Isolation and Function of a Clostridium perfringens Enterotoxin Fragment

YASUHIKO HORIGUCHI,‡ TETSUO AKAI, AND GENJI SAKAGUCHI

Department of Veterinary Science, College of Agriculture, University of Osaka Prefecture, Sakai-Shi, Osaka 591, Japan

Received 13 July 1987/Accepted 26 August 1987

A fragment was obtained by treating Clostridium perfringens enterotoxin with 2-nitro-5-thiocyanobenzoic acid, a reagent which specifically cleaves the amino-terminal peptide bond of cysteine residues. The fragment (molecular weight, 15,000) was purified by high-performance liquid chromatography. The fragment had no cytotoxic effect on Vero cells but competitively inhibited enterotoxin-induced 51Cr release. Binding of 125I-labeled fragment to Vero cells was comparable to that of enterotoxin. Moreover, 125I-labeled fragment did not bind to FL cells, which lack receptor for enterotoxin. We conclude that the fragment contains the binding domain of enterotoxin. The amino acid composition of the fragment suggests that it is located on the carboxyl-terminal part of enterotoxin.

Clostridium perfringens produces enterotoxin responsible for human diarrhea. The enterotoxin has been purified and demonstrated to be a single-chain polypeptide with a molecular weight of 34,000 (5, 21, 24). The enterotoxin specifically binds to sensitive cells, including enterocytes, hepatocytes, and cultured mammalian cells (7, 11, 19, 23, 26). The alteration of the permeability of the cell membranes occurs immediately after the binding (7, 8, 14-18, 25). Recently, we (8) and Matsuda et al. (14) found that the initial action of the toxin is alteration of the ion permeability of the cell membrane, while the subsequent leakage of macromolecules appears only in the presence of extracellular Ca2+.

Many bacterial toxins consist of at least two functionally distinct domains, the binding and the biologically active domains. It is not known whether C. perfringens enterotoxin also contains these domains. Many experimental results have indicated that the binding site of enterotoxin does not differ from the biologically active one: (i) alteration of membrane permeability occurred just after the binding of enterotoxin (7, 8, 14, 16); (ii) the polyclonal anti-enterotoxin antibody failed to neutralize enterotoxin bound to the cells (28); and (iii) Wnek et al. (28) failed to obtain monoclonal antibodies (MAbs) that neutralized enterotoxin without affecting the binding of the toxin.

One of the four MAbs we obtained (6), 3-B-2, barely inhibited the binding but neutralized the biological activity. We therefore proposed an antigenic structure consisting of two sites, the binding and biologically active sites. Moreover, a peptide which inhibited protein synthesis but had no effect on intact Vero cells was isolated by digesting enterotoxin with trypsin in the presence of sodium dodecyl sulfate (SDS) (3). Identification of the active and binding sites of C. perfringens enterotoxin is important to elucidate the molecular action of the toxin over target cells. To look for such a site with a single function on enterotoxin, the biologically active or binding site, we fragmented the enterotoxin with 2-nitro-5-thiocyanobenzoic acid (NTCB), a reagent which specifically cleaves the amino-peptide bond of cysteine residues (1, 10), and characterized the resulting fragment. This communication deals with evidence showing that the fragment contains the binding site of enterotoxin but not the biologically active site.

MATERIALS AND METHODS

C. perfringens enterotoxin and other reagents. C. perfringens enterotoxin was prepared by the method of Sakaguchi et al. (21). MAbs against enterotoxin were purified from ascitic fluid obtained as described elsewhere (6). NTCB was purchased from Sigma Chemical Co., St. Louis, Mo. Na125I and Na51CrO4 were from ICN Radiochemicals, Irvine, Calif. Other reagents were from Wako Pure Chemical Industries, Osaka, Japan.

Fragmentation of enterotoxin. Enterotoxin was treated with NTCB as described by Jacobson et al. (10), with slight modifications. Enterotoxin was dialyzed overnight against distilled water and lyophilized. The lyophilized preparation was dissolved at a concentration of 3.5 mg/ml in 0.05 M Tris hydrochloride buffer, pH 8.0, containing 4 M guanidine hydrochloride and 0.2 mM dithiothreitol. After incubation for 30 min at room temperature, NTCB at a final concentration of 0.5 mM was added to the enterotoxin solution and the incubation was continued for an additional 15 min. The pH of the sample solution was adjusted to 9.0 with 0.1 N NaOH, and the mixture was incubated for 6 h at 37°C. To stop the reaction, the mixture was dialyzed against 0.05 M Tris hydrochloride buffer, pH 8.0, or 0.01 M phosphate buffer, pH 6.5. After centrifugation at 100,000 x g for 2 h to remove insoluble aggregates formed during dialysis, the supernatant was used to characterize the fragment.

