**In Vitro Reconstitution of the Clostridium botulinum Type D Progenitor Toxin**

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*Clostridium botulinum* type D strain 4947 produces two different sizes of progenitor toxins (M and L) as intact forms without proteolytic processing. The M toxin is composed of neurotoxin (NT) and nontoxic-nonhemagglutinin (NTNHA), whereas the L toxin is composed of the M toxin and hemagglutinin (HA) subcomponents (HA-70, HA-17, and HA-33). The HA-70 subcomponent and the HA-33/17 complex were isolated from the L toxin to near homogeneity by chromatography in the presence of denaturing agents. We were able to demonstrate, for the first time, *in vitro* reconstitution of the L toxin formed by mixing purified M toxin, HA-70, and HA-33/17. The properties of reconstituted and native L toxins are indistinguishable with respect to their gel filtration profiles, native-PAGE profiles, hemagglutination activity, binding activity to erythrocytes, and oral toxicity to mice. M toxin, which contained nicked NTNHA prepared by treatment with trypsin, could no longer be reconstituted to the L toxin with HA subcomponents, whereas the L toxin treated with proteases was not degraded into M toxin and HA subcomponents. We conclude that the M toxin forms first by assembly of NT with NTNHA and is subsequently converted to the L toxin by assembly with HA-70 and HA-33/17.

The NT molecule (~150 kDa) is ordinarily part of a complex formed by noncovalent association with other proteins, including a single nontoxic-nonhemagglutinin (NTNHA) subunit and/or a member of a family of hemagglutinin (HA) proteins (3, 4). The complex, designated as the progenitor toxin, is found in three forms with molecular masses of 900 kDa (LL toxin for type A), 500 kDa (L toxin for types A to D and G), and 300 kDa (M toxin for types A to F) depending on the serotype (5, 6). Previous experiments have demonstrated that the progenitor toxin complex protects the toxin during exposure to harsh conditions. Most proteins are degraded into short peptides and amino acids in the stomach and small intestine during the process of digestion. However, the progenitor toxin is exposed to the acidic (pH 2) gastric juice containing the protease pepsin in the stomach and then enters the small intestine, where it encounters several more proteases. Despite these denaturing and proteolytic conditions, NT and other nontoxic components can subsequently be detected in the blood and circulatory systems (7, 8). In studies involving oral toxicities (9), endopeptidase activity of the NT (10), and therapeutic efficiency in medical treatment (11), the progenitor toxin has been found to be more effective than the 150-kDa NT subunit alone. Actually, most reports on the progenitor toxin indicate that auxiliary proteins probably play a role in protecting the NT from harsh conditions. However, there has been no work describing the formation mechanism or molecular organization of the progenitor toxin or the reasons for the existence of different sized progenitor toxin species in the same supernatant.

*C. botulinum* type A, B, C, and D strains produce two species of progenitor toxins, the 500-kDa L toxin (complex of NT, NTNHA, and three HA subcomponents) and the 300-kDa M toxin (complex of NT and NTNHA), in culture supernatants (12, 13). The NTs of the L and M toxins are split into a 50-kDa light chain (Lc) and a 100-kDa heavy chain (Hc) by the excision of several amino acid residues (14), and the ha-70 gene product is also split into 55-kDa and 22- to 23-kDa fragments by proteolytic processing after translation (15). The NTNHA of the M toxin are always found nicked at their N termini leading to a 15-kDa N-terminal fragment and a 115-kDa C-terminal fragment on SDS-PAGE, whereas the NTNHA of the L toxin remain intact (12, 16). Thus, the components of the mature progenitor toxins are nicked, leading to the appearance of many fragments on SDS-PAGE. Fortunately, during examination of the molecular composition of the progenitor toxins produced by many type C and D strains, we serendipitously found that a unique type D strain, strain 4947 (n-4947), produces intact M and L toxins without any nicking in the components of the complex. Although it has been technically difficult to isolate each component (especially HA subcomponents) from the entire complex (17), we recently succeeded in the isolation of viable...
HA subcomponents from the type C progenitor toxin (18). Thus, this newly established method which applies also to the isolation of the intact components from progenitor toxin of the Δ4947, encouraged us to examine the mechanism for the formation of the progenitor toxin via reconstitution experiments, especially in light of the fact that the reason two species of progenitor toxin exist in the same culture medium has not yet been elucidated.

