Mechanism of Substrate Recognition by Botulinum Neurotoxin Serotype A*

Received for publication, December 6, 2006, and in revised form, January 19, 2007  Published, JBC Papers in Press, January 23, 2007, DOI 10.1074/jbc.M611211200

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Botulinum neurotoxins (BoNTs) are zinc proteases that cleave SNARE proteins to elicit flaccid paralysis by inhibiting neurotransmitter-carrying vesicle fusion to the plasma membrane of peripheral neurons. Unlike other zinc proteases, BoNTs recognize extended regions of SNAP25 for cleavage; however, the molecular basis for this extended substrate recognition is unclear. Here, we define a multistep mechanism for recognition and cleavage of SNAP25 by BoNT/A. SNAP25 initially binds along the belt region of BoNT/A, which aligns the P5 residue to the S5 pocket at the periphery of the active site. Although the exact order of each step of recognition of SNAP25 by BoNT/A at the active site is not clear, the initial binding could subsequently orient the P4- residue of SNAP25 to form a salt bridge with the S4- residue, which opens the active site allowing the P1- residue access to the S1- pocket. Subsequent hydrophobic interactions between the P3 residue of SNAP25 and the S3 pocket optimize alignment of the scissile bond for cleavage. This explains how the BoNTs recognize and cleave specific coiled SNARE substrates and provides insight into the development of inhibitors to prevent 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 (HCT), and a C-terminal receptor binding domain (HCR) (7, 8). The tropism for neurons is due to the affinity of BoNT for receptors on peripheral neurons (7). BoNTs enter neurons via receptor-mediated endocytosis, and upon acidification of the early endosome LC is translocated into the cytosol. 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 (9). BoNT/C cleaves both SNAP25 and syntaxin (10), whereas the other BoNT serotypes and tetanus toxin cleave the vesicle-associated membrane protein (11).

Unlike other zinc proteases, BoNTs recognize extended regions of substrate for cleavage. The basis for this extended substrate recognition and the mechanism of BoNT substrate recognition are not clear. Recently, the structure of a mutated LC/A complexed with a truncated form of SNAP25 was solved (12). The complex structure provided insight into substrate recognition distanced from the active site, providing a model for exosite recognition of SNAP25 by LC/A, but had limited information on interactions between SNAP25 and the LC/A active site presumably due to structural perturbations caused by the mutations within LC/A that inactivated catalytic activity. The recently solved co-crystal structure of LC/A and l-Arg hydroxamate (l-ArgHX) provided an experimental starting point for functional mapping of the active site domain of LC/A (13), where a model for the orientation of the P1 and P1- residues (Gln197-Arg198) of SNAP25 within the active site of LC/A was presented. Earlier studies identified two regions within SNAP25 that comprised an active site (AS) region that represented the site of cleavage and a binding (B) region that conferred high affinity substrate binding. Within the AS region, P4’, P1’, P3, and P5 residues of SNAP25 were shown to contribute to scissile bond cleavage by LC/A (14). These findings prompted the present study to define how LC/A recognizes and cleaves SNAP25.

MATERIALS AND METHODS

Plasmid Construction and Protein Expression

BoNT LC/A was constructed by amplifying DNA encoding LC/A-(1–425) of C. botulinum serotype A1 Hall strain and subcloning into pET-15b. Plasmids encoding LCs were trans-
Multiple Step Recognition of SNAP25 by BoNT/A

formed into Escherichia coli BL21(DE3) RIL (Stratagene). Protein expression was achieved by culturing E. coli with 0.75 mM isopropyl-1-thio-β-D-galactopyranoside as previously described (15). SNAP25-(141-206) was expressed and purified as a glutathione S-transferase (GST) fusion protein in E. coli TG1 (14). Site-directed mutagenesis of LC/A or SNAP25 were performed using QuikChange (Stratagene) following the manufacturer’s instruction. Plasmids were sequenced to confirm the mutation.

Cleavage of SNAP25 by BoNT-LCs

Linear Velocity Reaction—Reactions (10 μl) performed were as follows. 5 μM SNAP25 was incubated with the indicated concentrations of LC/A or LC/A derivatives in 10 mM Tris-HCl, pH 7.6, with 20 mM NaCl. Reactions were resolved by SDS-PAGE, and the amount of SNAP25 cleavage was determined by densitometry.

