BoNTs (botulinum neurotoxins) are both deadly neurotoxins and natural toxins that are widely used in protein therapies to treat numerous neurological disorders of dystonia and spinal spasticity. Understanding the mechanism of action and substrate specificity of BoNTs is a prerequisite to develop antitoxin and novel BoNT-derived protein therapy. To date, there is a lack of detailed information with regard to how BoNTs recognize and hydrolyse the substrate VAMP-2 (vesicle-associated membrane protein 2), even though it is known to be cleaved by four of the seven BoNT serotypes, B, D, F, G and TeNT (tetanus neurotoxin). In the present study we dissected the molecular mechanisms of VAMP-2 recognition by BoNT serotype F for the first time. The initial substrate recognition was mediated through sequential binding of VAMP-2 to the B1, B2 and B3 functional domains: an N-terminal catalytic domain (LC, light chain), an internal translocation domain (heavy chain, HCT), and a C-terminal receptor binding domain (heavy chain, HCR) [5].

Owing to their extreme toxicity, the ease of production, handling, and delivery through aerosol or liquid route, BoNTs represent a potential biological warfare and have been classified as category A agent by the CDC (Center for Disease Control and Prevention) in the U.S.A. [6,7]. The first vaccine for human botulism is a Clostridium-derived, penta-serotype BoNT (BoNT/ABCDE) toxoid vaccine [8], which is currently used to immunize personnel at risk, but is in limited supply. In addition, due to the nature of BoNT intoxication, effective small molecular antitoxin is the most efficient way for the treatment of human botulism. The development of anti-botulism drugs is of utmost importance for BoNTs. However, little progress has been made due to a lack of understanding of the molecular mechanism of the action of BoNTs.

BoNT intoxication is reversible, and muscle will return to normal function upon the clearance of BoNTs in neuronal cells [5,9,10]. This reversible nature of intoxication has turned BoNTs from deadly agents into effective agents in some novel therapies for a range of neuromuscular conditions. BoNT/A was approved by the Food and Drug Administration to treat strabismus, blepharospam and hemifacial spasm as early as 1989, and then for cervical dystonia, cosmetic use, glabellar facial lines and axillary hyperhidrosis later [11]. The efficacy of BoNT/A in dystonia and other disorders related to involuntary skeletal muscle activity, coupled with its satisfactory safety profile, has prompted its empirical/offset-label use in a variety of opthalmological, gastrointestinal, urological, orthopaedic, dermatological, secretory, painful disorders and cosmetic use [12]. Future development to broaden the therapeutic applications of BoNT/A and discover novel BoNTs with new activity and longevity relies on the understanding of the mechanisms of action and substrate specificity of BoNTs.

Using protein crystallography, protein modelling and biochemical characterization of BoNTs and their substrate SNAP25 (synaptosome-associated protein of 25 kDa), the mechanisms of SNAP25 recognition by BoNT/A and BoNT/E have been addressed [13,14]. However, the extraordinary and unique substrate specificities of BoNTs require that the mechanisms of substrate recognition and specificity be thoroughly investigated for each serotype. Efforts have been made into understanding the recognition of VAMP-2 (vesicle-associated membrane protein 2) by BoNTs, much of which focussed on the characterization of VAMP-2 to understand the substrate requirements for efficient BoNT cleavage. In the case of LC/F (LC of BoNT serotype F), previous studies have identified the residues in VAMP-2 that are critical for efficient LC/F cleavage [15,16]. The recently resolved complex structure of LC/F bound

**INTRODUCTION**

BoNTs (botulinum neurotoxins) cause human botulism by interfering with vesicle fusion and neurotransmitter release in neuronal cells [1,2]. BoNTs are 150 kDa single chain proteins with typical A-B structure–function properties, where the B (binding) domain binds to surface components on the mammalian cell and translocates the A (active) domain to an intracellular location [3,4]. BoNTs are organized into three functional domains: an N-terminal catalytic domain (LC, light chain), an internal translocation domain (heavy chain, HCT), and a C-terminal receptor binding domain (heavy chain, HCR) [5].

**Key words:** botulinum neurotoxin serotype F (BoNT/F), mechanism of action, specificity, structure function, substrate, vesicle-associated membrane protein 2 (VAMP-2).
to a VAMP-2-based peptide inhibitor provides information on the mode of LC/F binding to VAMP-2 distal to the active site and proposes a three-exocite-pocket substrate recognition model [17]. However, these studies lack the detailed information with regard to how BoNTs recognize and hydrolyse their substrate VAMP-2 at the molecular level. The present study is based on the previous investigations on LC/F and extending these to comprehensively understand the molecular mechanisms of VAMP-2 substrate recognition and specificity by BoNT/F through a detailed characterization of the interactions between LC/F and VAMP-2. The present study, for the first time, addresses the mechanisms of VAMP-2 cleavage by BoNTs and provides insights into the development of therapies on BoNT intoxications and the engineering of novel BoNTs to extend their therapeutic interventions.

