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Probing BoNT/A Protease Exosites: Implications for Inhibitor Design and Light Chain Longevity

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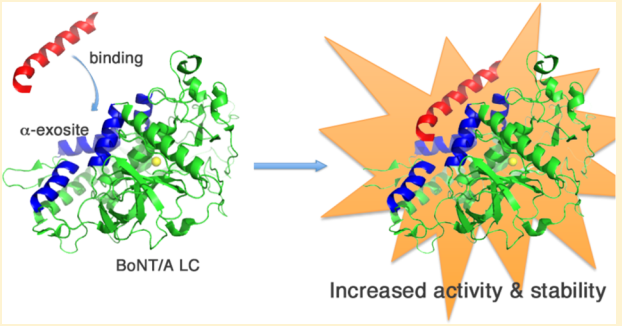
Supporting Information

ABSTRACT: Botulinum neurotoxin serotype A (BoNT/A) is one of the most lethal toxins known. Its extreme toxicity is due to its light chain (LC), a zinc protease that cleaves SNAP-25, a synaptosome-associated protein, leading to the inhibition of neuronal activity. Studies on BoNT/A LC have revealed that two regions, termed exosites, can play an important role in BoNT catalytic activity. A clear understanding of how these exosites influence neurotoxin catalytic activity would provide a critical framework for deciphering the mechanism of SNAP-25 cleavage and the design of inhibitors. Herein, based on the crystallographic structure of BoNT/A LC complexed with its substrate, we designed an α-exosite binding probe. Experiments with this unique probe demonstrated that α-exosite binding enhanced both catalytic activity and stability of the LC. These data help delineate why α-exosite binding is needed for SNAP-25 cleavage and also provide new insights into the extended lifetime observed for BoNT/A LC *in vivo*.



Botulinum neurotoxins (BoNTs) are a family of proteins produced by the bacterium *Clostridium botulinum* and are among the most lethal toxins known to man.[1] There are eight serotypes (A–H),[2] with the serotype A (BoNT/A) being the most potent with a lethal dose of less than 1 μg for a 70 kg person. Upon absorption, the toxin is internalized by endocytosis wherein the light chain (LC), a zinc metalloprotease, is released into the neuronal cytoplasm. BoNT/A LC cleaves SNAP-25, the key protein for membrane fusion, which is responsible for bringing the synaptic vesicle and plasma membrane together and controlling neuronal transmitter release.[3] The toxicity of BoNT/A is characterized by not only its potent lethal dose but also its long duration of action. Indeed, BoNT/A paralysis can last for months.[4] It has been nearly a century since BoNT was first purified in 1928;[5] however, there is still no satisfactory therapeutics available and the detailed mechanism of BoNT activity is still not entirely understood.

The SNARE protein, SNAP-25, as presented to the BoNT/A LC is a daunting substrate characterized by multiple binding regions and a very large binding surface that engages over 60 amino acids.[6] Foremost to our understanding of these protein–protein interactions between the BoNT/A LC and SNAP-25 has been the use of a series of truncated substrates.[7,8] These structural studies have revealed the importance of a helical motif approximately 30–50 amino acids away from the cleavage site of SNAP-25, which is interfaced with four light-chain α-helices (102–113, 310–321, 335–348, and 351–358) referred to as the α-exosite.[9] Of additional significance, a β-sheet region close to the active site was also found to interact with SNAP-25 and has been referred to as the β-exosite.[6,9] The dissection of SNAP-25 binding interactions and catalytic competence have marshalled a 66-amino acid (141–206) fragment to prominence. Indeed, it has been shown that this substrate interacts with all three critical regions, two exosites and the active site. We highlight that the use of the 66-mer fragment is a testimony to the importance of the α-exosite, because when the α-exosite-binding residues were omitted poor substrate turnover was observed. Similarly, mutations in the β-exosite caused reduction in catalytic activity (k_{cat}) of 40–80-fold.[6] Thus, exosites play an essential role in BoNT/A LC–SNAP-25 interactions.