Time Course Assay—5 μM SNAP25 was incubated with 2 nM wild type LC/A or LC/A derivatives. Aliquots were withdrawn at specified times and processed as described above.

Kinetic Parameters—Km and kcat determinations were performed for WT-LC/A and LC/A derivatives using SNAP25 as substrate. LC concentrations were adjusted to cleave <10% substrate at several concentrations of substrate ranging from 3 to 24 μM. The reaction was carried out in 10 μl of toxin reaction buffer, incubated at 37 °C for 10 min, and stopped by adding 10 μl of 2× SDS-PAGE buffer. The cleaved products were separated by SDS-PAGE, and the amount of cleaved product was calculated by densitometry. Reaction velocity versus substrate concentration was fit to the Michaelis-Menten equation, and kinetic constants were derived from the Lineweaver-Burk plot using the SigmaPlot program (Chicago, IL).

Mutation Complementation Assay—5 μM wild type SNAP25, SNAP(D193A), SNAP(R198A), or SNAP(I171A) were incubated with WT-LC/A (0.1–10 nM) or LC/A derivatives (0.01–10 μM). Reactions were subjected to SDS-PAGE analysis, and the amount of SNAP25 cleaved was determined by densitometry. The concentrations of WT-LC/A or LC/A derivatives that cleaved 50% of SNAP25 derivatives were calculated.

L-Arginine Hydroxamate Inhibition of LC/A Cleavage of SNAP25—WT-LC/A or LC/A derivatives, at concentrations that cleaved ~50% SNAP25-(141–206) in a linear velocity reaction, were incubated with 10, 1, or 0.1 mM ArgHX for 30 min on ice, followed by the addition of 5 μM SNAP25-(141–206). The reaction was subjected to SDS-PAGE to determine the amount of SNAP25 cleaved.

Trypsin Digestion of LC/A

5 μM WT-LC/A or LC/A derivative was incubated with 0.1 μM trypsin. The reactions were subjected to SDS-PAGE and stained to visualize the partial tryptic digestion. Mutations within LC/A had identical trypsin digestion profiles as WT-LC/A (Fig. 1), which indicated that these single mutations in LC/A had limited effects on the overall structural configuration of LC/A.

GST Pulldown Assay

GST pulldown assay (100-μl reaction) was performed by preincubating 3 μM GST-SNAP25(R198E) with 2 and 5 μM LC/A WT or LC/A(D370A, L175A) and then adding 30 μl of glutathione-Sepharose beads, preblocked in 10% bovine serum albumin. The beads were pelleted and washed, and proteins in the pellet were detected by Western blotting using anti-LC/A and anti-GST antibody (Sigma).

RESULTS

Molecular Modeling of the LC/A Active Site Domain—Saturation mutagenesis defined the organization and recognition of SNAP25 by LC/A, where an AS region defined substrate cleavage and a B region that was distanced from the AS defined high affinity binding by LC/A (14). Analysis of molecular models predicted a series of discontinuous interactions that first aligned the B region of SNAP25 to the active site and then optimized scissile bond cleavage through a series of interactions that involve four pockets (S5, S4, S1, and S3) that comprise the LC/A AS (Fig. 2, A–C). An overview of this model was shown by a stereo image (Fig. 2D).

Initial Interactions That Align the P5 Residue of SNAP25 to the LC/A Active Site—In the holotoxin structure, the N-terminal heavy chain (HC) loop forms a belt that wraps around the substrate binding cleft of the LC; unexpectedly, the region of the HC loop that bound LC/A aligned well with the region of SNAP25 that bound to LC/A (Fig. 3A). This prompted a comparison of SNAP25 and the HC loop of BoNT/A that revealed spatial and sequence homology between the two proteins. The alignment extended along a stretch of 38 amino acids of SNAP25 that abruptly ended at the P5 residue (Fig. 3, A and B). The residues that played an important role in substrate binding and recognition in SNAP25 with LC/A aligned with HC loop residues that interacted with LC/A (Fig. 3B). This suggested that the initial recognition of SNAP25 with LC/A was mimicking the binding of the HC loop within the binding cleft of LC/A in the native holotoxin. The biological function of the HC loop in BoNT/A may be as a pseudo-substrate that blocks the active site of LC/A to prevent auto-cleavage (16). Analysis of the
LC/A and SNAP25 complex structure revealed that LC/A residues Ile\textsuperscript{115}, Lys\textsuperscript{41}, Cys\textsuperscript{134}, and Val\textsuperscript{129} directly interacted with residues within the SNAP25 B region. Ala mutations to Ile\textsuperscript{115}, Lys\textsuperscript{41}, Cys\textsuperscript{134}, and Val\textsuperscript{129} each had a 4-fold increase in \(K_m\) and a 5-fold decrease in \(k_{\text{cat}}\) for the cleavage of SNAP25 (Table 1). Thus, the B region of SNAP25 performs a dual role as the initial site of LC/A recognition and as the first active site interaction between the P5 residue of SNAP25 and the S5 pocket of the LC/A active site. However, defining the significance of slight changes in \(K_m\) as being due to direct or indirect interactions is difficult. An Eadie Hofstee plot of wild type LC/A cleavage of SNAP25 is shown in Fig. 4.

