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Biophysical Characterization of the Stability of the 150-Kilodalton Botulinum Toxin, the Nontoxic Component, and the 900-Kilodalton Botulinum Toxin Complex Species

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Botulinum neurotoxin serotype A is initially released from the bacterium *Clostridium botulinum* as a stable 900-kDa complex. The serotype A 900-kDa complex is one of the forms of the toxin being used as a therapeutic agent for the treatment of various neuromuscular disorders. Previous experiments have demonstrated that the 900-kDa complex form of the toxin protects the toxin from the harsh conditions of the gastrointestinal tract. To provide molecular level details of the stability and equilibrium of the 900-kDa complex, the nontoxic component, and the toxic (botulinum neurotoxin) component, the three species have been investigated with a series of biophysical techniques at the molecular level (dynamic light scattering, proteolysis, circular dichroism, pH incubations, and agglutination assays). These experiments were conducted under harsh conditions which mimic those found along the gastrointestinal tract. Separately, exposure to denaturing and proteolytic conditions degrades both the botulinum neurotoxin and the nontoxic component. In the 900-kDa complex, the botulinum neurotoxin is protected during exposure to the gastrointestinal environment and the nontoxic component is slightly modified. Surprisingly, the toxin protects the ability of the nontoxic component to agglutinate erythrocytes. Contrary to previous reports, the purified 900-kDa complex did not have agglutination ability until after exposure to the proteolytic conditions. These experiments provide new evidence and detail for the theory that the nontoxic component and the toxic component protect one another during exposure to harsh conditions, and a molecular model is presented for the passage of the toxin through the gastrointestinal tract.

Botulinum neurotoxin is secreted by the anaerobic bacterium *Clostridium botulinum* as one of seven serotypes, classified A through G (35). The different serotypes proteolytically cleave specific proteins involved in synaptic vesicle docking that are necessary for cellular communication at the neuromuscular junction (12). Serotype A can be purified as a 900-kDa complex (BoNTA-HA) consisting of a toxic component (BoNTA [botulinum neurotoxin serotype A]) and a nontoxic component (HA [hemagglutinin]) (5–7). Studies involving relative oral toxicities (26, 33), intestinal absorption (34), and comparison to tetanus toxin (31) have indicated that the complex, not the botulinum toxin alone, is responsible for the extremely low amount of botulinum neurotoxin required in botulism poisoning.

The most common mechanism of botulism poisoning is through oral ingestion of the complex, which is found in food contaminated with *C. botulinum*. Ingested spores of the bacteria may also colonize and produce toxin in the intestinal tracts of infants, resulting in infant botulism (36). Previous experiments have demonstrated that the 900-kDa complex protects the toxin during its exposure to harsh conditions. Ohishi and coworkers (26) have demonstrated that the 900-kDa complex has a 360-fold-higher oral toxicity in rats than the 150-kDa botulinum neurotoxin component alone. Most proteins are broken down into peptides and amino acids in the stomach and small intestine during the process of digestion (2). However, the 900-kDa complex enters the stomach and withstands the acidic (pH 2) gastric juice containing the protease pepsin. The complex then enters the small intestine, where it encounters several more proteases (trypsin, chymotrypsin, and carboxypeptidase) that function at pH 7 to 8. Despite these harsh denaturing and proteolytic conditions, active botulinum neurotoxin (13, 22) and nontoxic component (34) can be detected in the lymph and circulatory systems.

The botulinum neurotoxin is comprised of a C-terminal 100-kDa heavy chain and an N-terminal 50-kDa light chain linked by a disulfide bond. From C terminus to N terminus, the protein can be further divided into three 50-kDa functional domains (3, 4, 18)—binding, translocation, and catalytic—which allow the protein to bind a cell surface receptor, pass across the membrane (29), and cleave a protein involved in synaptic vesicle docking (12), respectively. The nontoxic component is composed of several protein subunits and can be separated into nontoxic agglutinating proteins (14, 17, 32) and nontoxic nonagglutinating proteins (11, 14). Several subcomponents of the nontoxic component have been characterized. One of the nontoxic nonagglutinating proteins is a single peptide of 120 kDa and has been sequenced (11). The molecular weights of the subcomponents of the nontoxic agglutinating portion have also been determined (14, 32). However, the physiological role and molecular organization of the nontoxic component are not well understood. In addition, many of the studies of toxin exposure to harsh conditions have been conducted with crude cell supernatant. To understand the stability and equilibrium of the botulinum neurotoxin complex and its separated components at the molecular level, we conducted a series of biophysical experiments using pure preparations of botulinum neurotoxin, botulinum neurotoxin complex, and the nontoxic agglutinating portion.

