Cholera toxin (CT) and Escherichia coli heat-labile enterotoxin (LT) are causative agents of diarrhea and are structurally, functionally, and immunologically similar (1). Both toxins cause increase in cyclic AMP in target cells by catalyzing guanylyl cyclase in the cells, whereas the B subunit, consisting of 103 amino acids, binds the toxin to a receptor, GM₁-ganglioside, on the cell surface.

A mutant isolated after treatment of E. coli producing heat-labile enterotoxin with N-methyl-N'-nitro-N-nitrosoguanidine produces a B subunit that is unable to bind to ganglioside. This subunit was purified and its primary amino acid sequence was determined. It differed from the native B subunit in only one amino acid at position 33; namely it had aspartate instead of glycine at position 33 from the N terminus.

Thus glycine at position 33 from the N terminus of the B subunit is important for binding the B subunit to the ganglioside receptor.

Characterization of B subunit mutants that cannot bind to GM₁-ganglioside receptor was accomplished by subjecting mutants producing a different B subunit of LT (LT-B') that could not bind to GM₁-ganglioside receptor to centrifugation, washed once with minimal medium, and suspended in 2 ml of minimal medium. Then 0.6 ml of the mutagenized cell suspension was mixed with 3 ml of minimal medium and suspenesed for 3 h at 37 °C with shaking. Penicillin and tetacycline were then added at final concentrations of 1000 μg/ml and 20 μg/ml, respectively, and after incubation for 16 h, 0.1 ml of the culture were plated on brain heart infusion agar (Difco). The plates were incubated at 37 °C for 1 day, and then the sensitivities of the colonies to tetacycline were tested on brain heart infusion agar plates containing tetacycline.

**Materials and Methods**

Bacteria—A porcine strain of enterotoxigenic E. coli WT-1 producing LT (14, 15) was used.

**Isolation of Tetacycline-sensitive Mutants**—Mutagenesis was induced by the method of Koyama (16). A single colony of E. coli WT-1 was picked up, inoculated into 5 ml of minimal medium (16), and incubated with shaking for 8 h at 30 °C. Then 15 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine (NGD) was added and the culture stood for 15 min at 30 °C. The cells were then harvested by centrifugation, washed once with minimal medium, and suspended in 2 ml of minimal medium. Then 0.5 ml of the mutagenized cell suspension was mixed with 3 ml of minimal medium and suspended for 3 h at 37 °C with shaking. Penicillin and tetacycline were then added at final concentrations of 1000 μg/ml and 20 μg/ml, respectively, and after incubation for 16 h, 0.1 ml of the culture were plated on brain heart infusion agar (Difco). The plates were incubated at 37 °C for 1 day, and then the sensitivities of the colonies to tetacycline were tested on brain heart infusion agar plates containing tetacycline.

**Isolation of Mutants**—Mutagenesis of E. coli was induced with NGD by the method reported by Silva et al. (15). The tetacycline-sensitive mutants were isolated in 10 ml of CAYE broth (26) in Erlenmeyer flasks and incubated with shaking for 8 h at 37 °C. CAYE medium contained 2% casamino acids (Difco), 0.6% yeast extract (Difco), 0.25% NaCl, 0.87% KH₂PO₄, 0.25% glucose, and 0.1% (v/v) trace salt solution (5% MgSO₄, 0.5% MnCl₂, 0.5% FeCl₃, and 0.001% H₂SO₄). The bacteria were collected by centrifugation and resuspended in 4.5 ml of Tris-maleic buffer (pH 6.0) (17) containing 20 μg/ml of NGD.

After incubation for 30 min at 37 °C, the bacteria were collected by centrifugation and washed several times with a 0.9% sodium chloride solution (15). Then they were resuspended in 5 ml of 10% sucrose medium, and a sample of 0.5 ml was added into 5 ml of 0.9% sodium chloride solution. This culture was incubated with shaking at 37 °C for 16 h. After incubation, a sample (0.1 ml) was plated on brain heart infusion agar, containing 40 μg/ml of tetracycline and incubated at 37 °C for 2 days. Colonies that grew on the agar plate were picked up and the immunological difference of their LT from that of native LT was examined by the Biken test (18). Immunological differences were detected as spur formation between the precipitin lines of the parent and mutagenized cells on the Biken agar plates.

