Botulinum neurotoxins (BoNTs) are zinc proteases that cleave SNARE proteins to elicit flaccid paralysis by inhibiting the fusion of neurotransmitter-carrying vesicles to the plasma membrane of peripheral neurons. There are seven serotypes of BoNT, termed A–G. BoNT serotype A and serotype E cleave SNAP25 at residues 197–198 and 180–181, respectively. Unlike other zinc proteases, the BoNTs recognize extended regions of SNAP25 for cleavage. The basis for this extended substrate recognition and specificity is unclear. Saturation mutagenesis and deletion mapping identified residues 156–202 of SNAP25 as the optimal cleavage domain for BoNT/A, whereas the optimal cleavage domain for BoNT/E was shorter, comprising residues 167–186 of SNAP25. Two sub-sites were resolved within each optimal cleavage domain, which included a recognition or active site (AS) domain that contained the site of cleavage and a binding (B) domain, which contributed to substrate affinity. Within the AS domains, the P1, P3, and P5 sites of SNAP25 contributed to scissile bond cleavage by LC/A, whereas the P1’ and P2 sites of SNAP25 contributed to scissile bond cleavage by LC/E. These studies provide insight into the development of strategies for small molecule inhibitors of the BoNTs.

The Clostridium botulinum neurotoxins (BoNTs) are the most potent protein toxins for humans (1). There are seven distinguishable BoNT serotypes, A–G, with serotypes A, B, and E responsible for most natural human intoxications (2). BoNT serotypes are defined by the specificity of antibody neutralization where antibodies that neutralize one serotype fail to neutralize other serotypes. There are two licensed vaccines against botulism, a pentavalent vaccine against serotypes A–E (3) and a heptavalent vaccine against serotypes A–G (4). However, these vaccines are produced from chemically inactivated BoNT produced in C. botulinum and are currently in limited supply. Upon intoxication, BoNT intoxication has significant morbidity and mortality (5, 6). Thus, there is a need to develop more efficient vaccines and therapies against botulism.

BoNTs are zinc proteases that elicit flaccid paralysis by inhibiting the fusion of neurotransmitter-carrying vesicles to the plasma membrane of peripheral neurons. BoNTs are ~150-kDa single chain proteins that are activated by proteolysis to generate disulfide-linked di-chain proteins. BoNTs are organized into three functional domains: an N-terminal catalytic domain (light chain, LC), an internal translocation domain (heavy chain, HCT), and a C-terminal receptor binding domain (heavy chain, HCR) (7, 8). BoNTs enter neurons via receptor-mediated endocytosis. The tropism for neurons is due to the affinity of BoNT for receptors on peripheral neurons (7). BoNT-receptor interactions are sometimes enhanced by gangliosides, which act as co-receptors (9). In the acidic endosome, the HCT inserts into the endosome membrane to generate a pore that allows the catalytic LC to translocate into the cytosol (10). LCs cleave neurotransmitter vesicle fusion proteins. BoNT/A cleaves SNAP25 between residues 197 and 198, and BoNT/E cleaves SNAP25 between residues 180 and 181, inactivating SNAP25 on the plasma membrane (11). BoNT/C cleaves both SNAP25 and syntaxin (12), whereas the other BoNT serotypes and tetanus toxin cleave the vesicle-associated membrane protein (13).

Previous studies have addressed the specificity of substrate recognition to define residues and regions of SNAP25 that are required for cleavage by BoNT. Several amino acids that surround the scissile bond contribute to efficient cleavage of SNAP25 (14). Montecucco and coworkers (15) defined an amino acid repeat motif within SNAP25, termed the SNARE motif, which correlated to substrate recognition, whereas Binz and coworkers (16) defined regions and residues of SNAP25 required for optimal cleavage by BoNT/A, BoNT/E, and BoNT/C. Although the first co-crystal of BoNT/B and vesicle-associated membrane protein did not provide electron density of substrate (17, 18), a recent co-crystal structure of a non-catalytic LC/A and SNAP25-(146–204) (19) defined an α-exosite and β-exosite within SNAP25 that were implicated in substrate recognition. This study provides a reference for the functional mapping of substrate determinants required for catalysis.

