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ROLE OF ARGININE RESIDUES IN THE STRUCTURE AND BIOLOGICAL ACTIVITY OF BOTULINUM NEUROTOXIN TYPES A AND E

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Summary: When nicked types A and E as well as the unnicked (i.e., single chain) type E botulinum neurotoxins were treated with 1,2-cyclohexanedione, which specifically modifies the arginine residues in 0.2 M borate buffer, pH 8.0 i) both the nicked and unnicked neurotoxins were detoxified, ii) the unnicked single chain neurotoxin became resistant to nicking with trypsin, and iii) the serological reactivity of type A (type E was not tested) was altered. Reversal of the arginine modification partially restored toxicity. In the electroimmunodiffusion test the modified type A neurotoxin appeared as 2 cones; the height of one cone increased and the other decreased as the modification reaction progressed. These results indicate that i) at least one arginine residue is involved in maintaining the toxigenic structure of types A and E neurotoxins; ii) the site of nicking in type E is an arginyl bond; and iii) arginine residue is critical for at least one antigenic determinant of type A neurotoxin.

Clostridium botulinum produces pharmacologically similar but immunologically distinct neurotoxin types. Of these the type A, B, C, D, E or F neurotoxin is a single chain protein (unnicked), or a dichain (nicked) molecule composed of two chains (H- and L-chains of mol. wt. ∿100,000 and ∿50,000, respectively) that are held together by an interchain -S-S- bond(s) or a mixture of the single and dichain molecules (1,2,3). The ratio of single to dichain molecules in a purified preparation of neurotoxin depends on the neurotoxin type, physiology and conditions of culturing the organism. The single chain molecule is nicked endogenously in cultures of some (e.g., type A) but not in all (e.g., type E) types. Trypsinization of the single chain neurotoxin of any type results in its nicking and generally also in the increase in its specific toxicity (i.e., activation); type E neurotoxin, invariably a single chain protein in its native state, is activated more than any other type (1,2,3).

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Types A, B and F neurotoxins undergo endogenous activation as well as nicking.

Although speculations were made about the mechanism of nicking and activation (1,3), it is not known what bond is cleaved during nicking and if nicking is the primary cause for activation. Substrate specificity of trypsin suggests that the site of nicking is either an arginyl or a lysyl bond. The role of arginine residue(s) in type E neurotoxin was therefore examined with 1,2-cyclohexanedione (CHD) which selectively modifies arginine residues (4). The type A neurotoxin was included in this study because two of its properties are converse to those of type E; type A is a dichain molecule and trypsinization does not activate it.

Type E neurotoxin (see ref. 1 for purification method) and CHD (Aldrich Chemical Co.), both dissolved in 0.2 M boric acid-sodium borate buffer, pH 8.0, were mixed and incubated at 35°C (final concentration of protein, A_{278} = 0.317 and CHD, 0.058 or 0.116 M). The neurotoxin incubated without CHD served as a control. Aliquots of 0.6 ml were withdrawn from the reaction mixture after 0, 30, 60, 90 and 120 min and added to 0.1 ml of PB buffer (0.1 NaH_2PO_4-Na_2HPO_4, pH 6.0 + 0.005 M boric acid) containing 0.2 M arginine. The diluted sample was immediately dialyzed to stop the reaction and remove the excess CHD from the reaction mixture. The dialysis was done at 7°C, first against chilled PB buffer containing 0.2 M arginine for 1 hr and then against PB buffer for 3 hours with hourly changes of the buffer. The dialyzed samples were divided into two portions. Trypsin (EC 3.4.4.4., tosylamido-2-phenylethyl chloromethyl ketone treated), freshly dissolved in PB buffer, was added to one portion and PB buffer to the other (final concentration of trypsin, 30 μg/ml, volume of the enzyme solution and buffer were 1/10 that of the dialyzed protein solution). The trypsinized and untrypsinized samples were incubated at 35°C for 45 min and then assayed for toxicity by the i.v. injection of mice (5) and analyzed by polyacrylamide gel electrophoresis

in presence of sodium dodecylsulfate (PAGE-SDS) (6). The preparation of samples for electrophoresis and electrophoresis has been described (7). The destained gels appeared as follows: the neurotoxin (a) not treated with CHD (control) and (b) treated with CHD for zero min appeared as one band; (c) trypsinization or (d) reduction (with mercaptoethanol) of these samples did not change pattern of the band. The band in the cases of (a), (b) and (d) was the single chain molecule, and in the case of (c) the dichain molecule of mol. wt. 147,000. Reduction of the trypsinized samples resulted in the disappearance of the single band corresponding to 147,000 mol. wt. and appearance of the H- and L chains of mol. wt. 102,000 and 47,000, respectively. A photograph of the gels is not presented here because Fig. 1A of ref. 7 is representative. The neurotoxin treated with CHD for 15,30, 60, or 120 min appeared as single band corresponding to 147,000 mol. wt.; trypsinization or reduction of these samples did not change the pattern of the band in any gel; reduction of the trypsinized samples generated three bands of mol. wt. 147,000; 102,000 and 47,000 in each gel. Intensity of the band corresponding to mol. wt. 147,000 (nick resistant molecule) relative to the H- and L chains, was related to the duration of modification reaction; longer reaction produced more intense band. Higher concentration of CHD in the reaction, 0.116 vs. 0.058 M, also produced more intense band of nick resistant molecule. Presence of L-arginine, but not L-lysine in the modification reaction mixture, in molar excess over CHD, inhibited formation of the nick resistant molecules.