**Chromatography**—Gel filtration was carried out at 4°C on a column of Bio-Gel A-5m (Bio-Rad) equilibrated with TEAN buffer, composed of 50 mM Tris-HCl, 1 mM EDTA, 3 mM Na₂SO₄, and 0.2 M NaCl (pH 7.4). Material was eluted with the same buffer. Then the material was subjected to DEAE-Sepharose (Pharmacia Fine Chemicals) column chromatography. The B' subunit was eluted with 20 mM Tris·HCl (pH 7.1), suspended in 20 mM Tris·HCl (pH 7.1) containing 0.14 M NaCl and applied to anti-LT coupled Sepharose 4B prepared by the method reported previously (19, 20). The column was washed with 0.14 M NaCl containing 20 mM Tris·HCl (pH 7.1), 0.5 M NaCl containing 20 mM phosphate buffer (pH 7.4), and 6 M urea containing 20 mM phosphate buffer (pH 7.4), and then the B' subunit was eluted with 0.1 M propionic acid containing 6 M urea. Fractions containing the B' subunit from the columns...
were prepared as described by Crestfield et al. (22). Excess reagents were removed by dialysis against 10 mM ammonium bicarbonate containing 6 M guanidine HCl. Solid succinic anhydride (60-fold molar excess per amino group) was added and the pH of the solution was maintained at between 8.0 and 9.0 by adding 5 M NaCl as described previously (23).

Chymotrypsin Digestion — The carboxymethyl-succinyl-B and B' subunits were digested with chymotrypsin as described previously (28).

Peptide Preparation — The digests of the B and B' subunits were each subjected to Gilson high performance liquid chromatography (HPLC) in 0.1% trifluoroacetic acid and the peptides were eluted with a gradient of 0-90% acetonitrile in 0.15 M trifluoroacetic acid. Elution of peptides was monitored as absorbance at 220 nm. Fractions of peptides were pooled and lyophilized.

Amino Acid Sequence Determination — Amino acid sequences from the N terminus were determined by manual Edman degradation (29) using samples of about 0.1 µm peptides. Phenylthiohydantoin amino acids were identified by HPLC as described elsewhere (25).

Circular Dichroism — CD spectra were obtained from 205 to 260 nm with a Jasco J-500. In calculation of the mean residual ellipticity, [θ], the mean residue weight was taken to be 103 for the B and B' subunits.

RESULTS

Immunological Character of LT Produced by the Mutant — A crude extract of cells of the mutant grown in CAYE broth (1.51) was examined by the double gel diffusion test. As shown in Fig. 1a, LT produced by the mutant formed a precipitin line against anti-B subunit serum but not against anti-A subunit serum. Fig. 1b shows spur formation between the precipitin lines of the B and B' subunits with antisera prepared against LT or the B subunit. These data suggest that the mutant produces only a B subunit (B') that is immunologically different from the B subunit.

Reaction of the B' Subunit with Ganglioside in Gel — The binding ability of the B' subunit to ganglioside was examined by the double gel diffusion test. As shown in Fig. 1c, the native B subunit formed a precipitin line against ganglioside mixture type III (Sigma Chemical Co.), but the crude B' subunit did not, suggesting that the ability of the B' subunit to bind to the ganglioside was lost.

Purification of the B' Subunit — For more detailed analysis of the B' subunit, we tried to purify it. As shown in Table I, the crude sample prepared from cells in 10 liters of CAYE medium as described previously (20) was precipitated with ammonium sulfate (70% saturation). The precipitate was dissolved in TEAN buffer and subjected to Bio-Gel A-5m column chromatography in TEAN buffer. Although native LT and the B subunit were adsorbed to a Bio-Gel A-5m column (2 x 90 cm), the B' subunit was not adsorbed. Fractions (300 ml) containing the B' subunit in 3 liters of 20 mM Tris-HCl buffer (pH 7.1) were applied to a column of DEAE-Sephalac equilibrated with 20 mM Tris-HCl at a flow rate of 1 ml/min. The B' subunit passed through this column, whereas most other proteins were adsorbed. Fractions (500 ml) containing the B' subunit were mixed with NaCl at a concentration of 0.14 M and applied to an anti-LT immunoglobulin-coupled Sepharose 4B column equilibrated with 0.14 M NaCl at an elution speed of 1 ml/min. After absorption of the B' subunit, the column was washed with 20 mM Tris-HCl (pH 7.1) containing 0.14 M NaCl, 20 mM phosphate buffer containing 0.5 M NaCl, and 20 mM phosphate buffer containing 6 M urea and 20 mM phosphate buffer containing 6 M urea at a pH of 7.4. The eluate with 0.1 M propionic acid in 6 M urea was concentrated on PM-10 (Amicon) and applied to a Sephadex G-75 column equilibrated with 0.1 M propionic acid containing 6 M urea pH 2.8. Material was eluted with the same buffer. In this way we obtained 0.5 mg of purified B' subunit.

