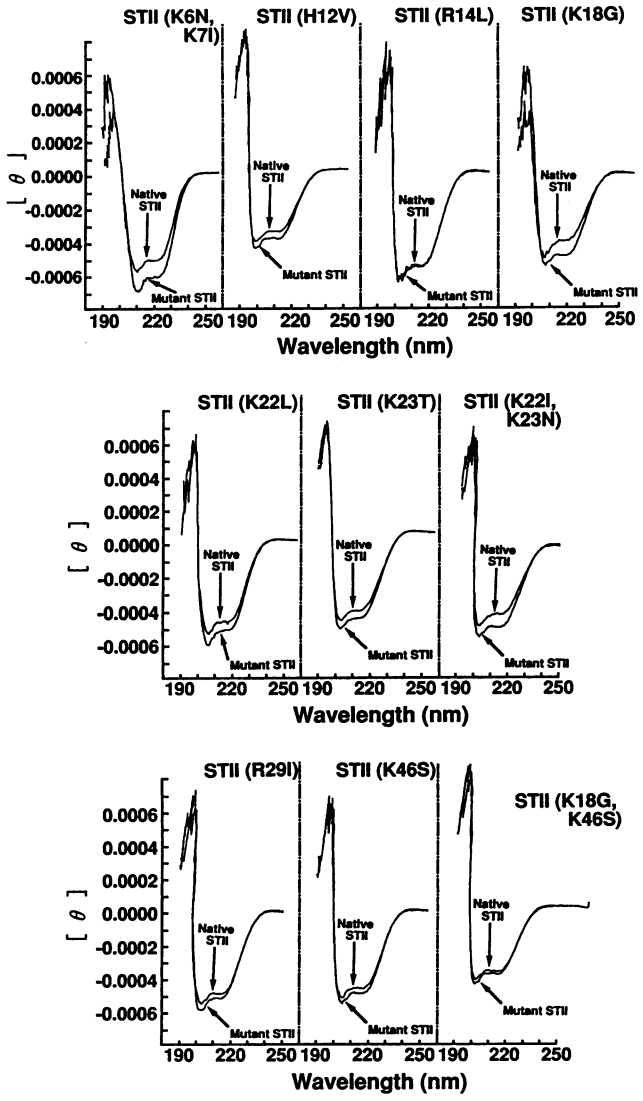

FIG. 3. CD spectra of purified STII. The CD spectra of purified STII dissolved in distilled water at a concentration of 50 μg/ml were recorded under the conditions described in the text. To compare the spectrum of the mutant STII with that of native STII, the pattern of the mutant STII was superimposed over that of native STII.