**FIGURE 2. Recognition of SNAP25 by LC/A.** A, recognition of P5 and P3 sites by the S5 and S3 pockets. The S5 pocket is formed by Leu\textsuperscript{175}, Thr\textsuperscript{176}, and Arg\textsuperscript{177}, and the S3 pocket is formed by the main chain of Glu\textsuperscript{164}, Cys\textsuperscript{165}, and Lys\textsuperscript{166}. B, interaction of P4-Lys\textsuperscript{201} with S4'-pocket residue Glu\textsuperscript{257}. This interaction packs loop\textsuperscript{250} to loop\textsuperscript{370} and broadens the active site cavity of LC/A, which allows LC/A to dock the P1'-site into S1'-pockets. C, recognition of P1'-Arg\textsuperscript{198} by the LC/A S1'-pocket. The S1'-pocket is formed by Asp\textsuperscript{370}, Thr\textsuperscript{220}, Phe\textsuperscript{194}, and Phe\textsuperscript{163}. Asp\textsuperscript{199} and Phe\textsuperscript{194} contribute to the direct recognition of the P1'-Arg\textsuperscript{198}. These structural models were made using the complex structure of LC/A-SNAP25 (1XTG) as template and prepared by PyMol. (Green structure represents LC/A; red structure, SNAP25; blue residues, basic residues; red residues, acidic residues). D, stereo image of LC/A bound to SNAP25(Lys\textsuperscript{189} > Lys\textsuperscript{201}) of SNAP25. Note the large acidic cavity representing the S1'-pocket of LC/A (red, acidic; blue, basic). The image was generated by Z. Fu as described in Ref. 13.

**FIGURE 3. Structural alignment of BoNT/A heavy chain loop with SNAP25.** A, structural alignment of the interactions of the HC loop of BoNT/A and SNAP25 with LC/A. The left panel shows the alignment from the C terminus of the HC loop of BoNT/A and SNAP25 on the face of LC/A that continues in the right panel along the back of LC/A toward the N termini. The yellow ribbon represents the HC loop; the white ribbon represents SNAP25. Asp\textsuperscript{193} of SNAP25 and Glu\textsuperscript{528} of the HC loop interactions with LC/A S5 pocket ends the similarity between SNAP25 and the HC loop of BoNT/A. B, sequence and spatial similarity of HC loop of BoNT/A and residues of SNAP25 that interact with LC/A is indicated by (1) or partial overlap (.), which was determined by manual alignment of the two structures. The highlighted residues in SNAP25 contribute to the substrate cleavage.
Interaction between the S5 Pocket Residue of LC/A and SNAP25 P5 Site Residue—The LC/A-SNAP25 structure showed that Leu175, Thr176, and Arg177 were organized as a pocket that surrounded the P5 residue, Asp193 (Fig. 2A). Mutation of each residue to Ala reduced LC/A hydrolysis activity between 60- and 100-fold (Table 1) with a greater effect on k_{cat} than k_m. This suggested that the S5 pocket contributed to the proper alignment of the scissile bond for cleavage rather than contributing to substrate affinity. The loss of catalytic activity by S5 pocket residue mutations can be complemented by mutation of the P5 site of SNAP25, Asp193, supporting a direct role for S5 pocket residues on P5 recognition. While LC/A(L175A), LC/A(T176A), and LC/A(R177A) possessed a reduced capacity to cleave WT-SNAP25, ~75, ~50, and ~50-fold reduction in cleavage activities, respectively, these three mutated LC/A cleaved SNAP25(D193A) at ~3- to 5-fold lower than WT-LC/A. These results suggest that P5 Asp^{193} was recognized by S5 pocket residues, but this recognition may not be a direct interaction between S5 pocket residues and Asp^{193} where electrostatic interactions between the basic S5 pocket and negatively charged Asp^{193} contribute to this interaction (Table 2). The S5 pocket residues appeared specific, because mutation of other LC/A residues adjacent to the P5 residue of SNAP25, LC/A(Q162A) and LC/A(H269A), did not affect hydrolytic activity (Table 1). Interactions between the S5 pocket of LC/A and P5 residue of SNAP25 orient the next step in SNAP25 recognition, the binding of the P4’ residue of SNAP25 to the LC/A active site.

