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Characterization of the Neurotoxin Isolated from a *Clostridium baratii* Strain Implicated in Infant Botulism

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Botulism is widely known to result from ingestion of food containing botulinum neurotoxin produced in situ by certain strains of *Clostridium botulinum*. Infant botulism caused by *C. botulinum*, unlike the food-borne intoxication, is the toxicoinfectious form of botulism (S. S. Arnon, p. 331–345, *in* G. E. Lewis, ed., *Biomedical Aspects of Botulism*, 1981). The strain of *Clostridium baratii* implicated in infant botulism produced a neurotoxin that was neutralized with antiserum for botulinum neurotoxin serotype F (J. D. Hall, L. M. McCroskey, B. J. Pincomb, and C. L. Hatheway, J. Clin. Microbiol. 21:654–655, 1985). We developed a procedure to culture the toxigenic *C. baratii* (strain 6341) in dialysis bags and a simple purification scheme (precipitation of 900-ml culture supernatant with ammonium sulfate and two anion-exchange chromatographic steps at pH 5.5 and 8.0) that yielded up to 150 μg of purified neurotoxin. It is an ~140-kDa single-chain protein and has the following sequence of amino acid residues at the N terminus: Pro-Val-Asn-Ile-Asn-Asn-Phe-Asn-Tyr-Asn-Asp-Pro-Ile-Asn-Asn-Thr-Thr-Ile-Leu. Comparison of this amino acid sequence with those of the botulinum neurotoxin serotypes A, B, and E showed 40 to 50% identical residues in comparable positions. The specific toxicity of the neurotoxin, ~2×10^6 50% lethal doses for mice per mg of protein injected, was not enhanced significantly by mild trypsinization, although the protease cleaved the neurotoxin within a disulfide loop that generated at least two primary fragments, ~47 and ~86 kDa, that remained linked by an interchain disulfide. These two fragments resembled the light and heavy chains of the well-characterized neurotoxin serotypes A, B, C, D, E, and F produced by *C. botulinum*.

Clostridium botulinum and *C. tetani* have been well recognized, until recently, as the only two bacterial species that produce the two extremely poisonous proteins—botulinum and tetanus neurotoxins (18). The former causes flaccid paralysis in botulism, and the latter causes spastic paralysis in tetanus (16). Botulinum neurotoxin is found in nature as seven antigenically distinguishable proteins (serotypes A, B, C_1, D, E, F, and G); tetanus neurotoxin occurs as a single serotype. The neurotoxins of these two *Clostridium* species have strikingly similar primary structures, structure-function relationships, and modes of action; however, their primary sites of action are different. Botulinum neurotoxin acts at neuromuscular junctions, and tetanus neurotoxin acts at inhibitory synapses in the central nervous system (for reviews of the pharmacology of these two proteins, see references 16 and 27).

Certain strains of *C. baratii* and *C. butyricum* responsible for human infant botulism (2) cases were isolated around 1985. The neurotoxins produced in cultures of these clostridial species were neutralized by anti-botulinum neurotoxin sera (17, 24); the genetic identities of the *C. baratii* and *C. butyricum* strains were confirmed (32). These findings have profound implications, one of which is that non-*C. botulinum* species found in the normal flora of infant intestines may acquire and express the neurotoxin gene. This elicited for us an immediate question: how do the neurotoxins produced by *C. baratii* and *C. butyricum* compare structurally with those produced by *C. botulinum*? The neurotoxin elaborated by *C. butyricum* was purified (15), and its partial amino acid sequence (13) and molecular topography (28) were determined; these and a few other features were compared with those of botulinum neurotoxin type E (13, 15, 21, 28). We now report partial characterization of the neurotoxin produced by *C. baratii* 6341 (to be referred to henceforth as baratii neurotoxin) in terms of (i) molecular size, (ii) partial amino acid sequence, (iii) toxicity (50% lethal doses [LD_{50}] for mice per mg of protein injected), and (iv) enhancement of toxicity (activation) after mild trypsinization. We also compare these features with those of the botulinum neurotoxins.