EXPERIMENTAL

Plasmid construction for protein expression

Plasmids for the expression of BoNT LC/F-(1–446) was constructed by amplifying the DNA encoding the LC from Clostridium botulinum serotype F strain NCTC 10281 (GenBank® code X81714.1). The product was subcloned into the pET-15b vector and transformed into Escherichia coli BL21(DE3)-RIL (Stratagene Biochemicals). Protein expression and purification was achieved as described previously [18,19]. Human VAMP-2-(1–97) was constructed by PCR amplifying a cDNA clone purchased from A.T.C.C. (accession code NM014232) and subcloning into pGEX-2T. VAMP-2-(1–97) is a soluble form of VAMP-2 which lacks the transmembrane spanning region of full-length VAMP-2. pGEX-VAMP-2-(1–97) was transformed into E. coli BL21 (DE3). VAMP-2 expression was achieved as described previously [18].

Alanine-scanning mutagenesis of VAMP-2 and LC/F mutagenesis

The optimal LC/F substrate is VAMP-2-(22–63). Alanine-scanning mutagenesis was performed to residues 22 to 63 of VAMP-2 using QuikChange® (Stratagene) protocols as described previously [18] with pGEX-VAMP-2-(1–97) as a template. The point mutations of LC/F were also performed using QuikChange® protocols. Plasmids were sequenced to confirm the mutation and that additional mutations were not present within the open reading frame of VAMP-2 and LC/F. Mutated proteins were produced and purified as described above for VAMP-2-(1–97) and LC/F.

Linear velocity and kinetic constant determinations for LC/F and its derivatives

Linear velocity reactions took place in 10 μl of 5 μM VAMP-2 or the indicated VAMP-2 derivatives, with various concentrations of LC/F or its derivatives, in 10 mM Tris/HCl (pH 7.6) with 20 mM NaCl at 37°C for 15 min. Reactions were stopped by adding SDS/PAGE buffer, and the substrate and cleaved product were resolved by SDS/PAGE (12 % gels). Three independent experiments were performed for each reaction and the average of the three experiments was used for each data point. The amount of VAMP-2 cleaved was determined by densitometry. \( K_m \) and \( k_{cat} \) determinations were performed using the same assay where VAMP-2 concentrations were adjusted to between 1 and 300 μM to achieve <10% cleavage by LC/F. Reaction velocity against substrate concentration was fitted to the Michaelis–Menten equation and kinetic constants were derived using the GraphPad program.

Molecular modelling

The complex structure of LC/F–VAMP-2 was modelled and analysed using SWISS-MODEL and refined by PyMol (http://www.pymol.org/) as described previously [13].

RESULTS

Different buffers and reaction conditions were tested for the optimization of LC/F substrate cleavage. LC/F showed slightly more efficient cleavage of substrate VAMP-2 in a buffer that contained 10 mM Tris/HCl and 20 mM NaCl (pH 7.6) than 20 mM Heps and PBS at the same pH, and LC/F was 2–3-fold more active than in buffer containing higher concentrations of NaCl (50–100 mM), consistent with the previous study of LC/A (LC of BoNT serotype A) [20]. The kinetic constants of LC/F in this buffer system were determined to be \( K_m \sim 7.1 \pm 1.1 \mu M \) and \( k_{cat} \sim 220.8 \, s^{-1} \), which is ∼3-fold lower and ∼10-fold higher than the previously reported \( K_m \) and \( k_{cat} \) of LC/F respectively (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/433/bj4330277add.htm) [16]. The higher activity of LC/F on VAMP-2 could be due to the presence of Trition X-100, which was carried in the elution buffer of the recombinant substrate GST (glutathione transferase)–VAMP-2 into the assay, as seen in some previous studies [20,21]. Compared with other LCs of BoNT in the same buffer system, LC/F was ∼12-, ∼50-, ∼7- and ∼780-fold more active on their substrate hydrolysis than LC/A, LC/B (LC of BoNT serotype B), LC/E (LC of BoNT serotype E) and LC/TeNT (LC of tetanus neurotoxin) respectively [13,14,16,18,19,22].