In this study, we succeeded in the in vitro reconstitution of the L toxin with almost identical properties to the native L toxin, via assembly of intact M toxin and HA subcomponents from the unique strain Δ4947 progenitor toxin. In addition, nicking of reconstituted progenitor toxin by treatment with exogenous proteases can reproduce the formation of mature progenitor toxins, which are usually observed in type D culture medium.

**EXPERIMENTAL PROCEDURES**

Production and Purification of Progenitor Toxin—C. botulinum Δ4947 cultivation and purification of progenitor toxins with SP-Toyopearl 650S ( Tosoh, Tokyo, Japan) and Hiload Superdex 200 pg 26/60 (Amersham Biosciences, Inc., Uppsala, Sweden) column were performed as previously described (18).

Isolation of the HA Subcomponents from L Toxin—The concentrated L toxin, a 250-mg pellet precipitated by centrifugation at 18,000 x g for 5 min, was dissolved in 0.7 ml of 20 mM Tris-HCl (pH 7.8) containing 4 mM guanidine hydrochloride (Gdn buffer) and incubated at 21 °C for 4 h. The treated sample was applied to a Hiload Superdex 200 pg 16/60 gel filtration column equilibrated with the Gdn buffer. After elution, fractions containing HA-70 or HA-33/17 were pooled separately and further purified by a repetition of gel filtration chromatography. The fractions corresponding to each HA subcomponent were diluted to 0.05 absorbance at 280 nm for HA-70 and 0.1 for HA-33/17 with Gdn buffer, and then dialyzed against 20 mM Tris-HCl (pH 7.8) at 4 °C for 15 h. Each sample was concentrated to 1.5 ml using VIVAPURE10 (Sartorius, Gottingen, Germany) and then kept in ice water until used.

PAGE Analysis—SDS-PAGE was performed as described by Laemmli (19) using a 13.6% polyacrylamide gel in the presence of 2-mercaptoethanol. PAGE under nondenaturing condition (native-PAGE) was carried out using the method of Davis et al. (20) at pH 8.8 using a 5–12.5% polyacrylamide linear gradient gel. The separated protein bands were detected with Coomassie Brilliant Blue R-250.

N-terminal Amino Acid Sequence Analysis—Separated progenitor toxin components were electroblotted onto a polyvinylidene difluoride membrane using a semi-dry blotting apparatus (Nippon Eido, Tokyo, Japan) (21). The N-terminal amino acid sequences of the subcomponents were determined using an automated sequence analyzer (Model 477A/120A, Applied Biosystems, Foster City, CA).

Preparation of M Toxin by Treatment with Trypsin—For the preparation of the nicked M toxin, 2.1 ml of M toxin (770 μg/ml) was mixed with 2 μg of trypsin and incubated at 37 °C for 1 h. After protease inhibitor (phenylmethylsulfonyl fluoride (PMSF)) was added to the mixture to a final concentration of 2 μM to stop the reaction, the mixture, diluted with 50 mM acetate buffer (pH 4.0), was applied to a MonoS HR5/5 column (Amersham Biosciences, Inc.). Fractions containing nicked M toxin were eluted with a linear gradient of NaCl ranging from 0.2 to 0.8 M.

Reconstitution of L Toxin Using Isolated HA and M Toxins—For L toxin reconstitution reactions, purified HA-70, HA-33/17, and intact or nicked M toxin were used at a protein ratio of 12:12:5, respectively. The combinations of HA-70 + HA-33/17, HA-33/17 + M toxin, and HA-70 + M toxin were mixed at protein ratios of 1:1, 1:1, and 3:1, respectively. The stoichiometry of each protein was determined roughly based on molecular sizes determined by gel filtration analysis and densitometric analysis of the Coomassie Brilliant Blue R-250 staining bands visualized by SDS-PAGE, as described previously (15). Reconstitution buffer was added to the mixture to make up final concentrations of 5 mM sodium phosphate, 20 mM KCl, 20 mM MgCl2, 6 mM 2-mercaptoethanol, and 0.15 μM PMSF. The final protein concentration of these mixtures was from 0.4 to 0.5 mg/ml and the final pH in the mixtures was from 5.8 to 6.0. After incubation at 27 °C for 21 h, the complexes were analyzed by gel filtration.

