The C Terminus of the Catalytic Domain of Type A Botulinum Neurotoxin May Facilitate Product Release from the Active Site*

Received for publication, January 24, 2013, and in revised form, June 11, 2013. Published, JBC Papers in Press, June 18, 2013, DOI 10.1074/jbc.M113.451286

Rahman M. Mizanur1, Verna Frasca2, Subramanyam Swaminathan3, Sina Bavari4, Robert Webb5, Leonard A. Smith6, and S. Ashraf Ahmed2

From the 4Integrated Toxicology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702, GE Healthcare Life Sciences, Northampton, Massachusetts 01060, Biology Department, Brookhaven National Laboratory, Upton, New York 11973, and Office of the Chief Scientist, Fort Detrick, Maryland 20854

Background: The function of C terminus of botulinum neurotoxin catalytic domain is unknown.
Results: Synthetic C-terminal peptides competitively inhibited but at stoichiometric concentrations stimulated serotype A proteolytic activity.
Conclusion: C terminus interacts with the active site and may function by removing a product.
Significance: The inhibition and product removal appear to be a unique feature of type A botulinum neurotoxin among catalytic proteins.

Botulinum neurotoxins are the most toxic of all compounds. The toxicity is related to a poor zinc endopeptidase activity located in a 50-kDa domain known as light chain (Lc) of the toxin. The C-terminal tail of Lc is not visible in any of the currently available x-ray structures, and it has no known function but undergoes autocatalytic truncations during purification and storage. By synthesizing C-terminal peptides of various lengths in this study, we have shown that these peptides competitively inhibit the normal catalytic activity of Lc of serotype A (LcA) and have defined the length of the mature LcA to consist of the first 444 residues. Two catalytically inactive mutants also inhibited LcA activity. Our results suggested that the C terminus of LcA might interact at or near its own active site. By using synthetic C-terminal peptides from LcB, LcC1, LcD, LcE, and LcF and their respective substrate peptides, we have shown that the inhibition of activity is specific only for LcA. Although a potent inhibitor with a \( K_I \) of 4.5 \( \mu \)M, the largest of our LcA C-terminal peptides stimulated LcA activity when added at near-stoichiometric concentration to three versions of LcA differing in their C-terminal lengths. The result suggested a product removal role of the LcA C terminus. This suggestion is supported by a weak but specific interaction determined by isothermal titration calorimetry between an LcA C-terminal peptide and N-terminal product from a peptide substrate of LcA. Our results also underscore the importance of using a mature LcA as an inhibitor screening target.

Functions of catalytic and regulatory proteins are largely dictated by their three-dimensional structures, which are dependent on their primary sequences. Recent years have witnessed a tremendous proliferation of three-dimensional structure determinations by the advent of high throughput x-ray crystallography soon after the sequence and adequate expression of a protein became available (1, 2). In some proteins, no electron density can be observed for stretches of the amino acid sequence especially at the N or C terminus (3–8). Thus, a functional role for such regions is not always discernible from their three-dimensional structures. The catalytic domain of botulinum neurotoxin (BoNT)3 belongs to this category of proteins. BoNT and tetanus neurotoxins are a unique class of zinc endopeptidases that act selectively at discrete sites on three synaptosomal proteins of the neuroexocytotic apparatus (for reviews, see Refs. 45 and 48). These neurotoxins are the most potent of all known toxins. Seven serotypes of BoNT, designated A–G, produced by immunologically distinct strains of Clostridium botulinum may cause death by flaccid muscle paralysis at the neuromuscular junction. These neurotoxins are expressed as 150-kDa single chain polypeptides. Post-translational proteolytic cleavage generates a dichain molecule consisting of a 100-kDa C-terminal heavy chain and a 50-kDa N-terminal light chain (Lc or Lc) of ~450 amino acids connected by a disulfide bond. The LC contains the zinc endopeptidase catalytic domain. The 100-kDa heavy chain can be further proteolyzed into a 50-kDa N-terminal membrane-spanning domain (Hn) and a 50-kDa C-terminal receptor-binding domain (Hc).

The first x-ray structure determined for the 150-kDa BoNT/A accounted for only the first 431 amino acids only of

* This work was supported in part by Defense Threat Reduction Agency-Joint Science and Technology Office for Chemical and Biological Defense Grant JSTOCD03.10012_06_RD_B (to S. A. A.).
1 Supported by the National Research Council through the research associateship program.
2 To whom correspondence should be addressed: Integrated Toxicology Division, United States Army Medical Research Inst. of Infectious Diseases, 1425 Porter St., Fort Detrick, MD 21702. Tel.: 301-619-2699; Fax: 301-619-2348; E-mail: syed.ahmed@amedd.army.mil.
3 The abbreviations used are: BoNT, botulinum neurotoxin; BoNT/A, botulinum neurotoxin serotype A; BoNT/B, botulinum neurotoxin serotype B; Lc or Lc, light chain; LcA, LC of serotype A; LcB, LC of serotype B; LcC1, LC of serotype C1; LcD, LC of serotype D; LcE, LC of serotype E; LcF, LC of serotype F; SNAP-25, synaptosome-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; ITC, isothermal titration calorimetry.
C Terminus of BoNT/A Light Chain

418–NFTGLFEFYKL–
LCVRIIITSKT–
KSLDKGYNK–448

FIGURE 1. Space-filling representation of LcA structure looking down the active site pit. Active site residues (Glu164, His223, Glu224, His227, Arg363, and Tyr366) in green are shown around the bound zinc atom (blue). For clarity, the C-terminal residues (red) 415–431 having electron density are shown in a ribbon representation. Only $\beta$-exosite residues 252–257 in the 250 loop (15), shown in yellow, are visible in this orientation. Not visible are $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha4$ in the $\alpha$-exosite and 370 loop in the $\alpha$-exosite residues (15) that are located on the other face of the molecule. Because the first BoNT/A structure (Protein Data Bank code 3BTA (9)) identified the maximum number of C-terminal residues in any reported LcA crystal structure, its coordinates for residues 415–431 are included in this figure along with the high resolution 1.5-Å substrate-bound structure of LcA showing residues 1–423 (Protein Data Bank code 3DDA (17)) that clearly identified the active site and catalytic and exosite residues. The figure was generated by Cα alignment of the first 1–423 residues (root mean square deviation of 1.3 Å except in the 200 and 250 loops) from both structures using the program VMD 1.9 beta 1 (47). Red letters in the C-terminal sequence shown are residues whose peptide bonds are sites of autocatalysis.

the N-terminal LC domain (9) in addition to residues of the heavy chain either due to no electron density of its highly mobile Lc C terminus or its proteolytic removal during purification. The structure was thus short by 17 residues from the full-length BoNT/A LC, by 10 residues from that of a proposed mature 444-residue BoNT/A LC (10), or by seven residues from the mature 438-residue BoNT/A LC (11) based on their isolation from culture filtrates of C. botulinum. After attempts to crystallize the full-length 448-residue light chain of serotype A (LcA) failed, investigators turned to C-terminally truncated LcA to determine its high resolution structures (12–15). Thus, we gained no knowledge on the structural importance or role of the C-terminal sequence on the function of the proteins. Moreover, although mutagenesis and x-ray crystallographic studies have unequivocally defined the S1–S5 sites of the catalytic domain (12, 15–17), none of residues involved in either substrate interaction or catalysis, all of which are located in a 20–24-Å-deep pit, were identified beyond Phe$^{423}$ of the 448-residue protein. In addition, the two exosites involved in the large substrate recognition (15), although located at the surface, are well removed from the C terminus of LcA. Fig. 1 shows some of these structural features.

