Substitutions of Cysteine Residues of *Escherichia coli* Heat-Stable Enterotoxin by Oligonucleotide-Directed Mutagenesis

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The *Escherichia coli* 18-amino-acid, heat-stable enterotoxin STp has six cysteine residues linked intramolecularly by three disulfide bonds. These disulfide bonds are important for toxic activity, but the precise role of each bond is not clear. We substituted cysteine residues of STp in vivo by oligonucleotide-directed site-specific mutagenesis to dissociate each disulfide bond and examined the biological activities of the resulting mutants. The Cys-6→Ala and Cys-17→Ala mutations caused a complete loss of toxic activity. The Cys-5→Ala, Cys-10→Ser, and Gly-16, Cys-17→Cys-16, Gly-17 mutations caused a large decrease in toxic activity. These results mean that all three disulfide bonds formed at fixed positions are required for full expression of the biological activity of STp. However, a weak but significant toxicity still remained after three mutations, Cys-5→Ala, Cys-10→Ser, and Gly-16, Cys-17→Cys-16, Gly-17. This indicates that STp has some flexibilities in its conformation to exert toxic activity and that the role of each disulfide bond in exerting toxic activity is not quite the same.

Enterotoxigenic *Escherichia coli* strains elaborate several enterotoxins which cause intestinal secretion and diarrhea (19, 24). One of these toxins is heat-stable enterotoxin (ST). *E. coli* ST falls into two classes: one is composed of 18 amino acid residues, and the other is composed of 19 amino acid residues. The former toxin is designated STp and the latter is designated STh, since they originate from porcine and human strains of enterotoxigenic *E. coli*, respectively (1, 21, 24). In addition to enterotoxigenic *E. coli*, some enteric bacteria such as *Yersinia enterocolitica* (14-16) and *Vibrio cholerae* non-O1 (2, 22) are known to produce STs. These STs have common biological properties and share antigenic determinants with *E. coli* ST (15, 17, 22). The amino acid sequences of these STs were determined, and the sequences at the C termini showed high similarity, especially in the position of six cysteine residues as shown in Fig. 1 (1, 21-24). These six cysteine residues link intramolecularly with three disulfide bonds. The positions of the three disulfide linkages of STh were recently determined by analyzing chemically synthesized STh analogs formed between cysteines at positions 6 and 11, 7 and 15, and 10 and 18 (Fig. 1). This disulfide arrangement is common to other STs (19a; Y. Shimonishi, personal communication). The tertiary structure formed by three disulfide bonds is very important for expression of the biological activities of STs (8). The role of each disulfide bond, however, has not been clarified. Recently, the development of the oligonucleotide-directed mutagenesis method has permitted the construction of mutants of many proteins in which individual amino acid residues of potential interest have been modified (4). Using this method, we replaced the cysteine residues of *E. coli* STp with another amino acid to eliminate each disulfide bond at specified positions and changed the position of cysteine residues to understand the role of each disulfide linkage.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *E. coli* HB101 was used as the host strain in all experiments and was cultured in Luria broth (13). Plasmid Tc-1, which carries the *E. coli* STp gene, was kindly provided by M. So. Plasmid Tc-1 contains the 350-base-pair *TaqI-Hinfl* fragment of Tn1681 at the HindIII site, and its size is approximately 4.7 kilobase pairs (20).

**Oligonucleotide-directed mutagenesis and DNA sequence.** Oligonucleotide-directed mutagenesis was performed on plasmid Tc-1, using the plasmid method reported by Inouye and Inouye (10). Oligonucleotides were synthesized on an Applied Biosystems model 380b DNA synthesizer by the phosphoramidite method. Purification of oligomer was performed by denaturing polyacrylamide gel followed by DEAE-cellulose column chromatography as described by Vlasuk et al. (25). The oligonucleotides were phosphorylated by using T4 polynucleotide kinase (Takara Shuzo Co., Japan). Figure 2 shows the relevant mutagenic oligonucleotides for production of five mutants. Asterisks denote mismatches used to produce desired amino acid substitutions. Oligonucleotide 6 was used as the probe for screening the mutant directed by oligonucleotide 5. Mutations were confirmed by DNA sequence determination by the procedure of Maxam and Gilbert (12).

**Resistance to tetracycline.** Cultures were grown to a density of 5 × 10⁷ cells per ml in the presence of ampicillin (50 μg/ml). Approximately 100 cells were plated on freshly poured Luria agar plates containing ampicillin (50 μg/ml) and tetracycline (10 and 20 μg/ml). After cultivation at 37°C for 15 h, the number of formed cells was counted.

**Toxin production and assay for ST.** *E. coli* HB101, harboring appropriate plasmids, was inoculated into 3 ml of Luria broth containing ampicillin (50 μg/ml). After 18 h of incubation at 37°C with shaking, the culture was centrifuged. ST activity of the culture supernatant was assayed in suckling mice as reported previously (16). Briefly, samples of 0.1 ml with Evans blue as marker dye were administered by gastric

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tube into the stomachs of 2- to 3-day-old suckling mice. After 3 h, the mice were killed and the entire intestines were removed. A ratio of intestine weight to body weight of 0.083 was considered to be a positive response. One unit of ST activity was defined as the amount of ST producing positive response, and the enterotoxin titer was expressed as the reciprocal of the highest dilution that gave 1 U of enterotoxin activity. Five mice were used for determination of the ST activity of each sample.