Relative to thermolysin, the BoNTs recognize a larger region of substrate for optimal cleavage. The large regions required for substrate recognition of vesicle fusion proteins, such as SNAP25, may be due to the limited structure of the SNARE proteins which may require an extended contact region for high affinity binding. There is limited understanding of how the SNAP25 is recognized for specific cleavage, which prompted a detailed study to identify how these BoNT/A and BoNT/E recognize SNAP25.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Protein Expression**

BoNT LC/A and LC/E were constructed by amplifying DNA encoding LC fragments from Clostridium botulinum serotype A1 Hall strain (20) and E. coli (21), respectively, and subcloning into pET-15b. Plasmids encoding LCs were transformed into Escherichia coli BL21(DE3) RIL (Strategene). Protein expression was achieved by culturing E. coli at 16 °C overnight with 0.75 mM isopropl-1-thio-β-D-galactopyranoside as previously described (22). Briefly, cells were broken with a French press. His-LCs were purified sequentially from cell lysates by nitritolactacetic acid-affinity chromatography, gel filtration, and DEAE chromatography. SNAP25 was...
constructed by amplifying DNA encoding the indicated region of SNAP25 and subcloning into pGEX-2T. Plasmids encoding SNAP25 were transformed into E. coli TG1. Protein expression was achieved by culturing E. coli at 30 °C for 2 h with 0.75 mM isopropyl-1-thio-β-D-galactopyranoside. GST-SNAP25 was purified using glutathione-Sepharose 4B affinity chromatography (Amersham Biosciences).

**Mutagenesis of SNAP25**

Random and directed mutagenesis was performed to achieve saturation mutagenesis of SNAP25. Mutations are designated N(XXX)N, where the first N is the native amino acid, the XXX is the residue mutated, and the second N is the mutation. Random mutagenesis of SNAP25 was performed with GeneMorph II Random Mutagenesis Kit (Stratagene) by Mutazyme II DNA polymerase during DNA amplification. Briefly, amplification was performed in a 50-μl reaction containing 1 ng of pGEX-SNAP25-(141–206), 2.5 units of Mutazyme II DNA polymerase, 40 μM dNTPs, and 50 pmol of 5′-GATCGGAGCTC and 5′-AGTCACGATGAATCCATCGATCC. The reaction was preheated at 95 °C for 2 min, and then incubated for 35 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and then finishing by incubating at 72 °C for 10 min. Amplified products were purified, digested, and subcloned into pGEX-2T. Recombinant plasmids were sequenced to identify the mutations within SNAP25. Site-directed mutagenesis was performed to mutate DNA encoding residues that were not mutated by random mutagenesis. Site-directed mutagenesis was performed by QuikChange (Stratagene) following the manufacturer’s instruction, using pGEX-SNAP25-(141–206) as a template. For example, the G168R, R176C, D179N, I181E, A195S, and R198A point mutations were generated by random mutagenesis that had >50% inhibition of LC cleavage were engineered with more conserved mutations, G168A, R176A, D179A, I181A, A195S, and R198A, and tested for sensitivity to cleavage by LC/A and LC/E.

**Cleavage of SNAP25 by BoNT-LCs**

**Linear Velocity Reactions**—Reactions contained (10 μl): 5 μM of the indicated SNAP25 incubated with LC/A or LC/E in 10 mM Tris-HCl (pH 7.6) with 20 mM NaCl. Reactions were run at 37 °C for 10 min, stopped by adding SDS-PAGE buffer and heating for 5 min, and subjected to SDS-PAGE. Gels were stained, and the amount of SNAP25 cleavage was determined by densitometry.

**Kinetic Parameters**—Km and kcat determinations were made for LC/A with the indicated SNAP25 (146–206). LC was adjusted to cleave <10% substrate in various concentrations of substrates. Reaction velocity versus substrate concentration was fit to the Michaelis-Menten equation, and kinetic constants were derived, using the EnzFitter program (Elsevier, UK).

**Molecular Modeling**

Proteins were modeled with Swiss Model software from the Brookhaven National Laboratories (23).

**RESULTS**

**Residues That Define Substrate Recognition of SNAP25 for LC/A and LC/E**

Initial attempts to identify the molecular basis for the substrate recognition of BoNT, by exchanging the surface determinants within LC/A and LC/E were not successful. In these experiments, chimeric proteins were generated that contained complementary loops of the two LCs (at residues 50, 170, 250, and 370 of LC/A and the corresponding region of LC/E) but yielded chimeric proteins that were either inefficient or inactive in SNAP25 cleavage. This prompted the reciprocal strategy to define substrate specificity through the mutagenesis of SNAP25.