Kinetic measurements with GST-fused SNAP-25 as well as a 13-residue FRET peptide substrate by Baldwin et al. (18) on several C-terminally truncated BoNT/A LCs demonstrated that residue 1–425-containing LcA was equally active as its full-length 448-residue counterpart. However, when the catalytic activity was measured on an intermediate-sized peptide substrate, a 1–425-residue LcA displayed only 25% of the activity, a 1–424-residue construct displayed 25% of the full-length LcA activity as well (12). Thus, it is important that this anomaly is more thoroughly investigated. The importance of determining the optimum length of a fully active LcA is more evident from the fact that some active site inhibitors showed nanomolar $k_i$ (19) when assayed with a short version of LcA but displayed micromolar $k_i$ (20, 21) when assayed in its full-length, 448-residue version. Additionally, active site peptide inhibitors bound the full-length LcA with higher affinity than its shorter, 1–425-residue versions (22). Such discrepant results have the potential to mislead in therapeutic development efforts against this deadliest toxin. These results also suggested that the C terminus of LcA might interact with other parts of the molecule. Thus, there is a clear need (a) to establish the length of an LC that will show optimum catalytic activity and (b) decipher the mechanism of interaction of this C terminus with other parts of the molecule that affects catalysis.

In this study, we have addressed these issues by using a series of peptides representing the C terminus of LcA to investigate their effects on the catalytic activity of the enzyme. Our strategy was based on the fact that a 30-residue C-terminal stretch of LcA underwent autocatalytic processing at least at five sites: Thr$^{420}$, Leu$^{429}$, Cys$^{430}$, Arg$^{432}$, and Lys$^{438}$ (Fig. 1) (23, 24), but the rate of the autocatalytic reaction was much slower than the rate of the catalytic reaction (25). Thus, a C-terminal sequence comprising these sites might act as a competitive inhibitor of LcA activity, and the extent of inhibition should correspond to its length for effective interactions with the LcA molecule. We deduced a minimum length of 444 residues for LcA that is needed for optimum activity. C-terminal sequences of LcB, LcC1, LcE, and LcF have an insignificant effect on their respective catalytic activities, suggesting uniqueness of the LcA C terminus. Our results of stimulating the LcA activity by near-stoichiometric addition of its C-terminal peptide are consistent with an important product removal role of the C terminus in the reaction mechanism of the enzymes.

EXPERIMENTAL PROCEDURES

Materials—Recombinant BoNT LCa and light chain of serotype B (LcB) were purified as described (25–27), and similar purification of that of serotype D (LcD) will be published elsewhere. Recombinant LcE (BBTech, Inc., Dartmouth, MA) and LcF (LIST Biological Laboratories, Campbell, CA) were commercial products. Truncated versions of LcA containing the first 420 (LcA420) (28) and 424 (LcA424) residues (12) and of LcC1 containing the first 430 residues (29) were purified as described. Sequence-derived substrates were as follows: from SNAP-25 for LcA and LcC1, SNKTRIDEANQATKML; for LcE, MGEIDTNQRQIDRIKEMADSNKTRIDEANQATKML (30); from VAMP for LcB, LSELDDRADALQAGASQFETSAAKLRKYYWKNLKD; and another VAMP

V. Roxas, personal communication.
sequence-derived substrate peptide for LcD and LcF, IQLQQTQAQ VDEVVDIMR VNKvLerDQKLSeLDD (32). All substrate peptides that were N-terminally acetylated and C-terminally amidated were custom synthesized and purified to >95% by Quality Controlled Biochemicals (Hampton, MS). The products of LcA reaction on the 17-mer, N-acetylated SNKTRIDEANQ (not C-amidated) and C-amidated RATKML (not N-acetylated), were also from Quality Controlled Biochemicals. All other peptides with free N and C termini listed in Table 1 and used elsewhere in this work were custom synthesized by Peptide2.0 Inc. (Chantilly, VA).

**Enzymatic Activity Assays**—Activity assays were based on UPLC™ (ultraperformance liquid chromatography) separation and measurement of the cleaved products from a 17-residue SNAP-25 peptide for LcA and LcCl, 35-residue VAMP peptide for LcB, and 34-residue VAMP peptide for LcD (32). A master reaction mixture lacking the Lc was prepared, and its aliquots were stored at -20 °C. Stocks of 0.05–0.07 mg/ml Lc, 0.041 mg/ml LcA424, 0.148 mg/ml LcCl, 0.00023 mg/ml LcD, 0.0011 mg/ml LcE, or 0.016 mg/ml LcF), 0.0016 mg/ml LcB, 0.148 mg/ml LcC1, 0.2 mg/ml BSA, 0.0026 mg/ml LcA (or 0.017 mg/ml LcA424, 0.041 mg/ml LcA424, 0.0016 mg/ml LcB, 0.148 mg/ml LcCl, 0.00023 mg/ml LcD, 0.0011 mg/ml LcE, or 0.016 mg/ml LcF), 0.25 mM ZnCl2, 5 mM dithiothreitol, and 50 mM Na-HEPES, pH 7.4. After 5 or 10 min (depending on the particular experiment) at 37 °C, reactions were stopped by adding 90 μl of 1% trifluoroacetic acid (TFA). The amounts of uncleaved substrate and the products were measured after separation by a Waters Acquity UPLC system equipped with Empower Pro software using a reverse-phase C18 column (2.1 × 50 mm, 1.7-μm particle size) with 0.1% TFA as solvent A and 70% acetonitrile, 0.1% TFA as solvent B at a flow rate of 0.5 ml/min (32). LcA and LcCl substrate and products were resolved by UPLC with a 0–42% gradient of solvent B over 2 min followed by column regeneration for 0.7 min. LcB and LcE substrate and products were resolved by UPLC with a 0–100% gradient of the solvents over 2 min then held at 100% B for 0.5 min followed by column regeneration for 0.5 min (32). LcD and LcF substrate and products were resolved by UPLC using the following solvent conditions: 10–25% B over 1 min, 25–55% B for 0.5 min, held at 55% B for 10 s and then at 100% B for 1.1 min followed by column regeneration for 0.7 min (32).

**UV-Visible Absorption, Circular Dichroism, and Fluorescence Measurements**—To determine protein concentration and to assess purity, UV-visible absorption spectra were recorded at 22 °C with a Hewlett-Packard 8452 diode array spectrophotometer. Lc concentration was determined using an A0.1% (1-cm light path) value of 1.0 at 278 nm (23) or by BCA assay (Pierce) with BSA as standard.

Circular dichroism spectra were recorded at 20 °C with a Jasco 718 spectropolarimeter with quartz cuvettes of 1-mm path length. An average of five scans was recorded to increase signal-to-noise ratio at a scan speed of 20 nm/min with a response time of 8 s. In all measurements, a buffer blank was recorded separately and subtracted from sample recordings. Tryptophan fluorescence emission spectra were recorded at 20 °C in a PTI QuantaMaster spectrofluorometer, Model RTC 2000 equipped with a Peltier controlled thermostat and Felix software package. Emission and excitation slit widths were set at 1 nm, and excitation wavelength was set at 295 nm. Each spectrum was an average of five scans.