In seeking to develop therapeutics for the reversal of BoNT/A intoxication, the design of inhibitors against the protease has become a centerpiece of research efforts.[10] Indeed, several nonpeptide active site ligands,[11,12] as well as compounds binding to LC exosites, such as caftaric acid, chicoric acid, and lomofungin,[13,14] have been shown to possess excellent protease inhibitory activity. The active site of BoNT/A LC is governed

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by zinc and while metalloprotease inhibitors are plentiful, their liabilities are well-documented.[15,16] Hence, exosite targeting is warranted, including clarifying the role of these exosites, which could provide a linchpin to further our understanding on the mechanism of BoNT/LC toxicity and thus the design of new inhibitors.

To explore how α-exosite binding influences the activity of BoNT/A LC, a probe was designed based on the published crystal structure of BoNT/A LC complexed with SNAP-25 (Figure 1, PDB 1XTG).[6] The probe consisted of the helical

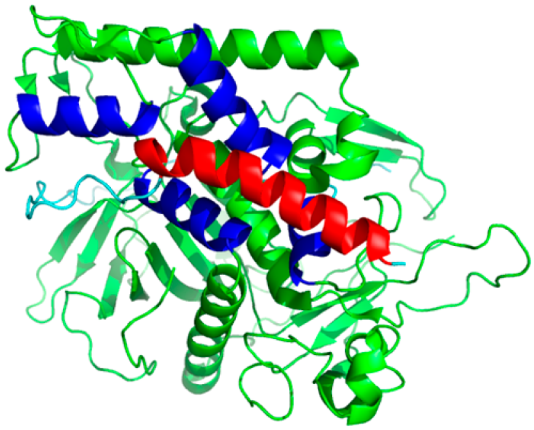

Figure 1. Structure of BoNT/A-LC (424 a.a. resolved, green) and SNAP-25 fragment (64 a.a. C-terminal, 59 a.a. resolved). BAP-24 region is shown in red, while the remaining SNAP-25 residues are shown in cyan and the α-exosite in blue (PDB 1XTG).

portion of SNAP-25 that binds to the α-exosite, spanning 24 amino acids, which we term BAP-24 (BoNT/A LC α-exosite probe 24). Additionally, within BAP-24, we highlight that the C-terminal alanine was replaced by aminoisobutyric acid, a well studied effective inducer of α-helical structure,[17] which assists BAP-24 to adopt a helical structure (Figure 2). As anticipated, BAP-24 on it own is not a substrate for the BoNT/A LC because it does not contain the sequence recognized by the active site. Accordingly no cleavage product was detected in any of our experiments.

MATERIALS AND METHODS

Synthesis of BAP-24. BAP-24 was synthesized on PL-AMS resin with Rink Amide linker by means of the standard Fmoc strategy and *N,N′*-diisopropylcarbodiimide/hydroxybenzotriazole (DIC/HOBT) protocol. The N-terminus was acetylated with 50% acetic anhydride in dichloromethane (DCM) for 15 min. The peptide itself and all protecting groups were cleaved from the resin with trifluoroacetic acid (TFA) containing ethanedithiol (2.5%), triisopropylsilane (1%), and water (2.5%) for 3 h. Upon deprotection, the peptide was precipitated using diethyl ether and centrifuged at 700 rpm for 15 min. This crude peptide was dissolved in TFA with 20 equiv of ammonium iodide and 20 equiv of dimethylsulfide to reduce the partially oxidized product. The final product (BAP-24) was purified by HPLC (see Supporting Information) and its mass was confirmed by LC-MS.

The 66-mer and truncated peptides including 50-, 45-, and 40-mers were synthesized on an automatic peptide synthesizer (C S Bio. Co., USA). The approach employed to make each of these peptides was predicated upon the principles of peptide synthesis using Boc-protecting strategy, and the final materials were cleaved with HF, as described previously.[18]

α-Exosite Binding Assays. The 66-mer (5.0 μM) was mixed with 0, 5.0, 25, 50, 75, 100, or 125 μM BAP-24; 0.2 nM BoNT/A LC was added, and the mixture was incubated for 20 min. The reaction was terminated with 20% TFA, and then 1 μM C-13 labeled cleavage product was added as internal standard. Samples were analyzed using LC-MS.[19]

To confirm α-exosite binding, a series of truncated C-terminal 66-mers (40-mer, 45-mer, or 50-mer) were used in tandem with BAP-24 in the assay described, *vide supra*. For the 40-mer, typical assay conditions did not allow reliable quantification; in that case, concentrations of 50 μM 40-mer and 10 nM BoNT/A LC were used in the assay.