NTCB-treated enterotoxin in 0.01 M phosphate buffer, pH 6.5, containing 4 M guanidine hydrochloride was subjected to high-performance liquid chromatography (HPLC) on a TSK gel-G2000SW column (Toyoda Soda Co., Tokyo, Japan) with a Jasco HPLC system (Japan Spectroscopic Co., Tokyo, Japan) at a flow rate of 1 ml/min. The eluate was collected and concentrated by dialysis against Ficoll 400 (Pharmacia, Uppsala, Sweden).

SDS-PAGE and immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in a 15% gel by the method of Laemmli (12). For immunoblotting, the samples that migrated on the gel were transferred electrophoretically to TM-2 nitrocellulose sheets (Toyo Roshi, Tokyo, Japan) by the method of Towbin et al. (27). The sheet was soaked...
overnight in phosphate-buffered saline (PBS)-3% bovine serum albumin, immersed in fresh PBS-3% bovine serum albumin containing each MAb or antisera against enterotoxin, and incubated for 30 min at room temperature. After the sheet was washed with PBS, it was treated for 30 min with peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.) diluted 500-fold in PBS-3% bovine serum albumin. After the sheet was washed, the bound peroxidase was allowed to react on 3,3'-diaminobenzidine (0.5 mg/ml)-0.003% H₂O₂ in PBS.

Other methods. Enterotoxin-induced ³¹Cr and ⁸⁶Rb release from Vero cells was determined by methods reported before (6-8). Maximal release of the isotopes was determined with samples ruptured with 0.5% saponin. Toxin-induced release and maximal release were corrected by subtracting spontaneous release.

Preparation of ¹²⁵I-labeled fragment and measurement of binding to Vero cells were performed as reported elsewhere (6-8). The amount of labeled enterotoxin bound was calculated by subtracting the counts associated with the cells previously treated with the unlabeled enterotoxin (100 μg/ml) from the total count.

Protein contents were determined by the method of Lowry et al. (13), with bovine serum albumin as the standard. Amino acid analysis was done with a Jasco amino acid analysis system (Japan Spectroscopic Co.). Hydrolysis under vacuum was performed in 6 N hydrochloric acid at 125°C for 24, 48, and 72 h.

RESULTS

Fragmentation of enterotoxin with NTCB. Enterotoxin formed high-molecular-weight aggregates in the presence of SDS (Fig. 1, lane 2), as reported before (2). Treatment of enterotoxin with NTCB in the presence of 4 M guanidine hydrochloride resulted in separation into the aggregated forms and a band with a molecular weight of 15,000 (NTCB fragment) (Fig. 1, lane 3). Immunoblotting analyses showed that all four MAbs (2-B-4, 2-H-2, 3-B-2, and 3-G-10 [6]) bound to the NTCB fragment.

Four major protein peaks were eluted by HPLC of NTCB-treated enterotoxin. SDS-PAGE showed that the fraction eluted first contained aggregates of enterotoxin and a small amount of NTCB fragment (Fig. 1, lane 4). The second and third peak fractions aggregated as did that of the first peak in SDS-PAGE (Fig. 1, lanes 5 and 6). The last peak contained only NTCB fragment (Fig. 1, lane 7) and therefore was used in the following experiments. Before use, the NTCB fragment in peak 4 was rechromatographed and its purity was confirmed by SDS-PAGE.