The type E single chain neurotoxin was affected by CHD in two other ways (Table I): i) The neurotoxin was detoxified. ii) Activation of the neurotoxin by trypsin was quenched; e.g., while the untreated neurotoxin (control) was activated by 44 fold (toxicity increased from 1×10^2 to 4.4×10^3 LD /ml corresponding to 152 vs. 51 min survival time) the product from 30 min of modification was activated 9.4 fold (increase in toxicity from 1.9×10 to 1.8×10^2 LD /ml or 240 min vs. 119 min

TABLE I

EFFECT OF C H C ON TYPE E NEUROTROXIN

	TOXICITY (SURVIVAL TIME)	
	NOT TRYPSINIZED	TRYPSINIZED
CONTROL	152 MIN.	51 MIN.
REACTION		
30 MIN.	>240 "	119 "
60 "	>16 HRS.	193 "

TABLE II

EFFECT OF C H D ON TYPE A NEUROTOXIN

REACTION TIME	TOXICITY					
	SURVIVAL TIME	LD_{50}/ml	LOSS %	SURVIVAL TIME	LD_{50}/ml	GAIN
0 MIN.	38 MIN.	4.8×10^5	--	REVERSAL EXPT.		
30 "	60 "	1.0×10^5	79.0			
60 "	117 "	8.9×10^3	98.0	68 MIN.	6.8×10^4	7.6 X
90 "	153 "	3.3×10^3	99.3	105 "	1.3×10^4	3.9 X
CONTROL 90 MIN.	39 "	4.5×10^5	--	39 "	4.5×10^5	--

To reverse the modification (type A neurotoxin was modified with CHD as in case of type E) neurotoxin samples, after appropriate length of modification reaction, were divided into two 0.3 ml portions; to one portion 0.6 ml of 0.1 M NaH_2PO_4-Na_2HPO_4 buffer, pH 7.2 + 0.005 M boric acid was added, and it was dialyzed against 100 ml of this buffer at 7°C; to another portion 0.6 ml of 0.2 M TRIS-HCl buffer, pH 7.2 was added and it was dialyzed against 100 ml TRIS buffer at 30°C. Each buffer was changed four times during the 24 hours of dialysis in dark. The dialyzed samples were assayed for toxicity.

survival time). In separate experiments the activated neurotoxin in dichain form (produced by trypsinizing the single chain neurotoxin) was detoxified when treated with CHD.

That this detoxification is not unique with type E is shown in Table II. Availablility of a steady but small supply of type A neurotoxin (see ref. 1 for purification method) and rabbit antiserum (prepared

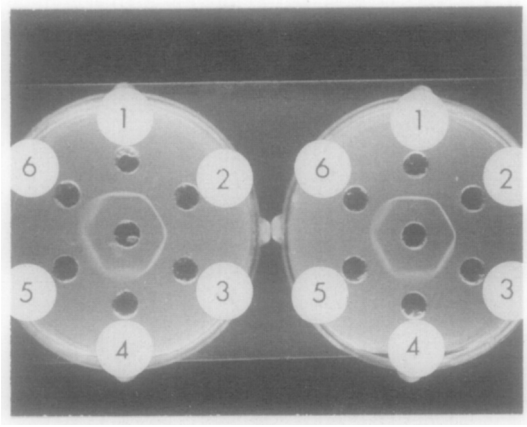

Fig. 1. Ouchterlony gel diffusion analysis of type A neurotoxin not treated with CHD (well 1); treated with CHD for zero min (about 20-30 sec) (well 2), 30 min (well 3), 60 min (well 5) and 90 min (wells 4 and 6). Rabbit antitype A neurotoxin serum (#133) 1:2 diluted in central well. Each of the peripheral wells had 32.7 µg protein in 20 µl. Duplicate plates were made with 1.5% agar in 0.05 M borate buffer, pH 7.4 + 0.85% NaCl.

against the neurotoxin detoxified with formalin) allowed the study of: i) the restoration of toxicity (lost after modification with CHD) by reversing the modification, and ii) the effect of CHD on serological reactivity of the neurotoxin. Part of the lost toxicity could be restored (Table II); e.g., the sample that had lost 98% toxicity after 60 min of modification gained 7.6X toxicity after subjecting it to the reversal conditions (4, and legend of Table II). Conditions optimum for higher or complete recovery of toxicity have not been established yet.