Previous deletion analysis resolved residues 141–202 as the minimal region of SNAP25 that can be efficiently cleaved by LC/A (15, 16). Initial mapping showed that deletion of the NARE motif (residues 145–154) did not affect the ability of LC/E to cleave a SNAP25 and had 5-fold lower rate for cleavage by LC/A (Table 1). Further mapping showed that SNAP25-(167–202) was efficiently cleaved by LC/E, whereas LC/A cleavage was less efficient, suggesting that the binding domain for LC/E was shorter than LC/A. This also indicated that residues 146–155 played a limited role in SNAP25 recognition by LC/A or LC/E and prompted a saturation mutagenesis approach to residues 156–202 of SNAP25 to define cleavage properties of LC/A and LC/E. Fig. 1A shows the differential effect of representative single amino acids mutations within residues 156–202 of SNAP25 on the cleavage capacities of LC/A and LC/E. A composite of the effect of individual point mutations within residues 156–202 of SNAP25 on cleavage by LC/A and LC/E is presented in Fig. 1B. Mutation of 18 of 48 residues did not affect LC/A or LC/E cleavage of SNAP25, mutations of 11 of 48 residues inhibited the ability of LC/A and LC/E to cleave SNAP25, whereas 18 of 48 residues had differential effects on SNAP25 cleavage by LC/A or LC/E. A195S reduced LC/A cleavage efficiency 5000-fold. Mutation to I178A in SNAP25 reduced LC/E cleavage of the mutated SNAP25. In linear velocity reactions, mutation of R198E in SNAP25 abolished the ability of LC/A to cleave SNAP25, whereas the more conserved mutation, R198A, reduced LC/A cleavage efficiency ~1000-fold. Mutation of A195S reduced LC/A cleavage efficiency ~5000-fold.

Relative to LC/A, mutation to fewer residues in SNAP25 inhibited LC/E cleavage. Mutations that influenced LC/E cleavage included residues 178–183 within the AS domain and residues 167–173 within the B domain. Mutation to I181E in SNAP25 abolished LC/E cleavage, whereas the more conserved mutation, I181A, reduced LC/E cleavage efficiency ~100-fold. Mutation to I178A in SNAP25 reduced LC/E cleavage efficiency ~60-fold.

Thus, two domains in SNAP25 were required for optimal LC/A and LC/E cleavage. The AS domain included the respective cleavage sites for LC/A and LC/E, and the B domain included residues that were 12 or 5 amino acids upstream of the respective cleavage site domain for LC/A and LC/E. Limited trypsin digestion patterns for point mutated SNAP25

| SNAP25 derivatives | LC/A* cleavage | LC/E* cleavage | Reference |
|--------------------|----------------|----------------|-----------|
| (146–206) | 1 | 1 | (16, 26) |
| (146–202) | 1 | 1 | (16, 25) |
| (146–199) | 1 | 1 | This study |
| (146–186) | 1 | 1 | (16, 25) |
| (141–182) | <0.01 | 1 | This study |
| (93–206) | <0.01 | 1 | This study |
| (93–206) | 0.2 | 0.5 | This study |
| (93–206) | 0.01 | 0.5 | This study |
| (93–206) | <0.01 | 1 | This study |
| (93–206) | <0.01 | 1 | This study |

* Activity relative to WT, which was designated as 1.
  1 designates that activity was within 2-fold of WT.
  <0.01% is the approximate limit of resolution of the gel detection with the amount of LC used in the analysis relative to cleavage of wild-type SNAP25.
that were cleaved by LC/A or LC/E by <50% cleavage relative to wild type yielded three identical SNAP25-dependent peptides for the mutated and wild-type SNAP25, suggesting that the point mutations that had the greatest effect on cleavage did not have gross conformational effects on SNAP25 structure (data not shown).

**AS Domain Contributes to Catalytic Efficiency, whereas B Domain Contributes to High Affinity Binding of SNAP25 by LC/A and LC/E**

Cleavage of SNAP25 by LC/A—Kinetic constants were determined for seven point mutations within SNAP25: K201N, R198E, R198A, A195D, A195S, and D193A in the AS domain and I171A in the B domain. These mutations were chosen based upon their capacity to reduce the catalytic efficiency of SNAP25 cleavage by LC/A. Point mutations K201N, R198E, R198A, and D193A reduced the $k_{cat}$ but had limited effect on the $K_m$, which indicated that residues in this region contributed to SNAP25 binding to LC/A. Thus, the AS domains contributed to catalysis while the B domains contributed to the affinity of SNAP25 by LC/A and LC/E.