For large-scale purification of reconstituted L toxin to examine its properties, a mixture (6–7 ml) containing 0.5 mg of intact M toxin, 1.2 mg of HA-70, and 1.2 mg of HA-33/17 was incubated at 27 °C for 21 h. After incubation, the mixture was diluted 2.4-fold volume with 50 mM acetate buffer (pH 4.0) and then filtered with a 0.45-μm pore size filter unit to remove insoluble material. The sample was applied to a MonoS HR 5/5 column equilibrated with the same buffer, and the absorbed protein was eluted by a linear concentration gradient of NaCl from 0.2 to 0.8 M. The reconstituted L toxins were further purified by gel filtration chromatography, and the fraction corresponding to the reconstituted L toxin was concentrated to minimum volume by ultrafiltration using a YM-10 membrane (Amicon, Beverly, MA).

**RESULTS**

**Molecular Composition of the M and L Toxins**—The two different sized progenitor toxins, M and L, were separated as two distinct peaks by a cation exchange column, and each toxin was further purified by gel filtration. Purified L toxin showed a single peak in gel filtration chromatography (Fig. 1A, trace 1) and five bands of 150, 130, 70, 33, and 17 kDa on SDS-PAGE as shown in Fig. 1 (lane 1). The N-terminal amino acid sequences of the bands were identical to those of the deduced sequences from the Δ4947 progenitor toxin gene (GenBankTM accession number AB037920); the 150- and 130-kDa bands corresponded to the NT and the NTNHA, respectively, and the remaining 70-, 33-, and 17-kDa bands corresponded to the NTNHA, the second peak to NTNHA and HA-70, and the third peak to HA-70 complex digestion, 140 μg of each toxin and 14 μg of trypsin was incubated in 50 mM phosphate buffer (pH 6.0) at 37 °C for 3 h. For the M toxin-HA-70 complex digestion, 14 μg of trypsin was incubated at 37 °C for 30 min against 140 μg of the complex in the reaction mixture (490 μl). Incubations were stopped by the addition of 1 mM PMSF. For pepsin digestion, 140 μg of each toxin and 14 μg of pepsin was incubated in 50 mM sodium acetate-HCl (pH 2.7) buffer at 37 °C for 3 h. The digestion was terminated by the addition of 1 mM PMSF. The degree of proteolytic degradation of the L toxins and M toxin-HA-70 complex was then examined by gel filtration chromatography at pH 6.0 and SDS-PAGE.

**Isolation and Properties of HA Subcomponents from the L Toxin**—When the purified L toxin treated with Gdn buffer was applied to a gel filtration column equilibrated with the same buffer, three peaks were eluted; the first peak corresponded to NT and NTNHA, the second peak to NTNHA and HA-70, and the third peak to HA-33 and HA-17, as determined by SDS-PAGE. The second and third peak fractions were collected separately and further purified by a repetition of gel filtration column chromatography. As shown in Fig. 1 (A, trace 3 and B, lane 3), the HA-70 was purified as a single component, whereas...
the third peak was purified as the HA-33/17 complex, as determined by gel filtration elution profile and SDS-PAGE (Fig. 1A, trace 4 and 1B, lane 4). Separation of HA-33 and HA-17 from the complex by increasing the denaturant concentration failed because of irreversible precipitation during dialysis.

The molecular size of isolated HA-70 was estimated to be 74 kDa by analytical gel filtration chromatography at pH 6.0 (Table I), indicating that HA-70 might be present as a monomer. The molecular size of the HA-33/17 complex was also estimated to be 46 kDa, indicating a dimer complex with molecular ratio 1:1.