**Mass Spectroscopy**—An ABI Sciex TOF/TOF 5800 instrument (ABS Sciex) was used to perform a matrix-assisted laser desorption ionization (MALDI)-TOF experiment for analysis of LcA C-terminal peptide. The peptide sample was spotted on a MALDI plate, dried, and then mixed 1:1 with the MALDI matrix, 4-hydroxycinnamic acid (10 mg/ml). Samples were analyzed in positive ion reflectron mode in the range of m/z 500–5000. The data were processed using the TOF/TOF Series Explorer software supplied by ABI Sciex.

**Isothermal Titration Calorimetry**—Isothermal titration calorimetry (ITC) experiments were performed on a Microcal ITC200 (Northampton, MA) instrument. The solutions of peptides were prepared in 50 mM HEPES, adjusted to pH 7.3, centrifuged to remove any residual debris, and warmed to 20 °C before use. Titrant solution containing acetyl-SNKTRIDEANQRATKMLamide, acetyl-SNKTRIDEANQ, or RATKML-amide (0.5 mM) was added from a 50-μl microsyringe at an interval of 150 s into a 1-nl microsyringe at an interval of 150 s into a 1-nl microsyringe at an interval of 150 s into a 1-nl microsyringe at an interval of 150 s into a 1-nl microsyringe at an interval of 150 s into a 1-nl microsyringe at an interval of 150 s into a 1-nl injection followed by 19 consecutive 2-μl injections at 20 °C. Data were analyzed by Origin 7.0 ITC analysis software using the standard, one-binding site model.

**RESULTS AND DISCUSSION**

**Effect of the C Terminus on LcA Catalytic Constants and LcA Stability**

In the past, we reported that an LcA construct, termed LcA424, that is devoid of the C-terminal 24 residues had only
at the active site, it undergoes nonspecific cleavage at a number of sites (Fig. 1) albeit very slowly compared with the intrinsic catalytic activity on the SNAP-25 substrate (23, 25). Integration of the C terminus could also destabilize the whole protease structure.

**Inhibition of LcA Activity by Its C-terminal Peptides Using 17-mer Substrate**

We had demonstrated previously that in addition to the main cleavage between Tyr²⁵¹ and Tyr²⁵², a 30-residue stretch of the C terminus of LcA also undergoes autocatalytic truncations at several sites (23). Because the rate of the autocatalytic reaction was much slower than its catalytic reaction on a 17-residue SNAP-25 substrate (25), we reasoned that peptides representing the C terminus of LcA might act as inhibitors of LcA. Therefore, we synthesized (Table 1) a 32-residue C-terminal peptide (LcA-1) comprising all the autocatalytic cleavage sites and looked for inhibition of LcA activity by this peptide. As expected, a concentration-dependent inhibition of LcA activity was observed such that more than 50% of LcA activity was lost in the presence of 10 μM LcA-1 peptide, and at 200 μM concentration, more than 95% of the activity was lost (data not shown). The assays were conducted with a 0.8 mM concentration of the SNAP-25 peptide substrate for 10 min. During this period, cleaved peptide products from only the substrate were produced by LcA. No cleaved product from the 32-mer peptide was detected either by MALDI-TOF or by UPLC. Thus, the C-terminal peptide behaved only as an inhibitor under our assay conditions and produced no detectable autocatalytic peptide fragments.

Because there were at least five autocatalytic cleavage sites including that at the 10th residue from the C terminus of LcA (23), it was expected that residues surrounding these cleavage sites would interact with the active site residues of the protein, which might interfere with normal catalysis. We therefore investigated the effects of successively removing five residues from the C terminus of the 32-mer C-terminal peptide on LcA activity (Fig. 3). The LcA-1 peptide (residues 417–448) abolished 85% of the LcA activity when the peptide (50 μM) was incubated with the 17-mer substrate (0.8 mM). Upon removal of

---

TABLE 2

| LcA form              | \(k_{cat}\) | \(K_m\) |
|-----------------------|------------|--------|
| Full-length LcA (1–448) | 3.6 ± 1.4 | 38.1 ± 12.1 |
| Truncated LcA424 (1–424) | 2.0 ± 0.4 | 4.2 ± 0.7 |
| Truncated LcA420 (1–420) | 3.5 ± 0.6 | 1.3 ± 0.2 |

**FIGURE 2.** Thermal unfolding of LcA448 (circles), LcA424 (rectangles), and LcA420 (triangles) in 50 mM sodium phosphate, pH 6.5 as monitored by measuring circular dichroism at 222 nm. The protein concentration in these experiments was 0.17–0.2 mg/ml. The midpoints of thermal transition, \(T_m\), are noted in the inset, mDeg, millidegrees.

---

25% catalytic activity of the full-length LcA (12), suggesting a role of the C terminus in enzyme catalysis. To investigate whether this role was due to an interaction with the substrate, we determined its steady-state kinetic constants using a 17-mer SNAP-25 substrate and compared them with those of the full-length LcA (Table 2). The results showed that there was a 9-fold reduction in the \(k_{cat}\) (4.2/s versus 38.1/s) compared with very little change in the \(K_m\) (3.6 versus 2.0 mM).

We also looked at the catalytic constants of a shorter version of the LcA having only the first 420 residues (LcA420) (15). This version was already known to have very low specific activity (32), and Table 2 shows that its \(k_{cat}\) is only 3% of that of the full-length LcA without considerable change in \(K_m\). From these results, two properties of LcA are evident. (a) Shortening the protein from the C terminus by at least 24 residues reduces the \(k_{cat}\) 9-fold, and further reduction in the length of the C terminus tremendously affects the activity. (b) Substrate \(K_m\) is not significantly affected, however, suggesting that the C terminus of LcA did not interact with the substrate to adversely affect its \(K_m\).

Although the active site pocket of LcA has been described as highly flexible (33), one of the reasons for very low catalytic activity of LcA420 may be the fact that it lacks Phe⁴²₃, and as a result, it may not form a substrate-binding pocket (13, 17). Phe⁴²₃ forms the 1) S3 pocket in the RRGC/LcA structure (17), 2) S4 pocket in the CRATML-LcA structure (13), and 3) S5 pocket in the RRATKM-LcA structure (17). However, \(K_m\) of the two shorter versions of the LcA used in this study was little affected compared with the effect on \(k_{cat}\) by the C-terminal truncations (Table 2). Thus, Phe⁴²₃ might play only an auxiliary role in forming the S3, S4, and S5 pockets (13, 17), or flexibility of the active site (33) could compensate for the missing Phe⁴²₃.

Fig. 2 shows the temperature-dependent change in the ellipticity at 222 nm, indicating \(\alpha\)-helical content, of the three forms of LcA. The midpoint of these thermal transitions (\(T_m\)) increased sequentially from 43 °C for the full-length LcA (LcA448) to 53 °C for the 24-residue-shorter LcA (LcA424) to 56 °C for the 28-residue-shorter LcA (LcA420). The results clearly show that although the C-terminally truncated LcA forms have poor catalytic activities they are more thermostable than their full-length counterpart. Such thermostability of the shorter versions provides experimental evidence that the C terminus of full-length LcA is highly mobile in nature. We hypothesize that this mobility allows the C-terminal tail to reach into and interact with its own active site. Once favorably juxtaposed at the active site, it undergoes nonspecific cleavage at a number of sites.
five residues from the C terminus (LcA-2), there was a very small (4 ± 3%) drop in the extent of inhibition, suggesting an insignificant role of the five C-terminal residues. Removal of 10 and 15 residues (LcA-3 and LcA-4) resulted in a much larger decrease in inhibitions of 18 and 35%, respectively, and removal of 20 residues (LcA-5) led to complete abolition of the inhibitory property. These results suggest that the residues in the sequence stretch of NTRKSLD443 appear to be the minimum length of the C terminus of LcA that shows substantial inhibition of LcA activity. Assuming that the length of the peptide exerting maximum inhibition corresponds to the minimum C-terminal length of LcA, our results establish that at least residues 1–444 will be needed to display maximum catalytic activity by LcA.