In order to determine the molecular mechanisms of substrate recognition and specificity, alanine scanning was performed on the LC/F optimal substrate VAMP-2-(22–63) to identify the VAMP-2 residues that contributed to substrate cleavage by LC/F. Protein modelling was performed to create the complex structure of LC/F–VAMP-2 using the complex structure of LC/F bound to a peptide inhibitor (PDB accession number 3FIE) as a template (Figure 1). The residues/pockets in LC/F that specifically recognized VAMP-2 residues were identified and characterized to address the molecular mechanisms of substrate recognition and specificity of LC/F. In order to make sure that a single mutation in LC/F did not affect the overall conformation of LC/F, partial trypsin digestion was performed on LC/F mutants. All LC/F mutants showed an identical digestion profile and some of the representative ones are shown in Supplementary Figure S2 (at http://www.BiochemJ.org/bj/433/bj4330277add.htm). In addition, CD spectroscopy analysis was performed to all mutants and they showed very similar far UV-CD spectra except for P25D and W44A, which showed significantly different spectra from the Wt (wild-type) LC/F. The altered CD spectrum of P25D compared with Wt LC/F may suggest a significant protein unfolding or decreased protein stability caused by the mutation of P25D. The significant spectrum change of W44A may indicate structural change or the influence of the aromatic side chain removal. The representative CD spectra were shown in Supplementary Figure S3 (at http://www.BiochemJ.org/bj/433/bj4330277add.htm). These results indicated that all point mutations in LC/F showed no effect on the overall conformation of LC/F, except for P25D.
Mechanisms of VAMP-2 recognition by BoNT/F

LC/F Active site substrate recognition

Alanine scanning of VAMP-2 identified three important residues, Leu60, Lys59 and Asp57, around the scissile bond (active site region), designated P2′, P1′ and P2 sites. The mutation of L60A, K59A and D57A in VAMP-2 decreased the LC/F substrate cleavage efficiency by ~30-, ~1000- and ~500-fold respectively (Table 1). Utilizing the modelled complex structure of LC/F–VAMP-2, three pockets, S2′, S1′ and S2, designated according to the designation of P sites, which showed potential interactions with the P2′, P1′ and P2 sites of VAMP-2, were identified and characterized.

S2′ pocket

The S2′ pocket that specifically recognizes the hydrophobic P2′ site Leu59 comprised several hydrophobic residues such as Tyr368, Phe369, Ile52 and Tyr168 (Figure 2a). The Y368A mutation in LC/F showed no effect on Kat, but a ~5000-fold decrease in substrate catalysis (κm) (Table 2), suggesting the important role of the residues in the S2′ pocket in substrate catalysis. The simultaneous mutations of Y368A, F369A, I370A and Y372A in LC/F completely abolished the catalytic activity of LC/F, suggesting that the hydrophobic interaction between the P2′ site and S2′ pocket stabilized the scissile bond arrangement for an efficient substrate cleavage (see Supplementary Table S1 at http://www.BiochemJ.org/bj/433/bj4330277add.htm).

S1′ pocket

The S1′ pocket that specifically recognized the positively charged P1′ site Lys59 was composed of residues Glu200 and Ser224. The VAMP-2 mutation K59A decreased the LC/F hydrolysis rate by ~1000-fold, suggesting the significant role that the P1′ site plays on VAMP-2 substrate recognition and cleavage (Table 1). The LC/F S1′ pocket residue Glu200 formed a salt bridge with VAMP-2 Lys59 (Figure 2a). The mutation E200A had no effect on the Km, but produced a ~15,000-fold reduction in substrate catalysis (Table 2). A conservative mutation E200Q showed less reduction in substrate catalysis, ~100-fold, whereas a charge reversal replacement E200R completely abolished LC/F catalysis (Supplementary Table S1). Ser224, the other residue in the S1′ pocket, might form a side-chain hydrogen bond with the P1′ site residue Lys59. The mutation of S224A also showed no effect on the Km, but a ~50-fold reduction in substrate catalysis, whereas the mutation of S224D showed a ~600-fold reduction in substrate hydrolysis (Table 2 and Supplementary Table S1). Similar to other serotypes of BoNTs, the role that the P1′–S1′ interaction played in substrate recognition in LC/F, which ensured the stabilization of the P1′–P1 scissile bond over active site Zn for efficient hydrolysis, was also significant.

S2 pocket

The S2 pocket that specifically recognized the P2 site of VAMP-2 Asp57 comprised two positively charged residues, Arg240 and Arg263. These two residues were located at both sides of the negatively charged P2, Asp57 (Figure 2a). The VAMP-2 P2 site also played a very important role in LC/F substrate recognition and the mutation of the P2 site D57A decreased substrate hydrolysis by ~500-fold (Table 1). The LC/F S2 pocket residues Arg240 and Arg263 might form a salt bridge and a side-chain hydrogen bond with the P2 site Asp57 and the mutations R240A and R263A in LC/F showed no effect on substrate affinity (Km), whereas substrate catalysis was reduced by ~200- and ~500-fold respectively (Figure 2a and Table 2). The mutations R240D and R263D showed a ~4000- and ~5000-fold reduction in substrate hydrolysis respectively. The double mutations of both R240A and R263A reduced the substrate hydrolysis rate by ~20,000-fold, whereas the double mutations of R240D and R263D in LC/F completely abolished the hydrolysis of substrate even at a very high concentration of enzyme (see Supplementary Table S1). This suggested that the P2–S2 substrate recognition may play an important role in LC/F substrate recognition through the stabilization of the P1 site for an efficient scissile bond hydrolysis.

