Reduction of Enterotoxic Activity of *Escherichia coli* Heat-Stable Enterotoxin by Substitution for an Asparagine Residue

KEINOSUKE OKAMOTO,* KYOKO OKAMOTO, JUN YUKI TAKE, AND AKIO MIYAMA

Department of Microbiology, School of Medicine, Fujita-Gakuen Health University, Kutsukake-cho, Toyoake-city, Aichi-ken 470-11, Japan

Received 6 January 1988/Accepted 3 May 1988

The *Escherichia coli* heat-stable enterotoxins (STs) are small peptide toxins consisting of 18 (STp) or 19 (STh) amino acids. STp and STh share biologically active sequences which reside in the C-terminal 13 amino acid residues, but the role of each amino acid in the active sequences is not clear. We substituted in vivo Asp, Tyr, His, Gln, Lys, and Arg for the Asn residue at position 11 of STp by oligonucleotide-directed site-specific mutagenesis and examined the biological activities of the resulting mutants. All mutant STs reacted with both monoclonal and polyclonal antibodies, demonstrating that the amino acid substitutions at position 11 did not cause a significant change in the conformation of STp. However, the substitutions invariably caused a significant decrease in enterotoxic activities. The most remarkable decrease was observed with Asn-11→Lys-11 and Asn-11→Arg-11 mutations; that is, enterotoxic activity could not be detected in the culture supernatant of either of these mutant strains. These results indicate that Asn-11 of STp plays an essential role in the enterotoxic activity. The amide group and the length of side chain of Asn-11 seem to be especially important for enterotoxic activity.

Enterotoxigenic *Escherichia coli* elaborates two kinds of heat-stable enterotoxins (STs) which cause intestinal secretion and diarrhea (16, 18). One of these toxins, STI, can be either an 18- or a 19-amino-acid peptide. The 18-amino-acid toxin is designated STp and the 19-amino-acid toxin is designated STh, since they originate from porcine and human strains, respectively (17, 19). The enterotoxigenic and receptor-binding functions of STI have been mapped to the C-terminal 13-amino-acid segment (5, 17, 23). Homologous sequences at the C termini have been reported for similar enterotoxins produced by some strains of *Yersinia enterocolitica* and *Vibrio cholerae* non-O1 (Fig. 1) (20, 21). The amino acid residues common to these enterotoxins are considered to play an important role in expression of enterotoxigenicity, but the exact role of these amino acids has not been clarified.

It is of interest that STI shares sequence homology with the sea snail neurotoxin, conotoxin (7, 13). All except one of the boxed amino acids shown in Fig. 1 are common to all of these toxins. The exceptional amino acid is His-4 of conotoxin GI; the amino acid at the corresponding position of the other toxins is Asn. This means that the amino acid at position 4 of conotoxin has some variability, because both conotoxins (GI and GII) possess the same level of activity (7). This leads us to guess that Asn-11 of STp (Fig. 1) can also tolerate some variability. We substituted Asn-11 of STp with other amino acids by oligonucleotide-directed site-specific mutagenesis and examined the biological activities of the resulting mutant STs.

**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 (12). 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*-HindIII fragment of Tn1681 at the HindIII site, and its size is approximately 4.7 kilobase pairs (18).

Oligonucleotide-directed mutagenesis and DNA sequencing. Mutagenic oligonucleotides were synthesized and purified as described previously (15). Figure 2 shows the relevant mutagenic oligonucleotides for production of six mutants; asterisks denote mismatches used to produce the desired amino acid substitutions. Oligonucleotides 201, 202, 203, 204, 205, and 206 were designed to direct the mutations of Asn-11 to Asp-11, Tyr-11, His-11, Gln-11, Lys-11, and Arg-11, respectively. The oligonucleotides were phosphorylated by using T4 polynucleotide kinase (Takara Shuzo Co., Kyoto, Japan).