Recognition of the P4’-Residue of SNAP25 by the S4’-Pocket Residue of LC/A—The loop250 of LC/A (residues 242–259) was initially identified as the site of auto-cleavage (17) and subsequently observed to pack next to LC/A loop370 (residues 359–370) when LC/A bound SNAP25 (12). Whereas earlier studies proposed that the loop250 residue Met^{202} represented the primary contact of SNAP25 to LC/A, subsequent analyses showed that mutations of SNAP25-Met^{202} had a limited effect on catalysis (12) but that mutations at SNAP25-Lys^{201} yielded poor substrates for cleavage by LC/A (14). Examination of the LC/A-SNAP25 structure showed a potential salt bridge between SNAP25-Lys^{201} and LC/A-Glu^{257} (Fig. 2B). Supporting a role of Glu^{257} in SNAP25 recognition/cleavage was the determination that mutations at Glu^{257} had a 60- to 600-fold slower rate of SNAP25 cleavage, reflected in a lower k_{cat} than WT-LC/A (Table 1). Sequential binding at the S5 and S4’-sites aligns SNAP25 within the active site such that the P1’-residue of SNAP25, Arg^{198}, initiates interactions with the S1’-pocket of LC/A.

Recognition of the P1’-Site Residue of SNAP25 by S1’-Pocket Residues of LC/A—LC/A-Asp^{270} forms a salt bridge with the inhibitor L-ArgHX (13). Examination of the structure of LC/A and L-ArgHX identified an additional LC/A residue that contacted the inhibitor Phe^{163} and several residues that aligned

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**TABLE 1**

| LC/A site designation | LC/A derivative | K_m (μM) | k_cat (s⁻¹) | k_cat/K_m (s⁻¹/μM) |
|-----------------------|----------------|----------|-------------|--------------------|
| WT                    |                | 16 ± 0.6 | 60          | 3.7                |
| Binding               |                |          |            |                    |
| L175A                 |                | 14 ± 0.5 | 1.2 × 10⁻¹ |                    |
| T176A                 |                | 12 ± 0.3 | 1.1 × 10⁻¹ |                    |
| R177A                 |                | 9 ± 0.2  | 1.0 × 10⁻¹ |                    |
| S5                    |                | 12 ± 0.3 | 1.1 × 10⁻¹ |                    |
| S4’                   |                | 9 ± 0.2  | 1.0 × 10⁻¹ |                    |

**TABLE 2**

| LC/A | 50% cleavage of SNAP25 | WT/LC/A derivative | 50% cleavage of SNAP25(D193A) | WT/LC/A derivative | 50% SNAP25(L175A) | WT/LC/A derivative |
|------|------------------------|--------------------|-------------------------------|--------------------|-------------------|--------------------|
| WT   | 0.003                  | 1                  | 0.4                           | 1                  | 0.8               | 1                  |
| L175A| 0.2                    | 75                 | 2.0                           | 5                  | >10               | ND                 |
| T176A| 0.15                   | 50                 | 1.3                           | 3                  | >10               | ND                 |
| R177A| 0.15                   | 50                 | 1.2                           | 3                  | >10               | ND                 |