MATERIALS AND METHODS

Culture media. Four different media of the following compositions were used to maintain the stock culture and to produce the neurotoxin: medium A, 12.5% solid meat media (Difco), 0.3% glucose, 0.2% soluble starch (Difco), and 0.05% sodium-thioglycolate (Sigma); medium B, 2% Trypticase-peptone (BBL, Becton Dickinson Microbiology Systems), 1% glucose, 0.5% yeast extract (BBL), 0.2% soluble starch, and 0.05% sodium-thioglycolate; medium C, 1% glucose and 0.05% sodium-thioglycolate; medium D, 4% Trypticase-peptone, 1% yeast extract, and 0.05% sodium-thioglycolate. In each case all the ingredients were dissolved (wt/vol) in 0.1 M sodium potassium phosphate buffer, pH 7.0.

Bacterial culture. One milliliter of *C. baratii* (strain 6341), grown in cooked-meat medium and kindly provided by Charles Hatheway (Centers for Disease Control, Atlanta, Ga.), was inoculated into 250 ml of medium A in a 500-ml flask and incubated for 5 days at 37°C. The bacterial cells in the culture fluid were packed into a small volume by centrifugation (10,000 × *g*, 20 min), suspended in sterile 20% skim milk (Difco; dissolved in water), and then distributed in 2-ml

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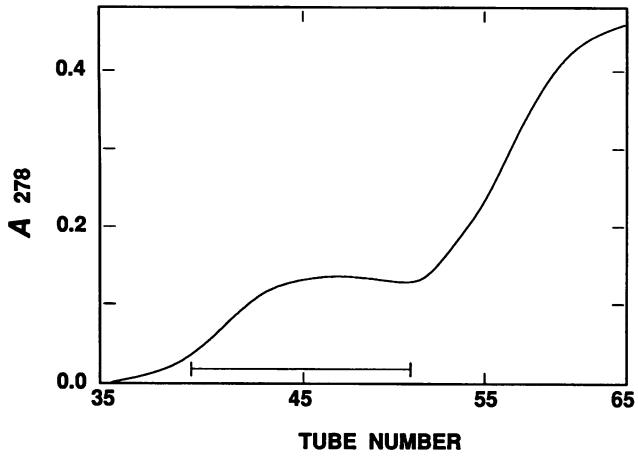

FIG. 1. Chromatography of culture supernatant concentrate on a DEAE-Sephadex A-50 column (2.5 by 60 cm) equilibrated and eluted with 50 mM sodium citrate buffer, pH 5.5. Chromatography was done at room temperature. The flow rate was 25 ml/h, and the fraction volume was 2.4 ml. The fractions containing the first peak were pooled and are indicated with a bar.

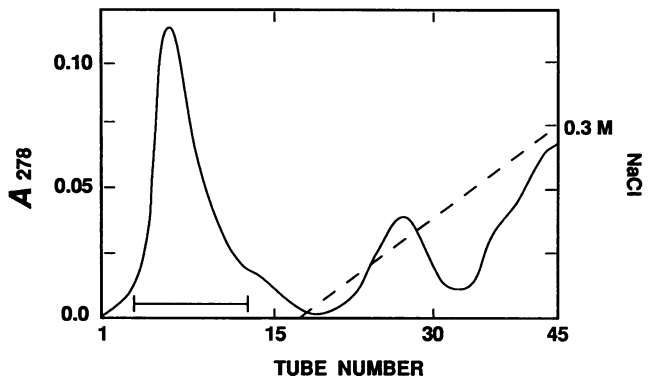

FIG. 2. Chromatography on a DEAE-Sephadex A-50 column (1.8 by 8 cm) equilibrated and eluted at 4°C with 20 mM sodium phosphate buffer (pH 8.0). Flow rate was 12 ml/h, and the fractions were of 2 ml. The salt gradient, indicated with a dashed line, was made with 50 ml of 20 mM sodium phosphate buffer, pH 8.0, and 50 ml of the same buffer but containing 0.3 M NaCl. The neurotoxin eluted isocratically and was recovered in the first peak (neurotoxin pool is indicated with a bar).

vials as stock culture. These were stored at −70°C or lyophilized and stored at 4°C.