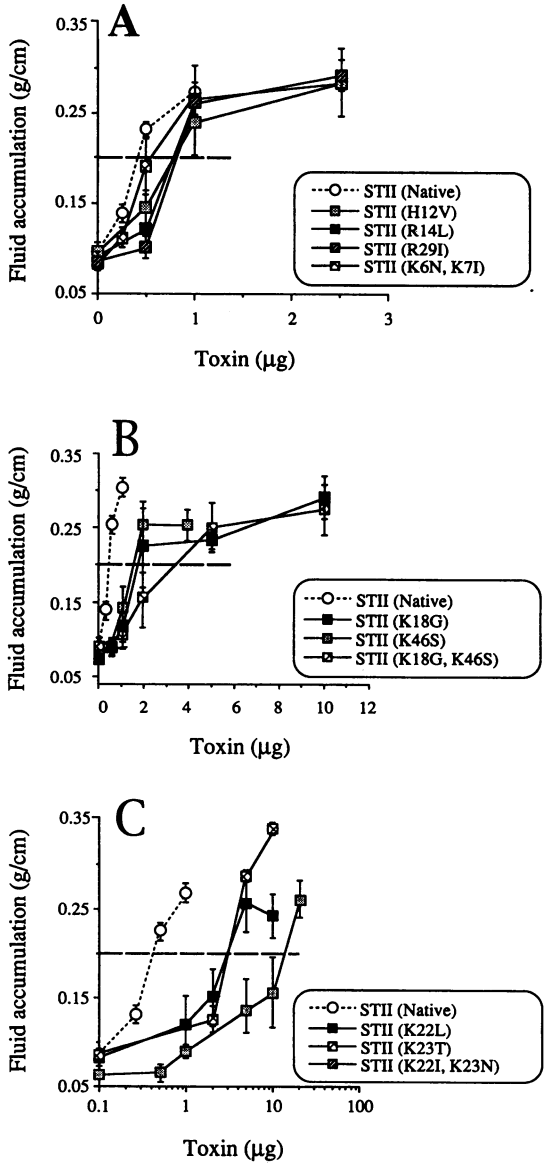

FIG. 4. Dose responses to purified STII in the mouse intestinal loop assay. The indicated amounts of purified toxins were administered into intestinal loops and incubated for 3 h. The sample activities were measured and are expressed as the ratio of the weight of the loop (in grams) to its length (in centimeters). Values represent means ± standard errors for five determinations. A ratio above 0.20 was regarded as a positive response.

the substitution of the desired amino acid residue with the correct amino acid residue was confirmed in every mutant STII (data not shown).

The structural changes induced by these mutations were analyzed by measuring the CD spectra (14). All STIIs examined had similar CD spectra (Fig. 3), showing that serious change of the backbone structure of STII was not induced by these mutations.

Enterotoxic activity of mutant STII. The enterotoxic activities of the purified mutant STIIs were determined in the mouse intestinal loop assay and compared with that of native STII. The results are shown in Fig. 4.

Native STII induced a positive response at a dose of more than 0.5 μg. Three mutant STIIs with substitutions at arginine and histidine residues, i.e., STII(H12V), STII(R14L), and STII(R29I), did not induce a positive response at the same dose. However, 1.0 μg of these mutant STIIs induced a positive response (Fig. 4A). This showed that the substitutions for arginine and histidine residues caused a twofold reduction of STII activity (the reduction size was estimated from the ratio of 1.0 to 0.5 μg as described above). We do not attach any significance to the twofold reduction because the intestinal loop assay is not very accurate.

Similarly, the substitution for lysine residues at positions 6 and 7 did not significantly affect the enterotoxic activity of STII (Fig. 4A). STII(K6N, K7I) induced a positive response in some of the loops at a dose of 0.5 μg. Every loop responded positively to 1.0 μg of this mutant STII.

On the contrary, the amount of STII(K18G) and STII(K46S)

required to induce a positive response was 2 μg. Five micrograms of mutant STII with substitutions for both of these lysine residues [STII(K18G, K46S)] was required to evoke a positive response (Fig. 4B). The reduction of the enterotoxic activity evoked by the substitution of Lys-18 and Lys-46 was more notable than that induced by the substitutions for Lys-6, Lys-7, His-12, Arg-14, and Arg-29 (Fig. 4A). It can be inferred from these results that lysine residues at positions 18 and 46 play a fairly important role in the expression of the enterotoxic activity of STII.

The enterotoxic activities of the mutant STIIs substituted at lysine residues 22 and 23 are shown in Fig. 4C. The minimal amounts of STII(K22L), STII(K23T), and STII(K22I, K23N) that induced a positive response were 5, 5, and 20 μg, respectively. This shows that substitutions for lysine residues at positions 22 and 23 caused a 10- to 40-fold reduction in enterotoxic activity (the reduction sizes were estimated from the ratios of these toxin amounts to 0.5 μg). These reductions were higher than those observed in other mutant STIIs. Therefore, of the basic amino acid residues of STII, the lysine residues at positions 22 and 23 are thought to contribute most to the active structure of STII.

DISCUSSION

In this study, we examined the role of the basic amino acid residues of STII in the expression of enterotoxicity. The isoelectric point of STII was 9.7. This value shows that some side chains of basic amino acid residues in STII project outside the molecule. To clarify the role of these basic amino acid residues in enterotoxicity, we chemically modified these basic amino acid residues of STII. After modification, the diluent of the reaction mixture was inoculated into a mouse intestinal loop. It was suspected that the modifying agent remaining in the diluent might affect the secretory response in the loop. To clear the suspicion, the action of STI in causing the intestinal fluid accumulation in the presence of these modifying agents was examined. The concentration of the agents used was the same as that used for the STII experiment. The fluid accumulation was induced by STI in the presence of the agents (data not shown), showing that the agent existing in the sample did not damage the secretory response of mouse intestine. Therefore, we think that the amounts of the fluid accumulated in the intestinal loops induced by the diluent (Fig. 1) reflect the activity of the chemically modified STII.

