Partial Characterization of the Enzymatic Activity Associated with the Binary Toxin (Type C2) Produced by Clostridium botulinum

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Clostridium botulinum produces a binary toxin that possesses a heavy chain (~100,000 daltons) and a light chain (~50,000 daltons). The heavy chain is a binding component that directs the toxin to vulnerable cells, and the light chain is an enzyme that has mono(ADP-ribosyl)ating activity. A number of experiments have been done to help characterize the enzymatic activity of the toxin. The data reveal that the enzyme has a pH optimum within the range of 7.0 to 8.0. It is not inhibited or stimulated by physiological concentrations of sodium, potassium, calcium, or magnesium. The enzyme is inhibited by high concentrations of salt, however, as well as high concentrations of nicotinamide, thymidine, theophylline, and histamine; and it is stimulated by histone and lysolecithin. Boiling irreversibly denatures the light chain of the toxin, but denaturation caused by guanidine and urea is partially reversible. Enzymatic activity is not altered by short exposure to lysosomal proteases, including cathepsin B, cathepsin H, dipeptidyl aminopeptidase, and catheptic carboxypeptidase B.

Two classes of mono(ADP-ribosyl)ating toxins have been well described. Diphtheria toxin (8) and Pseudomonas aeruginosa exotoxin (9) catalytically modify elongation factor 2 in eucaryotic cells. By virtue of modification of this translo-
case, they inhibit protein synthesis and may cause cell death. Cholera toxin (3, 7), Escherichia coli enterotoxin (16), and pertussis toxin (12) catalytically modify regulatory proteins that govern adenylate cyclase. This action disrupts the ability of eucaryotic cells to modulate cytoplasmic levels of cyclic AMP, but it does not ordinarily cause cell death.

Recently, an additional group of mono(ADP-ribosyl)ating toxins has been found. Clostridium botulinum (22) and Clostridium perfringens (30) produce binary toxins that are similar in molecular weight, macrostructure, and biological actions. Both toxins possess a light chain that mono(ADP-ribosyl)ates synthetic substrates such as homo-poly-L-argi-
ine (26, 28). There is preliminary evidence that the natural substrate is a form of actin (1, 24).

It is interesting that there are several classes of microbial toxins that act preferentially to mono(ADP-ribosyl)ate sub-
strates in eucaryotic cells. This is especially true in light of the discovery that certain eucaryotic cells possess their own mono(ADP-ribosyl)ating enzymes. Moss and Vaughn (20) have isolated a ribosyltransferase from turkey eryth-
rocytes, Lee and Iglewski (13) have found a related enzyme in transformed hamster kidney cells, and Sitkov et al. (29) have reported endogenous ADP-ribosylation in a rabbit reticulocyte preparation. These findings prompt questions about the commonalities of origin and similarities of action of microbial and eucaryotic ADP-ribosyltransferases.

The three classes of microbial toxins have different pre-
ferrred substrates (i.e., elongation factor 2, nucleotide-
bounding proteins, and actin), and the eucaryotic enzymes
have not been fully characterized with regard to substrate. However, most mono(ADP-ribosyl)ating enzymes act on synthetic substrates, such as homo-poly-L-arginine and ag-
matine. This makes it practical to compare the various enzymes under identical experimental conditions. In the
present study, several characteristics of the botulimum bi-

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nicotinamide, and ADP-ribose. The elution times were as follows: NAD, ~6 min; nicotinamide, ~10 min; ADP-ribose, ~21.5 min.

Biological materials. The light chain of the botulinum binary toxin (also referred to as C2 toxin) was isolated from the same organism and by the same methods described previously (22). Lysosomal proteases were kindly provided by Robert Metrione (Jefferson Medical College, Philadelphia, Pa.).

Reagents. Unlabeled NAD and homo-poly-L-arginine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Tritiated NAD labeled in the adenine moiety ([4-adenine-3H]NAD; 30 Ci/mmol) was obtained from ICN Pharmaceuticals Inc. (Irvine, Calif.), and tritiated material labeled in the nicotinamide moiety ([2,4-nicotinamide-3H]NAD; 3.2 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, Ill.).

RESULTS

Assay for enzyme activity. Mono(ADP-ribosyl)ation is typically assayed by two techniques: incorporation of an ADP-ribose group into the substrate or release of a free nicotinamide group. Time- and concentration-dependent incorporation of labeled ADP-ribose into polyarginine has previously been reported for the catalytic chains of binary toxins (26, 28). The binary toxin from C. botulinum also produces time- and concentration-dependent release of nicotinamide (Fig. 1).