As shown in Table I, the hemagglutination activity of the isolated HA-70 was negative at 150 µg/ml, and the isolated HA-33/17 showed titers of 2^2, which were much lower than that of parent L toxin (titer of 2^10). However, the reconstituted HA-70/33/17 complex showed a titer of 2^10, which was higher than that of parent L toxin.

The binding activities of isolated HA-70 and HA-33/17 to horse erythrocytes were analyzed by immunoblotting. As shown in Fig. 2, both components bound to erythrocytes at levels comparable to the native L toxin, indicating that both HA-70 and HA-33/17 maintained binding activity.

Reconstitution of Progenitor Toxin from Various Combinations of Components—Mixtures containing M toxin, HA-70, and HA-33/17 were incubated under the conditions described and then subjected to gel filtration. As shown in Fig. 1A (trace 5), the peak that corresponded to the elution volume of the native L toxin (estimated at 670 kDa) showed an identical banding pattern on SDS-PAGE as that of the native L toxin (660 kDa) (Fig. 1B, lane 5). Other peaks contained components that could not assemble to the L toxin, as determined by SDS-PAGE.

To understand the assembly process of the L toxin, reconstitution tests were also attempted by mixing other combinations of the components. As shown in Fig. 1A (traces 6 and 7), new peaks corresponding to the sum of the sizes of combinations of components in the mixtures, M toxin plus HA-70, and HA-70 plus HA-33/17, appeared on gel filtration elution profiles. Because the peaks contained each component responsible for the reconstituted complex as determined by SDS-PAGE (Fig. 1B, lanes 6 and 7), it was concluded that new complexes had been assembled. On the other hand, when M toxin and HA-33/17 were mixed, no new peaks at larger molecular sizes than that of the M toxin appeared, indicating that these components did not assemble into complexes (Fig. 1A, trace 8). These results strongly suggest that HA-70 is required for the formation of the L toxin and formation of links between M toxin and HA-33/17.

Effect of the Nicked M Toxin on Reconstitution—When the M toxin was treated with trypsin (designated as nicked M toxin), the NT and NTNHA subcomponents were nicked. As shown in Fig. 3B (lane 2), the single-chain NT split into the 50-kDa Lc N-terminal and the 100-kDa Hc C-terminal fragments on SDS-PAGE, and the cleavage was found at one or two sites K^142/N^143 and R^445/D^446, based on the N-terminal amino acid sequence and our previous data (14). However, the single-chain NTNHA was cleaved at a unique site leading to the formation of the 15-kDa N-terminal small fragment and the 115-kDa C-terminal large fragment, on SDS-PAGE. The large fragment had mixtures of three different N-terminal amino acid sequences beginning with Leu^135, Val^139 and Thr^140 (Fig. 3B, lane 2), similar to those observed in type C and D mature M toxins (16). However, both intact and nicked M toxins had similar molecular sizes by elution volume on gel filtration, indicating that the two fragments of the NTNHA form a binary complex. The reconstitution test, mixing the nicked M toxin, HA-70, and HA-33/17, was also attempted. As shown in Fig. 3A (lanes 3–5), neither the combination of nicked M toxin plus HA-70, nor that of nicked M toxin plus HA-33/17 and HA-70, gave peaks corresponding to the molecular sizes of their native assembled complex by gel filtration. This suggests that the intact M toxin can form L toxin when associated with HA-70 and HA-33/17, but that the nicked M toxin cannot.

Properties of the Reconstituted L Toxin—Native-PAGE of both native and reconstituted L toxins at pH 8.8 also gave identical banding patterns in which the L toxin dissociated into NT and an NTNHA complex (data not shown). This implies that the net charges and molecular size of the reconstituted L toxin are very similar to those of the native L toxin.

As shown in Table I, the hemagglutination activity of the reconstituted L toxin had a titer of 2^2 to 2^6, which is similar to that of the native L toxin, and no significant differences were found between the oral toxicities of reconstituted and native L toxins.