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MATERIALS AND METHODS

Purification of proteins. For all experiments, proteins were obtained as ammonium sulfate precipitates and were purified by ion-exchange chromatography as previously described (5).

Light scattering. The hydrodynamic radius, estimated molecular weight, and polydispersity of protein samples were determined by using a Dynapro-801 dynamic light scattering instrument (Protein Solutions, Charlottesville, Va.). Samples of botulinum neurotoxin (0.18 mg/ml), nontoxic component (0.20 mg/ml), and 900-kDa complex (0.22 mg/ml) were incubated for 30 min at 4°C over a range of pHs in 10 mM buffers containing 100 mM sodium chloride. Buffers were citric acid (pH 1 to 5) (citric acid has three pK_as at 25°C; $pK_1 = 3.128$, $pK_2 = 4.761$, and $pK_3 = 6.396$), bis-Tris [bis(2-hydroxyethyl)imino-tris(hydroxymethyl) methane; pH 6], HEPES (pH 7 to 8), and CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid] (pH 9 to 10). Individual samples were recorded for at least 5 min, during which 12 to 13 data points were taken and then analyzed by using a monomodal fitting program. The polydispersity served as the deviation of the size distribution based on the mean hydrodynamic radius of each sample. Experiments were performed in triplicate.

Test for pH effects. Protein samples were incubated at the appropriate pH for 48 h at 4°C. Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel, and the bands were visualized with silver stain (10).

Proteolysis. Proteins were incubated at various pHs and with several protease mixtures. Each protein sample contained 2 ml of protein (0.42 mg/ml) in dialysis tubing with a 50-kDa pore size. Dialysis tubing containing the protein sample was placed into the appropriate protease mixture. The mixture was incubated with gentle stirring for 2 h at 37°C. Pepsin incubations were performed with 0.4 mg of pepsin per ml in 10 mM hydrochloric acid (pH 2). Intestinal cocktail consisted of trypsin (0.02 mg/ml), chymotrypsin (0.03 mg/ml), and carboxypeptidase A (0.02 mg/ml) in 50 mM sodium bicarbonate–100 mM sodium chloride buffer (pH 8). Protease concentrations used were chosen based on reported values (15, 16), though the physiological protease concentration depends on the feeding state of the organism. After incubation, proteases were quenched with 0.5 ml of pepstatin (1 mg/ml) or phenylmethylsulfonyl fluoride (17.4 mg/ml) and 1 ml of 0.5 M EDTA. Proteins were also exposed to incubation of pepsin followed by intestinal cocktail or incubation at pH 2 followed by intestinal cocktail. Gels were 12% polyacrylamide, and protein bands were visualized with silver stain (10).

Circular dichroism. Circular dichroic spectra were gathered on a model J-600 spectropolarimeter (Jasco, Easton, Md.) at 37 and 25°C. The bandwidth used was 1 nm, and the step resolution was 2 nm. Four scans of each sample were made, using a time constant of 1 s and a scan rate of 50 nm/min. The cell volume was approximately 1 ml, with a path length of 0.1 cm. The cell was jacketed for temperature adjustment and controlled by using a water bath. Proteins at a concentration of 0.025 mg/ml were dialyzed extensively in their buffers before being examined on the spectropolarimeter. The pH 6 and pH 8 buffers were 100 mM sodium phosphate containing 100 mM sodium chloride. The pH 2 buffer was 60 mM hydrochloric acid-potassium chloride buffer containing 100 mM NaCl. No appreciable difference in signal was observed at 37 and 25°C; thus, only 37°C data are shown.