In determining the type of inhibition, we varied the substrate concentration to measure LcA activity at several fixed 32-mer peptide inhibitors (LcA-1) concentrations. Double reciprocal plots of activity versus substrate concentration yielded a series of straight lines that can be best described as intersecting on the y axis (Fig. 4), indicating a competitive inhibition. This result showed that the inhibitor binds at the active site of LcA, suggesting that when this peptide inhibitor is part of LcA it might have a propensity of reaching its own active site (23). The calculated \( k_i \) of 4.5 \( \mu M \) is almost 3 orders of magnitude smaller than the substrate \( K_m \) of 3.6 mM. Although much smaller tetrapeptide inhibitors of LcA with \( nM \) \( k_i \) values have been described (30), the significance of the 32-mer peptide as an inhibitor lies in the fact that it is a part of the LcA itself, not resembling the true substrate. Recently, three hydroxamate-containing small molecule inhibitors having 5–6 \( \mu M \) \( k_i \) values were found to induce the most compact catalytic pocket in LcA (34).

The results with synthetic C-terminal peptides described above suggest that the C terminus of one molecule of LcA should interact with its own active site (intramolecular) or with that of another molecule of LcA (intermolecular). One way to probe an intermolecular interaction is to investigate for any inhibition of catalytic activity of the full-length LcA by its catalytically inactive counterpart(s). We therefore included the inactive full-length mutant Y365N (16) in a standard enzymatic activity assay mixture (“Experimental Procedures”). As shown in Fig. 5, the mutant (3 \( \mu M \)) inhibited the LcA448 (9 nM) activity to 70% of the control. Similarly, another full-length but catalytically inactive mutant, R230L (3 \( \mu M \)) (16), inhibited the LcA448 (9 nM) activity to 72% of the control. Thus, although these results with both of these inactive mutants support an intermolecular interaction of LcA C terminus with the active site, they do not disprove any intramolecular interaction.
C Terminus of BoNT/A Light Chain

**FIGURE 5. Inhibition of full-length LcA (LcA448) activity by full-length inactive LcA mutants Y365N and R230L.** 8.6 nM wild type LcA was incubated in the absence and presence of 3.2 μM mutant LcA and 0.8 μM 17-mer substrate peptide at 37 °C for 60 min. During this period, wild type LcA alone converted 30% of the substrate into products. This value was treated as 100% as depicted in the y axis. Standard deviations for the average results of three determinations ranged from 0 to 3 in each case.

**FIGURE 6. Inhibition of LcA-catalyzed cleavage of SNAP-25 by the LcA-1 peptide.** SNAP-25 (40 μM) was incubated with 1 nM LcA448 and the indicated concentrations of LcA-1 peptide, 0.2 mg/ml BSA, 5 mM DTT, and 0.25 mM ZnCl₂ in 50 mM Na-HEPES, pH 7.4 at 37 °C for 10 min in a total volume of 30 μl. The products were analyzed by HPLC (32). Error bars represent S.D.

**TABLE 3.**

| Peptide         | Inhibition of LcA Activity (%) |
|-----------------|--------------------------------|
| LcA-1           | 100                            |
| LcB-1           | 90                             |
| LcC1-1          | 80                             |
| LcD-1           | 70                             |
| LcE-1           | 60                             |
| LcF-1           | 50                             |

**Specificity of Inhibition of LcA Activity by Its C-terminal Peptide**

The C-terminal peptide of LcA showing substantial inhibition is a large peptide of 32 residues. It is therefore likely to have nonspecific interaction with other proteases, especially with those of other BoNT serotypes. One way to probe the specificity is to include C-terminal sequences from other Lc serotypes in the LcA assay mixtures and vice versa. Another way is to include the peptide substrate of one serotype in the assay mixture of other serotypes. We therefore designed the C-terminal peptide sequences of five other serotypes based on multiple alignment of predominant subtypes of all seven serotypes, keeping the positions of the two essential, interchain disulfide-forming cysteines fixed (36). Thus, the serotype-dependent length of the C terminus varied from 32 residues for LcA to 21 residues for LcB and LcD (Table 1).

LcA-1 Peptide—As shown in Table 3, LcA activity was significantly reduced to 15% by the peptide from LcA. On the other hand, this peptide failed to inhibit the activities of LcB, LcC1, LcE, and LcF (Table 3). The situation with LcD activity was quite different in that its activity was stimulated more than 2-fold by the LcA peptide (addressed below). Thus, except for LcD, inhibition by the LcA peptide is highly specific for LcA.

LcE-1 Peptide—The LcE peptide did not have any significant effect on LcE activity but reduced LcA activity to 35% and LcB activity to a much lesser extent. The inhibitory effect of this peptide on LcA activity may have some significance from the fact that LcA and LcE share the same SNAP-25 substrate albeit at distinct sites. This peptide did not affect the activities of LcC1, LcE, and LcF (Table 3).

LcB-1, LcC1-1, LcD-1, and LcF-1 Peptides—These peptides did not have any adverse effect on their own activity or on the activities of the other serotypes (Table 3). Because these C-terminal peptides did not inhibit the catalytic activities of LcB, LcC1, LcD, LcE, and LcF on their respective VAMP- and SNAP-25-derived substrates, it can be reasonably argued that the C-terminal regions of these LcS do not play any role in modulating activities analogous to that observed with LcA. Thus, potential interaction of the C-terminal peptide with LcA may be unique among the six serotypes tested in this experiment. All of the above results show that (a) the LcA peptide is very specific in inhibiting its own enzymatic activity and that (b) although the LcE peptide did not affect its own activity, it did inhibit the LcA activity.

LcA, LcB, LcC1, LcD, LcE, and LcF Substrate Peptides—Specificity of the LcA peptide in inhibiting its own activity was further investigated by including the peptide substrates of other serotypes in the LcA activity assay mixtures. Compared with the 21–32-residue C-terminal peptides that represent sequences of the Lc proteins, the substrate peptides of 17–35-residue length will serve as control peptides. In addition, due to highly conserved overall and active site structures and commonality of their substrates, the substrate peptides may have auxiliary roles in either inhibiting or stimulating the activity of LcA. However, none of the three substrate peptides of other serotypes had a significant effect on LcA or other serotype activity (Table 3).