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**Fig. 1.** Reconstitution of progenitor toxin from various combinations of components. A. Superdex 200 HR 10/30 gel filtration column chromatograms of the components and reconstitution of various combinations of the components. Isolated components and reconstituted complexes are indicated with a number. Dotted lines on the chromatograms show elution volume of the complexes and components. Molecular masses, estimated by the elution volume of the size marker proteins, are indicated at the bottom. Peak fractions indicated by arrows were subjected to SDS-PAGE analysis. B. SDS-PAGE banding patterns of the complexes and components. The numbers of the lanes correspond to the numbers indicated by the arrows on chromatograms illustrated in panel A. In lane M are the molecular size marker proteins. The N-terminal amino acid sequences of the components determined are shown.
As illustrated in Fig. 2, the reconstituted L toxin also showed the same intensity of binding activity to erythrocytes as that of the native L toxin, indicating that the reconstituted L toxin maintains binding ability.

### Effects of Protease Treatment on the L and M Toxins and M Toxin/H18528 HA-70 Complex

To examine the possibility that the M toxin can be formed by the proteolytic digestion of the L toxin, native and reconstituted L toxins were treated with trypsin and pepsin at 37 °C for a ratio of L toxin to proteases of 10:1. Elution profiles of the digestion mixtures were compared with those of intact M and L toxins by gel filtration, as shown in Fig. 4A. Both native and reconstituted L toxin yielded a single peak on gel filtration profile with no significant new peak appearance (Fig. 4A, traces 2–5). The single peak fractions corresponding to the L toxin were converted to a typical nicked L toxin form upon protease treatment and were composed of seven fragments, Hc and Lc derived from NT, HA-55, and HA-22–23 derived from HA-70, and un-nicked NTNHA, HA-33, and HA-17 (Fig. 4B, lanes 2–5). Accordingly, both L toxins showed resistance to proteolysis with trypsin and pepsin with no degradation to the M toxin and HA components, although the NT and HA-70 were nicked by these enzymes.

Because the NTNHA was still intact during digestion of the L toxin with trypsin, but nicked NTNHA was obtained from the trypsin digestion of the M toxin, the participation of HA-70 in nicking of the NTNHA was also examined. When the M toxin-HA-70 complex was treated with trypsin, no degradation of the complex to M toxin and HA-70 was observed (Fig. 4B, lane 7). As this lane shows, SDS-PAGE analysis of the complex clearly showed that the NTNHA was still in an intact form but that NT and HA-70 were nicked at their specific sites. These results strongly suggest that the proteolytically modified M toxin-HA-70 is a complex with five fragments bound tightly to each other, and the trypsin cleavage site of NTNHA is protected by the binding of HA-70 protein.

### DISCUSSION

C. botulinum progenitor toxin genes have been found to form gene clusters; cluster 1 contains the nt and ntnha genes, and cluster 2 contains three genes, ha-33, ha-17, and ha-70 (24), that produce both the hemagglutination-positive and -negative progenitor toxins (3, 12, 25, 26). According to the gene organization, it was expected that botulinum toxin would consist of...
five components. Most purified L toxin preparations demonstrated seven bands with sizes of 17, 22–23, 33, 50, 55, 100, and 130 kDa on SDS-PAGE (15) and most M toxins consisted of NT and nicked NTNHA with molecular masses of 115 and 15 kDa on SDS-PAGE (12, 16). The nicking is thought to be due to a trypsin-like protease in the culture medium, by the cleavage site specificity (12, 15). Similarly the same nicking was observed in type A 12S progenitor toxin (corresponding to our M toxin) (4, 27). These facts may simply explain why the different sized progenitor toxins exist in the same culture. However, the process of formation of the two types of progenitor toxins in the culture medium has not yet been elucidated (15).