Effect of α-Exosite Binding on BoNT/A LC Activity. SNAPtide (5.0 μM) was mixed with 100 μM BAP-24, then 10 nM BoNT/A LC was added, and fluorescence at 523 nm was measured continuously for 4 h with the excitation wavelength of 490 nm. Collected data were fitted with a *one-phase association* equation in Prism 6.0 with $R^2 > 0.99$. The first derivative of the fitted curve was plotted as the rate of cleavage.

BAP-24 as a Probe for α-Exosite Binding Determination. The 66-mer cleavage assay was run in the presence of a series of concentrations of BAP-24 combined with chicoric acid or lomofungin; $1/v_0$ versus chicoric acid or lomofungin concentration was plotted with linear fitting. Sets of parallel curves indicate mutually exclusive inhibition, while intersecting curves indicate nonmutually exclusive inhibition.

RESULTS AND DISCUSSION

Kinetic Studies Delineating BAP-24 and Its α-Exosite Interactions. The truncated C-terminal 66-mer fragment of SNAP-25 is an excellent substrate for the BoNT/A LC, making it a widely used substrate for *in vitro* investigations.[19] Although, BAP-24 is not a substrate for the BoNT/A LC, the binding of BAP-24 to the light chain was evidenced by a competition experiment between BAP-24 and the 66-mer substrate. As shown in Figure 3a, a decrease in the 66-mer cleavage rate was observed as a function of BAP-24 concentration. A similar but less pronounced effect was also observed with a truncated subset of the 66-mer, 45- and 50-mer substrates, which bear a

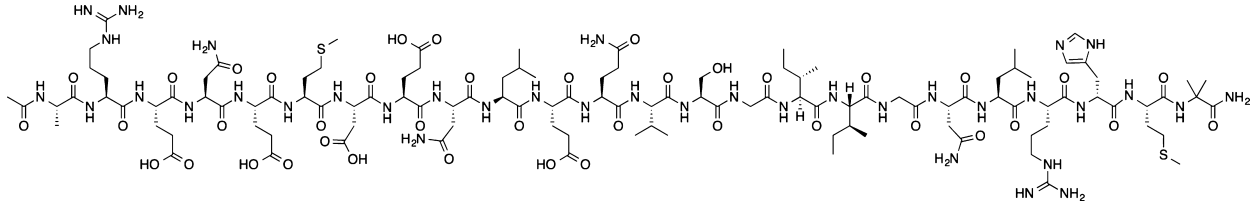

Figure 2. Sequence of BAP-24. It is important to note that the C-terminal alanine is replaced by α-aminoisobutyric acid (Aib) for α-helix induction.

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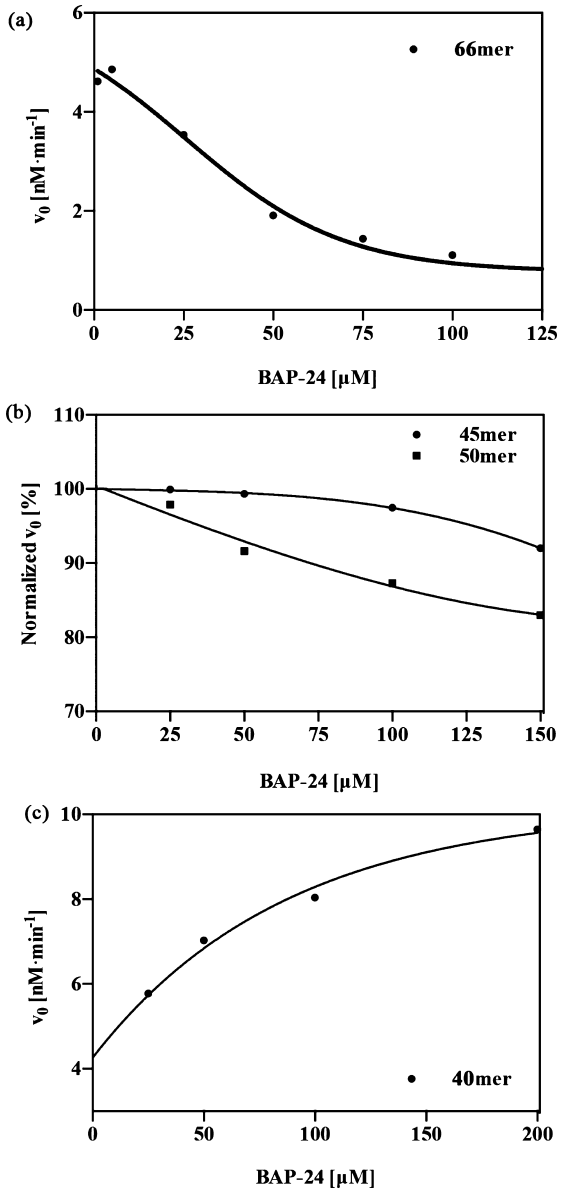