ELISA. Botulinum neurotoxin (0.01 mg/ml) was incubated in pepsin (0.4 mg/ml) and intestinal cocktail (see above) for 2 h at 37°C. The concentration of substrate protein used was the minimum amount of protein necessary to give strong enzyme-linked immunosorbent assay (ELISA) signal. Using single-chain variable fragments of mouse or of human monoclonal antibodies, ELISAs were performed on proteolyzed samples as described by Chen et al. (5).

Surface plasmon resonance. Binding of botulinum neurotoxin and 900-kDa complex to lipid was monitored by using surface plasmon resonance on a BIAcore 2000 (Biosensor, Piscataway, N.J.). Botulinum neurotoxin (84 to 1,300 nM) or 900-kDa complex (52 to 832.5 nM) was injected over a lipid monolayer containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). The lipid monolayer was formed by incubating a solution of POPC liposomes (2 mg/ml) on a BIAcore sensorchip (type HPA) overnight in a moist environment. Liposomes were made by sonicating a solution of POPC in a buffer of 10 mM Tris (pH 7.0) containing 100 mM sodium chloride and 2 mM sodium azide. The experiments were performed at a flow rate of 40 ml/min for 20 min in running buffer of 10 mM Tris (pH 7.0) containing 100 mM sodium chloride and 2 mM sodium azide.

Sugar binding. Agglutination assays were performed as previously described (20). Erythrocytes (RBC) from rabbits were washed twice with saline (68 mM sodium citrate, 146 mM sodium chloride [pH 7.4]), spun at 4,000 × *g*, and diluted to 0.5% for each assay. Protein solution (0.5 ml) at twice the desired concentration was added to a disposable semimicrocuvette. Then 0.5 ml of the 0.5% RBC was added to the protein, resulting in the desired protein concentration and a 0.25% RBC solution. The mixture was incubated at 37°C for 2 h, and the transmittance was measured at 405 nm in a Shimadzu UV-160 spectrophotometer. The saline control contained 0.25% RBC incubated in saline for 2 h at 37°C. Each sample was performed in triplicate. The 900-kDa complex and nontoxic component were also proteolyzed and then used in an agglutination assay. The saline control was subtracted from the values for the other samples. The agglutination ability of the unproteolyzed HA was scaled to a value of 100. The agglutination abilities of the other samples represent their percent transmittances relative to the percent transmittance by unproteolyzed HA. Previous reports (20) show that the 900-kDa BoNTA-HA complex is capable of agglutination at lower temperatures. When we performed the agglutination assay at a lower temperature (12°C), we observed that the complex showed an increased ability to agglutinate RBC, consistent with the literature but not physiologically relevant.

RESULTS AND DISCUSSION

Light scattering. Dynamic light scattering was used to determine the aggregation state and stability of the three species under a variety of conditions simulating the environments in the gastrointestinal tract. This technique measures the diffusion coefficient of particles in solution, allowing a radius and estimated molecular weight to be calculated. A molecular weight calculated to be higher than expected indicates a deviation from a spherical shape. This deviation may be due to nonspherical species, denatured protein, or the presence of large aggregates of many proteins. The distribution of particle sizes is indicated by the polydispersity value. A polydispersity of zero signifies that there is only one particle size in solution. A large polydispersity value indicates the presence of different-size particles in solution. Examination of the radii of the three molecular species between pH 1 and 10 yielded a number of intriguing results. From the hydrodynamic radii the corresponding molecular weights were calculated, assuming an approximate, spherical shape for the protein.