We were the first to find that, unlike most type C and D strains, strain d-4947 produces both L and M toxins composed of intact components without any nicking. The d-4947 M and L toxins are stable complexes under acidic conditions lower than pH 6.8, similar to those of other nicked-type progenitor toxins. When M toxin is transferred into alkaline conditions, it easily separates into NT and NTNHA and then can reassemble under acidic conditions. Similarly, the L toxin splits into NT and an NTNHA-HA complex under alkaline condition and also reassembles under acidic conditions. However, an attempt to separate NTNHA from the complex in the presence of denaturants failed because of irreversible aggregation during dialysis to remove denaturants. In the present study, we obtained pure HA-70 and HA-33/17 complexes from d-4947 L toxin in the presence of denaturants using a method recently described by Kouguchi et al. (18, 28), although HA-33 and HA-17 could not be separated from the complex as viable forms. Because the NT and NTNHA derived from either M or L toxins were identical (12), we attempted an in vitro reconstitution experiment of the L toxin by combining the M toxin and the isolated HA subcomponents from the L toxin.

We were able to achieve the in vitro reconstitution of L toxin using a stoichiometric mixture of isolated HA subcomponents and M toxin. The reconstituted L toxin was indistinguishable from the parent L toxin with respect to gel filtration profiles, electrophoretic patterns on native-PAGE, hemagglutination activities, binding activity to horse erythrocytes, oral toxicity to mice, and tolerance for limited proteolysis with proteases. During reconstitution experiments with various combinations of the components, it was demonstrated that the HA-70 subcomponent bound to the M toxin but HA-33/17 complex did not. Because assembly of an HA-70 and HA-33/17 complex was also observed, it was expected that HA-70 would interact directly with the components of M toxin, probably NTNHA, supported by the observation that the NTNHA-HA complex derived from the L toxin under alkaline condition was still stable.

It is curious that the M toxin containing trypsin-nicked NTNHA could no longer be reconstituted into L toxin. This was further confirmed by the observation that no M toxin was formed from the proteolytic treatment of either native or reconstituted L toxins with trypsin or pepsin, implying that the
M toxin observed in the culture medium is not a degradation product of the L toxin. This fact clearly provides an answer to the question of why M and L toxins coexist in the culture medium and led to the conclusion that nicking in the NTNHA component defines an alternative formation of two type progenitor toxins. In fact, no nicking of NTNHA in the L toxin was observed through proteolysis, although nicking occurred at specific sites of the other components, NT and HA-70, as usually observed in mature progenitor toxins of other strains in culture medium. The role of nicking in NTNHA may explain the same phenomenon observed in three types of progenitor toxins produced by type A strains (4, 27). On the other hand, type E (29) and F (30) strains, in which no genes encoding HA components have been identified, produce only M toxin. Additionally, 33 amino acid residues were deleted from the specific region of both NTNHA molecules corresponding to the nicking site observed in those of type A, C, and D strains. Interestingly, the properties of the NTNHA may imply that types E and F, having no ability to assemble with HA components, have lost the ha genes, or that other strains having the ability to form L toxin have gained ha genes over evolutionary time.

It would be of interest to know the specifics of the interaction between the cleavage site of NTNHA and the HA-70 binding region. When reconstituted M toxin-HA-70 complex was exposed to proteases, the NTNHA remained intact without nicking; indicating that protection from protease nicking in NTNHA seems to be due to the binding of HA-70. HA-70 may interact specifically and cover the particular nicking site of NTNHA in such a way that it remains inaccessible to proteolytic attack. During limited proteolysis, HA-70 in the M toxin-HA-70 complex was cleaved at a specific site leading to formation of 22- to 23-kDa N-terminal and 55-kDa C-terminal fragments, as ordinarily found in L toxin types C and D. On the other hand, limited proteolysis of the single HA-70 subcomponent with a lower concentration of trypsin gave, instead of two fragments, a ladder of fragments (data not shown). This also implies that HA-70 was protected from random cleavage with one or more endogenous proteases in culture medium by direct binding to the NTNHA of M toxin. Thus it was expected that the HA-70 subcomponent plays a key role in the constitution of the L toxin in culture medium. According to our recent report on reconstitution of functional HA of the type C strain 6814 (18), HA-70 (especially the C-terminal region) was also primarily responsible for aggregation of erythrocytes in cooperation with HA-33 and HA-17.

