Botulinum Neurotoxin Serotype F: Identification of Substrate Recognition Requirements and Development of Inhibitors with Low Nanomolar Affinity

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Toxins

ABSTRACT: Botulinum neurotoxins (BoNTs A–G) are zinc metalloendoproteases that exhibit extraordinary specificities for proteins involved in neurotransmitter release. In view of the extreme toxicities of these molecules, their applications in human medicine, and potential for misuse, it is of considerable importance to elucidate the mechanisms underlying substrate recognition and to develop inhibitors, with the ultimate goal of obtaining anti-botulinum drugs. We synthesized peptides based on vesicle-associated membrane protein (VAMP) to investigate the substrate requirements of BoNT F, which cleaves VAMP between residues Q58 and K59. The minimum substrate was a peptide containing VAMP residues 32–65, which includes only one of the two VAMP structural motifs thought to be required for botulinum substrate recognition. BoNT F exhibited a strict requirement for residues D57 (P₂), K59 (P₁'), and L60 (P₃'), but peptides containing substitutions for R56 (P₃), Q58 (P₁), and S61 (P₁') were cleaved. Therefore, the P₂, P₁', and P₃' residues of VAMP are of paramount importance for BoNT F substrate recognition near the scissile bond. Kᵢ values of uncleavable analogues were similar to Kᵢ values of the substrate, suggesting that substrate discrimination occurs at the cleavage step, not at the initial binding step. We then synthesized inhibitors of BoNT F that incorporated D-cysteine in place of glutamine 58, exhibited Kᵢ values of 1–2 nM, and required binding groups on the N-terminal but not the C-terminal side of the zinc ligand.

The latter characteristic distinguishes BoNT F from other zinc metalloendoproteases, including BoNTs A and B.

The clostridial neurotoxins consist of tetanus toxin, secreted by Clostridium tetani, and the seven serologically distinct botulinum toxins, designated types A–G, produced by various strains of Clostridium botulinum, Clostridium baratii, and Clostridium butyricum (1–3). They are the most toxic substances known (4). Each neurotoxin is synthesized as a single-chain protein with an Mₑ of ~150000. Endogenous proteases then act to produce the dichain structure, consisting of an Mₑ ~ 100 000 heavy chain and an Mₑ ~ 50 000 light chain, covalently linked by a disulfide bond. The dichain is thought to be the active form of the toxin (5). In the United States, there are ~200 cases of human botulism each year, due to ingestion of toxin-contaminated food, wound infections, or colonization of the intestinal tract by C. botulinum (5–7). However, much of the current interest in BoNTs stems from their use as tools in research on the mechanisms of neurotransmission (1, 8, 9), an ever-expanding number of applications in the treatment of human muscle dysfunctions (10–13), and their potential for use as bioterrorist weapons (14).

Intoxication resulting in the flaccid paralysis of botulism or the spastic paralysis of tetanus has been described as a four-step process, consisting of (1) toxin binding to specific receptors on neurons, (2) receptor-mediated toxin endocytosis, (3) translocation through the endosomal membrane, and (4) proteolytic inactivation of neuronal proteins critical to the mechanism of neurotransmitter release. The first three steps are mediated by the heavy chain, while the fourth is effected by the zinc metalloprotease activity of the light chain (15, 16). In the development of improved treatments for clostridial neurotoxin poisoning, each of the steps represents a potential target for therapeutic intervention. However, use of drugs directed against any of the first three (17–20) requires an awareness of toxin exposure before symptoms appear, which is seldom the case. In contrast, drugs based on inhibitors that inactivate toxin metalloprotease activity could be effective at any stage of disease progression. Such inhibitors must exhibit strong binding characteristics (Kᵢ values in the low nanomolar range, or better) and a high degree of specificity for clostridial neurotoxin(s) and must include an intracellular delivery mechanism for acute-stage therapy. With respect to the first two qualifications, previously identified inhibitors of other metallopeptases had only weak effects on BoNT and tetanus activities (21–23), while zinc chelators were too toxic and nonspecific for in vivo use (24).