The neurotoxin was produced in dialysis bags (31) with 32- or 100-mm-flat-width and 100-cm-long dialysis tubings (Spectrapor; D1615-2, 12- to 14-kDa cutoff, or 132670, 6- to 8-kDa cutoff limit). Approximately 30 ml of medium B in a 50-ml screw-cap glass tube was inoculated with 0.2 ml of stock culture (thawed or rehydrated with medium B). After ~12 h of incubation at 37°C, 1 ml of this culture was transferred to 30 ml of medium B in a glass tube and further incubated overnight at 37°C. One milliliter of this culture was inoculated into 150 ml of medium C contained in a dialysis bag which was immersed in a 1,000-ml glass cylinder containing 500 ml of medium D. After 5 days of incubation at 37°C, the contents of dialysis tubes from six parallel cylinders were pooled and centrifuged (10,000 × *g*, 60 min). The supernatant was 60% saturated with ammonium sulfate (390 mg/ml) and kept at 8°C overnight. Our one attempt to harvest more neurotoxin from a larger volume of culture fluid by using the larger-diameter dialysis bag (100 versus 32 mm) of identical length (100 cm) gave lower yield. Porosity of the bags (i.e., molecular size cutoff) and volumes inside and outside of the bags were different; whether these were factors in the neurotoxin yield remains to be explored.

Neurotoxin purification. The material precipitated with ammonium sulfate was recovered by centrifugation (10,000 × *g*, 45 min); dissolved in 15 ml of 50 mM citric acid-disodium citrate buffer, pH 5.5; and then dialyzed against this buffer for several hours at 4°C. The dialyzed solution (~25 ml) was centrifuged (15,000 × *g*, 60 min at 25°C); the supernatant was loaded into a DEAE-Sephadex A-50 column (2.5 by 60 cm), equilibrated, and eluted with the 50 mM citrate buffer, pH 5.5. The UV absorbing material (indicated with a bar in Fig. 1) eluting as a wide peak ahead of a large peak was pooled; dialyzed against 30 mM sodium phosphate buffer, pH 7.0, for 5 h at 4°C; and finally precipitated with ammonium sulfate (390 mg/ml). After 16 h at 4°C, the precipitate was recovered by centrifugation (10,000 × *g*, 30 min, 4°C); dissolved in 5 ml of 20 mM sodium phosphate buffer, pH 8.0; and dialyzed against this buffer for 16 h at 4°C. Turbidity that appeared during dialysis was removed by centrifugation. The clear supernatant was loaded onto a DEAE-Sephadex A-50 column (0.9 by 6 cm) equilibrated at 4°C with the pH 8.0 buffer. The UV absorbing material that did not bind to the column and emerged as the first peak (Fig. 2) was pooled, precipitated with ammonium sulfate (390 mg/ml), and stored at 4°C.

SDS-PAGE. Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% acrylamide, 0.89% cross-linking) in a discontinuous buffer (22) and with the stain Coomassie blue R-250 (Sigma). Their molecular masses were estimated from the relative electrophoretic migration of marker proteins (phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase, which were 94, 67, 43, and 30 kDa, respectively [Pharmacia]) as well as the well-characterized botulinum neurotoxin serotypes A, B, and E (5).