LC/F B3 pocket

The pockets that specifically recognize the B sites of VAMP-2 were designated to be B pockets. In the complex structure of LC/F–VAMP-2, immediately downstream of the active site, VAMP-2 assumes a helical secondary confirmation that allows the hydrophobic residues to form a surface to interact with the hydrophobic B3 pocket in LC/F. The VAMP-2 B3 region was constituted of three major hydrophobic residues, Leu54, Val53 and Ile45, which were located on the same surface of the helix. The mutations of VAMP-2, L54A, V53A and I45A, showed ~30-, ~10- and ~20-fold reduction in hydrolysis by LC/F respectively (Table 1). The LC/F B3 hydrophobic pocket comprised three major hydrophobic residues, Tyr368, Ile52 and Tyr168 (Figure 2b). The mutations of Y26A, I52A and Y168A showed a ~10-, ~5- and ~12-fold increase in Km, and a ~1000-, ~70- and ~400-fold reduction in substrate catalysis respectively (Table 2). The mutations Y26D, I52D or Y168D completely abolished the hydrolytic activity of LC/F (Supplementary Table S1). Leu73,
Figure 2  The LC/F B and active site pocket substrate recognition

(a) At the active site (AS) of LC/F, the S2′, S1′ and S2 pockets specifically recognize the P2′, P1′ and P2 sites of VAMP-2. The S2′ pocket that specifically recognizes the hydrophobic P2′ site Leu60 is formed by several hydrophobic residues including Tyr368, Phe369, Ile370 and Tyr372. The S1′ pocket that specifically recognizes the positively charged P1′ site Lys59 comprises two major residues, Glu200 and Ser224. The S2 pocket that specifically recognizes the P2 site Asp57 comprises the two positively charged residues Arg240 and Arg263.

(b) The hydrophobic B3 pocket that comprises residues Tyr26, Ile52, Tyr168 and Leu173 specifically recognizes the VAMP-2 hydrophobic residues Leu54, Val53 and Ile45, which are located on the same surface of the helix. In addition, residue Arg171 also forms hydrogen bonds with the amide oxygens on the main chain of the VAMP-2 residues Val53 and Lys52. The mutation of R171A increased $K_m$ by $\sim 12$-fold and reduced $k_{cat}$ by $\sim 400$-fold, suggesting that the main-chain hydrogen-bond interactions stabilized the hydrophobic interaction at the B3 pocket. This result is consistent with the finding that the mutation R171K reduced the LC/F hydrolytic activity by $\sim 60$-fold [17].

The kinetic constant analysis indicated that the mutations of residues in the B3 pocket showed a lower extent of effect on substrate affinity, but a greater effect on substrate catalysis. This suggested the dual roles of the B3 pocket in substrate binding and in stabilizing the active site for substrate catalysis.
LC/F B2 pocket

The B2 pocket followed the B3 pocket and was located at the edge of the LC/F active site surface (Figure 2c). The B2 pocket in LC/F, which specifically recognized the VAMP-2 B2-binding region residues Glu41, Val39, and Gln38, comprises the residues Arg133, Lys172, Pro25, and Ser47 (Figure 2c). Mutations of the VAMP-2 residues Glu41, Val39 and Gln38 inhibited LC/F cleavage by ∼50-, ∼100- and ∼30-fold respectively (Table 1). The LC/F B2 pocket residues Arg133 and Lys172 formed a salt bridge and hydrogen bond with VAMP-2 Glu41, and the mutations of R133A and K172A increased the $K_m$ by ∼20- and ∼10-fold, and reduced $k_{cat}$ by ∼40- and ∼2-fold respectively (Table 2). A charge reversal replacement of R133E and K172D completely abolished the substrate hydrolysis and reduced the hydrolysis rate by ∼20000-fold respectively (Supplementary Table S1). The role of Arg133 in LC/F substrate recognition is also consistent with the finding that the mutation of R133K reduced the substrate hydrolysis by ∼50-fold [17]. Residues Pro25 and Val39 formed a deep hydrophobic pocket, where the hydrophobic side chain of VAMP-2 Val9 was docked (Figure 2c). The mutation of V137A increased the $K_m$ by ∼20-fold and reduced $k_{cat}$ by ∼50-fold, and the mutation of V137D reduced the substrate hydrolysis by ∼100000-fold (Table 2 and Supplementary Table S1). In addition, the mutation of P25A increased the $K_m$ by ∼25-fold and reduced $k_{cat}$ by ∼100-fold, which suggested the role of Pro25 on Val39 recognition even though it is hard to interpret the P25D mutation effect due to the protein unfolding indicated by CD spectrum analysis (Table 2 and Supplementary Table S1). Taken together, the mutational analysis data suggested the significant role that the B2 pocket played in both substrate binding and catalysis. From the complex structure of LC/F–VAMP-2, the LC/F residue Ser47 might form a hydrogen bond with the Gln38 of VAMP-2 (Figure 2c). However, the mutational analysis showed that the mutation of S147A showed only a ∼6-fold increase in $K_m$, and no effect on substrate catalysis was observed (Table 2).

