Mechanism of Tryptic Activation of Clostridium botulinum Type E Toxin

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Abstract

Gerwing, Julia (University of British Columbia, Vancouver, B.C., Canada), Claude E. Dolman, and Arthur Ko. Mechanism of tryptic activation of Clostridium botulinum type E toxin. J. Bacteriol. 89:1176-1179. 1965.—The toxic peptide of tryptic-activated Clostridium botulinum type E toxin was purified by chromatography through columns packed with SephadeX G-75 and G-50. The molecular weight of the active peptide was estimated to lie between 10,000 and 12,000. Amino acid analyses indicated that the active peptide had lost at least 18 of the amino acid residues present in the original protein. The active peptide and the original protein were found to have different N-terminal amino acid residues. The mechanism of tryptic activation apparently involves chiefly the removal of amino acids from the N-terminus of the toxin molecule.

Sakaguchi and his associates speculated about the mechanism of tryptic activation of Clostridium botulinum type E toxin. Initially they postulated a toxin “precursor,” whose toxic moiety was inhibited by a masking group apparently linked to ribonucleic acid (Sakaguchi and Sakaguchi, 1959, 1961). In their view, the main action of trypsin was to disrupt this linkage, thus releasing nucleic acid and the postulated inhibiting substance, and leaving behind the unmasked, “activated” toxin. However, this hypothesis was abandoned after Gerwing, Dolman, and Arnott (1961, 1962) showed that their partially purified type E toxins, both before and after trypsin treatment, were devoid of nucleic acid, and also that the process of trypsin activation involved some degree of fragmentation with formation of a smaller, more active molecule.

Recently, Sakaguchi, Sakaguchi, and Imai (1964) contended that both the toxin “precursor” and the activated toxin to which this becomes converted by the action of trypsin have molecular weights around 200,000. However, in our laboratory, highly purified type E toxin was shown to have a molecular weight less than one-tenth of this figure (Gerwing et al., 1964). Similarly, purified preparations of type E toxin have been used in experiments designed to elucidate the trypsin activation phenomenon. The data presented here strengthen the evidence in support of the molecular fragmentation theory. The activated peptide appears to contain a lesser number of amino acid residues than the purified toxin, and to have a different N-terminal amino acid.

Materials and Methods

Toxin production. The bacterial strain, culture media, and methods for toxin production, assay, nitrogen determination, and purification have been described previously (Gerwing et al., 1961, 1964).

Tryptsin activation. Trypsin (twice crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio) dissolved in sodium acetate buffer (pH 5.8) was added to the pure toxin in an approximate molar ratio of 1:120 (assuming the molecular weight of the toxin to be 18,000 and that of trypsin to be 24,000), i.e., equivalent to 1% trypsin by weight. The pH of the preparation was adjusted to the required level, which was either 5.8 or 7.5. The mixtures were incubated at 37 C for a 24-hr period to determine when maximal activation occurred and to delineate the degradation curve. At various time intervals (5, 15, and 30 min; 1, 2, 3, 4, 5, 6, 9, and 24 hr), samples were removed for titration. They were diluted 1:10 in 0.05 M sodium acetate buffer (pH 4.0) to inhibit further trypsic digestion.

Chromatographic techniques. Columns (0.8 by 110 cm) were packed to a height of 45 cm with SephadeX G-75 in 0.05 M sodium acetate buffer (pH 4.5). The resin bed was allowed to settle overnight at room temperature, after which the height had shrunk to 42 cm. SephadeX G-50 in the same buffer was then superimposed to a final height of 105 cm, and the column was washed with 500 ml of buffer. (Sephadex was obtained from...
Pharmacia Fine Chemicals, Uppsala, Sweden.) Trypsin-treated samples were added in 1.0-ml quantities, each containing between 3 and 5 mg of the toxic material. Elution was carried out with use of the same buffer. Flow rate was regulated at 7.5 ml/hr, and 2.5-ml fractions were collected in a model V-10 Fraction Collector (Gilson Medical Electronics, Middleton, Wis.) Fractions were analyzed for the presence of 290 m\(\mu\)-absorbing and ninhydrin-positive material.

Ninhydrin reaction. Ninhydrin (5% in acetone) was added in a 1:1 ratio to the samples to be tested (usually 1.0 ml of each). The tubes were then placed in a boiling-water bath for 15 min and thereafter were immediately cooled. The reaction tubes were diluted 3:2 with 96% ethyl alcohol and read at 570 m\(\mu\) on a Beckman B spectrophotometer.

