Disulfide–Toxicity Relationship of Botulinal Toxin Types A, E, and F (37372)

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Toxin types A, B and F which are purified from proteolytic *Clostridium botulinum* cultures are of MW 150,000–167,000. They are "nicked" proteins in the sense this term is applied to diphtheria toxin (1, 2): one (or more) peptide bond of the toxin is cleaved by a protease(s) produced by the culture (3). The resulting two-chain structure is not demonstrable directly since the component polypeptides are linked by disulfide bridge(s). When these toxins are treated with a S–S reducing agent and analyzed by polyacrylamide gel electrophoresis, the two polypeptide chains can be separated and visualized [(4) and unpublished data for type F].

The toxin isolated from nonproteolytic type E cultures is also of MW 150,000 but it behaves as a single-chain protein. The protein is a progenitor toxin which does not manifest its full potential specific toxicity until it is activated by an appropriate enzyme such as trypsin (5). Although a causal relationship has not yet been shown, the activation of toxicity with trypsin is accompanied by nicking of the protein between two cystine residues which form an intrachain disulfide bridge (4). The resulting toxin form is comparable in its subunit structure to the naturally activated toxin isolated from proteolytic cultures. Nicked form of all toxin types studied have a larger polypeptide which is approximately twice the size of the smaller (*i.e.*, approximate MW 100, 000 and 50,000, respectively; Refs. 4, 6, and unpublished data for type F).[1]

The important role played by disulfide(s) in keeping intact the two unit nicked toxin molecules is evident. In this work, the possible relationship between disulfides and the biological activity of the toxins was examined. When neurotoxin of several antigenic types were treated with the disulfide reducing agent dithiothreitol (DTT), they lost almost all of the original toxicity.

Materials and Methods. Type A toxin and type E progenitor toxin preparations were similar to those used previously (4) and were of MW about 150,000. Type F toxin was an essentially pure preparation of similar MW (unpublished). Crystalline type A toxin is the neurotoxin–hemagglutinin complex of MW 900,000.

Type E progenitor toxin was activated (nicked) by preparing a solution in 0.2% gelatin-M/15 phosphate buffer, pH 6.2, adding a solution made of crystalline trypsin (grade A, Calbiochem, Los Angeles, Calif.) to give final 0.02% enzyme concentration, and incubating the mixture at 37° for 30 min. Tryptic action was terminated by adding a volume of soybean trypsin inhibitor (Worthington Biochemical, Freehold, N. J.) solution that contained inhibitor equal to twice the weight of enzyme being used to activate progenitor. This mixture was adjusted to pH 7.4 with 1 *M* NaOH before use in the actual experiment. Trysinization increased specific toxicity about 70-fold.

Other toxic forms were prepared directly in diluent of 0.2% gelatin-M/15 phosphate buffer, pH 7.4. Gelatin was used to minimize loss of toxicity that can accompany manipulation of toxin dissolved in otherwise

[1] The presentation in most of this report will be simplified by considering nicked toxins as being composed solely of the two polypeptide chains which have been demonstrated (4). However, the possibility exists that more than one peptide bonds have been cleaved; if so, these toxic proteins could have small peptide fragment(s) which have not been detected by the electrophoretic analysis.

protein-free buffers; the particular pH was chosen because the disulfide reducing efficiency of DTT is greater at this pH than at the slightly acidic range in which botulinal toxins are usually maintained.

Each of the toxic samples was tested in the same manner. The control portion was diluted with an equal volume of gelatin–phosphate buffer diluent and the test portion with an equal volume of diluent containing 0.08 *M* DTT. The samples were placed immediately in a 37° bath; after 30 min, the samples were diluted, the control with plain diluent and the test mixture with diluent containing 0.04 *M* DTT. Each of the twofold serial dilutions expected to cover the 100 to 0% lethality range was injected ip into four mice (0.5 ml/animal). Total elapsed time from start of incubation to finish of injections was about 60 min. LD_{50}/ml was calculated (7) from deaths occurring within 4 days of challenge.

Results. Table I shows that the nicked forms of all three botulinal toxin types lost more than 99% of their toxicity when treated with DTT. Essentially the same degree of inactivation occurred with type E progenitor toxin. The neurotoxin in the crystalline type A toxin complex was as susceptible to loss of biological activity as the neurotoxin tested in the purified form; presence of the hemagglutinin protein did not affect the results.