Oligonucleotide-directed mutagenesis was performed with plasmid Tc-1, using the plasmid method described by Inouye and Inouye (9). Two kinds of linearized fragments of Tc-1, fragment 1 and fragment 2, were obtained by digesting plasmid Tc-1 with *PstI* and HindIII, respectively. Fragment 1 contains the STp gene, and fragment 2 does not contain the gene. After treatment of fragments 1 and 2 with the Klenow fragment of DNA polymerase I and bacterial alkaline phosphatase, respectively, these fragments were mixed with each mutagenic oligonucleotide. The mixture was incubated at 100°C for 3 min and then cooled to allow the denatured DNA fragments to reanneal. Some DNA fragments formed heteroduplexes during the reannealing procedures. The mutagenic oligonucleotides hybridized to the single-stranded region of the heteroduplex DNA. After treatment of the DNAs with Klenow fragment of DNA polymerase I, T4 ligase, and four deoxyribonucleoside triphosphates, the mixture was used for transformation. Mutations were confirmed by DNA sequence determinations by the procedure of Maxam and Gilbert (11).

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 or 20 μg/ml). After cultivation at 37°C for 15 h, the number of 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, the culture was centrifuged. The ST activity of the culture supernatant was assayed in suckling mice as described previously (14). The minimal amount of ST giving a fluid accumulation of more than 0.083 (ratio of intestine weight to body weight) was designated 1 U, 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.

**Production of anti-STp antiserum.** Synthetic STp (200 μg) was coupled to keyhole limpet hemocyanin (2 mg; Sigma Chemical Co., St. Louis, Mo.) with glutaraldehyde as described by Alderete and Robertson (2). The coupling was performed at 4°C for 3 h in 0.1 M phosphate buffer (pH 7.4). The conjugate was emulsified with an equal volume of Freund complete adjuvant, and the emulsion (5 μg of STp) was injected into mice four times intraperitoneally at 1-week intervals. The antiserum was obtained 1 week after the final injection and inactivated at 56°C for 30 min.

**Immunological assays.** Competitive enzyme-linked immunosorbent assay was performed to detect the toxin in culture supernatants by using polyclonal anti-STp antibody and monoclonal anti-ST antibody.

The following method was used to detect the toxin with polyclonal antibody. The synthetic ST was dissolved in coating buffer (15 mM Na2CO3, 35 mM NaHCO3, 0.02% NaN3, pH 9.6) at a concentration of 6 μg/ml. Aliquots of 50 μl of the solution were added to each well of polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) and incubated at 37°C for 3 h. The contents of the wells were discarded by inversion, and the wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween). One hundred microliters of 1% ovalbumin in PBS containing 0.02% NaN3 was added to each well, and after incubation at 37°C for 2 h, the wells were washed three times with PBS-Tween. Sample and antiserum, which were appropriately diluted with PBS-Tween, were mixed, and 100 μl of the mixture was added to each well. After incubation overnight at 4°C, the wells were washed with PBS-Tween. One hundred microliters of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.) diluted with PBS-Tween was added to each well. After another incubation at 37°C for 1 h, the wells were washed; then 100 μl of p-nitrophenylphosphate (1 mg/ml; Sigma) in diethanolamine hydrochloride (pH 9.8) containing 0.02% NaN3 and MgCl2 was added, and the optical density at 405 nm was spectrophotometrically measured.

The assay kit for *E. coli* ST (COLI STET EIA) produced by Denka Seiken Co., Ltd., Niigata, Japan, was used for examination of reactivities of culture supernatants with monoclonal antibody. The monoclonal antibody used in the kit can neutralize ST activity in the suckling mouse assay (22). The kit supplied a microtiter plate coated with synthetic ST and all reagents, including peroxidase-conjugated monoclonal antibody, a substrate solution consisting of o-phenylene diamine and H2O2, and a washing solution. The assay procedure with the kit was almost the same as that described above. Culture supernatants were diluted with Luria broth. Sample (200 μl) and conjugated monoclonal antibody (10 μl) were added to each well, and the plate was incubated at 30°C for 90 min. The contents were discarded, and the wells were washed. One hundred microliters of the substrate solution was added to each well, and the plate was kept in the dark at 30°C for 30 min. After the reaction was stopped by addition of 100 μl of 1.5 N H2SO4, the optical density at 492 nm was spectrophotometrically measured.