The light chain of the binary toxin (10^-8 M) was incubated with [2,4-nicotinamide-3H]NAD in the presence of polyarginine. At the end of various times, the reaction mixture was injected into the high-performance liquid chromatograph. Unreacted NAD emerged at approximately 6 min, and it was essentially homogeneous in nucleotide. The NAD that was reacted with toxin in the presence of substrate was converted to free nicotinamide. The ADP-ribose moiety attached to polyarginine eluted in the void volume.

The data indicate that mono(ADP-ribosyl)ation by the C. botulinum binary toxin can be detected either by monitoring the incorporation of adenine-labeled material into the substrate or by monitoring the liberation of nicotinamide-labeled material. Therefore, both methods were used, depending on which one was more appropriate for a particular experiment.

pH optimum. Enzyme activity was assayed in dimethylglutaric acid buffer and in boric acid-citric acid-phosphate buffer. The former has an effective buffer range from about pH 4.0 to 8.0. The latter has an effective buffer range from pH 2.0 to 12.0.

Enzyme activity was assayed by monitoring the incorporation of ADP-ribose into polyarginine. Maximal activity for both buffers was obtained in the pH range of 7.0 to 8.0. Although the shapes of the curves were the same for both buffers, the absolute values were slightly different. Enzyme activity was approximately 20% higher in the dimethylglutaric acid buffer.

Exposure of the enzyme to low pHs for short periods of time did not produce irreversible loss of activity. The enzyme (10^-8 M) was incubated in dimethylglutaric acid buffer (pH 5.0) for 30 min, after which the pH was adjusted to 7.0, polyarginine was added, and adenine-labeled NAD was provided. Compared with enzyme that was maintained at pH 7.0, enzyme that was exposed to a low pH and then adjusted to pH 7.0 possessed 85 to 90% of its original activity (two assays done in duplicate).

Effects of physiological salts. Calcium, magnesium, sodium, and potassium were tested for their ability to alter the activity of the enzyme (10^-8 M). All four cations exerted an inhibitory effect (Fig. 2). The divalent cations were more effective than the monovalent cations, but the difference could not be explained solely on the basis of charge. The IC50 for the divalent cations was approximately 25 mM, and the IC50 for the monovalent cations was approximately 160 mM. It should be pointed out that the inhibitory effect was not due simply to increased tonicity. The presence of 33 mM calcium in a 100 mM dimethylglutaric acid buffer produced greater than 50% inhibition of enzyme activity. When dimethylglutaric acid was increased to 133 mM in the absence of calcium, there was negligible inhibition (<10%) of enzyme activity.

In addition to assays done in the absence of added cation, experiments were also done in the presence of EDTA (1

![FIG. 1. Mono(ADP-ribosyl)ation was monitored by following the release of nicotinamide from NAD. The high-performance liquid chromatographic method was used, as described in the text. The reaction mixture contained tritiated NAD plus unlabeled NAD to give final concentrations of 10^-6 to 10^-3 M. Results of a representative experiment done with 10^-3 M NAD and 10^-8 M toxin are illustrated. Within the limits of resolution of the assay, there appeared to be a stoichiometric relationship between the loss of NAD and the appearance of nicotinamide.](image-url)
mM). This chelator had no effect on enzymatic activity (two assays done in duplicate).

Enhancement of enzyme activity. The light chain of the binary toxin (10⁻⁸ M) was incubated with adenine-labeled NAD and polyarginine. The incorporation of ADP-ribose was measured in the presence of various concentrations of histone and lysolecithin. Both substances produced concentration-dependent enhancement of enzyme activity (Fig. 3). ADP-ribosylation was enhanced almost twofold.

Antagonism of enzyme activity. Several agents that are known to be inhibitors of mono(ADP-ribosyl)ation were tested for their effects on the binary toxin. When tested at 10⁻³ M, nicotinamide, thymidine, theophylline, and histamine all produced substantial inhibition. The respective percent inhibition values (two assays done in duplicate) were as follows: nicotinamide, 84 ± 4; thymidine, 61 ± 7; theophylline, 85 ± 8; histamine, 91 ± 3.

Dithiothreitol was tested both for its ability to reduce enzyme activity and for its ability to reduce any intrachain disulfide bonds. When the enzyme was exposed to the reducing agent (50 mM) for 30 min at pH 7.0, residual biological activity was between 90 and 100% of control values. When the treated enzyme was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, it migrated as a single band that was not distinguishable from the native protein. When the enzyme was exposed to larger amounts of dithiothreitol (250 mM), there was some inhibition of catalytic activity (~30%), but the protein still migrated as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Denaturation of the enzyme. The botulinum binary toxin was heat sensitive. When the enzyme was boiled (three assays done in duplicate), it lost 50% or more of its activity within 1 min. When the enzyme was exposed to a temperature of 50°C, it lost 50% of its activity within 5 min. When the enzyme was exposed to 30°C, it lost less than 10% of its activity within 100 min.