RESULTS

Mutations. Oligonucleotide-directed site-specific mutagenesis was performed on the Tc-1 plasmid by using the heat-reanneal technique (10). E. coli transformants were screened by colony hybridization with the corresponding 32P-labeled mutagen as a probe. Plasmids pKK101, pKK102, pKK103, pKK104, and pKK105 were obtained by mutation using oligonucleotides 1, 2, 3, 4, and 5, respectively (Table 1). To determine the nucleotide sequence around the mutated STp gene, the AluI-HindIII fragments of plasmids Tc-1, pKK101, pKK102, pKK104, and pKK105 were purified and labeled at the HindIII 3' end. In the case of plasmid pKK103, the MboII-HindIII fragment was used instead of the AluI-HindIII fragment because the AluI site was introduced by mutation using oligonucleotide 3. The substitution of nucleotide sequences was observed in the predicted region (Fig. 3).

Resistance to tetracycline. There were no differences among the strains with regard to tetracycline resistance; strains harboring plasmids Tc-1, pKK101, pKK102, pKK103, pKK104, and pKK105 were equally resistant to tetracycline at 10 µg/ml but sensitive to 20 µg/ml.

ST activity of culture supernatant. ST activities in the culture supernatants of the wild type and mutants are shown in Table 2. E. coli HB101(pKK104) and E. coli HB101 (pKK105) were active but quite low in enterotoxin titer compared with that of the wild type [E. coli HB101(Tc-1)]. On the other hand, E. coli HB101(pKK102) and E. coli HB101(pKK103) were completely inactive. The intestine weight/body weight ratio of mice administered the culture supernatant of E. coli HB101(pKK101) was invariably 0.078 ± 0.004 and considered a negative value in the assay. However, this value was very close to the positive value and was consistently higher than that of the negative control [E. coli HB101(pBR322)]. It seemed likely that the ST analog produced by E. coli HB101(pKK101) carried an extremely low ST activity. These results indicate that at least the structure involving two disulfide linkages is important for exerting ST activity.

Heat stability of toxin. The culture supernatants of strains harboring plasmids Tc-1, pKK104, and pKK105 were diluted to contain 2 U/0.1 ml and heated at 100°C for 5 or 10 min. The remaining activity was assayed in suckling mice. The activity was not significantly reduced by heating.

DISCUSSION

We modified cysteine residues of E. coli STp by site-directed mutagenesis. E. coli HB101 harboring the Tc-1 plasmid was used as the wild type. The strain is resistant to tetracycline (20). The direction of the tetracycline resistance gene is the same as that of the STp gene, and both genes are
FIG. 3. Determination of the mutant nucleotide sequences. The plasmid DNA was digested with HindIII and labeled with $[^{32}P]dATP$ by Klenow enzyme. The labeled DNA was cleaved with AluI (Tc-1, pKK101, pKK102, pKK104, and pKK105) or MboII (pKK103). The fragments carrying ST structure genes were purified and used for sequencing by the method of Maxam and Gilbert (12). The chemical cleavage products were applied to a denaturing 10% polyacrylamide gel and subjected to electrophoresis. STp, 1, 2, 3, 4, and 5 indicate the sequences around ST structure genes of plasmids Tc-1, pKK101, pKK102, pKK103, pKK104, and pKK105, respectively. Asterisks at the side of the gel indicate the bases that are different from the sequences of plasmid Tc-1.

considered to be controlled by the same promoter (20); that is, the tetracycline resistance gene of the Tc-1 plasmid is expressed by the identical transcript as the STp gene. The wild type and all obtained mutants were equally resistant to 10 μg and sensitive to 20 μg of tetracycline per ml. This means that the tetracycline resistance gene of the wild type and those of all mutants were expressed to the same extent. This leads us to believe that the amounts of ST peptide produced by all examined strains should be the same and that the ST titer of the culture supernatant reflects the specific activity of the peptide produced by each individual mutant.

Alanine and cysteine have $-\text{CH}_3$ and $-\text{CH}_2\text{SH}$ as their side chains, respectively. Substitution for cysteine with alanine may explain the role of the $-\text{SH}$ of the cysteine residue. Cys-5, Cys-6, and Cys-17 were replaced by alanine in plasmids pKK101, pKK102, and pKK103, respectively. Since three intramolecular disulfide linkages of $E$. coli STp are formed between cysteines at positions 5 and 10, 6 and 14, and 9 and 17, mutations brought about by use of oligonucleotides 1, 2, and 3 dissociate the disulfide bonds between Cys-5 and Cys-10, Cys-6 and Cys-14, and Cys-9 and Cys-17, respectively. These substitutions resulted in a remarkable decrease of ST activity (Table 2), indicating that each of the disulfide bonds was required for full expression of ST activity. However, toxins produced by $E$. coli HB101 (pKK104) in which the cysteine of position 10 was replaced by serine gave a weak but significant ST activity. Furthermore, $E$. coli HB101(pKK101) showed a value close to positive. These results strongly suggest that the conformation involving at least two disulfide linkages (Cys-6–Cys-14 and Cys-9–Cys-17) is essential, but the linkage between Cys-5 and Cys-10 seems not to be absolutely required for the