Figure 3. α-Exosite kinetic analysis. (a) The 66-mer cleavage rate examined with 0, 25, 50, 75, or 125 μM BAP-24. (b) The 45-mer and 50-mer cleavage rate tested with 0, 25, 50, 100, or 200 μM BAP-24. The results were normalized by setting the rates without BAP-24 at 100%. (c) The 40-mer cleavage rate examined with 0, 25, 50, 100, or 200 μM BAP-24.

smaller overlap (3 and 8 residues, respectively) with the sequence of BAP-24 (Figure 3b).

To confirm that BAP-24 is a unique exosite probe, we examined a 40-mer substrate that contains the BoNT/A cleavage site but does not overlap with the sequence of BAP-24, which makes the two sequences complementary rather than competing. In this case, a synergistic effect and an increase in cleavage of the 40-mer was discovered to be dependent on the concentration of BAP-24 (Figure 3c). Taken together, these results strongly suggested that BAP-24 binds at the α-exosite *in situ*.

Mechanistic Studies of α-Exosite Binding and Consequences of the Catalytic Activity. BAP-24 can both promote and inhibit BoNT/A LC catalysis. However, the exact mechanism of this increase in catalytic activity was unclear. The 40-mer was a relatively poor substrate even in the presence of BAP-24; thus, to further investigate the effect of this α-exosite binding and catalysis, we examined another short but highly active substrate, SNAPtide.[18] SNAPtide is a commercial substrate of the BoNT/A LC, a short 13-residue peptidomimetic comprising FITC and DABCYL fluorophores as the FRET system. BAP-24 should have no direct contact with SNAPtide because this FRET peptide resides exclusively within catalytic core of the protease; hence, results here would shine additional light upon BAP-24 exosite interactions and how these influence BoNT activity. The BoNT/A LC activity was measured by fluorescence for 4 h in the presence and in the absence of BAP-24 (Figure 4a). In the presence of BAP-24, the

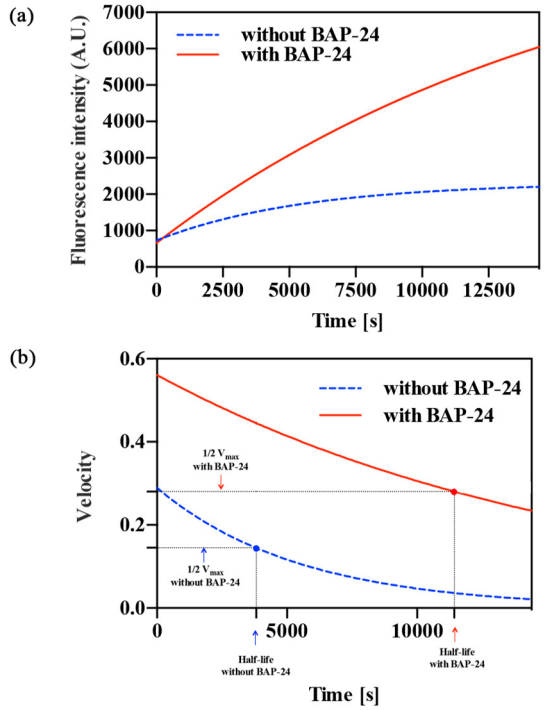

Figure 4. SNAPtide assay as conducted in the presence or absence of 100 μM BAP-24. (a) Fluorescence intensity over time was fit to a one-phase association equation. (b) The first derivative of the curves in panel a were calculated to yield the velocity of SNAPtide cleavage. The half-lives observed are based upon the velocity.