**Nonspecific Stimulation of LcD Activity by Peptides**

As noted above, the LcA C-terminal peptide stimulated the LcD activity more than 2-fold (Table 3). This was unexpected because in no other Lc serotype was such a stimulation of activity observed. Surprisingly, C-terminal peptide from all of the serotypes we tested also stimulated the LcD activity. Another interesting observation was that the LcD peptide itself was poorest in stimulating the LcD activity. Although the effect of the LcA peptide on LcD and LcA activities are opposite, it is also clear that stimulation of LcD activity by peptides is a nonspecific phenomenon. To support this nonspecific stimulation, all...
Therefore, we separately added a large excess of two peptide
peptide on LcA catalytic activity observed earlier (Table 3).
uct(s) might reverse the inhibitory effect of the LcA C-terminal
significant enough for a role of the LcA C terminus in product
favorable for optimum stimulation of activity. Detection of
they are an integral part of the whole LcA molecule) that are
C-terminal peptides in product removal. Most likely the free
that several unrelated peptides of comparable length (Table 3)
thermic or endothermic interaction between two molecules in
addition to providing an estimate of the binding energy and
interaction between the components is weak. Two practical
addition to the expectation of the light chains in the absence of the non-substrate peptides. The results are the average
five assays. When a peptide is a substrate for the Lc serotype, a
sign indicates no effect as the concentration of the substrate peptide was the same as in the control
plants, C-terminal peptides of six BoNT serotype Lcs on their catalytic activities
TABLE 3

| Peptide* | LcA | LcB | LcC | LcD | LcE | LcF |
|----------|-----|-----|-----|-----|-----|-----|
| None     | 100 ± 1 | 100 ± 2 | 100 ± 2 | 100 ± 1 | 100 ± 4 | 100 ± 2 |
| LcA C-terminal | 15 ± 1 | 98 ± 4 | 97 ± 0 | 213 ± 1 | 95 ± 0 | 109 ± 5 |
| LcB C-terminal | 92 ± 2 | 96 ± 1 | 93 ± 5 | 168 ± 2 | 91 ± 2 | 116 ± 11 |
| LcC C-terminal | 85 ± 1 | 95 ± 0.5 | 96 ± 1 | 172 ± 2 | 97 ± 2 | 98 ± 2 |
| LcD C-terminal | 92 ± 8 | 101 ± 4 | 99 ± 4 | 126 ± 1 | 97 ± 2 | 111 ± 3 |
| LcE C-terminal | 32 ± 3 | 82 ± 0 | 93 ± 0 | 220 ± 4 | 104 ± 1 | 110 ± 1 |
| LcF C-terminal | 106 ± 1 | 88 ± 1 | 104 ± 2 | 216 ± 2 | 93 ± 1 | 105 ± 1 |
| LcA/C substrate | – | 104 ± 6 | – | 143 ± 5 | 92 ± 1 | 107 ± 1 |
| LcB substrate | 99 ± 1 | – | 125 ± 2 | 178 ± 3 | 83 ± 2 | 113 ± 8 |
| LcD/F substrate | 110 ± 1 | 98 ± 1 | 150 ± 1 | – | 92 ± 4 | 114 ± 2 |
| LcE substrate | 108 ± 1 | 100 ± 1 | 102 ± 1 | 138 ± 4 | – | 120 ± 3 |

* Sequences of Lc C-terminal (LcA-1) and other C-terminal peptides are shown in Table 1, and those of the substrates are given under “Experimental Procedures.” A 50 μM concentration of each peptide was incubated with the light chains for 5 min at 25 °C. Enzyme reactions were initiated by addition of the respective substrates followed by incubation at 37 °C for 10 min. Percent activity is based on the control activity of the light chains in the absence of the non-substrate peptides. The results are the average

C Terminus of BoNT/A Light Chain

Stimulation of LcA Activity by the C-terminal Peptide of LcA

Because shortening the length of LcA from the C terminus reduced the $k_{cat}$ without affecting $K_m$ (Table 2), addition of a C-terminal peptide to the truncated LcA424 or LcA420 might stimulate activity by enhancing product release from the active site. Therefore, we tested the effects of various concentrations of the C-terminal peptide on the catalytic activities of all three forms of LcA. Contrary to the expectation and in accord with the results described above, Fig. 7, A–C, shows that the activities of all three LcA forms were inhibited by C-terminal peptides. However, a careful analysis of the concentration-dependent curves revealed that very low concentrations of the peptides indeed stimulated the activity (12–20%) of not only the two C-terminally truncated LcA forms but also the full-length LcA albeit to a lesser extent (7%) (Fig. 7D). Activity of the shortest LcA420 form was maximally stimulated more than 20% of the control activity. Ideally, the stimulation should have been about 9-fold for LcA424 and 30-fold for LcA420 (Table 2). However, (a) that this very low level of stimulation was consistently and repetitively observed with all the LcA forms and (b) that several unrelated peptides of comparable length (Table 3) did not show a similar stimulation may support a role of the LcA C-terminal peptides in product removal. Most likely the free peptides do not have the adequate structural features (when they are an integral part of the whole LcA molecule) that are favorable for optimum stimulation of activity. Detection of even a small stimulation of LcA420 and LcA424 activity may be significant enough for a role of the LcA C terminus in product removal.

Reversal of C-terminal Peptide-induced Inhibition of LcA Activity by a Product of LcA Enzymatic Reaction

If the C-terminal tail was indeed involved in the removal of one or both peptide products from the active site, the product(s) might reverse the inhibitory effect of the LcA C-terminal peptide on LcA catalytic activity observed earlier (Table 3). Therefore, we separately added a large excess of two peptide products to a preincubution mixture of LcA and its C-terminal peptide LcA-1. The two products of the LcA enzymatic reaction were the synthetic peptides acetyl-SNKTRIDEANQ and RATKML-amide that normally are generated from the synthetic substrate acetyl-SNKTRIDEANQRATKML-amide. The product peptides themselves did not have any significant effect on LcA activity (Table 4). Similarly, a preincubation of LcA with either of the product peptides followed by addition of the LcA C-terminal peptide failed to protect the LcA from loss of activity. On the other hand, the acetyl-SNKTRIDEANQ product peptide restored 25% of the lost activity of LcA that was preincubated with LcA-1 (Table 4). Our failure to restore 100% of the lost activity indicates that the affinity of the LcA C-terminal peptide for the product acetyl-SNKTRIDEANQ is much weaker than that with LcA. On the other hand, the RATKML-amide product peptide had no effect on the activity of LcA and its C-terminal peptide preincubation. Thus, the C-terminal tail of LcA might stimulate the LcA activity first by transiently binding with and then by releasing the N-terminal product of the 17-mer substrate (or SNAP-25) from the active site of LcA.

Isothermal Titration Calorimetry for the Binding of LcA C-terminal Peptide with a Product of the LcA Enzymatic Reaction