Both 2 M urea and 6 M guanidine hydrochloride caused loss of enzymatic activity, but the effect was somewhat reversible. For example, when the enzyme (10⁻⁸ M) was exposed to the denaturing agents for 30 min and then repeatedly washed and filtered (Centricon 10; Amicon Corp., Beverly, Mass.) with dimethylglyutaric acid buffer (pH 7.0), the residual activity was substantial. Enzyme treated with 2 M urea (two assays in triplicate) possessed 48% of control activity, and enzyme treated with 6 M guanidine hydrochloride (two assays in triplicate) had 63% of control activity.

Lysoosomal proteases. The light chain of the toxin (10⁻⁸ M) was exposed individually or collectively to four lyosomal proteases: cathepsin B, cathepsin H, catheptic carboxypeptidase B, and dipeptidyl aminopeptidase. Assays were done at pH 5.0 and 7.0, either in the absence or presence of dithiothreitol (20 mM). Each of the proteases was tested at a concentration of 10 μg/100 μl of reaction mixture. The exposure time was 30 min.

None of the proteases alone and none of the four proteases in combination significantly reduced enzyme activity (two assays done in duplicate). The greatest effect was found with the four enzymes in combination, and they produced an effect (~10% inhibition) that was not statistically significant.

**DISCUSSION**

*C. botulinum* produces two very potent toxins (27). The first of these is a neurotoxin that exists in at least seven serotypes (A, B, C, D, E, F, and G). The neurotoxin acts mainly at peripheral cholinergic junctions, where it blocks the release of acetylcholine. The precise intracellular action of botulinum neurotoxin has not been established. The second is a more ubiquitously acting toxin that modifies the structure and function of a variety of cells, but it does not have the property of being a cholinergic blocking agent (25). This substance has been labeled a binary toxin, and thus far it has been shown to exist in only one serotype (C₂).

The mechanism of action of the binary toxin produced by *C. botulinum* has been partially determined. The toxin is composed of two separate and independent polypeptide chains (10). The heavy chain is a binding component that targets the molecule to vulnerable cells (23, 25), and the light chain is an enzyme that possesses mono(ADP-ribosyl)ating activity (26; S. Leppila, personal communication). The substrate for the light chain is reported to be actin (1, 24).

The enzymatic activity of the binary toxin is generally similar to that of other ADP-ribosylating enzymes of bacterial toxin and eucaryotic cell origin. It possesses NAD-glycohydrolase activity (unpublished results), but this activity is minimal in the absence of substrate (21). The enzyme can be inhibited by a number of endogenous substances, including nicotinamide, AMP, ADP, ADP-ribose, and ATP (26); but the concentrations needed for half-maximal inhibition are not physiologically possible. The enzyme is also antagonized by high concentrations of thymidine, theophylline, and histamine.

The binary toxin differs in at least two respects from other ADP-ribosylating toxins. First, pertussis toxin is stimulated by adenine nucleotides (11, 19), but the binary toxin is inhibited by these substances. Second, the enzymatic chain of diphtheria toxin is very resistant to boiling (5), but the enzymatic chain of the binary toxin is heat sensitive. The fact that the binary toxin is antagonized by ATP makes it different from at least one of the eucaryotic ADP-ribosyltransferases (31). On the other hand, it is similar to eucaryotic enzyme in that it is enhanced by histone (17, 18) and lysolecithin (15).

Cations have been implicated in the mechanism of action of numerous toxins. Reported roles for monovalent and divalent cations include the following: (i) agents that promote binding, (ii) agents that promote internalization, (iii) essential cofactors, and (iv) regulators of enzymatic activity. The last two roles are relevant here. Enzymatic activity was assayed in the absence and the presence of sodium, potas-
sium, calcium, and magnesium. Activity was expressed in the absence of these cations, excluding the possibility that they act as cofactors. All of the cations depressed enzymatic activity, but the concentrations that produced a significant effect were too high to be suggestive of a regulatory function. Furthermore, the ion effect may have been due to modification of polyarginine rather than the toxin. These findings do not encourage a belief that sodium, potassium, calcium, or magnesium governs toxicity.

In the case of at least one clostridial toxin (C. difficile B toxin [6]), there has been a suggestion that lysosomal processing is needed before the protein expresses its biological activity. The presumed sequence of events is that the endosomes with inactive precursor fuse with lysosomes, after which lysosomal proteases activate the toxin. Brief exposure of the binary toxin to several lysosomal proteases, individually and in combination, neither enhanced nor inhibited activity. This result was obtained when the duration of exposure was about equivalent to what would be expected of a protein that undergoes lysosomal processing. However, this does not rule out the possibility that longer exposure or additional lysosomal proteases would alter the toxin.

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LITERATURE CITED

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