fluorescence intensity increased quickly, confirming its ability to accelerate substrate cleavage. To better define this increase in catalysis, the generated intensity curves were fitted with a one-phase association model and the first derivatives were calculated as a function of cleavage velocity (Figure 4b). In presence of BAP-24, the initial cleavage rate increased about 2 fold indicating an activation of the catalytic machinery.

A known phenomenon with the BoNT/A protease *in vitro* is the loss of catalytic activity over time.[20] Remarkably, while catalysis decreased, it did so more slowly in the presence of BAP-24 than without, indicating that BoNT/A LC was stabilized by the BAP-24 (Figure 4b). Impressively, the half-life of the BoNT/A LC was increased from 1 h to more than 3 h. Finally, the effect of BAP-24 activation and stabilization was also found to be sequence specific. Thus, a scrambled BAP-24 peptide containing the same set of amino acids but now randomized was found to be inactive in this SNAPtide assay (see Supporting Information). These results suggest that α-

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exosite binding can both enhance catalysis and stabilize BoNT/A protease.

Use of BAP-24 as a Probe for α-Exosite Kinetic Analysis. BAP-24 was confirmed to bind to the α-exosite, yet we wanted to further examine its value through kinetic testing in the form of mutually or nonmutually exclusive binding between BAP-24 and nonpeptidic inhibitors. Here we examined two inhibitors of BoNT/A LC, chicoric acid and lomofungin, which are known to bind α-exosite and β-exosite, respectively.[13,14] Curve fitting to a combination of BAP-24 and chicoric acid demonstrated mutually exclusive binding as evidenced from the observed parallel lines (Figure 5a and Scheme 1). However, BAP-24 and lomofungin in tandem gave a pattern of intersecting lines demonstrating nonmutually exclusive binding (Figure 5b and Scheme 2).[13] These data provide further worth of our BAP-24 probe because it can be used to validate the binding of small molecules to the α- or β-exosite of BoNT/A protease.

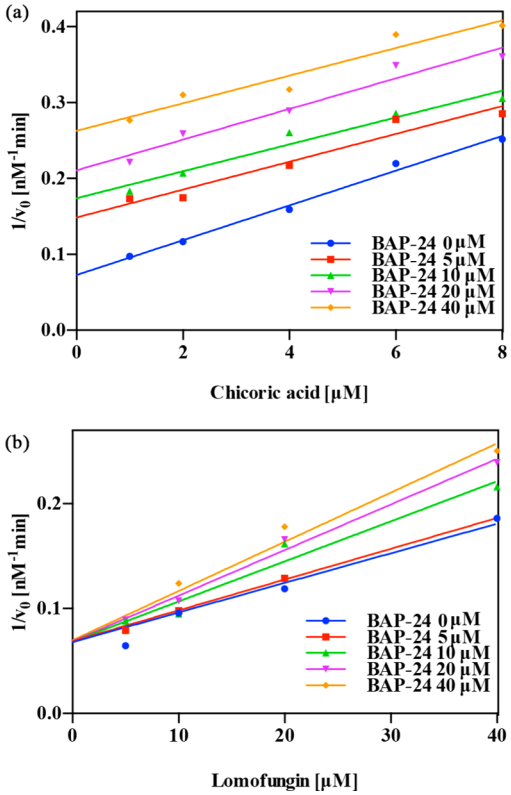


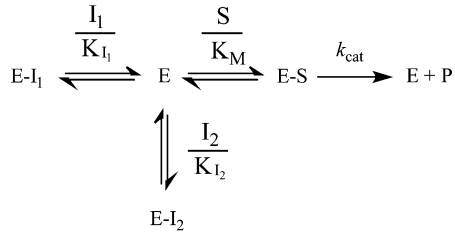
Figure 5. Inhibition experiments wherein BAP-24 was examined in the presence of chicoric acid or lomofungin to determine binding exclusivity. $1/v_0$ was plotted using a series of concentrations of chicoric acid or lomofungin in conjunction with gradient concentrations of BAP-24. BAP-24 displayed mutually exclusive binding with chicoric acid as shown in panel (a) and nonmutually exclusive binding with lomofungin as viewed in panel (b).