Between pH 10 and 4, the estimated molecular mass of BoNTA was determined to be approximately 150 kDa (Fig. 1A and D), corresponding to the expected molecular mass of a single BoNTA molecule (6). Upon incubation at pH 3 to 1, botulinum neurotoxin showed steadily higher values for molecular mass, from 150 to over 1,000 kDa, during the first 10 min in the corresponding buffer. The polydispersity also increased dramatically as the pH changed from 3 to 1. These higher values for polydispersity correlated with aggregation and acid-catalyzed degradation of the neurotoxin molecule. This result is consistent with botulinum neurotoxin retaining little toxicity at pH 3 to 1 (33). Examination of the nontoxic component at various pHs revealed behavior similar to that of the botulinum neurotoxin (Fig. 1B and D). The nontoxic component did not aggregate or disassemble between pH 10 and 5 and had an apparent estimated molecular mass of approximately 962 kDa. The molecular mass calculated by light scattering differs from that calculated by subtracting the molecular mass of the BoNTA from the molecular mass of the BoNTA-HA complex because the nontoxic component most likely is not spherical. Since the light scattering device calculates a molecular mass by a formula that assumes the protein is a sphere, deviations in sphericality will result in deviations in molecular mass. The nontoxic component aggregated, disassembled, or denatured when the pH dropped from pH 5 to 4, corresponding to a sharp increase in the polydispersity between pH 5 (0%) and 4 (polydispersity/radius = 18.1%). Below pH 4, the nontoxic component increased in size to over 2,000 kDa. In contrast to the botulinum neurotoxin and the nontoxic component, the entire BoNTA-HA complex was most stable and monodisperse between pH 1 and 4 (Fig. 1C and D), correlating with the observation that the BoNTA-HA complex retains over 60% of its toxicity at low pH (33). Between pH 5 and 7, the complex reached its maximum size. Near neutral pH, the complex reached its maximum polydispersity. A similar high polydispersity was observed upon ultracentrifugation at pH 7.5 (37). Above pH 8, the complex appeared as a more polydisperse species, suggesting the presence of more than one species in solution probably due to disassociation of the complex into the toxic and nontoxic components. This idea of dissociation at basic pH is supported by the procedure for

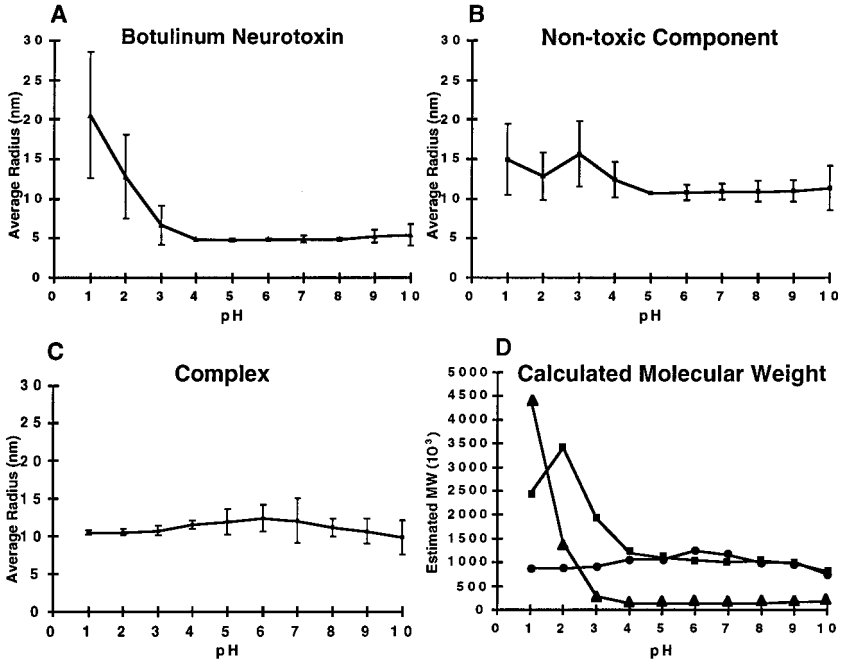

FIG. 1. Dynamic light scattering results, expressed as pH versus apparent radius and molecular weight of botulinum neurotoxin (A), nontoxic component (B), and 900-kDa complex (C) at various pHs. (D) Estimated molecular weights (MW) of botulinum neurotoxin (triangles), nontoxic component (squares), and 900-kDa complex (circles). The bar around each data point represents the average polydispersity value for three separate experiments. The polydispersity is the deviation of size distributions based on the mean radius measurement. Specimens containing aggregates or unfolded proteins have large polydispersity values.

purifying botulinum neurotoxin from the complex, since high pH is necessary for separation of toxic and nontoxic components (6, 7).