The type A neurotoxin whether modified or not appeared as a single band in PAGE-SDS (for a representative picture see Fig. 1B in ref. 7). The neurotoxin (a) not treated (control) and (b) treated with CHD for zero min (actually about 20-30 sec) produced one sharp precipitin band in Ouchterlony plate (Fig. 1). The band appeared increasingly diffused as the modification of the neurotoxin progressed. Electroimmunodiffusion analysis (Fig. 2) showed that (a) and (b) each produced two nearly superimposable small cones. Two clearly distinguishable taller cones emerged from 30, 60 and 90 min reaction products; difference in the height between the two tall cones from a sample widened as the modification reaction

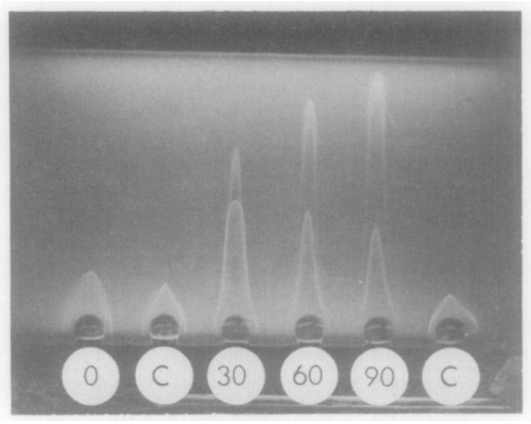

Fig. 2. Electroimmunodiffusion analysis of type A neurotoxin treated with and without CHD. Wells left to right: Neurotoxin treated with CHD for zero min (about 20-30 sec), neurotoxin not treated with CHD (control), neurotoxin treated with CHD for 30, 60, 90 min, and control. Each well received 3.6 µg protein in 20 µl. Plate (8 x 10 cm) is 0.6% agar in barbital-acetate buffer, pH 8.6 (see ref. 8 for composition of the buffer) containing antiserum (#133) 1:300 diluted. Current 10 mA (across 8 cm) for 5.5 hr at 7°C.

progressed, as if these three wells were loaded with increasing amounts of one and decreasing amounts of another antigen. Since each well received equal amounts of protein one explanation for the gain in the height of the two cones after 30 min of modification reaction and the progressive difference between their heights is as follows: The formalin treated neurotoxin induced the formation of antibodies of at least two different specificities. Arginine residue(s) is critical for one antigenic determinant and modification of the critical arginine damages the serological reactivity of this determinant. The spar formed between the 90 min reaction product (well 6) and the neurotoxin (well 1) in Fig. 1 (particularly in the left plate) indicated that the modified protein in well 6 is nearly devoid of an antigenic determinant that was present in wells 1 and 2. The neurotoxin acquired higher electrophoretic mobility only after reacting with CHD. Compared to the native neurotoxin the faster migration of CHD-modified neurotoxin towards the positive pole, also found in the immunoelectrophoresis plate (Fig. 3), is consistent with the chemistry of modification reaction. A CHD modified arginyl residue in the presence of borate forms a stable complex that acquires a negative

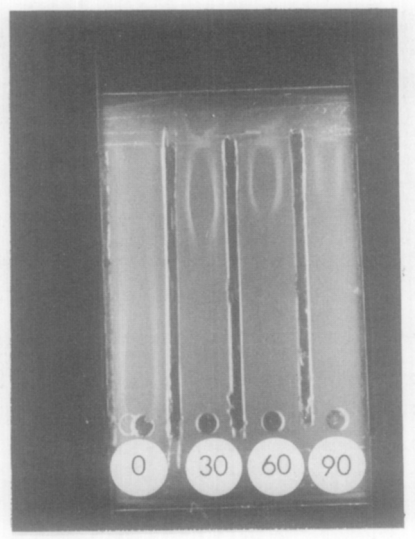

Fig. 3. Immunoelectrophoresis of type A neurotoxin. Wells from left to right: Neurotoxin treated with CHD for zero (about 20-30 sec), 30, 60 and 90 min. Each well received 16.3 µg protein in 10 µl. After 4.5 hours of electrophoresis at 7°C at 20 mA current (across 10 cm) the parallel troughs were filled with antiserum (#133) 1:4 diluted. Plate (8 x 10 cm) is 0.6% agar in barbital-acetate buffer, pH 8.6 (same as used in electroimmunodiffusion analysis).

charge (4); hence a protein becomes increasingly negatively charged as it undergoes modification. Fig. 3 also shows that the longer the neurotoxin was modified the faster it migrated. The two opposing effects on the heights of the two cones therefore appear to be due to: i) enhanced electrophoretic mobility of the neurotoxin resulting from the modification of arginyl residues that are critical and not critical for serological reactivity (the neurotoxin of 150,000 mol. wt. has approximately 37 arginine residues, ref. 1), and ii) damage of one serologically reactive site due to the modification of the critical arginyl residue(s).

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