Electroblotting and amino acid sequence determination. The baratii neurotoxin after electrophoresis on 6% linear acrylamide gel with SDS (1.6-mm-thick, 10- by 10-cm minislab; 40 mA for 90 min) by using the discontinuous buffer system (22) was electrotransferred (0.5 A, 60 min) to a polyvinylidene difluoride (Millipore, Bedford, Mass.) membrane by the method of Matsudaira (23) with the minor modifications reported before (12). The protein blotted onto the polyvinylidene difluoride membrane was stained with Coomassie blue, air dried, excised out with a razor blade, and stored at −20°C. For amino acid sequencing, Applied Biosystems Instrument pulse-liquid phase (models 475A and 477A) protein sequencers were used. Phenylthiohydantoin amino acids were identified with an on-line phenylthiohydantoin amino acid analyzer and a data analyzer system (Applied Biosystems model 120-A and 900-A, respectively).

Hemagglutination assay. Human and rabbit erythrocytes, recovered with citrate as an anticoagulant, were washed with 75 mM sodium phosphate buffer, pH 7.2, containing 75 mM NaCl. Microtiter plate (Dispo V plate; Scientific Products) wells were loaded with 50 μl of 0.5% blood cell suspension and 50 μl of test proteins serially diluted in the pH 7.2 buffer. The hemagglutinin protein chromatographically separated from botulinum type A neurotoxin (30) was used as positive control. The plates were read for hemagglutination after 8 h at 6°C.

Toxicity and neutralization with antisera. Toxicity was determined by intravenous injection of 0.1 ml of sample into the tail vein of white mice (~22 g); time (in minutes) until death of mice after injection is a function of the toxic potency (4). Ability of the antisera prepared against botulinum neurotoxin serotype A, B, E, or F to neutralize baratii neurotoxin was examined as follows: 50 μl of the neurotoxin (containing 25 LD_{50}) was incubated with 50 μl of anti-type A, B, E, or F serum (enough to neutralize 1,000 LD_{50} of the homologous neurotoxin) for 1 h at 37°C and then injected intravenously or intraperitoneally in mice. Anti-type A, B, and E neurotoxin sera were produced in rabbits as described before (8); anti-type F serum, produced in burros, was a gift from C. Hatheway.

Effects of trypsinization on covalent structure and toxicity. Baratii neurotoxin (~0.1 mg/ml) was incubated with trypsin (Sigma; type XIII, lot 67F-8045; ratios [wt/wt], 50:1, 25:1, 10:1, and 2:1) at pH 6.0 or 7.0 (0.1 M sodium-phosphate) or 7.5 or 8.0 (0.1 M Tris-hydrochloride) for 5 to 60 min at 30 to 35°C. Tryptic digestions were stopped with soybean trypsin inhibitor (Sigma; type I-S, lot 85C-8057); enzyme-to-inhibitor ratio was 1:3 (wt/wt). The digests and the control (neurotoxin incubated without trypsin) were diluted with 50 mM sodium phosphate buffer, pH 6.0, containing 0.2% gelatin (Difco) and then tested for toxicity. Trypsinized neurotoxins with and without dithiothreitol (DTT) reduction were analyzed by SDS-PAGE.

Protein concentration was determined with bicinchoninic acid (29) (by using the protein determination kit from Pierce, Rockford, Ill.). Bovine serum albumin was used as the reference protein.

RESULTS AND DISCUSSION

C. baratii 6341, cultured conventionally (i.e., without dialysis bag), produced amounts of the neurotoxin (<500 LD_{50}/ml) that were too low for biochemical studies. Dialysis bag culturing (31) improved the yield to >5,000 LD_{50}/ml, which was lower than the range from 10^5 to 10^8 LD_{50}/ml found in cultures of *C. botulinum* types A, E, and F (9, 14, 33). The baratii neurotoxin was purified (2×10^6 LD_{50}/mg) more than 1,000 times from the culture supernatant (1.5×10^3 LD_{50}/mg). The average yield of ~0.15 mg of purified baratii neurotoxin recovered from ~900-ml culture compared with ~1.0, ~1.5, and 0.63 mg of pure botulinum neurotoxin types A, E, and F per liter, respectively, from conventional culture (9, 14, 33) indicates low toxigenicity of *C. baratii* 6341 rather than poor recovery of the protein through purification steps.