We tried to capture the interaction between LcA and several of its C-terminal peptide ligands by measuring differences in thermal denaturation, far-UV CD, and tryptophan and tyrosine fluorescence spectroscopy of LcA before and after adding the ligands. None of these methods detected any change in the synthetic substrate acetyl-SNKTRIDEANQRATKML-amide. This indicates that the affinity of the LcA C-terminal peptide for the product acetyl-SNKTRIDEANQ is much weaker than that with LcA. On the other hand, the RATKML-amide product peptide had no effect on the activity of LcA and its C-terminal peptide preincubation. Thus, the C-terminal tail of LcA might stimulate the LcA activity first by transiently binding with and then by releasing the N-terminal product of the 17-mer substrate (or SNAP-25) from the active site of LcA.
problems arose with this situation. First, LcA with a high molecular mass of 51 kDa yields only an ~20 μM solution at 1 mg/ml, a relatively high concentration that often leads to its own precipitation. Second, titration in ITC uses constant stirring that leads to LcA precipitation (38) and a syringe, the metallic part of which most likely induces autocatalytic degradation (23, 25, 39). Therefore, we chose its C-terminal peptide LcA-1 in place of the full-length LcA. All three ligands, namely the substrate (acetyl-SNKTRIDEANQRATKML-amide), N-terminal product (RATKML-amide), generated very little heat change in these titrations (Fig. 8), except for the C-terminal product (acetyl-SNKTRIDEANQRATKML-amide), which was 50 μM, a relatively high concentration that often leads to its own precipitation. Second, titration in ITC uses constant stirring that leads to LcA precipitation (38) and a syringe, the metallic part of which most likely induces autocatalytic degradation (23, 25, 39). Therefore, we chose its C-terminal peptide LcA-1 in place of the full-length LcA. All three ligands, namely the substrate (acetyl-SNKTRIDEANQRATKML-amide), N-terminal product (RATKML-amide), generated very little heat change in these titrations (Fig. 8A) such that the data points obtained with the substrate (acetyl-SNKTRIDEANQRATKML) and the C-terminal product (RATKML-amide) could not be reasonably fitted with a theoretical curve. The titration data generated with the N-terminal product, however, were nicely fitted for a one-site model to a sigmoidal curve (exoergic), yielding a dissociation constant (K_D) of 135 μM, enthalpy change (ΔH) of 0.6 kcal/mol, and stoichiometry of binding (N) of 0.87 (Fig. 8B). A high K_D and a low ΔH denote a weak interaction between LcA-1 and the N-terminal product acetyl-SNKTRIDEANQ. However, a binding stoichiometry close to 1 and the absence of similar interaction of LcA-1 with the other two ligands (Fig. 8A) suggest that the interaction between LcA-1 and the N-terminal product is specific. Such weak interaction can also be expected between a highly mobile segment of a protein, such as the C terminus of LcA, and its ligand, such as the N-terminal proteolytically cleaved product.

Results of the ITC experiments (Fig. 8) reinforce the enzyme activity results (Fig. 7, D and E, and Table 4) for a product release role of the LcA C-terminal segment. This interpretation also suggests that the product release step may be the rate-limiting step of LcA catalysis.

No rapid reaction kinetic data are available for the LcA reaction. Thus, we do not know whether the substrate binding step, the product formation (cleavage) step, or the product release step is rate-limiting. The LcA protease is among the poorest of the biological catalysts. Its catalytic efficiency, k_cat/K_m, of ~0.04/s/M determined from steady-state kinetics (16, 18) compares with >10^6/s/M for fumarase and 9/s/M for chymotrypsin (40). Bacterial tryptophan synthase with a very low steady-state k_cat (product release) of 0.4/s (41, 42) catalyzes the product formation step much faster at ~10^3/s (43). In this latter case, the rate-determining product release step is facilitated by an active site lysine residue.

### Table 4

Reversal of C-terminal peptide-induced inhibition of LcA activity by a product of LcA enzymatic reaction

| Order of peptide additions to LcA | Activity | Restoration of lost activity |
|----------------------------------|----------|-----------------------------|
| Control, no addition             | 100 ± 1  | %                           |
| LcA C-terminal peptide 1         | 15 ± 2   | %                           |
| N-terminal product               | 102 ± 2  | %                           |
| C-terminal product               | 93 ± 6   | %                           |
| LcA C-terminal peptide 1, N-terminal product | 35 ± 4 (39 ± 1) | 23 (28) | % |
| N-terminal product, LcA          | 15 ± 3   | 0                           |
| C-terminal peptide               | 17 ± 5   | 2                           |
| LcA C-terminal peptide 1         |          |                             |
| C-terminal product               |          |                             |

* The values in parentheses were obtained by adding 1 mM N-terminal product.
A quick look at the sequences of the products suggested that acetyl-SNKTRIDEANQ with two acidic residues and two acid amides might have a preferential interaction with the highly positively charged LcA C terminus (KNFTGLFEFYKLLCVRGIITSKTKSLDKGYNK). Lack of electron density of LcA residues 420–448 in the crystal structure of BoNT/A (9) and failure of full-length LcA to form crystals (12, 13, 33) suggest that the LcA C terminus is highly mobile. Our results of the thermal denaturation experiment described in Fig. 2 provide direct evidence of such mobility. During catalysis, conformational changes of this highly mobile LcA C terminus could allow the two ionic/charged interactions to be transiently formed and broken. This will make the C terminus function by releasing a product from the active site so that a new cycle of catalysis can take place. The C-terminal product RATKML-amide, which has two basic residues, would not be expected to have any interaction, and indeed our biochemical and biophysical (ITC) experiments did not support any interaction between them.

Gradual truncation of the LcA C terminus will decrease the number of basic residues, reducing the already weak interaction with the N-terminal product, which will affect product release. If product release indeed is the rate-limiting step of LcA catalysis, shortening its C terminus will diminish but not abolish its \( k_{cat} \) as demonstrated with the C-terminally truncated LcA described in this study and elsewhere (17, 18). In support of this possibility, LcA molecules from BoNT/A subtypes 2, 3, and 4 have an acidic aspartic acid residue in place of the basic lysine at position 444 of subtype 1, which has maximum catalytic activity (44).

**Insight from LcB Structure**

What structural evidence do we have in support of the C terminus reaching the active site of LcA? Because the full-length LcA448 cannot be crystallized, the only crystal structures available are those of LcA1-acetyl-SNKTRIDEANQR (15), LcA424 (12), and LcA425 (13), which lack most of the flexible C-terminal segment. We therefore performed some analysis of the full-length LcB structure, 437 residues of which could be accounted for in the electron density (Protein Data Bank code 2ETF).5 The core structures of LcA and LcB are superimposable as are all serotypes with very little C/H root mean square deviation. The presence of the loop between residues 415 and 425 makes the long helix (residues 425–437) very floppy so it can assume any orientation in LcB. In some orientations, the helical region comes near the active site of LcB. The C-terminal helix can closely interact with the 60, 200, and 370 loops to change their orientations. The location of these loops in relation to the C terminus of LcB is shown in Fig. 9. In LcA424, residues in the latter two loops make interactions with substrate peptides (17). Taking

---

5 S. Eswaramoorthy and S. Swaminathan, crystal structure of full length botulinum neurotoxin (type B) light chain, RCSB Protein Data Bank, deposited December 6, 2005, revised February 24, 2009.
the LcB structure as a model, our results for an affinity of the synthetic C-terminal peptide for LcA (Fig. 4) can be explained either by (a) rotation of the C-terminal floppy region to pack against the active site of the same molecule or (b) approach of the C-terminal region of one molecule to the active site of a second LcA molecule. By using two catalytically inactive mutants, R230L and Y365N, in this study (Fig. 5), we have provided direct evidence for an intermolecular reaction. However, our kinetic analyses of the autocatalysis (23) suggested the occurrence of both intermolecular and intramolecular reactions (25).

When this manuscript was about to be submitted for publication, a study addressing the discrepant behavior of three inhibitors toward 2 LcA C-terminal variants (46) came to our attention. By determining the $K_i$ of inhibitors and by molecular dynamics simulations, the authors concluded that the flexibility of the “C terminus of full-length BoNT/A protease places additional flexibility on the loops” (i.e. 250 loop) “surrounding the active site” that is “responsible for the potency shifts of active-site proximally binding inhibitors.” We think analysis of the flexibility of the LcB C terminus discussed in the preceding paragraph is a more realistic comparison for the LcA C terminus than the molecular dynamics simulations. Nonetheless, this study (46) lends support to our conclusion for an interaction of the C terminus with the active site.