Development of effective BoNT or TeNT inhibitors must be based on an understanding of the extraordinary substrate specificities exhibited by the clostridial neurotoxins. Each cleaves only a single bond in its neuronal target protein:
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Botulinum neurotoxins (BoNTs A-G) are zinc metalloendoproteases that exhibit extraordinary specificities for proteins involved in neurotransmitter release. In view of the extreme toxicities of these molecules, their applications in human medicine, and potential for misuse, it is of considerable importance to elucidate the mechanisms underlying substrate recognition and to develop inhibitors, with the ultimate goal of obtaining anti-botulinum drugs. We synthesized peptides based on vesicle-associated membrane protein (VAMP) to investigate the substrate requirements of BoNT F, which cleaves VAMP between residues Q58 and K59. The minimum substrate was a peptide containing VAMP residues 32 - 65, which includes only one of the two VAMP structural motifs thought to be required for botulinum substrate recognition. BoNT F exhibited a strict requirement for residues D57 (P2), K59 (P1’), and L60 (P2’), but peptides containing substitutions for R56 (P3), Q58 (P1), and S61 (P3’) were cleaved. Therefore, the P2, P1’, and P2’ residues of VAMP are of paramount importance for BoNT F substrate recognition near the scissile bond. Ki values of uncleavable analogs were similar to Km of the substrate, suggesting that substrate discrimination occurs at the cleavage step, not at the initial binding step. We then synthesized inhibitors of BoNT F that incorporated D-cysteine in place of glutamine-58, exhibited Ki values of 1 - 2 nM, and required binding groups on the N-terminal but not the C-terminal side of the zinc ligand. The latter characteristic distinguishes BoNT F from other zinc metalloendoproteases, including BoNTs A and B.
might influence kinetics, because that substrate segments absent from the synthetic peptides inhibitors of BoNT A (invaluable in the development of specific, high-affinity (44) convenient HPLC-based assays (cleave synthetic peptides, and these developments led to several BoNT serotypes and tetanus toxin were shown to through assay systems (32)). Because identical bonds occur at multiple sites in the neuronal proteins and in many other proteins as well, substrate recognition must include sequences and structures both adjacent to and at some distance from the cleavage sites. In this regard, short helical segments called SNARE motifs have been identified in all three neuronal target proteins, and are said to be required for substrate recognition and cleavage (26, 27). Nevertheless, several BoNT serotypes and tetanus toxin were shown to cleave synthetic peptides, and these developments led to convenient HPLC-based assays (28–31) and to high-throughput assay systems (32–34) which have proven to be invaluable in the development of high-affinity inhibitors of BoNT A (35–38), BoNT B (39–43), and TeNT (44, 45). This approach is not invalidated by the possibility that substrate segments absent from the synthetic peptides might influence kinetics, because $K_i$ is independent of the substrate that was used to determine it.

Because of the extensive and unique BoNT substrate requirements, it is unlikely that a specific BoNT inhibitor will be highly effective against more than one serotype. Those that are multiseroype inhibitors might also inhibit other metalloproteases, an undesirable side effect. Therefore, detailed studies on the enzymatic characteristics of each BoNT serotype are needed. In this context, we have investigated the catalytic properties of BoNT F, identified its substrate requirements near the scissile bond, uncovered significant similarities and differences with respect to other serotypes, and developed specific inhibitors that bind to BoNT F with extremely high affinity. The latter exhibited a requirement for binding moieties on the N-terminal but not the C-terminal side of the zinc ligand, a highly unusual feature among metalloendoproteases.

MATERIALS AND METHODS

Peptide Synthesis. The peptide synthesizer was a model 431A system from Applied Biosystems (Foster City, CA). All peptides were C-terminal amides, and N-terminal amino groups were acetylated. Peptides were purified by reverse-phase HPLC with gradients of dilute trifluoroacetic acid and acetonitrile using equipment from Waters Associates (Milford, MA). To confirm that the appropriate fractions had been collected, purified peptides were subjected to mass spectrometry and results were compared to calculated values.