**RESULTS**

**Mutations.** *E. coli* transformants carrying the mutated plasmids were screened by colony hybridization, in each case using the same synthetic oligonucleotide which was used as the mutagen. The oligonucleotide labeled with 32P was used as the probe for screening. Plasmids pKK201, pKK202, pKK203, pKK204, pKK205, and pKK206 were obtained by mutation with oligonucleotides 201, 202, 203,
Tc-1 derivatives pKK201, 204, 205, and 206, respectively (Table 1). To confirm the correct mutation, the AluI-HindIII fragments of plasmids Tc-1, pKK201, pKK202, pKK203, pKK204, pKK205, and pKK206 were purified and labeled at the HindIII 3' end. The labeled fragments were subjected to DNA sequencing (11). The substitution of nucleotide sequences was observed in the predicted region (Fig. 3).

**Resistance to tetracycline.** There was no differences among the strains with regard to tetracycline resistance; strains harboring plasmid Tc-1, pKK201, pKK202, pKK203, pKK204, pKK205, or pKK206 were equally resistant to tetracycline at 10 μg/ml but were sensitive to tetracycline at 20 μg/ml.

**Reaction with antibodies.** The diluted culture supernatants were examined for their reactivities with monoclonal and polyclonal antibodies specific for *E. coli* ST. The supernatant from *E. coli* HB101(pBR322) (negative culture supernatant) did not react with either antibody (Fig. 4). The supernatants from all strains harboring Tc-1 or a mutant plasmid reacted with both polyclonal antibody (Fig. 4A) and monoclonal antibody (Fig. 4B) These results suggested that the substitutions for Asn-11 did not cause a significant conformational change in ST.

**ST activities of culture supernatants.** ST activities in the culture supernatants of wild-type and mutant strains are shown in Table 2. Strains of *E. coli* HB101 harboring plasmid pKK201, pKK202, pKK203, or pKK204 were active but low in enterotoxin titer compared with the wild type *E. coli*.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td><em>E. coli</em> STp gene cloned into pBR322</td>
<td>3, 18</td>
</tr>
<tr>
<td>Tc-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pKK201</td>
<td>Obtained by use of oligonucleotide 201</td>
<td>This work</td>
</tr>
<tr>
<td>pKK202</td>
<td>Obtained by use of oligonucleotide 202</td>
<td>This work</td>
</tr>
<tr>
<td>pKK203</td>
<td>Obtained by use of oligonucleotide 203</td>
<td>This work</td>
</tr>
<tr>
<td>pKK204</td>
<td>Obtained by use of oligonucleotide 204</td>
<td>This work</td>
</tr>
<tr>
<td>pKK205</td>
<td>Obtained by use of oligonucleotide 205</td>
<td>This work</td>
</tr>
</tbody>
</table>

FIG. 3. Determination of mutant nucleotide sequences. The plasmid DNA was digested with HindIII and labeled with [α-32P]dATP by Klenow enzyme. The labeled DNA was cleaved with AluI. The fragments carrying ST structural genes were purified and used for sequencing by the method of Maxam and Gilbert (11). The chemical cleavage products were applied to a denaturing 10% polyacrylamide gel and subjected to electrophoresis. STp, 201, 202, 203, 204, 205, and 206 indicate the sequences around the ST structural genes of plasmids Tc-1, pKK201, pKK202, pKK203, pKK204, pKK205, and pKK206, respectively. Asterisks indicate bases that are different from those in the sequence of plasmid Tc-1.
HB101(Tc-1)]. On the other hand, *E. coli* HB101(pKK205) and *E. coli* HB101(pKK206) were inactive.