The SDS-PAGE analysis shows progress through the purification steps (Fig. 3). The pool from the pH 5.5 column (Fig. 1) had 140-, 120-, and 60-kDa protein bands along with two or three smaller-size proteins (Fig. 3, lane 3). Chromatography at pH 8.0 (Fig. 2) removed the 120-kDa and most of the other proteins from the 140-kDa neurotoxin. The baratii neurotoxin did not bind to a DEAE-Sephadex A-50 column equilibrated with 20 mM sodium phosphate buffer, pH 8.0. We tested QAE-Sephadex A-50 and Mono-Q resin (Fast Protein Liquid Chromatography; Pharmacia) equilibrated with the same buffer. Both were unsatisfactory. The neurotoxin preparation partially bound to the QAE gel and eluted as a broad peak under a gradient of increasing NaCl concentration (not shown). The Mono-Q column bound the neurotoxin; an increasing salt gradient eluted a single peak containing a significant amount of impurities (not shown).

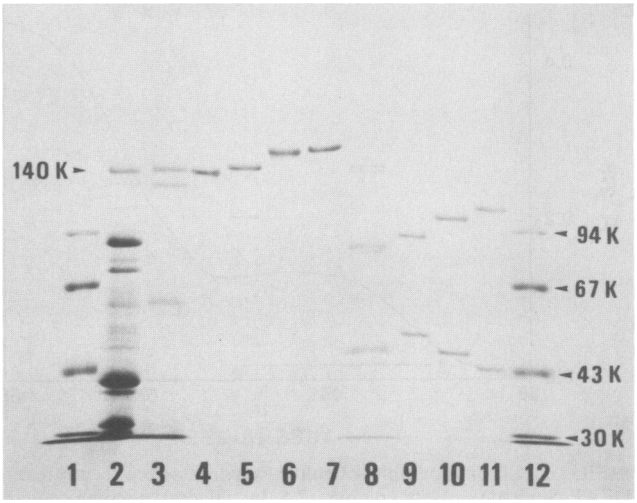

FIG. 3. PAGE pattern of toxin samples. Lanes: 1 and 12, molecular mass markers; 2, crude baratii neurotoxin before chromatography at pH 5.5 column; 3, neurotoxin purified through DEAE-Sephadex at pH 5.5; 4, neurotoxin from the pH 8 chromatography; 5, 6, and 7, botulinum type A, B, and E neurotoxins, respectively (the two extra bands below type A neurotoxin in lane 5 are impurities); 8, 10, and 11, baratii type B and E neurotoxins (same as in lanes 4, 6 and 7, respectively) after trypsinization and reduction with DTT; 9, botulinum type A neurotoxin reduced with DTT.

DEAE-Sephadex was therefore chosen for subsequent work.

The neurotoxin obtained from the DEAE-Sephadex column (Fig. 3, lane 4) did not change its electrophoretic migration after reduction with DTT. Thus, the 140-kDa neurotoxin appears to be a single-chain protein and not composed of two polypeptides linked by a disulfide bond(s). Determination of amino acid sequence of the neurotoxin prepared from two independent batches yielded Pro as the only N terminus (Table 1). The first preparation was analyzed for the first 32 residues, and the second batch was analyzed for the first 19 residues; identical results were obtained.