* ND, not determined.
near the inhibitor, including Phe\textsuperscript{194} and Thr\textsuperscript{220}. LC/A-D370A or the charge reversal mutation, LC/A-D370R, reduced the ability of LC/A to cleave SNAP25 by ~600- and 3000-fold, respectively (Table 1), whereas replacement of LC/A-Phe\textsuperscript{163}, LC/A-Phe\textsuperscript{194}, or LC/A-Thr\textsuperscript{220} with Ala each reduced $k_{cat}$ by ~100-fold without influencing the $K_m$ of LC/A for SNAP25. Thus, in addition to a salt bridge of the guanidinium group of Arg\textsuperscript{198} with the S1 pocket, Arg\textsuperscript{198} appears to be hydrophobic interactions between the aliphatic portion of the side chain of Arg\textsuperscript{198} and the hydrophobic S1’-pocket residues, in particular Phe\textsuperscript{194} (Fig. 2C). A direct role of Asp\textsuperscript{370} and Phe\textsuperscript{194} on substrate recognition was supported by the catalytic complementation of LC/A(Asp\textsuperscript{370}) and LC/A(Phe\textsuperscript{194}) by P1’-SNAP25 mutations. Whereas LC/A(D370A) and LC/A(F194A) cleaved WT-SNAP25 less efficiently than WT-LC/A, both mutated proteins cleaved the P1’-mutated SNAP25(R198A) at similar rates as WT-LC/A (Table 3).

The role of S1’-pocket residues on P1’-residue recognition was also supported by the change in sensitivity of S1’-pocket mutated LC/A to inhibition by L-ArgHX, an inhibitor of LC/A (18). Examination of the structure of the LC/A-ArgHX complex showed that the carbonyl- and N-hydroxyl groups of L-ArgHX formed a bidentate ligand with the zinc ion and the guanidinium group of the Arg side chain formed a salt bridge with the carbonyl R group of Asp\textsuperscript{370}, suggesting that the inhibitor-bound structure mimicked a catalytic intermediate for the P1’-residue Arg\textsuperscript{198} at the active site. The ability of L-ArgHX to inhibit catalysis by mutations in LC/A was used to probe the S1’-pocket. The residual hydrolytic activity of LC/A(D370A) and LC/A(D370R) for SNAP25 (Fig. 3) was not inhibited by 10 mM L-ArgHX. LC/A(F163A), LC/A(F194A), and LC/A(T220A) were also less sensitive to L-ArgHX inhibition than WT-LC/A and were only partially inhibited at 10 mM L-ArgHX (Fig. 5), which supported their role in P1’-residue interactions. In contrast, LC/A possessing mutations in S4’- and S5 pocket residues remained sensitive to the inhibition by L-ArgHX.

Substrate recognition by the S1’-residue has been proposed based upon characterization of BoNT/SNARE interactions (14, 19, 20) and solved crystal structures of BoNTs (17, 20–27), which share overall structure of the active site regions of the LCs. Within the active site, zinc is coordinated by the HEXXH motif, and a conserved Glu and a conserved Arg and Tyr lie in close proximity to the scissile peptide bond. However, conservation of amino acids within the active site domain is not extended to the S1’-pocket, which is consistent with the diverse chemical nature of P1’-residues of the substrates of various BoNT serotypes. Overall, S1’-pocket residues correlated in size and hydrophobicity with the cognate P1’-residue. In LC/A, each of the four residues that form the S1’-pocket appear to play different roles in substrate recognition. Asp\textsuperscript{370} is located underneath the S1’-pocket and forms a salt bridge with the guanidinium group of the Arg\textsuperscript{198} side chain. S1’-pocket residue recognition appears to be the primary mechanism of substrate recognition in LC/A and other neurotoxins (14, 28, 29).

Alignment of P3 Residue of SNAP25 to the S3 Pocket Residues of LC/A—Mutation of the P3 residue of SNAP25(A195S) had a 1000-fold reduction in hydrolysis relative to the cleavage of WT-SNAP25 by LC/A (14). Examination of the co-crystal structure of SNAP25 and LC/A revealed that the methyl side chain on Ala\textsuperscript{195} tightly fits into a pocket within LC/A (the S3 pocket) without enough space for the –OH of serine (12). Because of spatial constraints of the S3 pocket main chain interactions there were no conserved substitutions that were predicted to maintain the cavity of the S3 pocket, which discouraged subsequent manipulation of this site. The role of the S3 pocket residues of LC/A in the substrate recognition involves the alignment of the P3 residue of SNAP25, Ala\textsuperscript{195}, to the S3 pocket of LC/A, which sets up an optimal orientation for the interactions of the P1’-residue of SNAP25, Arg\textsuperscript{198}, to Asp\textsuperscript{370}.

