Amino Acid Residues in the Pro Region of *Escherichia coli* Heat-Stable Enterotoxin I That Affect Efficiency of Translocation across the Inner Membrane

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Escherichia coli heat-stable enterotoxin Ip (STIp), which is a typical extracellular toxin consisting of 18 amino acid residues, is synthesized as a precursor consisting of pre (amino acid residues 1 to 19), pro (amino acid residues 20 to 54), and mature (amino acid residues 55 to 72) regions. Though the pre region functions as a conventional leader peptide that guides the following region to cross the inner membrane, the role of the pro region in the maturation pathway remains to be elucidated. We previously indicated that the sequence from residues 29 to 38 in the pro region increases the efficiency of STI translocation across the inner membrane (H. Yamanaka, Y. Fuke, S. Hitotsubashi, Y. Fujii, and K. Okamoto, *Microbiol. Immunol.* 37:195–205, 1993). We therefore examined the amino acid residues in the sequence that are responsible for this function. We substituted several amino acid residues in the sequence by means of oligonucleotide-directed site-specific mutagenesis. We then evaluated the effect of the substitution on the efficiency of STI translocation across the inner membrane by determining the enterotoxic activity of the culture supernatant, the amount of a fusion protein consisting of STI and nuclease A released into the periplasm, and the amount of the labeled ST released into the periplasm after pulse-labeling with [³⁵S]cysteine. Substitution of the charged amino acid residues at positions 29 to 31 (K-E-K) with hydrophobic (I-V-L, F-W-F, or F-W-Q) or basic (K-K-K) residues significantly reduced these values in every assay. In contrast, the substitution of these amino acid residues with acidic amino acid residues (E-E-E) increased these values in all assays. This means that the negative charge near position 30 is important for STI to translocate efficiently across the inner membrane. A similar substitution of lysine residues at positions 37 and 38 showed that they are not involved in the translocation of STI across the inner membrane.

Escherichia coli heat-stable enterotoxin I (STI), elaborated by some enterotoxigenic *E. coli* strains after colonization of the small intestine, has been implicated as a causative agent of diarrhea in both infants and adults (1, 7, 11, 29). STI is an extracellular peptide consisting of 18 (STIp) or 19 (STIh) amino acid residues (2, 37). However, sequencing the STIp gene revealed that STIp is synthesized as a precursor of 72 amino acid residues in vivo (13, 33). Subsequent studies showed that the precursor of STIp consists of pre (amino acid residues 1 to 19), pro (amino acid residues 20 to 54), and mature (amino acid residue 55 to 72) ST regions (26). STIh is also synthesized as a precursor of 72 amino acid residues consisting of these three regions (13, 27).

The pre region of these STIs is composed of a conventional leader peptide which guides the following peptide to translocate across the inner membrane (26). The pre region is cleaved by signal peptidase in the inner membrane, and the resulting peptide, consisting of pro and mature regions, is released into the periplasm (26, 27). The released peptide is cleaved in the periplasm, and the generated peptide, consisting of the mature region, translocates across the outer membrane. The mature region released into the milieu acts as the enterotoxin elaborated by the cells (33, 37, 41). In this manner, the roles of the pre and the mature regions of STI in the maturation pathway

have been clarified. However, the role of the pro region in maturation remains to be elucidated.

To clarify the role of the pro region in the maturation pathway of STIp, we examined the translocation efficiency of hybrid proteins consisting of one or two of the three regions of STIp (pre, pro, and mature ST) and nuclease A (40). The results revealed that the mature STI region interferes with the translocation of the hybrid proteins across the inner membrane. Nevertheless, the mature STI region is secreted efficiently into the milieu by *E. coli*. We postulated that the pro region guides the mature STI region into the periplasmic space.