BoNT F Protease Assays. BoNT F was obtained from Metabiologics (Madison, WI). Toxin protease activity was preactivated by incubation in 20 mM HEPES, 0.05% Tween 20, 10 mM dithiothreitol, and 0.5 mM ZnCl$_2$ (pH 7.3) at 37 °C for 30 min. It was then divided into small aliquots and stored at $-70$ °C. Assay mixtures contained 2 mM dithiothreitol, 80 μM ZnCl$_2$, 1.5 μg/mL BoNT F (approximately 10 nM), 20 mM HEPES, and 0.05% Tween 20 (pH 7.3). Inclusion of Tween in assays led to a small (≈5–10%) but reproducible increase in BoNT F activity and improved reproducibility. As found for BoNT A (30), optimum BoNT F protease activity required addition of both dithiothreitol and zinc to assays. But unlike BoNT A assays (31), bovine serum albumin was not included because it had no effect on BoNT F protease activity. The samples were incubated at 37 °C for various times, depending on substrate and inhibitor concentrations, so that less than 10% of the substrate was hydrolyzed. Assays were stopped by acidification with trifluoroacetic acid and analyzed by HPLC (30, 31).

Data Analysis. Kinetic constants were calculated from nonlinear regression plots with Enzfitter (Biosoft, Cambridge, United Kingdom), including at least seven different substrate concentrations. $K_i$ values were calculated from slopes of Dixon plots using the equation $K_i = K_{cat}[(slope)W_{max}S]$, where $S$ is the substrate concentration (46). Because each inhibitor differed from the corresponding substrate peptide by only one residue, we assumed that inhibition was competitive. In the tables, each value is the average of three independent experiments. Standard deviations (not shown) were less than 20% in all cases.

RESULTS

Peptide Substrates for BoNT F Protease Activity. In earlier work, we reported that BoNT F could cleave a relatively short synthetic peptide consisting of VAMP residues 37–75 (33). BoNT F cleaved peptide 37–75 between Q58 and K59, the same site as in whole VAMP. In this work, we determined kinetic constants for this peptide when hydrolyzed by BoNT F, and then modified it to determine BoNT F substrate requirements near the cleavage site. The partial amino acid sequence of human VAMP 2, showing residues included in peptides described in this report. The BoNT F cleavage site is highlighted in bold, and SNARE motifs (26, 27) are underlined.
However, truncating five residues on the N-terminal side to yield peptide 32–75 eliminated BoNT F-catalyzed hydrolysis, clearly demonstrating that one or more residues of peptide 37–41 are required by BoNT F for substrate binding and/or catalysis.

Extending the peptide on the C-terminal side by 11 more VAMP residues to obtain peptide 37–86 did not yield a more efficient substrate. Compared to those of peptide 37–75, the $K_m$ was lower but $V_{\text{max}}$ was also reduced, emphasizing the fact that binding and catalysis are distinct events that can be independently influenced by substrate structural changes. Substrates that bind more tightly are not necessarily cleaved more rapidly. We concluded that VAMP residues 76–86 are not critical for substrate recognition by BoNT F.

In contrast, adding five N-terminal residues to give peptide 32–75 resulted in significantly lower $K_m$ and higher maximum velocity values, compared to those of peptide 37–75. This additional sequence is well outside any of the SNARE motifs in VAMP. Nonetheless, it was clear that one or more of VAMP residues 32–36 are important for substrate binding and catalysis by BoNT F. Within this five-residue segment, we then synthesized and tested substrates, adding amino acids one at a time to the N-terminus of peptide 37–75. The effect on kinetic constants was cumulative, indicating that no individual residue was predominantly responsible for the changes (data not shown). In this region, we have not yet examined the consequences of residue substitutions on substrate properties.

Further extension of the substrate on the N-terminal side had only small effects. Peptide 27–75 had a slightly lower $K_m$, compared to that of peptide 32–75, but its $V_{\text{max}}$ was identical.