The 120-kDa protein (the band migrating immediately ahead of the 140-kDa neurotoxin in lanes 2 and 3 of Fig. 3) found associated with the neurotoxin after chromatography at pH 5.5 was removed after chromatography at pH 8.0 (Fig. 3, lane 4). This protein appears analogous to the nonneurotoxic proteins possessing high, low, or no hemagglutinating activity and found tightly associated with all botulinum neurotoxin serotypes (25, 30). For example, the nonneurotoxic proteins associated with type A and F neurotoxins have high and low hemagglutinating activity, respectively; the 134-kDa protein associated with type E has no hemagglutinating activity (30). These nonneurotoxic proteins have other common characteristic features. They remain associated with the neurotoxins at acidic pH but can be separated at alkaline pH and high ionic strength, and also the nonneurotoxic proteins elute from an anion-exchange column at an ionic strength that is higher than that which elutes the neurotoxin. Baratii neurotoxin was freed of the 120-kDa protein with 20 mM phosphate buffer, pH 8.0. The 120-kDa protein was eluted with an increasing salt gradient (Fig. 2). No hemagglutinating activity was found in the bacterial culture, concentrated protein samples, or chromatographic fractions. Absence of hemagglutinating activity resembles

TABLE 1. N-terminal amino acid residues of various *Clostridium* neurotoxins

Neurotoxin	Reference	Amino acid residue 10	20	30	% Homology
Baratii[a]		P V N I N N F N Y N D P I N N T T I L Y M K M P Y Y Y D S N K Y			
Botulinum					
Type A	3	P F V N K Q F N Y K D P V N G V D I A Y I K I P N A G Q M Q P V			34
Type B	5	P V T I N N F N Y N D P I D N N N I I M M E P P F A R G M G R Y			53
Type E	5	P – K I N S F N Y N D P V N D R T I L Y I K P G G C Q E F Y K S			50
Butyricum	13	P – T I N S F N Y N D P V N N R T I L Y I K P G G X Q Q F Y K S			53
Tetanus	11	P I T I N N F R Y S D P V N N D T I I M M E P P Y C K G L D I Y			50

[a] The amino acid sequenator log is as follows: the theoretical initial yields (in picomoles) and combined amino acid repetitive yield (in percent) of the first batch (32 residues identified; LPS 1351) were 58 and 92%, respectively, and for the second batch (19 residues identified; MLK 5-7-90) were 10 and 91%, respectively.

the properties of products of type E culture. The relative proportions of baratii neurotoxin and the 120-kDa protein did not appear constant in the different preparations; the neurotoxin was always in a larger amount.

Specific toxicity of the purified baratii neurotoxin was ~2 × 10^6 LD_{50}/mg. Anti-botulinum type A or B serum did not neutralize toxicity. Complete neutralization of toxicity was attained with anti-type F serum, as expected (17). We also found that baratii neurotoxin is partially neutralized (delayed death in mouse lethality assay) with anti-type E serum (Hatheway also noted this [18a]).

Toxicity of the baratii neurotoxin was not significantly enhanced by trypsin digestion. Limited digestion of the neurotoxin with trypsin (20:1 [wt/wt], 30 min at 35°C) followed by reduction of disulfide bonds with DTT generated two major fragments (86 and 47 kDa) and at least three other minor fragments (<140, ~60, and ~43 kDa) as shown in Fig. 3 (lanes 4 and 8). Higher trypsin concentration or longer digestion produced further cleavage of the 140-kDa neurotoxin as well as the 86- and 47-kDa fragments.