### TABLE 3

| LC/A     | 50% cleavage of SNAP25 | WT/LC/A derivative | 50% cleavage of SNAP25(R198A) | WT/LC/A derivative |
|----------|------------------------|--------------------|-------------------------------|--------------------|
| WT       | 0.003 1.5 0.6          | 2.5 1.5 0.6       | 1.5 0.6                       | ND*                |
| D370R    | 7.5 2500 1.5           | 2.5 1.5 0.6       | 1.5 0.6                       | ND*                |
| D370A    | 1.5 500 1.5            | 2.5 1.5 0.6       | 1.5 0.6                       | ND*                |
| F194A    | 0.75 250 1.5           | 2.5 1.5 0.6       | 1.5 0.6                       | ND*                |
| T220A    | 0.6 200 >10            | ND                | ND                            | ND*                |
| F163A    | 0.45 150 >10           | ND                | ND                            | ND*                |

*ND, not determined.

**FIGURE 5.** ArgHX inhibition of the cleavage of SNAP25 by LC/A and LC/A derivatives. WT-LC/A and LC/A derivatives were incubated alone or with 0.1, 1, or 10.0 mM L-ArgHX for 30 min on ice when 5 μM SNAP25 (141–206) was added and incubated at 37°C for 10 min. Reactions were subjected to SDS-PAGE. The Coomassie-stained gel is shown. The percent of SNAP25 cleaved by WT-LC/A and LC/A derivatives was incubated alone or with 0.1, 1, or 10.0 mML-ArgHX for 30 min on ice when 5 μM SNAP25 (141–206) was added and incubated at 37°C for 10 min. Reactions were subjected to SDS-PAGE. The Coomassie-stained gel is shown. The percent of SNAP25 cleaved by WT-LC/A and LC/A derivatives was incubated alone or with 0.1, 1, or 10.0 mML-ArgHX for 30 min on ice when 5 μM SNAP25 (141–206) was added and incubated at 37°C for 10 min. Reactions were subjected to SDS-PAGE. The Coomassie-stained gel is shown.

**Alignment of P3 Residue of SNAP25 to the S3 Pocket Residues of LC/A**—Mutation of the P3 residue of SNAP25(A195S) had a 1000-fold reduction in hydrolysis relative to the cleavage of WT-SNAP25 by LC/A (14). Examination of the co-crystal structure of SNAP25 and LC/A revealed that the methyl side chain on Ala\textsuperscript{195} tightly fits into a pocket within LC/A (the S3 pocket) without enough space for the –OH of serine (12). Because of spatial constraints of the S3 pocket main chain interactions there were no conserved substitutions that were predicted to maintain the cavity of the S3 pocket, which discouraged subsequent manipulation of this site. The role of the S3 pocket residues of LC/A in the substrate recognition involves the alignment of the P3 residue of SNAP25, Ala\textsuperscript{195}, to the S3 pocket of LC/A, which sets up an optimal orientation for the interactions of the P1’-residue of SNAP25, Arg\textsuperscript{198}, to Asp\textsuperscript{370}.
and Phe\textsuperscript{194} of LC/A, resulting in the precise alignment of the scissile bond in the active site.

**Effect of Double Pocket Mutations on SNAP25 Cleavage by LC/A**—The functional relationships between residues within a single pocket or different pockets were assessed to test the predicted organization of LC/A for substrate recognition. The double pocket mutations LC/A(Leu\textsuperscript{175},Asp\textsuperscript{370}) and LC/A(Leu\textsuperscript{175},Phe\textsuperscript{194}) showed only residual capacity to cleave SNAP25 (reductions in specific activity were $>40,000$-fold), whereas double mutations within a single predicted pocket, LC/A(Phe\textsuperscript{194},Thr\textsuperscript{220}) and LC/A(Leu\textsuperscript{175},Lys\textsuperscript{177}), cleaved SNAP25 at rates similar to the respective individual mutations at these residues. GST pulldown experiments showed that LC/A(L175A,D370A) and LC/A(L175A,F194A) had similar affinity for SNAP25(R198E), a non-cleavable form of SNAP25, as WT LC/A (Fig. 6), supporting that the roles for these residues are in catalytic action, not in substrate binding. LC/A(F194A,T220A) and LC/A(L175A,K177A) had reduced $k_{\text{cat}}$ for SNAP25 cleavage as observed for individual pocket mutations (Table 1). Experiments were conducted at concentrations of LC/A and SNAP25 to yield a dose response for the amount of wild type LC/A in the pulldown (data not shown). Together, the data indicated that mutation of residues that lay in different pockets caused synergistic reduction in catalysis, whereas mutation of residues located within a single pocket had reduced catalytic activity that was similar to individual mutations within the respective pockets.