Finally, the effect of substrate truncation on the C-terminal side of the scissile bond was tested with substrate peptide 32–65. This change eliminated most of the residues in the second SNARE motif. Nonetheless, $V_{\text{max}}$ was unchanged, compared to that of peptide 32–75, and its $K_m$ was very similar. Both values were identical to those of peptide 27–75. Therefore, VAMP residues 66–75 are not required for substrate recognition and cleavage by BoNT F.

$\textbf{Hydrolysis of Substrate Analogues.}$ Peptides were synthesized to test the effects of amino acid substitutions on the kinetic properties of the substrate. Residues were changed, one per peptide, in a segment from R56 (P$_1$ with respect to the cleavage site) to S61 (P$_9$). In certain cases, peptides that were not cleaved by BoNT F were tested as inhibitors. Results are presented in Table 2.

Substituting alanine for arginine at P$_3$ (R56A) greatly reduced but did not eliminate hydrolysis. Therefore, the side chain of arginine is preferred but not required by BoNT F at this location. In contrast, substrate analogues containing conservative replacements for aspartic acid at P$_2$ were not cleaved by BoNT F. These replacements included lengthening the side chain by one methylene group (D57E), eliminating the negative charge (D57N), or eliminating the carboxyl group (D57A). When P$_2$-substituted peptides were tested as inhibitors, their $K_i$ values were similar to the $K_m$ of the native sequence substrate, indicating that these peptides bound to BoNT F, but the complexes did not proceed to the catalytic stage.

Changes to glutamine at P$_1$ (Q58N and Q58A) did not eliminate BoNT F-catalyzed hydrolysis, but peptides containing arginine or alanine in place of the native sequence lysine at P$_1$ were not cleaved. Testing the latter two analogues as inhibitors revealed a pattern similar to that seen for the P$_2$-substituted peptides, in that binding was not strongly affected but cleavage did not occur. Finally, substituting alanine for leucine (P$_5$) eliminated BoNT F-catalyzed hydrolysis, but replacing serine at P$_5$ with alanine had no substantial effect.

$\textbf{Substrate-Based Inhibitors of BoNT F Protease Activity.}$ Results from our earlier work with BoNT A and BoNT B inhibitors (34–36) suggested that introducing a zinc-binding moiety, the sulfhydryl group, near the scissile bond in peptide 37–75 would inhibit BoNT F protease activity. Therefore, analogues of substrate peptide 37–75 were synthesized with L- or D-cysteine substituted for glutamine at P$_1$ were not cleaved. Testing the latter two analogues as inhibitors revealed a pattern similar to that seen for the P$_2$-substituted peptides, in that binding was not strongly affected but cleavage did not occur. Finally, substituting alanine for leucine (P$_5$) eliminated BoNT F-catalyzed hydrolysis, but replacing serine at P$_5$ with alanine had no substantial effect.

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### Table 2: Effects of Single-Amino Acid Substitutions on Kinetic Constants

<table>
<thead>
<tr>
<th>VAMP peptide</th>
<th>Substitution</th>
<th>$K_m$ (mM)</th>
<th>$V_{\text{max}}$ (μmol min$^{-1}$ mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37–75</td>
<td>none</td>
<td>1.0</td>
<td>6.7</td>
</tr>
<tr>
<td>37–75</td>
<td>R56A</td>
<td>0.10</td>
<td>ND$^a$</td>
</tr>
<tr>
<td>37–75</td>
<td>D57E</td>
<td>0.0</td>
<td>0.83</td>
</tr>
<tr>
<td>37–75</td>
<td>D57N</td>
<td>0.0</td>
<td>1.8</td>
</tr>
<tr>
<td>37–75</td>
<td>D57A</td>
<td>0.0</td>
<td>0.47</td>
</tr>
<tr>
<td>37–75</td>
<td>Q58N</td>
<td>0.69</td>
<td>0.92</td>
</tr>
<tr>
<td>37–75</td>
<td>Q58A</td>
<td>0.27</td>
<td>1.8</td>
</tr>
<tr>
<td>37–75</td>
<td>K59R</td>
<td>0.0</td>
<td>3.5</td>
</tr>
<tr>
<td>37–75</td>
<td>K59A</td>
<td>0.0</td>
<td>0.17</td>
</tr>
<tr>
<td>37–75</td>
<td>L60A</td>
<td>0.0</td>
<td>ND$^a$</td>
</tr>
<tr>
<td>37–75</td>
<td>S61A</td>
<td>1.3</td>
<td>0.89</td>
</tr>
</tbody>
</table>

$^a$ With 0.5 mM substrate. $^b$ Not determined.