CONCLUSION

Based on the cocrystal structure of BoNT/A LC complexed with SNAP-25, we designed a peptide probe for α-exosite binding. With this probe, new data was acquired further defining the α-exosite's role in both catalysis and protein stability. In sum, α-exosite binding can be used to both increase LC catalytic activity and greatly extend the half-life of the protease. Engaging the α-exosite, we surmise, causes the protease tertiary structure to undergo a subtle conformational change resulting in a reorganization of the active site with the net result of increased catalysis. In addition, this allosteric binding may also "anchor" the enzyme in a state preventing destabilization or degradation. This finding may shed additional light on the protease's cryptic extended lifetime *in vivo*. Thus, we posit that if upon SNAP-25 cleavage the truncated SNARE complex remains intimately associated with the BoNT/A LC then its degradation would be retarded.

Finally, using the BAP-24 probe has also allowed further insights into the mechanism of α-exosite inhibition by a small molecule. In previous work from our group, we noted that chicoric acid granted a hybrid inhibition profile, both competitive and noncompetitive.[13] Based on data uncovered in the current study, α-exosite interactions of chicoric acid may not only deter substrate binding but also prevent realignment of the protease active site required for optimal catalysis. Further study on the dynamic changes of BoNT/A LC structure may give confirmation of this dual hypothesis.

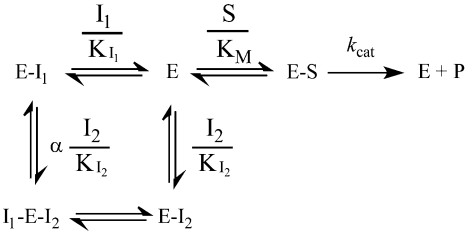
Scheme 1. Mechanism of Mutually Exclusive Inhibition[a]



$$\frac{1}{v} = \frac{1}{V_{max}}\left(\frac{I_1}{K_{I_1}} + \frac{I_2}{K_{I_2}} + \frac{S}{K_M}\right) \quad \text{(Eq. 1)}$$

[a]I1 and I2 are the concentrations of two inhibitors; K_{I1} and K_{I2} are K_I of I1 and I2 separately; S, K_M, k_{cat}, and v_{max} have their usual definitions. As shown in eq 1, at various concentrations of I2, curves of $1/v$ versus I1 have the same slope, giving parallel curves.

Scheme 2. Mechanism of Nonmutually Exclusive Inhibition[a]



$$\frac{1}{v} = \frac{1}{V_{max}}\left(\frac{I_1}{K_{I_1}} + \frac{I_2}{K_{I_2}} + \alpha\frac{I_1 I_2}{K_{I_1}K_{I_2}} + \frac{S}{K_M}\right) \quad \text{(Eq. 2)}$$

[a]Here α is a synergistic parameter reflecting the difference in affinity for I1 binding in the presence of I2; the other parameters have the same connotation as in Scheme 1. In eq 2, the slopes of the curves of $1/v$ versus I1 are function of I2, which gives sets of intersecting curves.

ASSOCIATED CONTENT

Supporting Information

Supplementary data, experimental procedures, and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

BoNT/A, botulinum neurotoxin serotype A; LC, light chain; SNAP-25, synaptosomal-associated protein 25; FITC, fluorescein isothiocyanate; DABCYL, 4-(4-dimethylaminophenylazo)benzoic acid; FRET, fluorescence resonance energy transfer; DIC, *N*,*N*′-diisopropylcarbodiimide; HOBT, hydroxybenzotriazole; DCM, dichloromethane; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography−mass spectrometry

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