The enterotoxic activity of cells harboring the plasmid carrying the mutant STIp gene in which the gene of the pro region was deleted was remarkably lower than that of cells harboring the plasmid carrying the gene for native STI (26, 40). We constructed three derivatives of Tc-1 (parental plasmid for the STIp gene) by deleting the gene fragments encoding amino acids (i) 22 to 57, (ii) 29 to 38, and (iii) 40 to 53 in the pro region of STIp and examined the enterotoxic activity of the cells harboring these derivatives. The results showed that deleting the sequence encoding amino acids 29 to 38 in the pro region caused a remarkable reduction of the enterotoxic activity of the cell (40). Of additional interest is the observation that the sequence encoding residues 29 to 38 is well conserved in every STI gene with a known nucleotide sequence (see Fig. 1). This result suggests that the sequence from residues 29 to 38 is mainly responsible for the function of the pro region. However, which amino acid residues in the sequence from residues 29 to 38 are important for the pro region to express the function remains unknown.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Features	Source or reference
Strains		
HB101	Wild type	Takara Co.
BL-21	Containing T7 RNA polymerase gene	34
Plasmids		
Tc-1	Derivative of pBR322 containing STIp gene (parental plasmid for STIp gene)	33
pKK601	Base substitutions to introduce <i>NspV</i> site at position 53 of STIp in Tc-1	26
pTB051	Base substitution for K-29-E-30-K-31 → K-Q-K in STIp gene in pKK601	This work
pTB052	Base substitution for K-29-E-30-K-31 → E-E-E in STIp gene in pKK601	This work
pTB053	Base substitution for K-29-E-30-K-31 → K-K-K in STIp gene in pKK601	This work
pTB054	Base substitution for K-29-E-30-K-31 → F-W-Q in STIp gene in pKK601	This work
pTB055	Base substitution for K-29-E-30-K-31 → F-W-F in STIp gene in pKK601	This work
pTB056	Base substitution for K-29-E-30-K-31 → I-V-L in STIp gene in pKK601	This work
pTB061	Base substitution for K-37-K-38 → A-V in STIp gene in pKK601	This work
pTB062	Base substitution for K-37-K-38 → I-S in STIp gene in pKK601	This work
pTB063	Base substitution for K-37-K-38 → E-E in STIp gene in pKK601	This work
pTB064	Base substitution for K-37-K-38 → L-E in STIp gene in pKK601	This work
pTB065	Base substitution for K-37-K-38 → E-L in STIp gene in pKK601	This work
pIN-III- <i>OmpA</i> -#98	Derivative of pBR322 containing staphylococcal nuclease A gene	36
pKK605	Constructed by insertion of nuclease A gene to the <i>NspV</i> site of STIp gene of pKK601	26
pKK651	As pKK605 but from pTB051 carrying STIp(²⁹ K ³⁰ E ³¹ K → KQK) gene	This work
pKK652	As pKK605 but from pTB052 carrying STIp(²⁹ K ³⁰ E ³¹ K → EEE) gene	This work
pKK653	As pKK605 but from pTB053 carrying STIp(²⁹ K ³⁰ E ³¹ K → KKK) gene	This work
pKK654	As pKK605 but from pTB054 carrying STIp(²⁹ K ³⁰ E ³¹ K → FWQ) gene	This work
pKK655	As pKK605 but from pTB055 carrying STIp(²⁹ K ³⁰ E ³¹ K → FWF) gene	This work
pKK656	As pKK605 but from pTB056 carrying STIp(²⁹ K ³⁰ E ³¹ K → IVL) gene	This work
pKK661	As pKK605 but from pTB061 carrying STIp(³⁷ K ³⁸ K → AV) gene	This work
pKK662	As pKK605 but from pTB062 carrying STIp(³⁷ K ³⁸ K → IS) gene	This work
pKK663	As pKK605 but from pTB063 carrying STIp(³⁷ K ³⁸ K → EE) gene	This work
pKK664	As pKK605 but from pTB064 carrying STIp(³⁷ K ³⁸ K → LE) gene	This work
pKK665	As pKK605 but from pTB065 carrying STIp(³⁷ K ³⁸ K → EL) gene	This work
pET11	Containing a T7 promoter under the control of the <i>lac</i> operator	34
pT7-Blue(R)	Cloning vector for PCR-amplified gene	Takara Co.
pET11-601	STIp structure gene of pKK601 was cloned into the <i>NdeI</i> site of pET11 to be under the control of the T7 promoter	This work
pET11-051	As pET11-601 but with STIp gene from pTB051	This work
pET11-052	As pET11-601 but with STIp gene from pTB052	This work
pET11-053	As pET11-601 but with STIp gene from pTB053	This work
pET11-054	As pET11-601 but with STIp gene from pTB054	This work
pET11-055	As pET11-601 but with STIp gene from pTB055	This work
pET11-056	As pET11-601 but with STIp gene from pTB056	This work

Here, we examined the amino acid residues in the sequence from residues 29 to 38 responsible for the enterotoxic activity of cells. We also examined whether these amino acids are involved in the action of the pro region in guiding the following region into the periplasmic space. These investigations were performed using fusion proteins and by means of pulse-chase studies.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The host strain with which we analyzed the STI and fusion proteins was *E. coli* HB101. Pulse-labeling proceeded in *E. coli* BL21, the chromosome of which contains the T7 RNA polymerase gene.

The wild-type plasmid Tc-1 containing the STIp gene was constructed by inserting a 350-bp fragment containing the STIp gene into the *HindIII* site of pBR322 (33). The transformant with the plasmid was selected with ampicillin. Plasmid pKK601 was prepared by substituting the nucleotides around position 53 of STIp in Tc-1 to introduce an *NspV* site (26). Plasmid pKK605, which carries the gene for the fusion protein consisting of the pre and pro regions of STIp and nuclease A, was constructed by inserting the nuclease A gene into the *NspV* site of pKK601 (26). Plasmid pIN-III-*OmpA*-#98 was the source of the staphylococcal nuclease A gene (36). Plasmid pET11, which contains the T7 promoter under the control of the *lac* operator, was used as a vector in the pulse-labeling study.

Oligonucleotide-directed site-specific mutagenesis. The amino acid residues at positions 29 to 31 and 37 to 38 were substituted by oligonucleotide-directed

site-specific mutagenesis as described by Inouye and Inouye (14). The mutagenesis was performed with plasmid pKK601 in which the STIp gene was mutated at position 53 to introduce an *NspV* site (26). The mutagenic oligonucleotides and replaced amino acid residues are shown in Table 2. The correct mutation was confirmed by sequencing the DNA from every mutant by means of dideoxy chain termination (31).

Fusion of the nuclease A gene to the STp gene. Plasmids carrying the DNA sequence encoding the hybrid proteins consisting of the amino terminus of the STIp precursor fused to nuclease A were constructed by fusing the nuclease A gene to the portion of the STIp gene encoding the first 54 amino acids of the STIp precursor. Plasmids (pTB051, pTB052, pTB053, pTB054, pTB055, pTB056, pTB061, pTB062, pTB063, pTB064, and pTB065) were cleaved with *NspV*, and the 5' single-stranded regions of the resultant linear DNA were filled in with Klenow polymerase. A fragment of about 1,300 bp including the nuclease A gene was obtained by *EcoRI*-*Sall* digestion of pIN-III-*OmpA*-#98, and both 5' single-stranded regions were filled in with Klenow polymerase. The fragment was cloned into the *NspV* site of each plasmid by blunt-end ligation. The reaction mixture was transformed into competent HB101 cells, and the transformants were selected on ampicillin-DNA agar plates (Nissui Co., Tokyo, Japan) for detecting nuclease activity as described elsewhere (40). The plasmids derived from pTB051, pTB052, pTB053, pTB054, pTB055, pTB056, pTB061, pTB062, pTB063, pTB064, and pTB065 were designated pKK651, pKK652, pKK653, pKK654, pKK655, pKK656, pKK661, pKK662, pKK663, pKK664, and pKK665, respectively (Table 1).