Homogeneity of the Purified B' Subunit — The homogeneity of the purified B' subunit was examined by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2, when about 5 µg of material was applied, the B' subunit (lane 2) gave a single band on the gel, suggesting that the preparation was almost homogeneous. The mobility of the purified B' subunit was almost the same as that of the B subunit of LT prepared according to the method previously reported (20, 26). Thus there seemed to be little difference between the molecular weights of the B and B' subunits.

Isoelectric Point — The isoelectric point of the B' subunit was determined by polyacrylamide gel electrophoresis, as shown in Fig. 3. The pI of the B subunit was calculated to be 10.0 and that of the B' subunit as 9.8.

UV Absorption Spectra of the B and B' Subunits — The UV absorption spectra of the B' and B subunits from 250 to 330 nm were compared. As shown in Fig. 4, spectra were very...
Receptor-binding Site in the B Subunit of E. coli Enterotoxin

According to the method previously reported (29), SDS-PAGE was performed. For dithiothreitol treatment, the samples were heated at 100 °C for 2-3 min in the presence of 10 mM dithiothreitol and analyzed with SDS-PAGE in 12% polyacrylamide containing 1% SDS. 1, LT; 2, B' subunit; 3, B subunit.

Isoelectric points were determined by polyacrylamide gel electrophoresis (30, 31) from pH 3 to 10. The marker proteins for pi were purchased from Oriental Yeast Co., Tokyo.

Similar, indicating that the B' and B subunits had similar contents of amino acids, especially tryptophan.

Comparison of the B' and B Subunits by GM1-ganglioside ELISA—The binding abilities of the B' and B subunits to ganglioside were examined in ganglioside ELISA.

As shown in Fig. 5, the B subunit showed positive absorption at a concentration of 10 ng/ml, but the B' subunit showed no absorption even at a concentration of 10,000 ng/ml in this ELISA test. Thus the binding ability of the B' subunit to GM1-ganglioside was at least 1000 times less than that of the B subunit. A crude sample of the B' subunit, which gave a precipitin line in the Ouchterlony test, did not react with the ganglioside in the ELISA test. Thus the mutant produces only the B' subunit, which cannot bind to GM1-ganglioside.

Analysis of Binding of the B' Subunit to Ganglioside by SDS-PAGE—Van Heyningen (27) reported that after treatment with GM1-ganglioside the CT-B subunit did not enter urea-PAGE. We used this principle to compare the binding abilities of the B' and B subunits to ganglioside by SDS-PAGE. As shown in Fig. 6, treatment with ganglioside influenced the mobility of the B subunit but not that of the B' subunit, again suggesting that the B subunit bound to GM1-ganglioside but the B' subunit did not.

Inhibitory Effects of the B' and B Subunits on LT Activity of CHO Cells—Fig. 7 shows that the dose-response curve of LT was affected by pretreatment of CHO cells with excess B subunit but not with excess B' subunit: 50% elongations of untreated CHO cells and cells pretreated with the B' subunit were observed with 63 and 100 pg/ml of LT, respectively, but after pretreatment of the cells with excess B subunit, 50% elongation was not observed even with more than 10 ng/ml of LT.

These data suggest again that the B' subunit does not combine with the binding site of LT.

Effects of Unlabeled B and B' Subunits on Binding of 125I-
polyacrylamide containing 1% SDS. B' subunit with ganglioside; were mixed with the ganglioside mixture (1 mg) and incubated at 

Receptor-binding Site in the B Subunit of E. coli Enterotoxin

a) 100

With B' subunit

b) 50

With B subunit

c) 0

Concentration of unlabeled B and B' subunit (log ng/ml)

Fig. 6. Analysis by SDS-PAGE of the B and B' subunits treated with the ganglioside mixture. B and B' subunits (20 μg) were mixed with the ganglioside mixture (1 mg) and incubated at 37 °C for 60 min. The samples were treated with dithiothreitol (10 mM) at 37 °C for 10 min and were analyzed with SDS-PAGE in 12% polyacrylamide containing 1% SDS. I, B' subunit with ganglioside; 2, B' subunit; 3, B subunit with ganglioside; 4, B subunit.