At present, the nontoxic components of the botulinum progenitor toxin are considered to be critical to elicit food poison: the NTNHA protects the NT from acidic conditions and proteases in the stomach (6) and the HA component facilitates effective absorption of the progenitor toxin to the epithelial cells in the intestine (31, 32). Thus the botulinum progenitor toxin is a unique example of a protein complex where nontoxic components protect the NT against the gastrointestinal tract. However, in oral toxicity experiments, the d-4947 L toxin was only about 2.6-fold more toxic than the M toxin according to early work by Ohishi and Sakaguchi (33). Recently, Cai et al. (10) reported that the endopeptidase activity of the type A progenitor toxin was severalfold higher than that of the pure form of the NT and suggested that the enhanced activity was likely due to direct interactions between NT and NTNHA-HA complex. Interestingly, the botulinum NT in the progenitor form with NTNHA and HA components is used as a therapeutic agent and is a more effective drug than the pure NT (11). Accordingly, our work on the isolation and reconstitution of botulinum progenitor toxin may help explain the molecular basis of the increased therapeutic efficacy of the NT complex in medical treatment.

It is believed that the progenitor toxin forms by spontaneous and random association of expressed proteins in the culture medium. By summarizing the results obtained here, we have proposed a model of the pathway for formation of the two types of botulinum progenitor toxins from individual gene products, as shown in Fig. 5. The observation that both intact M and L toxin were found in d-4947 culture medium could be explained by the model that the M toxin, as an intermediate, forms first by association of NT and NTNHA and then the M toxin and HA-70 form the L toxin by the assembly of the remaining HA subcomponents. On the other hand, nicked forms of M and L toxins observed in other proteolytic strains might be explained by a proteolytic pathway, as shown in the lower panel of Fig. 5. This is supported by experimental evidence that nicking at specific sites in HA-70 arises after complete assembly of the L toxin, because a key HA-70 subcomponent was highly sensitive to one or more proteases, and nicking of NTNHA induced by one or more endoproteases resulted in M toxin as a dead-end product in the course of assembly. This model may help in understanding the subunit structure of the progenitor toxins, which is closely related to their biological functions (34), and may provide information for x-ray crystallographic analyses of the progenitor toxin, which will be undertaken in the near future, because crystallographic analyses of botulinum NT have recently been made available (35, 36).

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REFERENCES

1. Montecucco, C., and Schiavo, G. (1993) Trends Biochem. Sci. 18, 324–327
2. Li, W., and Singh, B. R. (1999) Toxicon 38, 95–112
3. Suzuki, S. Mutoh, K. Ono, D. Nakano, and M. Sakurai for technical assistance with d-4947 cultivation.

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29. Fujii, N., Kimura, K., Yokosawa, N., Yashiki, T., Tsuzuki, K., and Ouma, K. (1993) *J. Gen. Microbiol.* 139, 79–86
30. East, A. K., and Collins, M. D. (1994) *Curr. Microbiol.* 29, 69–77
31. Fujinaga, Y., Inoue, K., Watanabe, S., Yokota, K., Hirai, Y., Nagamachi, E., and Oguma, K. (1997) *Microbiology* 143, 3841–3847
32. Fujinaga, Y., Inoue, K., Nomura, T., Sasaki, J., Marvaud, J. C., Popoff, M. R., Kozaki, S., and Oguma, K. (2000) *FEBS Lett.* 467, 179–183
33. Ohishi, I., and Sakaguchi, G. (1980) *Infect. Immun.* 28, 303–309
34. Mei, G., Di Venere, A., Baganza, M., Vecchini, P., Rosato, N., and Finazzi-Agro, A. (1997) *Biochemistry* 36, 10917–10922
35. Lacy, D. B., Tepp, W., Cohen, A. C., DasGupta, B. R., and Stevens, R. C. (1998) *Nat. Struct. Biol.* 5, 898–902
36. Swaminathan, S., and Eswaramoorthy, S. (2000) *Nat. Struct. Biol.* 7, 693–699
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