### Table 3: $K_i$ Values of Cysteine-Containing BoNT F Inhibitors

<table>
<thead>
<tr>
<th>VAMP residues</th>
<th>Substitution</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37–75</td>
<td>Q58C</td>
<td>78</td>
</tr>
<tr>
<td>37–75</td>
<td>Q58D–C</td>
<td>7.2</td>
</tr>
<tr>
<td>37–75</td>
<td>Q58D–C</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>37–75</td>
<td>Q58D–C</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>37–75</td>
<td>Q58C</td>
<td>11</td>
</tr>
<tr>
<td>37–75</td>
<td>Q58D–C</td>
<td>0.28</td>
</tr>
</tbody>
</table>
compared to a value of 11 μM for the peptide containing the L-isomer.

**High-Affinity Inhibitors of BoNT F Protease Activity.** A series of inhibitors was then synthesized, each with C-terminal d-cysteine in place of the native sequence glutamine, to test the effect of N-terminal chain length on binding affinity. Results are shown in Table 4. Eliminating residues 37–41 (peptide 42–58/Q58d-C) drastically inhibited binding, reflecting the previously described importance of these residues for substrate function. In contrast, extending the peptide toward the N-terminus of VAMP substantially strengthened inhibitor binding, compared to that of peptide 37–58/Q58d-C. Adding five residues (32–58/Q58d-C) yielded an 8-fold decrease in \( K_i \), while another five (27–58/Q58d-C) yielded a further 17-fold decrease. A small but reproducible improvement was obtained by adding residues 22–26, while including residues 17–21 had no effect. In sum, highly potent inhibitors of BoNT F protease activity with \( K_i \) values in the 1–2 nM range were obtained, based on VAMP residues 22–58, with d-cysteine in place of glutamine 58. The latter corresponds to the P₁ residue of the substrate.

Binding constants for the most effective inhibitors were calculated from experiments where inhibitor concentrations were close to that of BoNT F (Table 4). Therefore, the free inhibitor concentration could have been lower than its calculated value because a significant fraction was bound to the enzyme, resulting in \( K_i \) values that were too high. However, calculations of \( K_i \) from Henderson plots, which supposedly compensate for this situation (47), were not significantly different from those in Table 4 (data not shown).

**Requirement for the Sulphydryl Group.** For the BoNT F inhibitors described herein, the largest contribution to binding was presumed to result from interactions between the active site zinc of the enzyme and the sulfhydryl group of cysteine. To test this hypothesis, analogues of inhibitor 37–58/Q58d-C were synthesized, where d-cysteine was replaced with D- and L-glutamine, D- and L-aspartic acid, D- and L-histidine, D-alanine, D-serine, and D-2,3-diaminopropionic acid. None were effective inhibitors (\( K_i > 0.1 \) nM). Therefore, we concluded that the sulphydryl group of cysteine, which is most advantageously positioned in the d-isomer, probably binds to the BoNT F active site zinc, and this is responsible for much of the binding affinity.

**Specificity of BoNT F Inhibition.** BoNT D cleaves VAMP between residues K59 and L60, immediately C-terminal to the BoNT F cleavage site (I). BoNT D will also cleave VAMP peptide 37–75 (33). Therefore, an indication of specificity for BoNT F was determined by testing a BoNT F inhibitor against BoNT D, with peptide 37–75 as the substrate. Inhibitor 37–58/Q58d-C had a \( K_i \) value of 21 ± 3.0 μM when BoNT D was the test enzyme, 75-fold higher than the \( K_i \) for this inhibitor against BoNT F (Table 4). When the same peptide was tested against BoNT A and BoNT B, no inhibition was observed at concentrations up to 50 μM. It is reasonable to expect that a similar pattern would be found with the other BoNT F inhibitors in Table 4. In sum, the data suggested that the inhibitors described in this work exhibit a high degree of specificity for BoNT F, compared to other BoNT serotypes.