The mutations of the LC/F residues at the B2 pocket displayed a higher extent of effect on substrate binding than the B3 pocket residues, although the latter mutations still showed significant effects on substrate catalysis, suggesting the role of the LC/F B2 pocket on both substrate binding and catalysis.

LC/F B1 pocket

The B1 pocket was located on the LC/F surface opposite the active site and specifically recognized the VAMP-2 B1 region made up of the residues Glu33, Arg31 and Leu26. The mutations of VAMP-2 Q33A, R31A and L26A inhibited LC/F substrate cleavage by ∼10-, ∼10- and ∼5-fold respectively (Table 1). The B1 pocket of LC/F comprised residues Trp44, Arg31, Tyr113, Trp319 and Tyr322 (Figure 2d). The residue Trp44 might form a hydrogen bond with the VAMP-2 Gln33 and the mutation of W44A increased $K_m$ by ∼25-fold and reduced $k_{cat}$ by ∼30-fold, whereas no effect on substrate catalysis was observed (Table 2). Glu315 of LC/F formed a salt bridge and hydrogen bond with Gln38 of VAMP-2 (Figure 2d and Table 2). Residues Tyr113, Trp319 and Tyr322 constituted a hydrophobic pocket, where the hydrophobic side chain of Leu26 was docked (Figure 2d). The mutations Y113A, W315A and Y322A increased $K_m$ by ∼10-, ∼8- and ∼8-fold respectively, whereas no effect on substrate catalysis was observed (Table 2).

The mutations of residues in the B1 pocket primarily affected substrate binding, but showed no effect on substrate catalysis.
The extent of effect caused by the mutations of residues in the B1 pocket was much smaller than the effect of the mutations of residues on the B2 and B3 pockets, suggesting the less important role that the B1 pocket played on LC/F substrate hydrolysis.

**Molecular mechanisms of substrate recognition and specificity of LC/F**

Although the precise step-by-step mechanism of substrate recognition and hydrolysis remained to be resolved, the knowledge of the mechanism of substrate recognition by BoNT/A and current mutational analysis of the substrate recognition pockets of LC/F proposed the multiple-step substrate binding and recognition process (Figure 3). After internalization and translocation into the cytoplasm of neuronal cells, LC/F attacks the membrane-bound vesicle SNARE (soluble N-ethylmaleimidesensitive fusion protein-attachment protein receptor) protein, VAMP-2, through a series of accumulative interactions. The B1 pocket of LC/F recognizes the B1 region of VAMP-2 through hydrophobic, electrostatic and hydrogen-bond interactions. The B1 pocket substrate recognition facilitates the further recognition of the VAMP-2 B2 region by the B2 pocket of LC/F, which contributes to both the substrate binding and further substrate recognition. The B1 and B2 substrate recognition is further strengthened by the interactions between the hydrophobic B3 region of VAMP-2 and the hydrophobic B3 pocket of LC/F. At the B3 region, VAMP-2 maintains a helical confirmation as in the SNARE complex [17], which folds the hydrophobic residues into a surface. The primary role of the B3 pocket on substrate catalysis suggests that B3 substrate recognition may stabilize the active site substrate catalysis, where the P2, P1' and P2' sites are specifically recognized by the LC/F S2, S1' and S2' pockets. The S2–P2, P1'–S1' and S2–P2' substrate recognition stabilizes the P1–P1' scissile bond alignment over the active site Zn, which promotes the substrate hydrolysis. After substrate cleavage, the cleaved substrate disassociates from LC/F, which then can recognize and cleave other VAMP-2 proteins.

**DISCUSSION**

In the present study, alanine scanning was performed to identify residues that were specifically recognized by LC/F. In addition, the velocity of LC/F on these point mutants was determined in order to quantify the contribution of different VAMP-2 residues to LC/F substrate hydrolysis. The VAMP-2 mutagenesis analysis provided more comprehensive information to identify the pockets in LC/F that specifically recognized the residues in VAMP-2 than a previous report [16]. The current complex structure of LC/F bound to VAMP-2-derived peptide inhibitor provides information on the mode of VAMP-2 binding to LC/F distal to the active site, but it provides little information on the molecular mechanisms of substrate recognition and specificity [17]. Using protein modelling, the complex structure of LC/F–VAMP-2 was acquired, which enabled us to identify and characterize the substrate recognition pockets in LC/F to address the molecular mechanisms of substrate recognition and specificity by LC/F.