**DISCUSSION**

Although the precise mechanism for peptide bond cleavage by the BoNTs remains to be resolved, cleavage of the scissile peptide bond appears to follow a general base-catalyzed mechanism. Arg\textsuperscript{362} and Tyr\textsuperscript{365} interact with the carboxyl oxygens of the P1 and P1’-residues of SNAP25, respectively, and stabilize the oxyanion in the transition state (30, 31). Peptide bond cleavage is initiated by a water molecule that is polarized by the Glu within the zinc binding motif (HEXXH) and Zn\textsuperscript{2+}, which causes a nucleophilic attack on the carbonyl carbon of the scissile bond to form an oxyanion.

Peptide bond cleavage is likely achieved by a proton transfer from the attacking water mediated by the carboxyl group of the downstream Glu to form a protonated amine. Rate of catalysis will be affected by the relative contribution of particular residues in the docking and stabilization of substrate to the active site cavity. Upon substrate cleavage, the P4’-residue-S4’-residue interaction is disrupted, which initiates the dissociation of the C-terminal product of SNAP25 from LC/A (32, 33). Upon dissociation of the P4’-residue the AS returns to the original conformation that has a lower affinity for the P1’-residue, facilitating the dissociation of the C-terminal product of SNAP25 from LC/A. The N-terminal product of LC/A cleavage can associate with syntaxins, which yields unproductive syntaxin-SNAP25 complexes that impede vesicle exocytosis, resulting in BoNT/A poisoning (34–36).

The current study provides a molecular mechanism of LC/A recognition and cleavage of SNAP25 that involves sequential steps representing SNAP25 recognition and active site organization (Fig. 7).
interactions involve discontinuous surfaces between residues within the belt region of LC/A and the B region residues of SNAP25. The Velcro-like binding of SNAP25 to LC/A aligns the P5 residue Asp\(^{193}\) to form a salt bridge with Arg\(^{177}\), an S5 pocket residue at the periphery of one side of the active site. Although the exact order of each step of recognition of SNAP25 by BoNT/A at the active site is not clear, the initial binding could subsequently orient SNAP25 for the formation of a salt bridge between the P4'-residue SNAP25(Lys\(^{201}\)) and the S4'-residue LC/A(Asp\(^{257}\)). These interactions (12) broaden the LC/A active site cavity and dock Arg\(^{198}\), the P1'-residue, via electrostatic and hydrophobic interactions within the S1'-pocket. The fine tuning of the alignment of Arg\(^{198}\) into the S1'-pocket resulting in the precise alignment of the scissile bond is facilitated by the binding of the P3 residue, SNAP25-Ala\(^{195}\), into the hydrophobic S3 pocket of LC/A. The proper docking of the P1'-P1 sites into the AS initiates substrate cleavage. After cleavage, the P4'-residue dissociates from the S4'-residue of LC/A, which converts the AS to a smaller conformation, facilitating dissociation of the P1'-residue from the AS.

Understanding the mechanism of substrate recognition may provide insight into the development of serotype-specific inhibitors against botulism. In addition, BoNT is the most widely utilized protein for human therapy (37) for numerous neurological disorders from migraines to muscle trauma to physical disabilities (38). BoNT serotype A is used in these therapeutics, based upon the ability to produce relatively pure amounts of the holotoxin and the longevity of LC action in neurons (39, 40). Thus, understanding the substrate specificity of LC/A should also provide insight into the modification of BoNT to optimize therapeutic potential.

Acknowledgments—We acknowledge Michael R. Baldwin and members of the Barbieri laboratory for helpful discussions and Z. Fu of the Kim laboratory for making the model shown in Fig. 2D.

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