**DISCUSSION**

We substituted Asn-11 of *E. coli* STp with Asp, Tyr, His, Gln, Lys, and Arg by site-directed mutagenesis. The culture supernatants of all mutants reacted with monoclonal and polyclonal antibodies specific for *E. coli* ST (Fig. 4). The three-dimensional structure of ST formed by three intramolecular disulfide bonds is known to be very important for exerting ST activity (8, 17). We reported previously that dissociation of any of three disulfide bonds resulted in a remarkable decrease in ST activity (15), and we recently confirmed that such a mutant ST reacted poorly with polyclonal and monoclonal ST antibodies (unpublished data). Therefore, it was expected that the substitution for Asn-11 of STp performed in this study would not cause a great change in ST conformation.

Plasmid Tc-1 encodes the tetracycline resistance gene (18). The direction of the gene is the same as that of the STp gene, and both genes are thought to be controlled by the same promoter (15, 18); that is, the tetracycline resistance gene of the Tc-1 plasmid is expressed by the same transcript as expresses the STp gene. The wild type and all mutants obtained in this study were equally resistant to 10 μg of tetracycline per ml and sensitive to 20 μg of tetracycline per ml. This means that the tetracycline resistance genes of all strains used in this study are expressed to the same extent. Moreover, the mutations seem not to affect ST secretion from the cell, because, like the wild-type strain, no mutant whole-cell lysate reacted with the anti-ST antibodies (unpublished data). These results, with those shown in Fig. 4, suggest that the amounts of ST analog produced by the mutants should be the same and that the ST titer of the culture supernatant reflects the specific activity of the peptide produced by each mutant.
ST has been observed to bind with intestinal surface receptors and to activate the membrane-bound guanylate cyclase of intestines (6, 10), but the actual mechanisms whereby ST binds and activates guanylate cyclase have not been clarified. Moreover, the relationship between the structure of ST and its activity remains unclear. The mutant STs obtained here help to resolve these problems.

Substitution for Asn with Gln at position 11 resulted in a great loss of ST activity. Furthermore, the substitutions with Lys and Arg caused a significant loss of ST activity (Table 2). Reduction of enterotoxic activity was similarly observed with the partially purified toxin isolated by successive column chromatographies (data not shown). The side chains of Asn, Gln, Lys, and Arg are \(-\text{CH}_3\text{CONH}_2\), \(-\text{CH}_2\text{CONH}_2\), \(-\text{CH}_2\text{CH}_2\text{CONH}_2\), and \(-\text{CH}_2\text{CH}_2\text{CONH}_2\), respectively. It can be presumed that the length of the side chain affects ST activity; that is, the longer the side chain of the amino acid residue at position 11, the lower the enterotoxic activity of the ST. This means that a precise space at position 11 is required for ST activity.

As Asp has \(-\text{CH}_2\text{COOH}\) as the side chain, substitution for Asn with Asp may explain the role of \(-\text{NH}_2\) of the Asn residue. Such a substitution caused a decrease in ST activity to about 2% of that of the wild type (Table 2), suggesting that \(-\text{NH}_2\) at position 11 of STp plays an important role in ST activity. It has been said that the charge of amino acid side chains and the formation of hydrogen bonds play an essential role in determining the biological activity of an enzyme (1, 4). Therefore, it is possible that the amide group of Asn contributes to ST activity through the formation of hydrogen bonds with charged acceptors such as oxanion from the ST receptor and/or functional mediator of guanylate cyclase. It can be inferred that the low enterotoxic activity of ST (Asp-11) is caused by the inability to form a hydrogen bond between Asp-11 and the acceptor. The evidence that the ST analog with His-11 still retains a significant toxic activity (Table 2) may support this hypothesis, because the side chain of His is known to be charged positively in a neutral environment. The side chain of His-11 will be charged positively in the intestines and will form a hydrogen bond with the acceptor molecule of the ST receptor present in intestinal epithelial cells.

ACKNOWLEDGMENT

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture, Japan.

LITERATURE CITED