Construction of the plasmid for the pulse-labeling experiment. The STIp gene was selectively expressed by cloning it into pET11, which contains a T7 promoter under the control of the *lac* operator (34). A DNA fragment containing the STIp gene was amplified from the pKK601 plasmid by 25 PCR cycles of 30 s at 95°C

TABLE 2. Mutagenic oligonucleotides

Oligonucleotide	Nucleotide sequence ^a	Replacement of amino acid(s) ^b	Recognition site induced by mutation ^c
051	5' TCTTCAAAACAGAAAATTAC 3'	K-29-E-30-K-31 → K-Q-K	(-)
052	5' TCTTCAGAAGAGGAAATTACA 3'	K-29-E-30-K-31 → E-E-E	(-)
053	5' TCTTCAAAAAAGAAAATT 3'	K-29-E-30-K-31 → K-K-K	(-)
054	5' TTGACTCTTCATTTTGGCAAATTACATT 3'	K-29-E-30-K-31 → F-W-Q	(-)
055	5' GACTCTTCATTTTGGTTCATTACATTAG 3'	K-29-E-30-K-31 → F-W-F	(-)
056	5' GACTCTTCAATCGTGTGATTACATTAG 3'	I-29-V-30-K-31 → I-V-L	(-)
061	5' TTAGAGACTGCAGTGTGTGATGTT 3'	K-37-K-38 → A-V	<i>Pst</i> I
062	5' TTAGAGACTATCAGCTGTGATGTT 3'	K-37-K-38 → I-S	<i>Pvu</i> II
063	5' TTAGAGACTGAAGAGTGTGATGTT 3'	K-37-K-38 → E-E	(-)
064	5' TTAGAGACTCTCGAGTGTGATGTT 3'	K-37-K-38 → L-E	<i>Xho</i> I
065	5' TTAGAGACTGAGCTCTGTGATGTT 3'	K-37-K-38 → E-L	<i>Sac</i> I

^a The underlines indicate mismatches.^b Numbers indicate the positions from the amino-terminal residues of the ST precursor composed of 72 amino acids.^c (-), the recognition site was not generated by the mutation.

for denaturation, 1 min at 50°C for annealing, and 3 min at 72°C for elongation. The sequences of the oligonucleotides used as primers were (i) 5' GGGAGGT CATATGAAAAAGC 3' (sense) and (ii) 5' CCCTGGATCCTTTTAATAAC 3' (antisense). Primers i and ii extended from bases 276 to 295 and from bases 497 to 517 of the STIp gene reported by So and McCarthy (33), respectively. Primer i was designed to contain two mismatches at positions 283 and 285 (C and T indicated by the underlines) to create an *Nde*I site. The initiation codon (ATG) lies one base after the *Nde*I cleavage site. This allowed the structural STIp gene to be cloned into the *Nde*I site of pET11 downstream from the T7 promoter. Primer ii contained mismatches at positions 501, 502, and 505 (these bases are indicated by underlines) which created a *Bam*HI site. The PCR product was ligated into pT7-Blue(R) (Takara Co., Kyoto, Japan), which is the cloning vector for the PCR-amplified DNA fragment (Table 1). An *Nde*I-*Bam*HI fragment from the recombinant plasmid carrying the STIp gene was isolated and inserted into pET11 digested with *Nde*I and *Bam*HI. The correct insertion of the STIp structure gene was confirmed by sequencing the nucleotide of the flanking DNA of the cloned gene. The resulting plasmid was named pET11-601 (Table 1). Likewise, the structural genes of mutant STIps encoded in plasmids pTB052, pTB053, pTB054, pTB055, and pTB056 were cloned into pET11. These plasmids were named pET11-052, pET11-053, pET11-054, pET11-055, and pET11-056, respectively (Table 1).

Pulse-labeling. BL-21 cells transformed with pET11-derivative plasmids were cultured until they reached an optical density of 0.4 at 660 nm in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) (24) containing ampicillin (50 µg/ml) at 37°C with shaking. The culture was centrifuged, and the cell pellet was suspended in M9 minimal medium (24) containing an amino acid mixture (the final concentration of each amino acid was 50 µg/ml) without cysteine at an optical density of 3.0 at 660 nm. After addition of a final concentration of 2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), the suspension was shaken at 37°C for 20 min. A portion (1 ml) was labeled for 30 s at 37°C with 10 µCi of L-[³⁵S]cysteine (0.5 to 1 kCi/mmol; ARS-101; American Radiolabeled Chemicals, Inc., St. Louis, Mo.) per ml. Incorporation of the label was terminated with the addition of 250 µg of unlabeled cysteine. Immediately after the addition of the unlabeled cysteine, a portion of the culture (50 µl) was taken out and the cells were collected by centrifugation. The cells were mixed with sodium dodecyl sulfate (SDS) dye (21), heated at 100°C for 10 min, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (21).

The remaining culture solution (950 µl) was chased for 30 s. The culture was centrifuged, and the cell pellet was washed with M9 salts (24) and resuspended in 300 µl of distilled water. The periplasmic fraction of the cells and the inner membrane fraction of the cells were prepared by the polymyxin B and sarcosyl methods, respectively. The details of these methods are described in the next section. Ten microliters of each sample prepared was resolved by SDS-PAGE.

The radioactive bands were visualized by autoradiography using an imaging plate analyzer (BAS 2000; Fujix, Tokyo, Japan).