Fig. 7. Effects on LT activity of the B and B' subunits in the CHO cell assay system. CHO cell assay was performed as previously described (33, 34). The B and B' subunits were added to the culture medium of CHO cells at the final concentrations indicated. After 30 min incubation, toxin was added to the culture medium of the CHO cells. After 10 min incubation, the cells were washed with culture medium without toxin. The percent elongation of cells was determined by examination of 200 cells after further incubation for 18 h.

B Subunit to CHO Cell—CHO cells were incubated with a constant amount of 125I-B subunit (75 ng/ml) and different amounts of unlabeled B or B' subunit at 37 °C for 60 min. Then the cells were washed repeatedly and the amount of bound 125I-B subunit was measured. As seen in Fig. 8, unlabeled B subunit (25 ng/ml) inhibited binding of the 125I-B subunit, causing almost complete inhibition at a concentration of 28 μg/ml. In contrast, unlabeled B' subunit was not inhibitory even at a concentration of 6 μg/ml. These data also support the conclusion that the B' subunit differs from the B subunit in the region of the binding site of the later to GM1-ganglioside.

Formation of Hybrid Toxin from the Native A Subunit and B' Subunit—Hybrid toxin containing the native A subunit and B' subunit was constructed by the method reported previously (26). The mixture of A and B' subunits in 0.1 M propionic acid containing 6 M urea was dialyzed against TEAN buffer and then analyzed by disc electrophoresis. As shown in Fig. 9, the mobility of the B' subunit (lane B') was less than that of the A subunit (lane A). The reconstituted hybrid toxin gave a band (arrow) with a mobility intermediate between those of the B' and A subunits (lane Hy). As the B' subunit could form a hybrid toxin with the native A subunit, it must have the native conformation necessary for binding to the A subunit and thus not have a significantly different conformation from the B subunit.

As expected, the biological activity of the hybrid toxin, examined by CHO cell assay, was similar to that of the A subunit.

Circular Dichroic Spectra of B and B' Subunits—It was examined whether there was difference in secondary structure between B and B' subunits by obtaining each CD spectrum in the far ultraviolet region. As shown in Fig. 10, the pattern of CD spectrum of B' subunit was very similar to that of B subunit, suggesting that there was no significant change in secondary structure of B' subunit from native one.

HPLC Patterns of Chymotrypsin Digests of the B and B' Subunits—Samples of 3-5 mg of the B and B' subunits were carboxymethylated, succinylation, and then digested with chymotrypsin as described under "Materials and Methods." The digests were then subjected to HPLC and eluted with a gradient of 0–90% acetonitrile in 0.1% trifluoroacetic acid. As shown in Fig. 11, the elution profiles were similar except that peak 4 of the B' subunit was eluted later than that of the B subunit.

Comparison of Amino Acid Compositions of Peptides of the B and B' Subunits—The amino acid contents of the peptides in peaks 1–10 separated by HPLC were determined (Table II). Only the amino acid contents of the peptides in peak 4 of
Receptor-binding Site in the B Subunit of E. coli Enterotoxin

A Receptor-binding Site in the B Subunit of E. coli Enterotoxin

A Receptor-binding Site in the B Subunit of E. coli Enterotoxin

the B and B' subunits were found to differ: the content of aspartate was increased and that of glycine was decreased in the peptides of the B' subunit, indicating replacement of glycine by aspartate. As shown in Table II, the amino acid composition of peptide 3 was the same in the B and B' subunits but different from that reported by Dallas and Falkow (3); peptide 3 contained lysine but not methionine.

**Amino Acid Compositions of the B' and B Subunits**—The amino acid compositions of the B' and B subunits are determined in an amino acid analyzer (Irica Instruments, model A-3300), after hydrolysis of samples in 25% hydrochloric acid at 110 °C for 24 and 72 h (Table III). The numbers of various amino acid residues in the B and B' subunits are almost identical except for those of glycine and aspartate: the B' subunit has less glycine and more aspartate than the B subunit.

**Determination of Primary Sequence of Peak 4**—We determined the primary sequence of the peptide in peak 4 to be Thr-Glu-Ser-Met-Ala-Asp*-Lys-Arg-Glu-Met, as shown in Table IV. This sequence corresponded to residues 28-37 from the N terminus of the B subunit except for Asp* at position 33. This residue is glycine in the B subunit and aspartate in the B' subunit.

**DISCUSSION**

In this work mutagenesis of E. coli WT-1 (14) was induced with NGD as reported (15) and the revertant mutants were screened by the Biken test. We obtained 10 mutants that produce immunologically nonidentical LT. The mutant described here produces only a B subunit (the B' subunit) that differs immunologically from the native B subunit (Fig. 1b).