The mechanism of LC/F substrate recognition involves three steps of substrate binding distal to the active site, which facilitates the LC/F active site substrate recognition and catalysis. Each step of the binding is mediated by hydrophobic, electrostatic and hydrogen-bond interactions. However, the contribution of each step of binding is significantly different. The B1 interaction between LC/F and VAMP-2, which is further away from the active site, only contributes to substrate binding to a small extent, although the strength of interaction force is similar to the B2 and B3 interactions (hydrophobic, electrostatic and hydrogen bond). The B2 interaction contributes greatly to both substrate binding and catalysis, and the B3 interaction showed a higher extent of contribution to substrate catalysis and only a slight contribution to substrate affinity. At the active site, the P–S interactions only contribute to substrate binding and further substrate catalysis, but not substrate binding. Comparing with the known mechanisms of SNAP25 recognition by LC/A and LC/E, the mechanisms of substrate recognition by LC/F share similarities in the general strategy of substrate binding and recognition, but there are differences in the detailed step-by-step recognition process [13,14]. The latter provides more

### Table 2 Kinetic constants of LC/F and derivatives

<table>
<thead>
<tr>
<th>Function</th>
<th>VAMP-2 site/region (residue)</th>
<th>LC/F pockets</th>
<th>LC/F derivatives</th>
<th>( k_{cat} (\mu M) )</th>
<th>( k_{cat}/K_{m} (s^{-1}) )</th>
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<tr>
<td>Active site (catalysis)</td>
<td>P2′ (Leu57)</td>
<td>S2′</td>
<td>S2′</td>
<td>7.1 ± 1.1</td>
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<td></td>
<td>P1′ (Lys59)</td>
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<td>6.9 ± 1.3</td>
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<td></td>
<td>P2′ (Asp57)</td>
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<td>7.2 ± 1.2</td>
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<td>B3 region (Leu54, Val53, Ile65)</td>
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<td>6.9 ± 1.2</td>
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<td>83.8 ± 9.3</td>
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<td>Binding (affinity)</td>
<td>B2 region (Asp41, Val39, Gln38)</td>
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comprehensive information on understanding the unique features of BoNT substrate recognition and specificity.

Most of the metalloproteinases have a broad range of substrates and the substrate specificity is only determined by one or more P sites around the scissile bond [23–25]. These few P sites contribute to both substrate affinity and catalysis. In contrast, BoNTs recognize an extended substrate for cleavage and each of the BoNTs only recognize one substrate, except for BoNT/C which recognizes both SNAP25 and syntaxin 1a [26]. For BoNTs, substrate specificity is determined by the recognition of P sites at the active site region of substrate by the active site of BoNT LCs [18,22]. The P–S interactions at the active site of BoNT LC only contribute to substrate catalysis, but not substrate affinity. Therefore BoNT requires the binding region distal to the active site to contribute to an efficient substrate binding [13,14,16,18,19,22]. In LC/F, the B1, B2 and B3 pockets contribute to VAMP-2 binding. In addition, the B2 and B3 pockets also contribute to substrate catalysis, suggesting that substrate binding close to the active site may stabilize the active site substrate recognition for efficient substrate hydrolysis, which is also seen in both LC/A and LC/E recognition of the substrate SNAP25 [13,14]. The disruption of the B2 and B3 interactions in LC/F through point mutations completely abolishes the LC/F substrate hydrolysis, suggesting the significant role of substrate distal to the active site on BoNT LC substrate recognition.

The active site region of the BoNT substrate normally includes at least three P sites, a P′ site that is significant to the substrate recognition, an upstream P′ site for the stabilization of P1′ and a downstream P site for the stabilization of the P1 site for efficient substrate cleavage [13,14,16,18,19,22]. The different rates of substrate hydrolysis by different serotypes of BoNTs might be attributed to their active site substrate recognition. Comparing the active site substrate recognition of BoNTs, the P2′ and P2 sites contribute to the LC/E and LC/F P1′–P′ scissile bond stabilization respectively, and the P4′ and P5 sites, P2′ and P7 sites contribute to the LC/A, LC/B and LC/TeNT P1′–P′ scissile bond stabilization respectively (Supplementary Figure S4). The different arrangements of the P site for P1′–P′ scissile bond stabilization affect the substrate catalytic activity of BoNTs. The higher catalytic activity of LC/E and LC/F may be attributed to the tight stabilization of the P1′–P′ by the closest P sites, the P2′ and P2 sites. The relatively low catalytic activity of LC/B and LC/TeNT might be due to the weak stabilization of the P1′–P′ by the furthest P sites, the P2′ and P7 sites [13,14,16,18,19,22].