Preparations of periplasmic fraction and inner membrane fraction. The cells cultured in liquid medium were collected by centrifugation, and the cell pellet was suspended in 10 mM Tris-HCl buffer (pH 7.5). To prepare the periplasmic fraction, the cell suspension was incubated with polymyxin B at a concentration of 6,500 U/ml at 4°C for 15 min and centrifuged (12,000 × g for 15 min). The supernatant obtained was used as the periplasmic fraction.

To prepare the inner membrane fraction, the method described by Filip et al. (9) was used. Briefly, the cell suspension was intermittently sonicated on ice for 2 min and then centrifuged (150,000 × g). The pellet was suspended in 10 mM phosphate buffer (pH 7.1). An equal volume of 1% sodium lauroylsarcosine was added and incubated on ice for 5 min. The sample was centrifuged at 180,000 × g for 30 min. The supernatant obtained was used as the inner membrane fraction.

Assay of STI activity. STI activity was determined by means of the suckling mouse assay as described previously (25). Briefly, 0.1-ml samples were administered via a gastric tube into the stomachs of 2- to 3-day-old suckling mice, using 0.01% Evans blue dye as a marker. The mice were killed 3.0 h later, and a ratio of intestinal to body weight of more than 0.083 was considered positive. One unit was defined as the minimum effective dose that elicited a positive response, and the enterotoxin titer was expressed as the reciprocal of the highest dilution that gave 1 U of enterotoxin activity. The enterotoxin activity of each sample was determined in five mice.

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (21).

Protein determination. Protein was measured by the method of Lowry et al. (22) with bovine serum albumin as the standard.

RESULTS

Two stretches of charged amino acids were located at positions 29 to 31 and 37 to 38 in the sequence from residues 29 to 38 (Fig. 1). In some proteins, charged amino acid residues play an important role in the translocation of the protein across the membrane. However, they play only a minor role in other proteins (5, 16, 18). To clarify their role in the maturation pathway of STI, we substituted these charged amino acid residues by oligonucleotide-directed site-specific mutagenesis using the oligonucleotides shown in Table 2.

Role of the amino acid residues at positions 29 to 31. (i) Effects of amino acid substitutions on enterotoxigenicity. The plasmids carrying the STIp gene mutated at positions 29 to 31 (pKK601, pTB051, pTB052, pTB053, pTB054, pTB055, and pTB056) were introduced into HB101, and the transformants were shaken in L broth containing ampicillin (50 µg/ml) at 37°C to an optical density of 1.0 measured at 660 nm. The supernatant was separated from the cells by centrifugation. The STI activity of the supernatants was determined by the suckling mouse assay. The results are shown in Fig. 2A. The enterotoxin activities of the culture supernatant of cell harboring pTB056, pTB055, and pTB054 were 24, 30, and 32% of that of the parent cells (cells harboring pKK601, the parent plasmid). The amino acid residues at positions 29 to 31 of these mutant STIps produced by cells harboring pTB056, pTB055, and pTB054 were replaced with I-29-V-30-L-31, F-29-W-30-F-31, and F-29-W-30-Q-31, respectively, which are hydrophobic. This means that the replacement of the amino acid residues at positions 29 to 31 with hydrophobic amino acid residues induced a significant reduction of the enterotoxin activity, meaning that the hydrophilicity brought about by the sequence of K-29-E-30-K-31 plays an important role in the maturation pathway of STIp.

There are two positively charged amino acids (lysine resi-

	20				25					29	30	31			35		37	38		
STp (STA 1)	Gln	Ser	Thr	Glu	Ser	Leu	Asp	Ser	Ser	Lys	Glu	Lys	Ile	Thr	Leu	Glu	Thr	Lys	Lys	Cys
STh (STA 2)	-	Asp	Ala	Lys	Pro	Ala	Gly	-	-	-	-	-	-	-	-	-	Ser	-	-	-
STh (STA 3)	-	Asp	Ala	Lys	Pro	Val	Glu	-	-	-	-	-	-	-	-	-	Ser	-	-	-

	40				45					50			54		
STp (STA 1)	Asp	Val	Val	Lys	Asn	Asn	Ser	Glu	Lys	Lys	Ser	Glu	Asn	Met	Asn
STh (STA 2)	Asn	Ile	-	-	Lys	-	Asn	-	Ser	Ser	Pro	-	Ser	-	-
STh (STA 3)	-	Ile	Ala	-	Lys	Ser	Asn	-	Ser	Gly	Pro	-	Ser	-	-

FIG. 1. Comparison of the amino acid sequence of the pro region of STIs predicted from the nucleotide sequences. The STp (STA 1) sequence is from So and McCarthy (33), and the STh (STA 2) and STh (STA 3) sequences are from Guzman-Verduzco and Kupersztoch (13). For the STh (STA 2) and STh (STA 3) sequences, only the amino acids that differ from STp (STA 1) are shown; dashes in the sequences indicate identical amino acids.

dues at positions 29 and 31) and one negatively charged residue (glutamic acid at position 30) in the sequence from residues 29 to 31. Although the hydrophilicity of this sequence is conferred by these positively and negatively charged amino acid residues, it is not known which of the electric charges, positive or negative, contributes to the maturation of STI. To clarify the kind of charge contributing in the maturation, we substituted for the amino acid residues K-29-E-30-K-31 with K-29-K-30-K-31 and E-29-E-30-E-31 and examined the enterotoxic activity of the culture supernatant of the cells harboring these mutant plasmids. The enterotoxic activity of the culture supernatant of cells harboring pTB053, which produce STIp ($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{KKK}$), was about 60% of that of the wild-type strain (HB101/pKK601), meaning that the substitution with K-29-K-30-K-31 caused a 40% loss of enterotoxic activity (Fig. 2A). In contrast, the substitution with E-29-E-30-E-31

caused a 1.5-fold increase of enterotoxic activity, as shown by that of the cells harboring pTB052 (Fig. 2A). These results showed that the negative charge near position 30 is important for the precursor of STI to become the mature form.