Preparation of substrate. Toxic material to be used for chemical analysis was desalted either by dialyzing it against distilled water or by passing it through Sephadex G-75 columns in distilled water. It was then freeze-dried.

Hydrolysis procedures. Weighed samples to be used for either amino acid analysis or N-terminal residue identification were taken up in 1 to 2 ml of 6 N HCl in ampoules, which were sealed under vacuum. Hydrolysis was carried out for either 8 or 18 hr at 105 C, according to the requirements of the respective procedures. Samples to be used for amino acid analysis were then freeze-dried to remove residual HCl, and washed three times in distilled water alternately with flash evaporation at 40 C.

Performic acid oxidation. Performic acid was prepared by adding 1.0 ml of 30% \(\text{H}_2\text{O}_2\) to 9.0 ml of 88% performic acid. The mixture was allowed to stand for 1 hr and was then cooled to 0 C. Dried protein samples to be analyzed for cysteic acid content were oxidized with 2.0 ml of performic acid at 0 C for 18 hr. Excess performic acid was removed by the addition of 0.3 ml of 48% HBr and was subjected to subsequent freeze-drying and hydrolysis as described above.

Amino acid analysis. Quantitative amino acid analysis was carried out on a Technicon Autoanalyser.

N-terminal amino acid determinations. Sanger's technique for labeling N-terminal amino acid residues was employed, with use of method A described by Fraenkel-Conrat, Harris, and Levy (1955).

RESULTS

Effect of pH and time on the tryptic activation of purified type E toxin. Experiments were done to determine the influence of pH and time upon the action of trypsin on the purified toxin. These procedures were carried out three times, giving practically identical results on each occasion. Figure 1 shows a typical set of results obtained when toxin (titer 10,000 MLD/ml) was exposed to trypsin at pH 7.5 and 5.8, respectively. At pH 7.5, a maximal titer of 200,000 MLD/ml was reached in about 15 min. This was followed by an equally rapid decline in activity, which could be attributed either to instability of the toxin molecule at pH 7.5 or to progressive destruction of the toxic site by trypsin. At pH 5.8, considerably longer incubation of the trypsinized toxin (about 5 hr) was necessary to achieve maximal activation, but the resulting product was somewhat more potent, containing 300,000 MLD/ml. This latter method was selected for the preparation of the activated material used in further studies. Incidentally, the activation ratio obtained under these conditions was not only reproducible, but was also comparable to that observed when crude or partially purified toxic preparations were similarly treated.

Purification and analysis of trypsin-activated toxin. A column of Sephadex G-50 superimposed upon G-75, with a length-to-diameter ratio of 125:1, proved satisfactory for the separation of the active peptide from the portions of the toxin molecule removed by tryptic digestion. Quantitative amino acid analyses of both the active peptide thus separated and the purified toxin showed a lesser number of amino acid residues in the former product (Table 1). The toxicities of the peptide and of the toxin from which it was
TABLE 1. Type E purified toxin and activated peptide compared in terms of amino acid contents, N-terminal amino acids, and relative toxicities

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Toxin MLD/mg</th>
<th>Active peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteic acid</td>
<td>—*</td>
<td>2</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Threonine</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Serine</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Proline</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Glycine</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Alanine</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Valine</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Isoleucine</td>
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<td>6</td>
</tr>
<tr>
<td>Leucine</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Tyrosine</td>
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<td>4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Lysine</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Histidine</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

N-terminal residue... Glycine  Arginine
Toxicity (MLD/mg of N)... 7.5 × 10⁴  2.8 × 10⁴

* Not calculated.

dinitrophenylated peptide samples, showed the presence of only one N-terminal residue, which was identified as arginine, by use of the tert-amyl alcohol-phthalate chromatographic system and the Sakaguchi reaction (Fraenkel-Conrat et al., 1955) for confirmation. When dinitrophenylated samples of purified toxin were similarly examined, the N-terminal amino acid residue was identified as glycine.

**DISCUSSION**

Although trypsin specifically attacks peptide bonds containing the carboxyl groups of lysine and arginine, some of these bonds may be partially or totally resistant to action of this enzyme by virtue of the amino acid sequence or the tertiary structure of the protein (Thompson, 1960). Our results show that the intact toxin molecule possesses nine residues of lysine and three of arginine (Table 1). Hence, exhaustive tryptic digestion theoretically should give rise to 13 peptides. However, at the time of maximal activation, a considerable number of these residues (seven lysyl and two arginyl) still remain in the active peptide. At this juncture, the peptide linkages concerned obviously display relative resistance to cleavage by trypsin. However, continued digestion might be expected to reveal that certain of these linkages are only partially resistant, and would result in further degradation of the molecule with coincident decline in toxic activity.