In addition to active site substrate recognition, substrate binding distal to the active site can also affect the catalytic activity of BoNTs. Within BoNTs, there are two types of substrate-binding regions, a longer binding region in the case of LC/A and LC/F, and a relatively shorter binding region in the case of LC/B, LC/E and LC/TeNT (Supplementary Figure S4). The size of binding region shows little correlation with the substrate-binding affinity of the LCs [13,14,16,18,19,22]. The mechanism of the contribution of substrate catalysis through substrate binding distal to the active site of LCs is not clear, but it may be due to the following reasons. One reason is that distal substrate binding may change the LC active site confirmation. The superimposition of LC/A structure over the LC/A–SNAP25 complex structure and that of LC/F structure over the LC/F–peptide inhibitor complex structure indicated that only a minor confirmation change was seen at the active site after substrate binding. This mainly reflected at the side chain movement of the active site residues due to the substrate–LC interactions, suggesting that distal substrate binding showed a minor or no effect on the secondary confirmation of LC/A and LC/F active sites [17,27]. In addition, although a cooperative exosite-dependent cleavage of VAMP-2 by LC/TeNT is reported, where distal substrate binding can enhance the active site substrate cleavage, this effect is minor and not enough to support the argument that distal substrate binding affects the active site confirmation change [28]. Another reason is that substrate binding close to the active site contributes to the stabilization of active site substrate recognition and arrangement by affecting the substrate catalysis. The tightness of substrate binding close to the active site could stabilize the active site substrate interaction, recognition and further catalysis. LC/F and LC/E share similar organization of P–S interactions at the active sites, whereas LC/F shows higher hydrolytic activity than LC/E, which may be due to the B3 interaction of LC/F–VAMP-2 being closer to the active site than the B region interaction in LC/E–SNAP25, and the B1–B3 binding in LC/F–VAMP-2 is much stronger than the B region binding of LC/E–SNAP25 [13]. In addition, compared with LC/TeNT, LC/B shares similar organization of P–S interactions at the active sites as LC/TeNT; however, LC/B shows ~20-fold higher hydrolytic activity than LC/TeNT, which may be because the B region interaction of LC/B–VAMP-2 is closer to the active site than that of LC/TeNT.
In terms of the initiation of substrate recognition in BoNTs, the structural alignment of LC/A–SNAP25 with holotoxin of BoNT/A revealed that the initial recognition of SNAP25 by LC/A mimics the binding of the HC loop within the binding cleft of LC/A in the native holotoxin [14,17]. To check whether the LC/F initial substrate binding mimics the HC loop of holotoxin, BoNT/F holotoxin was modelled using available BoNT/A, BoNT/B and BoNT/E holotoxin structures as templates. The superimposition of VAMP-2 over the HC loop of the modelled BoNT/F structure indicated that, consistent with a previous report [17], VAMP-2 binding to LC/F is very unique and different from VAMP-2 binding to other serotypes of BoNT and TeNT. The different contributions of the B1–B3 regions to LC/F substrate recognition suggested that the initial substrate recognition of VAMP-2 is mediated by B1 and subsequent B2 and B3 binding, which leads to the active site substrate recognition and catalysis.

The present study is the first to comprehensively address how BoNT LC/F specifically recognizes its substrate VAMP-2, which also provides insights into the understanding of the mechanisms of substrate recognition of other VAMP-2-hydrolysing BoNTs. LC/D (LC of BoNT serotype D) recognizes the same substrate as LC/F and cleaves at a scissile bond next to the LC/F cleavage site. The detailed mapping of VAMP-2 for LC/D cleavage and the three-dimensional crystal structure of LC/D will enable us to propose the possible mechanism of VAMP-2 recognition by LC/D [16,21,29]. The hydrophobic pocket formed by Tyr and Leu can specifically recognize the P1 site in VAMP-2. The requirement of the short sequence, residues 39–62, of VAMP-2 indicates that the substrate recognition of LC/D will be different from LCF and there is no helical formation of VAMP-2 on the interaction interface of VAMP-2 and LC/D.

In conclusion, the understanding of the molecular mechanisms of LC/F substrate recognition provides insights to address the mechanisms of the extended substrate requirements and different hydrolytic activities of BoNTs as well as essential information for the development of antitoxins and engineering novel BoNTs to extend therapeutic interventions.

AUTHOR CONTRIBUTION
Sheng Chen designed and performed the research, analysed the data and wrote the manuscript. Hoi Ying Wan performed the research and analysed the data.