pH effects. The effect of pH on the neurotoxin's structural stability was examined by SDS-PAGE. A polyacrylamide gel of botulinum neurotoxin incubated at pH 2, 6, and 8 for 48 h indicated that the neurotoxin is susceptible to breakdown at pH 2 and 8 (Fig. 2). Table 1 shows that nonincubated neurotoxin runs as a 100-kDa heavy chain and a 50-kDa light chain. After 48 h of incubation at pH 6, we observed primarily the 100-kDa heavy chain and 50-kDa light chain of botulinum neurotoxin. A band at 150 kDa corresponded to a small quantity of unnicked neurotoxin. Although the 100- and 50-kDa bands predominated at pH 2 and 8, we observed a ladder of many lower-molecular-weight fragments. This fragmentation could be a result of acid-catalyzed hydrolysis, base-catalyzed hydrolysis, or proteolysis.

Proteolysis. Besides pH extremes, the other stresses that the 900-kDa complex must endure come from proteases. The effects of proteases on botulinum neurotoxin, nontoxic component, and 900-kDa complex were observed by performing protease incubations and assaying the results by gel electrophoresis (Table 1). Incubations consisted of pepsin at pH 2, intestinal cocktail (trypsin, chymotrypsin, carboxypeptidase) at pH 8, and pepsin at pH 2 followed by intestinal cocktail at pH 8. When incubated individually, both the neurotoxin and the nontoxic component were susceptible to all of the protease incubations (Table 1). In contrast, the 900-kDa complex demonstrated an amazing resistance to proteolysis. After incubation in pepsin at pH 2, the 900-kDa complex lost none of its bands. When the 900-kDa complex was incubated in intestinal cocktail, the 100- and 50-kDa bands of botulinum neurotoxin were proteolyzed along with the 120- and 106-kDa bands of the nontoxic component. However, incubating the 900-kDa complex in pepsin at pH 2 before exposing the complex to intestinal cocktail inhibited the proteolysis of the bands corresponding to botulinum neurotoxin. The bands which have been previously shown to be responsible for agglutination (14, 19, 21, 35, and 52 kDa) (14, 32) remained intact according to our gel whereas the band previously shown to be nontoxic-nonagglutinating (11) was destroyed.

Thus, under conditions that simulate the path of the 900-kDa complex through the gastrointestinal tract, the nontoxic component protected botulinum neurotoxin from proteolysis. This protection of the neurotoxin could be due to exposure to pepsin or to acidic pH. To determine if the protection of botulinum neurotoxin was a result of low pH, we performed an incubation without pepsin at pH 2, followed by an intestinal cocktail incubation. With these conditions, we observed that low pH was sufficient for protecting the complexed botulinum neurotoxin from proteolysis (data not shown). This protection of the botulinum neurotoxin in the 900-kDa complex was also seen upon incubations in rat gastric juice (33).

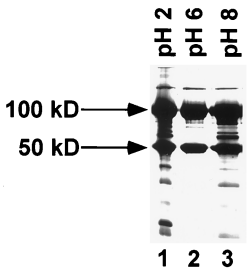

FIG. 2. SDS-PAGE of botulinum neurotoxin incubated at pH 2, 6, and 8. Arrows identify the heavy chain (100 kDa) and light chain (50 kDa) of botulinum neurotoxin. The band appearing at approximately 150 kDa is unnicked neurotoxin.

FIG. 3. Circular dichroism (CD) spectra of the 900-kDa complex at pH 8 (top), pH 6 (middle), and pH 2 (bottom) (A), the nontoxic component at pH 2 (top), pH 6 (middle), and pH 8 (bottom) (B), and botulinum neurotoxin at pH 8 (top), pH 6 (middle), and pH 2 (bottom) (C).