We demonstrated that this B' subunit could not bind to gangliosides by several methods. 1) The binding ability of the B' subunit to GM1-ganglioside in GM1 ELISA (Fig. 5) was at least 1000 times less than that of the native B subunit. 2) SDS-PAGE analysis showed that the B' subunit did not bind to ganglioside (Fig. 6). 3) The B' subunit did not form precip-
Receptor-binding Site in the B Subunit of E. coli Enterotoxin

Amino acid analyses were performed on 24-h hydrolysates (23). Values in parentheses are cited from Ref. 3.

| Table II | Amino acid compositions of fractionated peptides |
|-----------|-----------------------------------------------|
| Amino acid | B' B' B' B' | B' B' B' B' |
| Cys       | 3.30     | 1.01 (1) | 0.11 | 1.16 | 1.10 | 0.16 (1) | 2.11 | 1.71 (2) | 0.84 | 0.85 (1) | 0.00 |
| Asp       | 0.96     | 1.09 | 2.05 (2) | 1.25 | 1.25 (1) | 1.09 | 1.09 (1) | 1.97 | 1.85 (2) | 0.70 (1) | 0.14 (1) | 0.15 |
| Thr       | 1.11     | 0.96 (1) | 0.96 | 1.10 (1) | 0.10 | 0.10 (1) | 0.10 | 0.09 (1) | 0.94 | 0.09 (1) | 0.94 |
| Ser       | 0.92     | 0.96 (1) | 1.17 | 1.82 (1) | 0.11 | 0.11 (1) | 0.11 | 0.11 (1) | 0.94 | 0.94 (1) | 0.94 |
| Glu       | 0.18     | 0.19 | 2.28 | 0.50 (1) | 0.12 | 0.12 (1) | 0.12 | 0.12 (1) | 0.53 | 0.53 (1) | 0.53 |
| Pro       | 0.08     | 0.10 | 0.12 | 0.73 (1) | 0.13 | 0.13 (1) | 0.13 | 0.13 (1) | 0.83 | 0.83 (1) | 0.83 |
| Gly       | 0.80     | 0.87 | 0.15 (1) | 0.72 | 0.72 (1) | 0.72 | 0.72 (1) | 0.73 | 0.73 (1) | 0.73 |
| Ala       | 0.80     | 0.87 | 0.15 (1) | 0.72 | 0.72 (1) | 0.72 | 0.72 (1) | 0.73 | 0.73 (1) | 0.73 |
| Val       | 0.80     | 0.87 | 0.15 (1) | 0.72 | 0.72 (1) | 0.72 | 0.72 (1) | 0.73 | 0.73 (1) | 0.73 |
| Met       | 0.80     | 0.87 | 0.15 (1) | 0.72 | 0.72 (1) | 0.72 | 0.72 (1) | 0.73 | 0.73 (1) | 0.73 |
| Leu       | 0.80     | 0.87 | 0.15 (1) | 0.72 | 0.72 (1) | 0.72 | 0.72 (1) | 0.73 | 0.73 (1) | 0.73 |
| Ile       | 0.80     | 0.87 | 0.15 (1) | 0.72 | 0.72 (1) | 0.72 | 0.72 (1) | 0.73 | 0.73 (1) | 0.73 |
| Lys       | 0.80     | 0.87 | 0.15 (1) | 0.72 | 0.72 (1) | 0.72 | 0.72 (1) | 0.73 | 0.73 (1) | 0.73 |
| His       | 0.80     | 0.87 | 0.15 (1) | 0.72 | 0.72 (1) | 0.72 | 0.72 (1) | 0.73 | 0.73 (1) | 0.73 |
| Arg       | 0.90     | 0.96 (1) | 0.96 | 1.00 (1) | 0.90 | 0.90 (1) | 0.90 | 0.90 (1) | 0.90 | 0.90 (1) | 0.90 |
| Trp       | 0.90     | 0.96 (1) | 0.96 | 1.00 (1) | 0.90 | 0.90 (1) | 0.90 | 0.90 (1) | 0.90 | 0.90 (1) | 0.90 |
| Total     | 6        | 6     | 10     | 10     | 9     | 9     | 5     | 12     | 24     | 12     |

* Peptide.
* Residue number.
* Fraction 6 (residues 19-27) was contaminated about 20% (estimated from the value for Glu in the case of the B subunit) or 20% (estimated from the value for Glu in the case of the B' subunit) with another peptide (residues 49-67) and so its amino acid composition was calculated as total amino acids in fraction 6/10% or 20% of residues in peptide 49-67.
* Values for valine and isoleucine in fraction 7 were lower than those determined from the DNA sequence, because amino acid analysis was performed on a 24-h hydrolysate and the sequence Val-Ile-Ile was not hydrolyzed completely.
* We did not determine the tryptophan content of fraction 10, but this fraction should contain tryptophan and consistent with this, it gave a high peak on HPLC.