The negative charge is afforded by glutamic acid at position 30 in the wild-type STI. To confirm the involvement of this negative charge in maturation, glutamic acid was replaced with glutamine. The enterotoxic activity of cells producing STIp ($^{29}\text{K}^{30}\text{Q}^{31}\text{K}$) was 60% of that of the parent cells (HB101/pKK601) (Fig. 2A). This result supports the notion that the glutamic acid at position 30 is important for the precursor to become mature STI.

(ii) **Analysis of the fusion protein.** Previously, we constructed plasmids carrying the DNA sequences of hybrid proteins consisting of the constituent(s) of STI and nuclease A and analyzed the fusion protein produced by the cells harboring

FIG. 2. Enterotoxic activities of the culture supernatants of the strains harboring plasmids. *E. coli* HB101 transformed with the indicated plasmids was cultured in L broth containing ampicillin (50 $\mu\text{g}/\text{ml}$) with shaking at 37°C to an optical density at 660 nm. The culture supernatants were separated from the cells by centrifugation, and the enterotoxic activities of the supernatants were determined by the suckling mouse assay as described in the text. The parentheses show the amino acid substitutions induced by the site-specific mutation to generate the appropriate plasmid.

FIG. 3. Schematic representation of the structure of the fusion protein synthesized by cells harboring pKK651. The pre and pro regions consist of those of the mutant STIp(²⁹K³⁰E³¹K → KQK) precursor. The nuclease region consists of the mature region of the staphylococcal nuclease A (36). The solid box represents the extra sequences generated by the gene manipulations. The amino acids and nucleotides of the extra sequence are shown under the box. The residues are numbered from the amino-terminal residue of the peptide.

these plasmids. The results showed that the pro region of STI helped the mature STI region to translocate across the inner membrane (40). The mutational analysis described in section i above revealed that the sequence at positions 29 to 31 in the pro region is important in the maturation of STIp. Taking this result together with the previously described results (40), we assumed that the sequence from positions 29 to 31 was involved in the translocation of the precursor of STI across the inner membrane. To confirm this notion, we constructed plasmids carrying genes for hybrid proteins consisting of nuclease A and the pre and pro regions of which the nucleotide sequences at positions 29 to 31 were mutated. We then analyzed the fusion proteins synthesized from cells harboring these plasmids (pKK651, pKK652, pKK653, pKK654, pKK655, and pKK656) (Table 1). The structure of the product of the fusion gene encoded in pKK651 is schematically represented in Fig. 3. The amino acid sequences at positions 29 to 31 of other fusion proteins whose genes are carried by pKK652, pKK653, pKK654, pKK655, and pKK656 were mutated as shown in

Table 1. *E. coli* HB101 was transformed with these mutants and the parent plasmid (pKK605). The transformants were cultured in L broth containing ampicillin (50 μg/ml) with shaking at 37°C for 18 h. The materials in the periplasmic space were extracted with polymyxin B as described in Materials and Methods and separated by SDS-PAGE. The gels were stained with Coomassie brilliant blue (Fig. 4A). The position of the fusion proteins is indicated by the arrow in Fig. 4A. We demonstrated that the fusion protein synthesized by the cells harboring pKK605 is proteolytically processed between positions 19 and 20 and that the resulting peptide consisting of the pro region and nuclease A (pro-Nuc) was released into the periplasmic space (40). As shown in Fig. 4A, the mobility in SDS-PAGE of all fusion proteins examined was identical to that of the native fusion protein produced by the cells harboring pKK605. This means that all the mutant fusion proteins were cleaved between residues 19 and 20. This indicated that the mutation did not induce a significant alteration of the function and conformation of the leader peptide (the amino-terminal peptide of 19 amino acid residues).

FIG. 4. SDS-PAGE analysis of the fusion proteins accumulated in the periplasmic fraction. *E. coli* HB101 harboring the indicated plasmid was grown in L broth containing ampicillin (50 μg/ml) to an optical density at 1.0 measured at 660 nm. The periplasmic fraction was prepared and analyzed as described in the text. After electrophoresis, the acrylamide gel was stained by Coomassie brilliant blue R-250. Arrows indicate the positions of the fusion proteins. The parentheses show the amino acid substitutions induced by the site-specific mutation to generate the appropriate plasmid. Numbers along the side refer to the molecular masses of standard proteins (in kilodaltons).

FIG. 5. Detection of mutant STI with [³⁵S]cysteine in cells (A), in the periplasm (B), and in the inner membrane (C). Exponentially growing *E. coli* BL-21 cells harboring various plasmids were induced with IPTG and labeled for 30 s with [³⁵S]cysteine. The labeling was terminated by adding cold cysteine to the culture. Some cells were taken out immediately after the addition of cold cysteine and collected by centrifugation. The collected cells were mixed with SDS dye solution (21) and resolved by SDS-PAGE after heating at 100°C for 10 min. After electrophoresis, the radioactive bands were detected by image analysis as described in the text (A). The remaining cells were chased for 30 s. The cells were collected by centrifugation. The materials in the periplasmic space of the cells (B) and the materials in the inner membrane of the cells (C) were recovered by the polymyxin B and sodium lauroylsarcosine methods, respectively, as described in the text. The materials obtained were mixed with SDS dye, heated at 100°C for 10 min, and then resolved by SDS-PAGE. The parentheses show the amino acid substitutions induced by the site-specific mutation to generate the appropriate plasmid. Plasmid pET-11 is a vector that does not carry the STI gene.