There seems no need to postulate any qualitatively different effect of trypsin upon type E toxin at pH 7.5 and 5.8. At pH 7.5, the activation process is markedly accelerated, as demonstrated in Fig. 1. The particularly rapid drop in activity noted within 3 hr at pH 7.5 could be explained on the grounds that progressive digestion under these conditions breaks some of the lysyl and arginyl bonds more intimately involved with the active site of the molecule, in addition to the readily cleaved bonds responsible for the activation reaction. On the other hand, at pH 5.8, the rate of tryptic digestion is retarded. During the early stages of the reaction, only the most readily cleaved sites are acted upon—probably those involved in the activation phenomenon.

Chemical analysis of both the active peptide and the purified toxin have clarified some of the mechanisms involved in the activation process. The amino acid analyses of these materials, although not complete (tryptophan and methionine were not estimated) show clearly that at least 18 amino acid residues have been removed from the toxin molecule during trypsin activation. Calculated from the number of amino acid residues derived were 2.8 × 10⁴ and 7.5 × 10⁴ MLD/mg of nitrogen, respectively.

The probable homogeneity of the peptide was established by calculating the N-terminal amino acid residue. Examination of 8- and 18-hr hydrolysates, as well as the aqueous phase of the
residues still remaining, the molecular weight of the activated peptide lies between 10,000 and 12,000. Similarly calculated, the molecular weight of the pure toxin would be around 14,000 to 16,000, or roughly 20% less than the figure of 18,600 based on ultracentrifugal analysis, arrived at in a previous paper (Gerwing et al., 1964). In view of the sources of experimental error inherent in these methods, this can be regarded as a satisfactory degree of concordance. The observations reported here indicate a reduction in number of amino acid residues in the active peptide, coupled with an alteration in the identity of its N-terminal amino acid, and strongly suggest that these are the specific mechanisms of tryptic activation.

These conclusions appear irreconcilable with the claims of Sakaguchi and his associates. As regards the far higher molecular weight ascribed by them to type E toxin, we suggest that certain of their procedures, especially the extraction of the toxin from bacterial cell suspensions with molar sodium acetate, and its repeated precipitation with solid ammonium sulfate, are conducive to the formation of molecular aggregates. In our experience, both types A and E botulinus toxins exhibit a marked tendency to undergo molecular aggregation in the course of purification, and rigorous precautions must be taken against this contingency. Whatever the final explanation of the discrepancy between their findings and ours may prove to be, it seems axiomatic that the true molecular weight should approximate to the smallest figure derived from accurate and valid data bearing on material characterized by high purity and unimpaired potency.

Our other point of dispute with the Sakaguchis arises over their use of the term “toxin precursor” for an ill-defined entity whose presence is postulated by them in toxic but untrypsinized cultures or bacterial cell extracts of *C. botulinum* type E. In their view, apparently the true type E toxin molecule becomes fully manifest only after trypsin activation—a transformation which, according to their latest report (Sakaguchi et al., 1964), occurs without detectable loss in molecular weight. These authors appear uncertain whether to attribute the toxic activity of their untreated type E culture filtrates or cell extracts to the toxin precursor alone, or to the combined effects of a mixture of precursor (more or less inert) and of toxin already freed by some intrinsic mechanism unrelated to trypsin. In any event, neither of these variants of their precursor concept is compatible with our findings that the biological activity of highly purified type E toxin is enhanced to the same extent as that of crude or partially purified toxin when exposed to trypsin under the specified conditions. The terms “precursor,” “protoxin,” or “prototoxin,” which are coming into vogue in this context, would be unobjectionable if applied to the hypothetical nontoxic entity from which—presumably by processes of enzyme synthesis or breakdown, but without the intervention of trypsin—the type E toxin molecule derives. But, as we have already briefly suggested (Gerwing et al., 1964), the haphazard use of such terms is both semantically unsound and scientifically misleading. We feel called upon to reiterate this contention in the light of our present conclusions, that the toxic activity of untreated type E culture filtrates simply reflects the presence of a certain content of homogeneous toxic protein molecules, and that the process of activation by trypsin involves potentiation of the toxic sites through removal of a number of amino acids from the N-terminus of those molecules.

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**Literature Cited**