Although high mobility of N- and C-terminal segments of proteins is not uncommon, only a handful of cases have been documented for their participation in the catalytic cycle of an enzyme. Human glutathione transferase (3) and Neisseria meningitidis heme oxygenase (4) are two such proteins whose C-terminal segments carry out the product release steps. Participation of the C-terminal region of LcA in a product release role is different from these and others in the literature by the fact that it is an endopeptidase. With many unique features of BoNT/A and its LcA, the product release role of its C terminus may add another unusual facet of LcA among peptidases.

In conclusion, we have provided biochemical and biophysical evidence for a product release role of the C terminus of LcA during its catalytic cycle. This role may be specific for LcA compared with other serotypes. We have also defined the length of LcA showing optimum catalytic activity to consist of the first 444 residues. By defining a function of the C terminus, our results also underscore the importance of using a mature LcA as an inhibitor screening target.

Acknowledgments—We thank Robert Stafford for technical assistance in conducting the enzymatic reactions and HPLC analyses, Dr. Michael Lee for molecular graphics, and Dr. Sarah L. Norris for statistical analyses.

REFERENCES

1. Sugahara, M., Asada, Y., Shimizu, K., Yamamoto, H., Lokanath, N. K., Mizutani, H., Bagautdinov, B., Matsuru, Y., Taketa, M., Kageyama, Y., Ono, N., Morikawa, Y., Tanaka, Y., Shimada, H., Nakamoto, T., Sugahara M., Yamamoto, M., and Kunishima, N. (2008) High-throughput crystallization-to-structure pipeline at RIKEN SPring-8 Center. J. Struct. Funct. Genomics 9, 21–28

2. Liu, Z. J., Tempel, W. N., Li, D. L., Lin, D., Shah, A. K., Chen, L., Horanyi, P. S., Habel, J. E., Kataeva, I. A., Xu, H., Yang, H., Chang, J. C., Huang, L., Chang, S. H., Zhou, W., Lee, D., Praissman, J. L., Zhang, H., Newton, M. G., Rose, J. P., Richardson, J. S., Richardson, D. C., and Wang, B. C. (2005) The high-throughput protein-to-structure pipeline at SECSG. Acta Crystallogr. D Biol. Crystallogr. 61, 679–684

3. Nilsson, L. O., Edalat, M., Pettersson, P. L., and Mannervik, B. (2002) Aromatic residues in the C-terminal region of glutathione transferase A1-1 influence rate-determining steps in the catalytic mechanism. Biochim. Biophys. Acta 1598, 199–205

4. Peng, D., Ma, L. H., Ogura, H., Yang, E. C., Zhang, X., Yoshida, T., and La Mar, G. N. (2010) $^3$H NMR study of the influence of mutation on the interaction of the C-terminus with the active site in heme oxygenase from Neisseria meningitidis: implications for product release. Biochemistry 49, 5832–5840

5. Angelucci, F., Sayed, A. A., Williams, D. L., Boumis, G., Brunori, M., Dimastrogiovanni, D., Miee, A. E., Pauly, F., and Bellelli, A. (2009) Inhibition of Schistosoma mansoni thioredoxin-glutathione reductase by auranofin: structural and kinetic aspects. J. Biol. Chem. 284, 28977–28985

6. Kucera, N. J., Hodson, M. E., and Wolin, S. L. (2011) An intrinsically disordered C terminus allows the La protein to assist the biogenesis of diverse noncoding RNA precursors. Proc. Natl. Acad. Sci. U.S.A. 108, 1308–1313

7. MacDonald, J. A., Ishida, H., Butler, E. L., Ulke-Lemée, A., Chappellaz, M., Tulk, S. E., Chik, J. K., and Vogel, H. J. (2012) Intrinsically disordered N-terminus of calponin homology-associated smooth muscle protein (CHASM) interacts with the calponin homology domain to enable tropomyosin binding. Biochemistry 51, 2694–2705

8. Bartels, T., Ahlstrom, L. S., Leftin, A., Kamp, F., Haass, C., Brown, M. F., and Beyer, K. (2010) The N-terminus of the intrinsically disordered protein α-synuclein triggers membrane bending and helix folding. Biophys. J. 99, 2116–2124

9. Lacy, D. B., Tepp, W., Cohen, A. C., DasGupta, B. R., and Stevens, R. C. (1998) Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nat. Struct. Biol. 5, 989–902

10. DasGupta, B. R., and Dekleva, M. L. (1990) Botulinum neurotoxin type A: structure of botulinum neurotoxin type A: sequence of amino acids at the N-terminus and around the nicking site. Biochimie 72, 661–666

11. Kriegstein, K. G., DasGupta, B. R., and Henschen, A. H. (1994) Covalent structure of botulinum neurotoxin type A: location of sulfhydryl groups, and disulfide bridges and identification of C-termini of light and heavy chains. J. Protein Chem. 13, 49–57

12. Kumaran, D., Rawat, R., Ludivico, M. L., Ahmed, S. A., and Swaminathan, S. (2008) Structure- and substrate-based inhibitor design for Clostridium botulinum neurotoxin serotype A. J. Biol. Chem. 283, 18883–18891

13. Silvaggi, N. R., Wilson, D., Tzipori, S., and Allen, K. N. (2008) Catalytic features of the botulinum neurotoxin A light chain revealed by high resolution structure of an inhibitory peptide complex. Biochemistry 47, 5736–5745

14. Mizanur, R. M., Gorbet, J., Swaminathan, S., and Ahmed, S. A. (2012) Inhibition of catalytic activities of botulinum neurotoxin light chains of serotypes A, B and E by acetate, sulfate and calcium. JMBB 3, 313–321

15. Breidenbach, M. A., and Brungcr, A. T. (2004) Substrate recognition strategy for botulinum neurotoxin serotype A. Nature 432, 925–929

16. Ahmed, S. A., Olson, M. A., Ludivico, M. L., Gilford, J., and Smith, L. A. (2008) Identification of residues surrounding the active site of type A botulinum neurotoxin important for substrate recognition and catalytic activity. Protein F 27, 151–162

17. Kumaran, D., Rawat, R., Ahmed, S. A., and Swaminathan, S. (2008) Substrate binding mode and its implication on drug design for botulinum neurotoxin A. PLoS Pathog. 4, e1000165

18. Baldwin, M. R., Bradvshaw, M., Johnson, E. A., and Barbieri, J. T. (2004) The C-terminus of botulinum neurotoxin type A light chain contributes to solubility, catalysis, and stability. Protein Expr. Purif. 37, 187–195

19. Eubanks, L. M., Hixon, M. S., Jin, W., Hong, S., Clancy, C. M., Tepp, W. H., Baldwin, M. R., Malizio, C. J., Goodnough, M. C., Barbieri, J. T., Johnson, E. A., Boger, D. L., Dickerson, T. J., and Janda, K. D. (2007) An in vitro and in vivo disconnect uncovered through high-throughput identification of
botulinum neurotoxin A antagonists. Proc. Natl. Acad. Sci. U.S.A. 104, 2602–2607.

20. Burnett, J. C., Ruthel, G., Stegmann, C. M., Panchal, R. G., Nguyen, T. L., Hermone, A. R., Stafford, R. G., Lane, D. J., Kenny, T. A., McGrath, C. F., Wipf, P., Stahl, A. M., Schmidt, J. J., Gussio, R., Brunger, A. T., and Bavari, S. (2007) Inhibition of metalloprotease botulinum serotype A from a pseudo-peptide binding mode to a small molecule that is active in primary neurons. J. Biol. Chem. 282, 5004–5014.