Amino acid compositions of the B and B' subunits

After hydrolysis of samples in 25% hydrochloric acid at 110 °C for 24 and 72 h, the biological activity of LT (Fig. 7). Pretreatment of CHO cells with excess B subunit but not excess B' subunit inhibited the biological activity of LT (Fig. 7). In binding inhibition assay, unlabeled B' subunit did not inhibit the binding of 125I- B subunit to CHO cell (Fig. 8). All these data indicate that the B' subunit cannot bind to GM1-ganglioside, the receptor for LT.

Then we determined the primary sequence of the purified B' subunit. Samples of the B and B' subunits were carboxymethylated, succinylated, and digested with chymotrypsin. The digests were then applied to a HPLC column and eluted with an acetonitrile gradient. All the elution peaks of peptides of the B' subunit were identical with those peptides of the B subunit, except that of peak 4 (Fig. 11). The amino acid compositions of the peptides (Table II) in these peaks were compared with those of the peptides of the B subunit reported (3). All were the same, except for that of peak 4; peak 4 of the B' subunit contained more aspartate and less glycine than that of the B subunit. The amino acid sequence of the peptide in this peak was determined as Thr-Glu-Ser-Met-Ala-Asp*-Lys-Arg-Glu-Met. This sequence coincides with the sequence of positions 28-37 of the native B subunit reported except at position 33; the B' subunit has glycine instead of the N-terminal aspartate. Thus it is supposed that mutation of GGC encoding glycine to GAC encoding aspartate in the mutant could occur on treatment with NGD.

As a single amino acid exchange at position 33 from the N-terminus in the B subunit results in loss of ability to bind to ganglioside, we suppose that position 33 is important for binding activity. We also observed an immunological difference between B and B' (Fig. 1b), suggesting that the binding site of the B subunit to GM1-ganglioside is one of the epitopes of B subunit.

This 1 amino acid exchange at position 33 of the B subunit cannot cause some conformational change in the molecule that results in loss of ability to bind to ganglioside. However,
the fact that a hybrid toxin could be formed between the B' subunit and native A subunit suggests that the conformation of the B subunit for binding to the A subunit was not affected by the 1 amino acid exchange. Moreover, CD spectrum of B' subunit was very similar to that of B subunit (Fig. 10). Thus these data suggested that there was no significant conformational change in the B' subunit.

De Wolf et al. (11, 12) reported that the tryptophan residue at position 88 from the N terminus might be near or in the binding site of the B subunit of cholera toxin to GM₁-ganglioside. However, this tryptophan residue was not affected in the B' subunit.

The glycine at position 33 of the B subunit is very close to which was reported to be important for CT-B binding to ganglioside (13). Thus positions 33-35 from the N terminus of the B subunit seem to be important for binding of LT-B and CT-B to ganglioside receptor.

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### TABLE IV

**Comparison of amino acid sequences of the B' subunit and B subunit of LTₐ, LTᵢ, and CT**

| Subunit | Amino Acid Sequence |
|---------|---------------------|
| LTₐ     | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| LTᵢ     | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| CT      | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| LTₐ     | Asp Lys Ile Leu Thr Ser Tyr Glu Ser Met Ala  |
| LTᵢ     | Asp Lys Ile Leu Thr Ser Tyr Glu Ser Met Ala  |
| CT      | Asp Lys Ile Leu Thr Ser Tyr Glu Ser Met Ala  |
| LTₐ     | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| LTᵢ     | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| CT      | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| LTₐ     | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| LTᵢ     | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| CT      | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| LTₐ     | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| LTᵢ     | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |
| CT      | Ala Pro Gln Thr Ile Thr Glu Leu Cys   |

The glycine at position 33 of the B subunit is very close to which was reported to be important for CT-B binding to ganglioside (13). Thus positions 33-35 from the N terminus of the B subunit seem to be important for binding of LT-B and CT-B to ganglioside receptor.