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REFERENCES

SUPPLEMENTARY ONLINE DATA

Molecular mechanisms of substrate recognition and specificity of botulinum neurotoxin serotype F

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METHODS

Trypsin digestion of LC/F and its derivatives

His–LC/F (6 μg) or the indicated LC/F-derivatives were incubated with 25 nM of trypsin for 20 min at 37°C. The reactions were stopped and resolved by SDS/PAGE.

CD spectroscopy analysis

CD data were collected with a JASCO (J810) spectropolarimeter equipped with a computer-controlled temperature cuvette holder. Far UV-CD data in the range of 198–250 nm were obtained with a 0.1 mm path length cuvette containing 0.5–0.8 mg/ml protein in 5 mM Tris/HCl buffer (pH 7.2) supplemented with 10 mM NaCl. CD spectra were recorded at room temperature (21°C) at a speed of 6 nm/min with a response time of 2 s. The wavelengths (198–250 nm) were fitted to the molar ellipticity ×10^3 of each residue (degree cm^2/dmol). Far UV-CD spectra were generated by GraphPad using the CD data.

Figure S1 Kinetic constants of LC/F

The kinetic constant determinations were performed in LC/F enzyme assays at 37°C for 15 min in the buffer containing 10 mM Tris/HCl (pH 7.6) and 20 mM NaCl, where VAMP-2 concentrations were adjusted to between 1 and 300 μM to achieve <10% cleavage by LC/F. Reaction velocity against substrate concentration was fitted to the Michaelis–Menten equation and kinetic constants were derived using GraphPad software.

Figure S2 Trypsin digestion analysis of LC/F and its derivatives

His–LC/F (6 μg) or the indicated LC/F derivatives were incubated with 25 nM trypsin for 20 min at 37°C. The reactions were stopped and resolved by SDS/PAGE. No significant difference was found in the trypsin digestion profiles between the Wt-LC/F and the derivatives. The trypsin digestion profiles of other LC/F derivatives were the same as Wt-LC/F and are not shown.

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Figure S3  CD spectroscopy analysis of LC/F and its derivatives

CD data were collected with a JASCO (J810) spectropolarimeter equipped with a computer-controlled temperature cuvette holder. Far-UV CD spectra were generated by GraphPad using the CD data. No significant difference was found in the secondary structure between the Wt-LC/F and its derivatives, except for mutations of P25D and W44A, which showed significantly different spectra from Wt-LC/F. The CD spectra of other LC/F derivatives were very similar to Wt-LC/F and are not shown.

Figure S4  The recognition of active site and B regions of substrate by BoNT LCs

The active site region of BoNT substrate normally includes at least three P sites, a P' site that is significant to the substrate recognition, an upstream P' site for the stabilization of P' and a downstream P site for the stabilizations of the P1 site for efficient substrate cleavage. Within BoNTs, there are two types of substrate-binding regions, a longer binding region in the case of LC/A and LC/F, and a relatively shorter binding region in the case of LC/B, LC/E and LC/TeNT. The distance of active site to B regions is also different and significant for efficient substrate hydrolysis.
Table S1  The hydrolysis of VAMP-2 by LC/F and derivatives

The VAMP hydrolysis rate was the ratio of the amount of Wt-LC/F to cleave 50 % VAMP-2 against the amount of LC/F derivatives to cleave 50 % VAMP-2. NC, no cleavage under the assay conditions used.

<table>
<thead>
<tr>
<th>Function</th>
<th>VAMP site (residue)</th>
<th>LC/F pockets</th>
<th>LC/F derivatives</th>
<th>VAMP hydrolysis rate</th>
</tr>
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<tbody>
<tr>
<td>Active site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(catalysis)</td>
<td>P2' site (Leu60)</td>
<td>S2'</td>
<td>Y369A, F369A, I370A, Y372A</td>
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<tr>
<td></td>
<td>P1' site (Lys59)</td>
<td>S1'</td>
<td>E200Q</td>
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<td></td>
<td></td>
<td></td>
<td>E200R</td>
<td>NC</td>
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<td></td>
<td></td>
<td></td>
<td>S224D</td>
<td>1.7×10^-3</td>
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<td></td>
<td></td>
<td></td>
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<td>NC</td>
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<td>R240D</td>
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<td>R263D</td>
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<td>R263A, R240A</td>
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<td></td>
<td></td>
<td></td>
<td>R263D, R240D</td>
<td></td>
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<tr>
<td>Binding (affinity)</td>
<td>B3 region (Leu54, Val39, Ile45)</td>
<td>B3</td>
<td>Y26D</td>
<td>NC</td>
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<td></td>
<td></td>
<td></td>
<td>I52D</td>
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<td>L173D</td>
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<td></td>
<td>B2 region (Asp41, Val43, Gin38)</td>
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<td>R133E</td>
<td>NC</td>
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<td>P25D</td>
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