However, there were considerable differences in the amounts of the pro-Nuc in the periplasmic space among samples (Fig. 4A). The levels of three fusion proteins replaced with F-29-W-30-Q-31, F-29-W-30-F-31, and I-29-V-30-L-31 were very low compared with that of the native fusion protein (Fig. 4A, compare the fusion proteins in lanes 5, 6, and 7 with that in lane 1). These replaced amino acid residues were hydrophobic. As shown in section i above, the enterotoxic activities of the cells producing the mutant STIPs containing these hydrophobic amino acid residues at positions 29 to 31 [STIP(²⁹F³⁰V³¹L), STIP(²⁹F³⁰W³¹F) and STIP(²⁹F³⁰W³¹Q)] were lower than that of cells producing native STIP (Fig. 2A). Likewise, the substitution for K-29-E-30-K-31 with hydrophobic amino acid residues reduced not only of the enterotoxic activity of the cells but also the amount of the fusion protein released into the periplasmic space of the cells.

As shown in Fig. 4A, the amount of the mutant fusion protein replaced with K-29-K-30-K-31 (lane 4) was slightly smaller than that of the parental strain and that of the mutant fusion protein replaced with E-29-E-30-E-31 (lane 3) was larger than that of the parent strain. These alterations were also related to the enterotoxic activity of the culture supernatant of cells producing the corresponding mutant STIP (Fig. 2A).

As shown in lane 2 of Fig. 4A, the substitution for glutamic acid at position 30 with glutamine induced a slight but apparent reduction in the amount of the fusion protein. As described in section i above, this slight reduction was also induced in the enterotoxic activity of the cell by the substitution for glutamic acid at position 30 with glutamine.

These results showed that the mutation at positions 29 to 31 altered the amount of the fusion protein in the periplasm and that the amount of each mutant fusion protein was proportional to the enterotoxic activity of the culture supernatant of cells producing the mutant STI possessing the identical mutation at positions 29 to 31. As the amount of fusion protein in the periplasm reflects the efficiency of the translocation across

the inner membrane, the fact that the substitution of amino acid residues at positions 29 to 31 altered the levels of the fusion proteins in the periplasm means that the sequence from positions 29 to 31 is directly involved in the translocation of the fusion protein across the inner membrane. As described, there is a close relationship between the enterotoxic activity of cells producing the mutant STIP and the amount of the corresponding mutant fusion protein. This suggests that the sequence from positions 29 to 31 plays an important role in the translocation across the inner membrane of the mature ST region.

(iii) Analysis of labeled ST. To further confirm the involvement of the sequence from positions 29 to 31 in the translocation of the mature ST region across the inner membrane, we examined the amount of the intermediate of STI consisting of pro and mature ST regions in the periplasm by means of pulse-chase studies. Transformed BL-21 cells harbored pET11-601, pET11-051, pET11-052, pET11-053, pET11-054, pET11-055, and pET11-056, which produced native STIP, STIP(²⁹K³⁰E³¹K → KQK), STIP(²⁹K³⁰E³¹K → EEE), STIP(²⁹K³⁰E³¹K → KKK), STIP(²⁹K³⁰E³¹K → FWQ), STIP(²⁹K³⁰E³¹K → FWF), and STI(²⁹K³⁰E³¹K → IVL), respectively. *E. coli* BL-21 transformed with pET11, which does not produce STI, was the control. We pulse-labeled the cells with [³⁵S]cysteine for 30 s and terminated the labeling by the addition of cold cysteine. Immediately after addition of the cold cysteine, some of the cells was taken out. The total cellular proteins of the cells were resolved by SDS-PAGE and then visualized by autoradiography. As shown in Fig. 5A, the dense bands appeared in all lanes except for the lane containing the control sample (lane C, BL-21/pET11). Therefore, all these bands are considered to be STIPs. This shows that the STIPs were synthesized in all mutant cells examined in this study, indicating that the mutations did not affect the synthesis of STIP in the cells.

As shown in lane 2 of Fig. 5A, the migration position of STIP(²⁹K³⁰E³¹K → EEE) is different from those of other STIPs. As the sizes of STIPs which were examined are small

(M_r of the precursor consisting of pre, pro, and mature regions, ca. 8,000; M_r of the intermediate consisting of pro and mature regions, ca. 6,000), the site in the STIp in which SDS binds is thought to be restricted. Therefore, the amount of SDS adhering to STIp is strongly influenced by the composition of amino acid residues of STIp. The different migration position of STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{EEE}$) from other STIps might be induced by the characteristic of STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{EEE}$) in binding to SDS.

The remaining cells were chased for 30 s. The periplasmic materials of the chased cells were recovered with polymyxin B as described in Materials and Methods. The periplasmic materials were resolved by SDS-PAGE and then visualized by autoradiography (Fig. 5B).

Samples of cells harboring the derivative plasmids of pET11 contained the band(s) that was absent in lane C of Fig. 5B, which shows the control periplasmic materials (BL-21/pET11). These bands were thus intermediates of each STIp in the periplasm.

There are differences in the density of the band. The band was very faint in lanes 5, 6, and 7 of Fig. 5B [samples of cells producing STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{FWQ}$), STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{FWF}$), and STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{IVL}$), respectively]. The bands in lanes 3 and 4 [samples of cells producing STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{KKK}$) and STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{KQK}$)] were fainter than that in lane 1 (sample of parent cells). In contrast, the band in lane 2 [sample of cells producing STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{EEE}$)] was denser than that in lane 1. These differences in the densities of the bands corresponded with those shown in the amount of fusion proteins (Fig. 4A) and in the level of enterotoxigenic activity (Fig. 2A).