**Deletion Mapping SNAP25 Peptides as Substrates for LC/A and LC/E**

Saturation mutagenesis studies predicted optimal cleavage sites of SNAP25 that should support cleavage by LC/A or LC/E. Deletion mutations were engineered within SNAP25 to test the role of B domain and AS domain on efficiency and specificity of SNAP25 cleavage by LC/A and LC/E.

Previous studies that mapped the minimal domain required for the LC/A and LC/E cleavage of SNAP25 (16) were performed using GST fusion proteins. Our initial studies determined that GST-SNAP25-(155–206) is cleaved by LC/A and LC/E at a slow rate when the cleavage site resides were aligned close to the GST component of the fusion.
protein (data not shown). This indicated that the GST component of GST-SNAP25 fusion proteins hindered cleavage by BoNTs when the cleavage site was aligned near GST. To circumvent these complications, an extended version of GST-SNAP25 was engineered to include residues 93–206 of SNAP25, which placed the SNAP25 cleavage site more distanced from the GST component of the fusion protein than used in the earlier studies. In this platform, LC/A cleaved SNAP25-(93–206)Δ146–155 within 5-fold of the rate of SNAP25-(93–206) cleavage. Deletion of residues 146–166 reduced the ability of LC/A to cleave by ~100-fold, whereas removal of residues 146–186 reduced the ability of LC/A to cleave substrate by ~1000-fold relative to the cleavage of SNAP25-(93–206) (Table 1). Deletion of 199–206 of SNAP25 abolished LC/A cleavage, indicating the importance of C-terminal residues for substrate cleavage (Table 1). The efficiency of LC/A cleavage of deletion peptides of SNAP25 were consistent with the analysis of point mutations within SNAP25, where mutation to Ile156, Leu160, Met163, and Ala164 inhibited LC/A cleavage of SNAP25. This defined residues 156–202 of SNAP25 as an optimal cleavage site for LC/A.

LC/E cleaved SNAP25-(93–206)Δ146–155 and SNAP25-(93–206)Δ146–166 similar efficiencies, within ~2-fold, as SNAP25-(93–206). LC/E cleaved SNAP25-(146–186) at a rate similar as SNAP25-(93–206). LC/E did not cleave SNAP25-(141–182), implicating a role for residues 183–186 in substrate cleavage (Table 1). This defined residues 167–186 of SNAP25 as an optimal cleavage site for LC/E.

**Minimal AS Domains of SNAP25 That Can Be Cleaved by LC/A and LC/E**

The cumulative data indicated the AS domain contributed to the catalytic capacity and substrate specificity of SNAP25 by LC/A and LC/E. To test this model, GST fusion proteins were engineered that included two minimal LC/A AS domains, 192DEANQRATK200 and 196ANQRATK200 termed as SN/A1 and SN/A2, respectively, and two minimal LC/E AS domains for LC/E, 178IDRIMEKAD186 and 178IDRIME183 termed SN/E1 or SN/E2, respectively (Fig. 2A). LC/A cleaved SN/A1, but did not cleave SN/A2 at the highest concentration of LC/A tested, 10 μM. This indicated that 196ANQRATK200 was a minimal substrate for LC/A. LC/E cleaved SN/E1 more efficiently than SN/E2, but cleavage of SN/E2 was detected at the highest concentration of LC/E, 10 μM (Fig. 2B). This indicated that residues 184–186 enhanced substrate cleavage, but that 178IDRIME183 was a minimal cleavable substrate for LC/E. Controls showed that 10 μM LC/A did not cleave SN/E1 and that 10 μM LC/E did not cleave SN/A1 (Fig. 2C).

To test a minimal cleavage domain of SNAP25 for LC/E, a peptide, Y179NRQIDRIME183 (1466.7 Da), comprising SNAP-(174–184) was tested as a substrate for LC/E. Mass spectrometry analysis detected two peptide products with masses of 965.5 Da and 520.3 Da, indicating that LC/E cleaved the peptide at the scissile bond, between Arg180 and Ile181 of SNAP25.

**A Peptide Comprising Residues 167–181 of SNAP25 Inhibits LC/A and LC/E Catalysis**

Mutational analysis showed a common region (residues 167–181) that impacted the ability of LC/A and LC/E to cleave SNAP25 at their respective cleavage sites. A peptide comprising SNAP25-(167–181), MGNEIDTNQRQIDRINI, was synthesized and tested for the ability to inhibit LC/A and LC/E cleavage of SNAP25. The peptide inhibited 50% cleavage of SNAP25 by LC/E at ~400 μM and LC/A at ~600 μM. The concentration of peptide required for inhibition was similar to the $K_{m}$ of peptides that were previously identified as substrates for BoNT/A cleavage (14). The peptide did not inhibit cleavage of vesicle-associated membrane protein by LC/B, which indicated inhibition specificity. Mass spectrometry did not detect cleavage of the peptide by LC/E or LC/A (data not shown).