21. Burnett, J. C., Wang, C., Nuss, J. E., Nguyen, T. L., Hermone, A. R., Schmidt, J. J., Gussio, R., Wipf, P., and Bavari, S. (2009) Pharmacophore-guided lead optimization: the rational design of a non-zinc coordinating, sub-micromolar inhibitor of the botulinum neurotoxin serotype A metalloprotease. Bioorg. Med. Chem. Lett. 19, 5811–5813.

22. Molles, B. E., Collins, E. K., Levit, M. B., Sweeny, R. E., Zottola, M. A., Sunil-Datta, S., Tucker, W., Zeytin, F. N., Ruge, D., Oyler, G., Ahmed, S. A., Smith, L. A., Barbieri, J. T., Janda, K. D., O’Malley, S., and Adler, M. (2010) in Program and Abstract Book: 40th Inter-institute Botulinum Research Coordinating Committee Meeting, Interventions II Section, Dekatur, Atlanta, GA.

23. Ahmed, S. A., Byrne, M. P., Jensen, M., Hines, H. B., Brueggemann, E., and Smith, L. A. (2001) Enzymatic autocatalysis of botulinum A neurotoxin light chain. J. Protein Chem. 20, 221–231.

24. Dasgupta, B. R., Antharavally, B. S., Tepp, W., and Evenson, M. L. (2005) Botulinum neurotoxin types A, B, and E: fragmentations by autoproteolysis and other mechanisms including by O-phenanthroline-dithiothreitol, and association of the dinucleotides NAD+/NADH with the heavy chain of the three neurotoxins. Protein J. 24, 337–368.

25. Ahmed, S. A., McPhie, P., and Smith, L. A. (2003) Autocatalytically fragmented light chain of botulinum A neurotoxin is enzymatically active. Biochemistry 42, 12539–12549.

26. Jensen, M. J., Smith, T. J., Ahmed, S. A., and Smith, L. A. (2003) Expression, purification, and efficacy of the type A botulinum neurotoxin catalytic domain fused to two translocation domain variants. Toxicon 41, 691–701.

27. Gilksdorf, J., Gil, N., and Smith, L. A. (2008) Expression, purification, and characterization of Clostridium botulinum type B light chain. Protein Expr. Purif. 66, 256–267.

28. Segelke, B., Knapp, M., Kadkhodayan, S., Balhorn, R., and Rupp, B. (2004) Crystal structure of Clostridium botulinum neurotoxin protase in a product-bound state: evidence for noncanonical zinc protease activity. Proc. Natl. Acad. Sci. U.S.A. 101, 6888–6893.

29. Rawat, R., Ashraf Ahmed, S., and Swaminathan, S. (2008) High level expression of the light chain of botulinum neurotoxin serotype C1 and an efficient HPLC assay to monitor its proteolytic activity. Protein Expr. Purif. 60, 165–169.

30. Hale, M., Oyler, G., Swaminathan, S., and Ahmed, S. A. (2011) Basic tetrapeptides as potent intracellular inhibitors of type A botulinum neurotoxin protease activity. J. Biol. Chem. 286, 1802–1811.

31. Foran, P., Shone, C. C., and Dolly, J. O. (1994) Differences in the protease activities of tetanus and botulinum B toxins revealed by the cleavage of vesicle-associated membrane protein and various sized fragments. Biochemistry 33, 15365–15374.

32. Rowe, B., Schmidt, J. J., Smith, L. A., and Ahmed, S. A. (2010) Rapid product analysis and increased sensitivity for quantitative determinations of botulinum neurotoxin proteolytic activity. Anal. Biochem. 396, 188–193.

33. Silvaggi, N. R., Boldt, G. E., Hixon, M. S., Kennedy, J. P., Tai, S., Janda, K. D., and Allen, K. N. (2007) Structures of Clostridium botulinum neurotoxin serotype A light chain complexed with small-molecule inhibitors highlight active-site flexibility. Chem. Biol. 14, 533–542.

34. Thompson, A. A., Jiao, G. S., Kim, S., Thai, A., Cregar-Hernandez, L., Margosiak, S. A., Johnson, A. T., Van, G. W., O’Malley, S., and Stevens, R. C. (2011) Structural characterization of three novel hydroxamate-based zinc chelating inhibitors of the Clostridium botulinum serotype A neurotoxin light chain metalloprotease reveals a compact binding site resulting from 60/70 loop flexibility. Biochemistry 50, 4019–4028.

35. Chen, S., and Barbieri, J. T. (2006) Unique substrate recognition by botulinum neurotoxins serotypes A and E. J. Biol. Chem. 281, 10906–10911.

36. Lacy, D. B., and Stevens, R. C. (1999) Sequence homology and structural analysis of the clostridial neurotoxins. J. Mol. Biol. 291, 1091–1104.

37. Velazquez-Campoy, A., Leavitt, S. A., and Freire, E. (2004) Characterization of protein-protein interactions by isothermal titration calorimetry. Methods Mol. Biol. 261, 35–54.

38. Toth, S. I., Smith, L. A., and Ahmed, S. A. (2009) Extreme sensitivity of botulinum neurotoxin domains towards mild agitation. J. Pharm. Sci. 98, 3302–3311.

39. Ahmed, S. A., Ludivico, M. L., and Smith, L. A. (2004) Factors affecting autocatalysis of botulinum A neurotoxin light chain. Protein J. 23, 445–451.

40. Mathews, C. K., and van Holde, K. E. (1990) in Biochemistry, 1st Ed., pp. 339–377, The Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA.

41. Ahmed, S. A., Hyde, C. C., Thomas, G., and Miles, E. W. (1987) Micro-crystals of tryptophan synthase αβ2 complex from Salmonella typhimurium are catalytically active. Biochemistry 26, 5492–5498.

42. Ahmed, S. A., Martin, B., and Miles, E. W. (1986) β-Elimination of indole from L-tryptophan catalyzed by bacterial tryptophan synthase: a comparison between reactions catalyzed by tryptophanase and tryptophan synthase. Biochemistry 25, 4233–4240.

43. Weber-Ban, E., Hur, O., Bagwell, C., Banik, U., Yang, L. H., Miles, E. W., and Dunn, M. F. (2001) Investigation of allosteric linkages in the regulation of tryptophan synthase: the roles of salt bridges and monovalent cations probed by site-directed mutation, optical spectroscopy, and kinetics. Biochemistry 40, 3497–3511.

44. Henkel, J. S., Jacobson, M., Tepp, W., Pier, C., Johnson, E. A., and Barbieri, J. T. (2009) Catalytic properties of botulinum neurotoxin subtypes A3 and A4. Biochemistry 48, 2522–2528.

45. Montecucco, C., and Schiavo, G. (1995) Structure and function of tetanus and botulinum neurotoxins. Q. Rev. Biophys. 28, 423–472.

46. Silhár, P., Lardy, M. A., Hixon, M. S., Shoemaker, C. B., Barbieri, J. T., Struss, A. K., Lively, J. M., Javor, S., and Janda, K. D. (2013) The C-terminus of botulinum A protease has profound and unanticipated kinetic consequences upon the catalytic cleft. ACS Med. Chem. Lett. 4, 283–287.

47. Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: visual molecular dynamics. J. Mol. Graph. 14, 33–38.

48. Simpson, L. L. (2004) Identification of the major steps in botulinum toxin action. Annu. Rev. Pharmacol. Toxicol. 44, 167–193.