To examine the intermediates in the inner membrane, the inner membrane fractions of the cells chased for 30 s were prepared. These fractions were resolved by SDS-PAGE (Fig. 5C). The intermediate was detected in every sample except the sample from the control strain (BL-21/pET11) (lane C of Fig. 5C). The amount of the intermediate differed with the sample. As shown in Fig. 5C, the bands in lanes 5 and 6 [samples of cells producing STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{FWQ}$) and STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{FWF}$), respectively] were denser than that in lane 1 (sample of parent cell). That is, more intermediates of STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{FWQ}$) and STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{FWF}$) than of native STIp existed in the inner membrane.

In contrast, the densities of the bands in lanes 2, 3, 4, and 7 [samples of cells producing STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{EEE}$), STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{KKK}$), STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{KQK}$), and STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{IVL}$), respectively] were not significantly different from that in lane 1 (sample of parent cell).

Role of the amino acid residues at positions 37 and 38. We also examined the effect of the mutation at positions 37 and 38 on the efficiency of translocation of the following region across the inner membrane. The method was the same as that used to determine the role of the sequence from positions 29 to 31.

The cells transformed with the plasmids of pKK661, pKK662, pKK663, pKK664, and pKK665 (Table 1), carrying the gene encoding the fusion protein of the pre and pro regions of mutant STIp and nuclease A, were cultured, and the fusion proteins released into the periplasmic space were analyzed. As shown in Fig. 4B, the amount of the fusion protein did not vary among samples. This result shows that the amino acid residues at positions 37 and 38 did not play an important role in the translocation of the fusion protein across the inner membrane.

Subsequently, plasmids pTB061, pTB062, pTB063, pTB064, and pTB065 (Table 1) encoding STIps mutated at positions 37 and 38 were introduced into HB101. The enterotoxigenic activities of the culture supernatants of these transformants were deter-

mined as described in section i above, and the results are shown in Fig. 2B. None of the mutations at positions 37 and 38, except K-37-K-38 $\rightarrow$ E-37-E-38, induced a significant alteration of the enterotoxigenic activity. These results indicated that the amino acid residues at positions 37 and 38 do not play an important role in the maturation pathway of STI.

DISCUSSION

E. coli proteins that are destined for translocation across the inner membrane are synthesized as preproteins with amino-terminal extensions called leader or signal sequences ranging from 15 to 30 amino acids in length. The leader sequence is essential for efficient translocation of the protein across the inner membrane (6, 32, 39). However, some proteins possess determinants in the mature domain of preproteins which contribute to the translocation efficiency across the inner membrane (3, 4, 15, 17, 23, 28, 35, 42).

We studied the synthesis and secretion of STI *in vivo* and found that STI is synthesized as a precursor consisting of pre, pro, and mature regions, of which the pre region is composed of a leader sequence (26). Subsequently, we found that the pro region of STI helps the nascent STI to translocate across the inner membrane (40), as do the determinants of some proteins introduced above. Our further studies indicated that some amino acid residues in the sequence from positions 29 to 38 are responsible for this action of the pro region (40).

On the basis of these observations, we prepared several mutant plasmids by substituting the amino acid residues constituting the sequence from positions 29 to 38 by means of oligonucleotide-directed site-specific mutagenesis (Table 1) and determined the enterotoxigenic activities of cells harboring these mutant plasmids. As shown in Fig. 2A, substitution for the amino acid residues at positions 29 to 31 significantly altered the enterotoxigenicity, meaning that the amino acid residues at positions 29 to 31 are involved in the maturation pathway of STIp. The maturation of STI comprises many steps, such as translocation across the inner membrane, folding in the periplasm, resistance to protease in the periplasm, translocation across the outer membrane, and so on. Therefore, the step in which the sequence from positions 29 to 31 is involved remained unknown.

To examine the step(s) in which the sequence from positions 29 to 31 was involved during the maturation of STIp, we constructed plasmids containing the fusion gene and analyzed the fusion proteins produced by cells harboring these plasmids. Subsequently, we performed pulse-chase studies to examine the involvement of the sequence in the translocation. As shown in Fig. 4A, the mutation of amino acid residues at positions 29 to 31 caused alterations in the amounts of these fusion proteins released into the periplasm. The pulse-chase experiment also revealed that the mutation altered the amount of the STIp intermediates released into the periplasm (Fig. 5B). These results showed that the mutation at positions 29 to 31 changed the efficiency of the translocation across the inner membrane, meaning that the sequence from positions 29 to 31 is important for the nascent precursor STIp to cross the inner membrane.

As shown in Fig. 4A, substituting the amino acid residues at positions 29 to 31 with hydrophobic amino acid residues (F-29-W-30-Q-31, F-29-W-30-F-31, or I-29-V-30-L-31) remarkably reduced the amount of the fusion protein in the periplasm. In the pulse-chase experiment, the amount of the STIp released into the periplasmic space was dramatically reduced in the mutant cells producing STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{FWQ}$), STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{FWF}$), and STIp($^{29}\text{K}^{30}\text{E}^{31}\text{K} \rightarrow \text{IVL}$) (Fig. 5B). Stretches of hydrophobic amino acid residues

in a protein generally retard the rate at which the protein crosses the membrane (19, 30). This is called the stop-transfer effect (8, 20).