Generation of 86- and 47-kDa fragments from the 140-kDa protein by trypsinization and separation of the two fragments after reduction with DTT suggest that trypsin cleaved the neurotoxin at about one-third the distance from its N or C terminus and within a disulfide loop. We infer that 86- and 47-kDa fragments represent the heavy and light chains, respectively, of the neurotoxin. This inferred structure is consistent with the structures of different serotypes of botulinum neurotoxin as well as tetanus neurotoxin. Each of these neurotoxins is synthesized as an ~150-kDa single-chain protein. Proteolytic cleavage (nicking) converts these proteins to ~150-kDa dichain proteins, each composed of an ~100-kDa heavy chain and an ~50-kDa light chain that remain linked by noncovalent bonds and a disulfide bond, and the light chain retains the N-terminal segment of the parent single chain (5). Proteases endogenous to the bacteria or exogenous, such as trypsin and endoproteinase Lys-C, nick the single-chain neurotoxin to the dichain form (20). Botulinum neurotoxin serotypes A, B, and E isolated from 96-h-old bacterial cultures are, respectively, nicked (dichain), partially nicked (mixture of 80 to 90% single and 10 to 20% dichain), and unnicked (single-chain) proteins (5). In Fig. 3 the 140-kDa baratii neurotoxin (lane 4) is compared with ~150-kDa type A, B, and E neurotoxins (lanes 5, 6, and 7, respectively); lanes 9, 10, and 11 exhibit the heavy and light chains of types A, B, and E, respectively. (Type B and E neurotoxins were trypsinized to generate the dichain forms [26].) In lane 8 the 140-kDa band represents the residual amount of undigested baratii neurotoxin. Our limited attempts to generate a homogeneous population of dichain protein by confining cleavage of the single-chain neurotoxin at only one peptide bond were not successful; additional cleavages occurred. The thin band immediately below the 140-kDa band is probably a fraction of the neurotoxin population that was cleaved near N or C termini but was not nicked. One band located between the heavy and light chain and the one below the light chain are products of cleavages at sites other than nicking. Similar additional fragmentations, in addition to nicking, have been observed with type B and E neurotoxins treated with trypsin and endoproteinase Lys-C (20, 26).

A short stretch at one-third the distance from the N terminus of botulinum type A and E and tetanus neurotoxins is rich in Arg and Lys residues (1, 7, 12). Trypsin or the protease endogenous to the neurotoxin-producing bacteria cuts multiple bonds within a range of few residues. Homogeneity of the N terminus of the heavy chain therefore depends on precise conditions of proteolysis and the end point of proteolysis (1, 7, 12). The 86-kDa heavy and 47-kDa light chains of baratii neurotoxin produced by trypsinization (Fig. 3, lane 8) were heterogenous (SDS-PAGE showed more than a single band); therefore, we did not determine their N-terminal sequences.

The 86-kDa fragment generated by trypsinization (Fig. 3) is, we believe, the heavy chain segment of the neurotoxin. After verifications of its identity, this material could provide interesting opportunities in the structure-function relationship studies. Electrophoretic migration (Fig. 3) indicates that baratii neurotoxin heavy chain is smaller in molecular mass than those of types A, B, and E, while the baratii neurotoxin light chain is smaller than that of type A, similar to that of type B, and larger than that of type E. The heavy chain appears to have at least three roles in the neurotoxin's pathway of intoxication. (i) In the 150-kDa dichain neurotoxin the ~50-kDa light chain remains linked to the N-terminal half of the heavy chain; unless the heavy chain delivers it into the cytosol, the light chain cannot poison the neurotransmitter secretory cells. (ii) The C-terminal half of the heavy chain is thought to have the binding site for receptors on the target cell membrane (6, 19). (iii) After receptor-mediated endocytosis, the neurotoxin, inside the cell, initially remains encapsulated by the endosomes. The N-terminal half of the heavy chain forms channels in the endosomal membrane through which the light chain exits into the cytosol (see reference 6 and references therein). The baratii neurotoxin heavy chain is smaller than the heavy chains of type A, B, and E neurotoxins; therefore, its structure-function analysis would be highly instructive in defining which precise segments of the heavy chain play what role.

The baratii neurotoxin purified from 4-day-old culture was

in the single-chain form; this feature differs from the dichain type F neurotoxin isolated from 4- to 5-day-old culture of proteolytic strains of *C. botulinum* type F (10, 33, 34).

The mechanisms by which *C. baratii* (strain 6341) acquires the neurotoxin gene and expresses the protein are unknown. The amino acid sequence reported here can be exploited to construct DNA probes which could be valuable in answering some of the genetic questions regarding the location of the neurotoxin gene and its exchange or acquisition by the nonneurotoxic *C. baratii* and toxigenic *C. botulinum*.

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