Biological activities of the NTCB fragment. Even high concentrations of the NTCB fragment released neither ⁸⁶Rb nor ⁵¹Cr from Vero cells, but enterotoxin-induced ⁵¹Cr release was inhibited. The dose-response curves for enterotoxin with or without the NTCB fragment were compared in a ⁵¹Cr release assay (Fig. 2). Curves showing the cell response to enterotoxin had the same shapes and reached the same maxima in the presence or absence of the NTCB fragment; the NTCB fragment shifted the curve to higher enterotoxin concentrations. This behavior was regarded as evidence of competitive inhibition. To substantiate this presumption, a Schild plot was constructed from the response curves of enterotoxin and a set of curves obtained at different NTCB fragment concentrations (Fig. 3). From a

FIG. 1. SDS-PAGE of enterotoxin and its fractions isolated by HPLC. Lane 1, Low-molecular-weight markers (x10⁵) as indicated on the left; lane 2, enterotoxin; lane 3, enterotoxin treated with NTCB; lanes 4, 5, 6, and 7, fractions eluted in HPLC in the order of retention time. About 5 μg of protein was applied.

FIG. 2. Enterotoxin-induced ⁵¹Cr release in the presence of the NTCB fragment. Enterotoxin at various concentrations was incubated with ⁵¹Cr-labeled Vero cells in the absence (●) or the presence of the NTCB fragment at 4 (○) or 16 (△) μg/ml (n = 3).

FIG. 3. Schild plot of enterotoxin inhibition by the NTCB fragment constructed from a set of dose-response curves such as those in Fig. 2. IT/I' ratios were calculated for fragment concentrations of 0, 4, 8, and 16 μg/ml.
model for competitive inhibition, the following relationship was given (9): \( \frac{T}{T'} - 1 = \frac{1}{K_d} \), where \( T \) is the enterotoxin concentration in the absence of NTCB fragment at a given response, \( T' \) is the concentration that gives the same response in the presence of the NTCB fragment, \( I' \) is the concentration of the fragment, and \( K_d \) is the apparent dissociation constant of the fragment. For competitive inhibition, a plot of \( \log (\frac{T}{T'} - 1) \) versus \( \log I' \) is linear with a slope of 1 and an intercept of \(-\log K_d \). For the NTCB fragment, the slope was 1.2 and the apparent \( K_d \) was \( 1.1 \times 10^{-7} \) M. These findings suggest that enterotoxin and the NTCB fragment compete for a cellular target.

**Binding of NTCB fragment to cells.** The \(^{125}\text{I}-\text{labeled NTCB fragment bound to Vero cells (Fig. 4)}, and the binding was saturable. Scatchard plot analysis showed that the NTCB fragment bound to Vero cells in a single class of receptors with an apparent \( K_d \) of \( 9.2 \times 10^{-9} \) M (Fig. 4). The number of binding sites on one Vero cell was estimated at \( 3.74 \times 10^{6} \) molecules. The NTCB fragment did not bind to FL cells that have no receptor for enterotoxin (7).

**Amino acid analysis.** The amino acid composition of the NTCB fragment appears to be similar to that of the carboxy terminus of enterotoxin, from Cys-177 to the carboxyl-terminal end, especially with respect to the relative amounts of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, isoleucine, and leucine (Table 1).

**DISCUSSION**

Whether the binding and biologically active sites on the enterotoxin molecule are the same has not been determined. To answer this question, we fragmented enterotoxin and isolated a fragment (molecular weight, 15,000) by treating enterotoxin with NTCB. The NTCB fragment did not have a biological effect on Vero cells but competitively inhibited enterotoxin-induced \(^{51}\text{Cr} \) release. Moreover, it bound to Vero cells but not to FL cells that lack receptors for enterotoxin. The \( K_d \) value of and the number of binding sites for the NTCB fragment \( (9.2 \times 10^{-9} \) M and \( 3.74 \times 10^{6} \) sites per cell, respectively), obtained by Scatchard plot analysis, were in good agreement with those of enterotoxin \( (0.31 \times 10^{-9} \) to \( 2.3 \times 10^{-9} \) M and \( 0.56 \times 10^{6} \) to \( 2.5 \times 10^{6} \) sites per cell) (7, 8, 11). From these results, we conclude that the NTCB fragment binds to Vero cells via a specific enterotoxin receptor and that the binding site of enterotoxin is located on this fragment.

The \( K_d \) value \( (1.1 \times 10^{-7} \) M) of the NTCB fragment obtained by Schild plot analysis was about 1/10 that \( (9.2 \times 10^{-9} \) M) obtained by Scatchard plot analysis. When a MAb (2-B-4 or 3-G-10) inhibits about 50% of the binding of enterotoxin, inhibition of \(^{51}\text{Cr} \) release occurs (6). Similarly, to inhibit toxic action with the NTCB fragment, it might be necessary to place >50% of the receptors on the Vero cell. Therefore, a considerably larger amount of the fragment was required for competitive antagonism, and the lower \( K_d \) value was obtained by Schild plot analysis.