To clarify the stop-transfer effect of the hydrophobic amino acid residues at positions 29 to 31, we measured the amount of the mutant STIPs in the inner membrane of cells (Fig. 5C). As expected, the amount of STIPs in the inner membrane of cells producing STIP(²⁹K³⁰E³¹K → FWQ) and STIP(²⁹K³⁰E³¹K → FWF) (lanes 5 and 6 of Fig. 5C) was larger than that in the control cells producing native STIP (lane 1 of Fig. 5C). This shows that these substituted hydrophobic amino acid residues function to stop the transfer of STIP across the inner membrane. Therefore, the substitutions with these hydrophobic amino acids might induce the loss of the ability of the pro region to help the nascent STI with translocation across the inner membrane.

However, the replacement with I-V-L (these are also hydrophobic amino acid residues) did not induce a significant alteration in the amount of the STIP in the inner membrane (lane 7 of Fig. 5C). Judging from the result given above, it is likely that the mutation of K-29-E-30-K-31 to I-V-L also confers the stop-transfer property to the STIP. At present, the reason why the substitution I-V-L for K-E-K at positions 29 to 31 did not induce the alteration of the amount of the STIP in the inner membrane remains unknown.

As shown in lanes 7 of Fig. 5, the mutation of K-29-E-30-K-31 to I-V-L did not affect the synthesis of STIP in the cytoplasm (Fig. 5A), reduced the amount of STIP in the periplasm (Fig. 5B), and did not induce a significant alteration of the amount of STIP in the inner membrane (Fig. 5C). Therefore, we think that other properties in addition to the stop-transfer property generated in the mutant STIP, such as (i) the quick digestion of STIP(²⁹K³⁰E³¹K → IVL) entering into inner membrane by proteases and (ii) the low efficiency of the synthesized STIP(²⁹K³⁰E³¹K → IVL) in cytoplasm in entering the inner membrane, function to reduce the amount of the STIP in the inner membrane. On the other hand, the stop-transfer effect functions to increase the amount of the STIP in the inner membrane. It is likely that the amount of STIP(²⁹K³⁰E³¹K → IVL) in the inner membrane did not apparently alter because of these positive and negative effects. Further studies are necessary to determine the precise effect of the mutation of K-29-E-30-K-31 to I-V-L on the translocation of the STIP across the inner membrane.

Substituting the amino acid residues at positions 29 to 31 with basic amino acids (K-29-K-30-K-31) reduced the amounts of proteins in the periplasm in the fusion protein and pulse-chase experiments. On the other hand, substitution with acidic amino acids (E-29-E-30-E-31) increased the amount of these proteins (Fig. 4A and 5B). These results indicated that a negative charge near position 30 is favorable for the precursor of STIP to cross the inner membrane, meaning that the glutamic acid at position 30 plays an important role in the translocation across the inner membrane. This notion was supported by the finding that substituting glutamic acid at position 30 with glutamine significantly reduced the enterotoxicity of the culture supernatant (Fig. 2A), as well as the amounts of the fusion protein and the labeled STIP released into the periplasm (Fig. 4A and 5B). However, it is unknown how the negatively charged residue exerts its effects on the translocation of the precursor of STIP across the inner membrane.

The membrane potential across the inner membrane in *E. coli* is such that the positive charge is on the periplasmic surface (11). The membrane potential functions as the driving force in controlling the topology of membrane proteins such as leader peptidase (5, 16, 18) and M13 precoat protein (12, 38).

The membrane potential promotes the translocation across the inner membrane of the negatively charged domain of these proteins and impedes the translocation of the basic amino acid domain. Geller et al. have found that the translocation of the Sec-dependent pro-OmpA mutant protein, which is exported, is controlled by the driving force provided by the membrane electrical potential (10). Judging from these reports, it is likely that STI uses the membrane electrical potential as the driving force in crossing the inner membrane. As described, STIP translocation across the inner membrane was affected by the charge near position 30. That is, the membrane potential may promote the translocation across the inner membrane of the mutant STIP, of which the residues near position 30 are replaced with negatively charged amino acids. In contrast, the membrane potential may restrict the transfer of the mutant STIP in which the residues near position 30 are replaced with positively charged amino acids (Fig. 2A, 4A, and 5B).

However, these substitutions (K-29-E-30-K-31 to E-E-E, K-K-K, and K-Q-K) did not markedly alter the amount of STIP in the inner membrane (lanes 1, 2, and 3 of Fig. 5C). The sensitivity of the method used may be too low to measure quantitatively the amount of STIP which passes through the inner membrane. Detailed experiments should be done carefully to examine the effect of the membrane potential in the behavior of STI in the inner membrane.

As shown in Fig. 4B, substitution of the lysine residues at positions 37 and 38 did not alter the amount of the fusion protein in the periplasm, meaning that the mutations did not alter the translocation efficiency of the fusion protein across the inner membrane. This indicated that these lysine residues do not respond to the driving force provided by the membrane electrical potential. In other words, the membrane electrical potential functions upon specific charged amino acid residues. Further studies are necessary to clarify the factor that determines the specificity.

As shown in Fig. 2B, the enterotoxic activity of the culture supernatant of cells producing STIP(³⁷K³⁸K → EE) was about 45% of that of the wild-type strain. However, the mutation of K-37-K-38 to E-E did not reduce the amount of the fusion protein released into the periplasm (lane 3 of Fig. 4B), showing that the mutation did not affect the efficiency of the fusion protein translocation across the inner membrane. This indicated that the translocation of the nascent STIP across the inner membrane is not obstructed by the mutation of K-37-K-38 to E-E. The STI intermediate composing pro and mature STI regions released into the periplasm is processed by periplasmic enzymes. It is likely that the mutation of K-37-K-38 to E-E induced conformational changes of the intermediate STIP and that the enzymes involved in the processing could not act on the mutant STIP. This might reduce the enterotoxic activity of the culture supernatant of the cells producing STIP(³⁷K³⁸K → EE). Further studies on the conformation of the STI intermediates in the periplasm are in progress in our laboratory.

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