TABLE 1. Proteolysis and agglutination ability of BoNTA, nontoxic component (HA), and 900-kDa BoNTA-HA complex

Protein	Unproteolyzed		Pepsin (pH 2)		Intestinal cocktail (pH 8)		Sequential pepsin (pH 2)/ intestinal cocktail (pH 8)	
	No. of bands on 12% gel[a]	Agglutination[b]	No. of bands on 12% gel	Agglutination	No. of bands on 12% gel	Agglutination	No. of bands on 12% gel	Agglutination
BoNTA	2 (50, 100)	4.4	2 (30, 25)	—	4 (40, 45, 50, 100)	—	0	—
HA	7 (14, 19, 21, 35, 52, 106, 120)	100	7 (7, 14, 19, 21, 35, 52, 106, 120)	87.8	5 (14, 19, 21, 35, 52)	—	5 (14, 19, 21, 35, 52)	10.4
BoNTA-HA complex	9 (14, 19, 21, 35, 50, 52, 100, 106, 120)	1	9 (14, 19, 21, 35, 50, 52, 100, 106, 120)	9.4	5 (14, 19, 21, 35, 52)	—	7 (14, 19, 21, 35, 50, 52, 100)	37.9

[a] Numbers in parentheses indicate sizes (in kilodaltons) of bands appearing on 12% acrylamide gels under denaturing and reducing conditions.
[b] Percent transmittance by the protein relative to the percent transmittance by unproteolyzed HA, which was scaled to 100. Saline control contained 0.25% RBC incubated in saline for 2 h at 37°C. The saline control value was subtracted from the sample values before scaling to the unproteolyzed HA value. —, no activity.

Circular dichroism. Circular dichroic spectra of the botulinum neurotoxin, the nontoxic component, and the complex were taken at 37 and 25°C to determine the effects of pH on the structure and stability of the proteins. Temperature appeared not to have an effect on the spectra. At pH 2, 6, and 8, the 900-kDa complex shows little change in secondary structure (Fig. 3A). Thus, the increased protease resistance of the 900-kDa complex, upon exposure to pH 2, did not result from a visible conformational change in the secondary structure. When the nontoxic component was studied at pH 6 and 8, it likewise showed minor change in secondary structure (Fig. 3B). Upon exposure to pH 2, the nontoxic component showed a slight decrease in helicity. This loss of secondary structure is not large enough to indicate that the nontoxic component significantly unfolds. Since individual nontoxic components do not unfold, an increase in hydrodynamic radius, which corresponds to an increase in the size of the particle being observed, likely results from the aggregation of multiple nontoxic components. This aggregation explains the size increase of the nontoxic component observed by light scattering (Fig. 1B).

The greatest change in secondary structure occurred with the botulinum neurotoxin. Circular dichroism spectroscopy on botulinum toxin and its separated components at pH 8.1 have shown that approximately 70% of the amino acid residues exist in an ordered structure (30). Previous reports of circular dichroic data from pH 9 to 6 revealed a slight increase in alpha helicity (8). Our results for botulinum neurotoxin show an increase in helicity on moving from pH 8 to 6 (Fig. 3C). However, a larger increase in helicity occurred on moving from pH 6 to 2. This increase in helicity may be due to a conformational change in the translocation domain of the neurotoxin, which is known to form pores upon exposure to acidic pH (4). If the neurotoxin increases its helicity at pH 2, producing more secondary structure, then botulinum neurotoxin does not unfold in an acidic environment. Consequently, the huge increase in

hydrodynamic radius indicated by light scattering (Fig. 1A) is most likely caused by a conformational change in the molecular structure, followed by oligomerization or aggregation of the botulinum neurotoxin.

ELISA and surface plasmon resonance. The nontoxic component appears to prevent the vulnerable regions of the botulinum neurotoxin from protease attack, acid hydrolysis, and aggregation at low pH. Epitope mapping suggests that in the 900-kDa complex, the nontoxic component covers a large portion of the binding domain of botulinum neurotoxin (5). Previous experiments have shown that the binding domain is highly susceptible to trypsin cleavage (28). Therefore, the nontoxic component may play an important role in protecting the binding domain in the gastrointestinal tract. Furthermore, ELISA performed on botulinum neurotoxin after pepsin and intestinal proteolysis showed that antibodies no longer bind to it, suggesting that cleavage, denaturation, or both have occurred. In addition to being highly susceptible to proteolysis, botulinum neurotoxin appears to stick strongly and irreversibly to lipid monolayers such as POPC, a lipid commonly found in membranes of cells lining the gastrointestinal tract (9). Using surface plasmon resonance, we found that botulinum neurotoxin injected over POPC monolayers adsorbed strongly whereas the 900-kDa complex did not. Similarly, unless the toxic component was in its complexed form, it would probably adhere to lipid membranes along the gastrointestinal tract. Additional evidence for the association of botulinum neurotoxin with phospholipids has been seen by several researchers (17, 24, 25, 27). Thus, without the nontoxic component for protection, botulinum neurotoxin might never leave the gastrointestinal tract but instead be digested like most other proteins.