**DISCUSSION**

Understanding the mechanism of substrate recognition by the BoNT can provide insight for vaccine and therapeutic development (24–26). The recent co-crystal structure of a non-catalytic LC/A and SNAP25-(146–204) (19) exposed an α-exosite and β-exosite in substrate recognition. This structure provided a model for biochemical studies to define the functional interactions between LC and SNAP25. The current study utilized saturation mutagenesis and deletion mapping to define regions and residues within SNAP25 involved in substrate binding and catalysis. From these functional studies, an AS domain and a B domain are shown to be required for efficient cleavage and high affinity binding, respectively, of SNAP25 by LC/A and LC/E (Fig. 3).

**SNAP25 Cleavage by LC/A**—Previous studies implicated several regions and residues of SNAP25 that play a role in substrate recognition by LC/A. Three motifs have been implicated in substrate recognition, the SNARE motif, α-exosite, and β-exosite (15, 19). The SNARE motif and β-exosite lie outside residues 156–202, suggesting that neither play major roles substrate recognition, while several residues that are components of the α-exosite were identified as contributing to substrate recognition. The inability to detect mutations that influenced catalysis in residues throughout the α-exosite indicated that this region is localized and that discontinuous residues were utilized for substrate recognition. This is supported by the co-crystal of LC/A and SNAP25 (19), which showed that an extended region of SNAP25 in the α-exosite aligned, but that several residues were not bonded to LC/A. Non-contact residues within the α-exosite may provide proper alignment for rather than contact points in the association where Gln152 and Asp156 in
SNAP25 Recognition by BoNT/A and -/E

The hydrophobic and charge properties of these residues implicate roles for both hydrophobic and ionic interactions for the optimal alignment of the N-terminal portion of the α-exosite show direct interactions with LC/A.

Saturation mutagenesis showed that Ala\textsuperscript{195} (P3) and Arg\textsuperscript{198} (P1’) and to a lesser extent Asp\textsuperscript{193} (P5) and Lys\textsuperscript{210} (P4’) in the AS domain were required for efficient cleavage of the scissile bond of SNAP25 by LC/A. The hydrophobic and charge properties of these residues implicate roles for both hydrophobic and ionic interactions for the optimal alignment of the scissile bond for cleavage. Previous studies implicated a role for the P1’ residue in substrate cleavage by several BoNT serotypes, where Arg/Tyr is required for LC/A, Ile/Val for LC/E, Phe/Tyr for LC/B, and Lys for LC/F for optimal substrate cleavage (16, 26, 27). Other studies reported a role for the P2 site in the cleavage of substrate by LC/A and LC/F (28, 29), which was not observed in the current study. The identification of a role for the P2 residue in catalysis was made using a peptide as substrate for LC/A cleavage (14). The differential affect for mutations at the P2 site may be due to the different amino acids substitution characterized in the two studies or to the fact that the peptide substrate had a lower affinity than SNAP25 and thus, amino acids within the peptide may make different contacts than when presented in the context of SNAP25. Nonetheless, several similarities were found between both studies where multiple residues within the AS domain contributed to substrate cleavage and that the primary affect of mutations to these residues was to reduce the $k_{cat}$ of scissile bond cleavage. In addition, the peptide defined as the optimal peptide for BoNT/A cleavage represented the AS domain defined in the current mutagenesis studies.

SNAP25 Cleavage by LC/E—Relative to LC/A, there is limited understanding of how LC/E cleaves SNAP25. Residues 167–186 of SNAP25 represented an optimal cleavage domain for LC/E, while \textsuperscript{178}IDRIME\textsuperscript{183} was a minimal cleavable substrate for LC/E. Mutation to a residue within the B domain increased the $K_m$ for SNAP25, consistent with the B domain contributing to substrate affinity. The AS domain of SNAP25 for LC/E included three residues, Ile\textsuperscript{181} (P1’), Val\textsuperscript{179} (P2), and Ile\textsuperscript{178} (P4’), that when mutated decreased the efficiency of SNAP25 cleavage. Two other residues, Met\textsuperscript{182} (P2’) and Glu\textsuperscript{183} (P3’), also contributed to cleavage, but to a lesser extent. Previous studies implicated a role for the P1’ and P2 residues of SNAP25 in LC/E action (23).