Chemical modifications indicated that the arginine and histidine residues of STII were not important for expression of the enterotoxicity (Fig. 1A and B). On the contrary, the lysine residues were found to be important for expression of STII toxicity (Fig. 1C). However, we could not identify by chemical modification the position of the lysine residue which is most responsible for the toxicity because it is impossible to chemically modify a single, intended amino acid residue. Moreover, amino acid residues in addition to that intended are sometimes chemically modified as a result of side reactions (4, 32). It means that total certainty cannot be assumed from the results obtained after chemical modification.

We then substituted the basic amino acid residues of STII by oligonucleotide-directed site-specific mutagenesis to confirm the role of these basic amino acid residues in toxicity. All mutant STIIs prepared were purified to homogeneity. The following observations indicate that the serious change of the backbone structure of STII was not induced by these mutations. (i) The elution profile of the mutant STII on the reversed-phase column chromatography resembled that of native STII (Fig. 2). (ii) All mutant STIIs consisted of 48 amino acid residues. (iii) The CD spectra of the mutant STIIs resemble that of negative STII (Fig. 3). Since the backbone structure was not changed by the mutations, the reduction of the enterotoxic activity of STII caused by the mutations was thought to be attributable to the function of the substituted amino acid residues.

Figure 4A shows that arginine and histidine residues were not important for enterotoxicity. This result is in agreement with the result observed in chemical modification. However, Dreyfus et al. reported that the arginine residue at position 29 was important because a substitution of the residue with serine resulted in an 87.5% (about eightfold) reduction in enterotoxicity (6). They also proposed that the arginine residue at position 29 was involved in the binding of STII to the putative receptor since the mutant STII could not compete with native STII action. This result is contrary to our result. In our experiment, the substitution of the arginine residue at position 29 with isoleucine caused only a twofold reduction of the enterotoxic activity (Fig. 4A). Since the residue changes are not identical, care should be taken in comparing the two works. However, we question the size of the reduction induced by the replacement with a serine residue because Dreyfus et al. did not use the purified STII(R29S). A study using purified toxin is required to determine the activity of the toxin. An accurate determination of the toxicity of STII(R29S) will clarify our understanding of the role of the arginine residue at position 29 in the toxicity of STII.

Although the lysine residues were found to be important in the expression of toxicity by the chemical modification (Fig. 1C), the position of the important lysine residue could not be defined. We substituted for these lysine residues and determined the enterotoxic activity of these mutant STIIs. The results showed that the lysine residues which were most responsible for the enterotoxic activity were located at positions 22 and 23 (Fig. 4C). As the substitution of these lysines caused severe reduction (ca. 40-fold) in the toxic activity, we presume that these lysine residues are involved in receptor binding. Further analysis of the receptor by using the mutant STIIs prepared in this experiment would provide insights into the molecular basis of the toxin-receptor interaction.

STI and STII contain three and two intramolecular disulfide bonds, respectively. In STIp, which is a kind of STI, disulfide bonds are formed between cysteines at positions 5 and 10, 6 and 14, and 9 and 17 (11). Structural studies on STI revealed that the peptide spanning the region from the cysteine residue near the N terminus to the cysteine residue near the C terminus of STIp has full activity and the peptide spanning the region from the first to the fourth residues at the N terminus of STIp is not involved in the active structure (37). In STII, disulfide bonds of STII are formed between Cys-10 and Cys-48 and between Cys-21 and Cys-36 (8). The substitution of lysine residues at positions 6 and 7 did not reduce the enterotoxic activity (Fig. 4A). Furthermore, we confirmed that the substitutions of amino acid residues at positions 3 and 4 also did not reduce enterotoxic activity (data not shown). These results indicate that the peptide spanning the region from the cysteine residue near the N terminus to the cysteine residue near the C terminus has full activity and that the peptide consisting of seven amino acid residues at the N terminus is not involved in the active structure of STII, as observed in STI. Further studies on the structure-activity relationship of STII are in progress in our laboratory.

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