Sugar binding. The only known ability of the nontoxic component is its capacity to bind certain sugars (1). Upon binding these sugars, the nontoxic component is able to agglutinate, or clump together, cells (19). Whether this property aids the neurotoxin in reaching its target is under investigation. The clumping of RBC was monitored with agglutination assays (Table 1). Previous work has shown that the 14-, 19-, 21-, 35-, and 52-kDa bands are primarily responsible for agglutination (14, 32). Unproteolyzed nontoxic component containing these five bands was able to agglutinate RBC at 37°C (Table 1). After proteolysis by pepsin (pH 2), the nontoxic component experienced a slight decrease in agglutination ability (Table 1). Upon exposure to sequential protease incubations, the nontoxic component showed the largest decrease in the ability to agglutinate RBC (Table 1). This decrease appears to correlate with the loss of the 120- and 106-kDa bands (Table 1). The 106-kDa band was shown to be a proteolyzed portion of the 120-kDa band (14). Therefore, the 120-kDa band appears to be necessary for the nontoxic component to maintain optimal agglutination ability by protecting the other proteins but is not directly involved in agglutination. When unproteolyzed or exposed to pepsin, the complex showed a low ability to agglutinate (Table 1). Upon exposure to a sequential pepsin and intestinal cocktail incubation, the complex increased its ability to agglutinate RBC (Table 1). The sequentially proteolyzed complex contains the 14-, 19-, 21-, 35-, and 52-kDa bands plus the 100- and 50-kDa bands corresponding to botulinum neurotoxin (Table 1). Thus, the presence of botulinum neurotoxin appears to protect the ability of the complex to agglutinate after a sequential protease incubation.

These experiments indicate that in order for the nontoxic component to maintain optimal agglutination activity, it must be part of the 900-kDa complex while traveling through the gastrointestinal tract. After exposure to the conditions simulating the gastrointestinal tract, the 900-kDa complex appears to be modified so that the agglutination ability is activated. We propose that the 900-kDa complex may then interact with sugars on RBC and release the 150-kDa neurotoxin into the circulatory system. Although further experiments are necessary, experiments in which the 900-kDa complex was incubated with RBC that were subsequently pelleted by centrifugation and washed in saline solution (0.85% NaCl) showed that the neurotoxin was released and remained active in the supernatant (21). In addition, the neurotoxin has been shown to separate from the nontoxic component when the latter is bound to a sugar affinity column (23) and eluted with buffers at basic pH and ionic strength.

The 900-kDa complex form of botulinum neurotoxin is necessary for the delivery of botulinum neurotoxin in its most potent form. Using purified material and examining the results of the different toxin species at the molecular level, we have provided further evidence that the neurotoxin must exist in the 900-kDa complex to maintain its activity in conditions mimicking the environment of the gastrointestinal tract. In addition, a synergistic partnership between botulinum neurotoxin and the nontoxic component in which the nontoxic portion preserves the toxic ability of the toxic portion and the toxic portion protects the agglutinating ability of the nontoxic component seems to exist. In the 900-kDa complex, the neurotoxin and nontoxic component protect each other from pH extremes and gastrointestinal proteases. In contrast, when separated and exposed to simulated digestive conditions, each component is degraded. Although the molecular mechanism of the neurotoxin's journey to the neuromuscular junction is still unclear, these biophysical studies provide further evidence and detail as to the importance of the 900-kDa complex in the potent oral toxicity of botulinum neurotoxin.

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F. Chen and G. M. Kuziemko contributed equally to the work.

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