Recognition of SNAP25 Isoforms by LC/A and LC/E—Human SNAP25 is a substrate of LC/A and LC/E, whereas human SNAP23 is not cleaved by LC/A and LC/E and mouse SNAP23 is a poor substrate for LC/A and LC/E (16, 30). Sequence alignment shows that the B domain for LC/E binding is identical between SNAP25 and the two isoforms (Fig. 4). However, a Lys in human SNAP23 disrupts the “IDRI” sequence of the AS domain to interfere with LC/E cleavage. The mutation of K185D makes human SNAP23 a good substrate of LC/E. The presence of Gln\textsuperscript{184} in mouse SNAP23 shows less effect than Lys in this position because both Gln and Asp have similar sized R-groups (16). This explains why mouse SNAP23 is cleaved more efficiently by LC/E than human SNAP23.

LC/A does not cleave human SNAP23 and cleaves mouse SNAP23 poorly (16). While AS recognition of LC/A is identical between human SNAP25 and these isoforms, residues within the B domain of human SNAP25 vary with respect to human SNAP23 and mouse SNAP23, including Ile\textsuperscript{173}, Arg\textsuperscript{182}, Glu\textsuperscript{189}, Lys\textsuperscript{195}, and Thr\textsuperscript{196} of human SNAP23. Previous studies observed that the I173M and the R182P mutation enhanced the binding and cleavage of human SNAP23 by LC/A, which supports a role for these residues in SNAP recognition by LCs. The higher homology within the B domains of mouse SNAP23 and human SNAP25 than human SNAP23 and SNAP25 may be responsible for the relatively better cleavage of mouse SNAP23 by LC/A than human SNAP23.

AS Site Interactions of LC/A and LC/E with SNAP25—The known structures for BoNT LCs and tetanus toxin, along with the structure of thermolysin in complex with ZFPLA containing leucine at the P1’ residue (31) provided an opportunity to identify the putative S1’ of LC/A...
SNAP25 Recognition by BoNT/A and -E

and LC/E, using the assumption that the S1’ pocket of each LCs align with thermolysin and was complementary in size and hydrophobicity to the cognate P1’ residue (31, 32). In case of LC/A (Fig. 3), the S1’ pocket, is formed by Phe183, Phe194, Thr220, Asp179, and P1’ Arg198 and may include salt bridge with Asp158 within the pocket. Insertion of P1’ side chain into S1’ pocket and the formation of hydrogen bond of LC/A Arg263 with carbonyl oxygen of P1’ R198 may stabilize SNAP25 docking and contribute to substrate specificity. P3, Ala195, may form a main-chain interaction to contribute to catalysis or Ala195 function as a bridging molecule, since Arg231 is at the corner of the S3 pocket. The smaller overall cavity of LC/E may also contribute to substrate recognition where insertion of P5 Asp192 into the basic S5 pocket may stabilize substrate-LC/A interactions.

A similar pocket model can be predicted for LC/E cleavage of SNAP25 (Fig. 3). The S1’ pocket of LC/E is formed by Thr159, Phe194, Thr208, and Tyr254 and is shallower and more hydrophobic than the S1’ pocket of LC/A, which may reflect the nature of the P1’ Ile181 of SNAP25 for LC/E cleavage. Insertion of the P1’ Ile181 side chain into the S1’ pocket of LC/E where a hydrogen bond may form between LC/E Arg247 and main-chain carbonyl oxygen of P1’ to stabilize the substrate docking and contribute to substrate specificity. P2 Asp179 of SNAP25 may contribute to substrate specificity through ionic interactions with Lys222 in the S2 pocket of LC/E. The smaller overall cavity of LC/E relative to LC/A may be explained by the P1’ and P2 sites specify of LC/E versus the P1’, P3, and P5 site specify of LC/A. Relative to thermolysin, several of the A5 interactions of LC/A and LC/E are unique and possibly a property common to the BoNT family proteins that can be exploited in designing small molecule inhibitors that specifically target BoNTs.

Acknowledgments—We acknowledge Michael Baldwin and the other members of the Barbieri laboratory for helpful discussion. We also acknowledge C. Pier and E. A. Johnson (University of Wisconsin-Madison) for supplying the LC constructs for subcloning.

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