All MAbs bound to the NTCB fragment. MAbs were characterized as described before (6). 2-B-4 and 3-G-10 neutralized enterotoxin by inhibiting its binding to Vero cells. 2-H-2 inhibited neither the binding nor the cytotoxic action of the toxin. 3-B-2 only barely inhibited the binding, but neutralized enterotoxin. It is conceivable that 2-B-4 and 3-G-10 bound to the NTCB fragment on which the binding domain is located. That 3-B-2 and 3-H-2 also recognize the NTCB fragment may indicate that the fragment contains not only the epitopes associated with the binding domain but also some other epitopes and that the binding domain is in a much restricted portion of the NTCB fragment. Why 3-B-2 neutralized enterotoxin without affecting the binding of the toxin remains unknown.

NTCB specifically cleaves the amino-terminal peptide bond of cysteine residues (1, 10). Of 309 amino acid residues of enterotoxin, there is only one cysteine and it is residue 177 from the amino-terminal end of enterotoxin (20). It seems that the amino-terminal bond of Cys-177 was cleaved and the fragment was released. The amino acid composition and the molecular weight of the NTCB fragment suggest that it is located on the carboxyl-terminal part of enterotoxin (Table 1). However, more evidence is needed to confirm this.

**TABLE 1. Amino acid composition of NTCB fragment and amino and carboxy termini of enterotoxin**

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>NTCB fragment*</th>
<th>Enterotoxin*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Residues/molecule</td>
<td>N term</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>25.2</td>
<td>22</td>
</tr>
<tr>
<td>Threonine</td>
<td>9.9</td>
<td>13</td>
</tr>
<tr>
<td>Serine</td>
<td>10.8</td>
<td>23</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>13.2</td>
<td>17</td>
</tr>
<tr>
<td>Proline</td>
<td>4.8</td>
<td>5</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.3</td>
<td>13</td>
</tr>
<tr>
<td>Alanine</td>
<td>8.3</td>
<td>6</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Valine</td>
<td>6.6</td>
<td>11</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.8</td>
<td>2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.8</td>
<td>16</td>
</tr>
<tr>
<td>Leucine</td>
<td>14.9</td>
<td>16</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>7.8</td>
<td>9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.0</td>
<td>4</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.8</td>
<td>9</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.8</td>
<td>1</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.6</td>
<td>4</td>
</tr>
</tbody>
</table>

* Based on molecular weight of 15,000.
* Calculated from the amino acid sequence (20). The N term is the peptide from N-terminal Met at AUG-3 and the C term is from Cys-177 to C-terminal Phe.
* Extrapolated to zero time.
* ND. Not determined.
* From 72-h hydrolysis.

FIG. 4. Binding of \(^{125}\text{I}-\text{labeled NTCB fragment to Vero cells and Scatchard plot (inset). Vero cells (10^6) were mixed with}^{125}\text{I}-\text{labeled fragment (0.07 mCi/mg of protein). Each plot is the mean of the bound radioactivities (n = 4).**
Granum (3) isolated a peptide with a molecular weight of 16,000 by treating enterotoxin with trypsin in the presence of 0.05% SDS. He indicated that the 16,000-dalton peptide (16K peptide) aggregated in the gel of SDS-PAGE (3) while the NTCB fragment did not (Fig. 1), which may suggest that the 16K peptide and the NTCB fragment are located at different sites. Furthermore, Granum considers the 16K peptide to be on the amino terminus of the toxin (personal communication).

Granum's 16K peptide inhibited protein synthesis in a cell-free system but did not kill Vero cells. It is not known whether the 16K peptide affects the target cell membrane. Further characterization of the 16K peptide and the NTCB fragment and isolation of some other fragment which directly acts on the cell membrane seem essential both to the conclusion that enterotoxin consists of the distinct binding and active sites and to elucidation of the mode of action of enterotoxin.

ACKNOWLEDGMENT

This work was supported by a grant-in-aid for scientific research (61790242) from the Ministry of Education, Science and Culture, Japan.

LITERATURE CITED