Equivalent LD_{50} values were obtained when the individual toxins, not treated with DTT, were titrated in normal mice and in those treated three hours previously by an ip injection of 0.5 ml of the gelatin-buffer containing 0.04 *M* DTT. Only minor reduction of toxicity occurred when treatment of toxins with DTT was performed in pH 6.4 buffer. These observations indicate that the toxicity loss shown in Table I resulted from an effect on the toxins and not from the reducing agent making the mice less susceptible to toxin. The greater loss when toxin was treated at pH 7.4 than at 6.4 makes more likely the probability that the effect of DTT is due to reduction of the toxins' S–S linkages.

The residual toxicities observed could result because not all the disulfides critical for toxicity are reduced. Such a possibility is suggested by the consistent findings of about twofold higher toxicity when toxins that had been incubated for S–S reduction were diluted for assays with plain diluent instead of DTT-containing diluent. Alternatively, toxins whose readily reactive S–S are completely reduced may still be toxic; if so the specific toxicity is several magnitudes less than the original untreated toxin.

Discussion. The data indicate that the biological activity of the several nicked botulinal toxin types depends on the integrity of at least one disulfide bond. Available information does not permit definite conclusion that the critical disulfide(s) is the interchain one(s), but some support for this possibility can be adduced in the case of type A toxin.

If it is accepted that freshly purified type A toxin has only one disulfide per molecule (8), the toxin of the crystalline complex (immediate source of the purified toxin) should have but one disulfide per molecule.

TABLE I. Toxicity Losses Accompanying Treatment of Botulinal Toxin Types with Disulfide Reducing Agent Dithiothreitol.

Toxin		LD_{50}/ml		Loss of toxicity (%)
Type	Form[a]	Not reduced	Reduced	
A	crystalline	2.4×10^7	8.0×10^3	99.9
A	purified	2.0×10^7	1.0×10^3	99.9
F	purified	1.1×10^6	2.4×10^3	99.8
E	purified progenitor	6.4×10^4	4.2×10^2	99.3
E	purified, activated	2.6×10^5	3.7×10^2	99.8

[a] MW of crystalline type A toxin is 900,000; that of all others is 150,000. Type E progenitor toxin is form not treated with trypsin and activated type E is form obtained by trypsinization of progenitor.

Since reductive treatment is needed to demonstrate the two-chain nature of the toxin, this single disulfide must be an interchain link. On this basis the two polypeptide fragments of this nicked toxin molecule are individually nontoxic or have very low toxicity in comparison to the intact molecule.

Type E progenitor toxin is not nicked; it becomes a two-chain protein when trypsinized and reduced. Nevertheless, toxicity lost during its treatment with DTT is proportionately equal to that found with the nicked toxins. The inferences are that the disulfide(s) critical for toxicity is (i) intramolecular S–S group(s) which becomes intermolecular bridge(s) during trypsinization of the progenitor toxin, (ii) intramolecular disulfide(s) other than those becoming intermolecular during trypsinization, or (iii) that connecting a yet to be identified small peptide to the otherwise intact protein.

Types A and B toxin lose toxicity when treated with reagents which react with sulfhydryl groups (6, 8). Although the precise relationship of these findings to those of the present study is not clear, the detoxification in both instances is most likely due to changes in the molecular configuration of the toxin molecule. Any treatment which alters the requisite three-dimensional shape of the protein could affect the unique activity of the botulinal toxin.

An earlier, generally unknown, report in abstract form (9) indicates that crude type A toxin was inactivated by agents (unspecified) destroying disulfide bonds. The present data confirm this observation with purified type A toxin and extend the findings to other botulinal toxin types. A possible clue to the mechanism involved is that disulfides are important in maintaining the two chain structure of activated botulinal toxin (4).

Summary. Botulinal toxins (types A, E and F), in forms of two chain proteins of MW 150,000, lost more than 99% of their toxicity when incubated with the disulfide reducing agent dithiothreitol. Type E progenitor toxin, which is a single chain protein of MW 150,000, showed a comparable loss of toxicity during similar treatment. Results indicate the importance of disulfide bonds in holding these proteins in the configuration that is critical for their biological activity.

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