**DISCUSSION**

The BoNT F substrate peptide, VAMP 37–75 in Table 1, includes both of the SNARE motifs in VAMP, residues 39–47 (V1) and 63–71 (V2). In earlier work, Pellizzari et al. (27) studied the ability of BoNT F to cleave whole recombinant VAMP containing mutations in V1 or V2. Replacing any of the acidic residues in V1 with the corresponding amide eliminated hydrolysis by BoNT F. Analogous changes in V2 diminished but did not abolish BoNT F-catalyzed cleavage. The authors concluded that the carboxylates of V1 are major substrate recognition factors for BoNT F, while those of V2 are important, but to a lesser degree. In this work, we found that VAMP peptide 42–75, which does not contain the first three residues of V1, was not hydrolyzed by BoNT F, confirming the importance of these residues for substrate function. We then determined the minimum substrate for efficient BoNT F catalysis to be the 34-amino acid peptide, VAMP 32–65 (Table 1). This substrate includes the V1 SNARE motif, but only the first two residues of V2 are present. Therefore, the complete second motif cannot be required for substrate recognition and cleavage by BoNT F. Finally, the 2-fold decrease in \( K_m \) and the 3-fold increase in \( V_{max} \) afforded by the addition of residues 32–36 to the substrate suggest that, although they are not part of any SNARE motif, these residues are important not only for binding of the substrate to BoNT F but also for efficient catalysis.

Peptide 32–65 is almost identical in size to the minimum substrate for BoNT B, VAMP 60–94 (29), but longer than the 16-residue BoNT A substrate, SNAP 25 187–202 (31). In the BoNT F minimum substrate, 27 of the required residues are on the N-terminal side of the scissile bond, while only seven are needed on the C-terminal side. The latter situation is similar to that of the BoNT A substrate, with only five residues C-terminal to the cleavage site (31), but differs from that of BoNT B, where 18 residues are needed (29).

Studies on BoNT F substrate analogues that contained substituted residues revealed important similarities and differences, compared to the enzymatic properties of BoNTs A, B, and E, with regard to residue requirements near the scissile bond. For clarity, these characteristics are summarized in Table 5. For each of the four serotypes in Table 4, the calculated value because a significant fraction was bound to the enzyme, resulting in \( K_i \) values that were too high.
5, the most striking feature is a strict requirement for the $P_1'$ residue: arginine or tyrosine is required by BoNT A (31, 48), phenylalanine or tyrosine by BoNT B (29), isoleucine or valine by BoNT E (48), and lysine by BoNT F (this work). This observation is significant because it suggests that the properties of clostridial neurotoxin $S_1'$ binding pockets are one of the most important determinants of substrate specificity for these neurotoxins.

In addition to stringent $P_1'$ specificities, BoNTs A and F exhibited strong requirements for their respective $P_2$ residues. Peptides containing conservative replacements at this site, such as glutamine for asparagine in the BoNT A substrate (31) or glutamate for aspartate in the F substrate (Table 2), could not be hydrolyzed by the respective serotypes. Therefore, we concluded that the $S_2$ binding pockets on BoNTs A and F are probably well-defined and are major contributors to substrate specificities for these two serotypes.

Because glutamine is the $P_1$ residue in substrates for BoNTs A, B, and F and TeNT, a requirement for this particular residue at that location might be implied. Nonetheless, it could be replaced without a loss of function in substrates for the three BoNT serotypes (refs 29 and 31 and this work). Similarly, analogues containing $P_3$ and $P_1'$ replacements were hydrolyzed by BoNTs A, B, and F. Changes at $P_2'$ could be made in BoNT A and B substrates, but not in the F substrate. Taken together, the data indicated that aspartic acid at $P_2$, lysine at $P_1'$, and leucine at $P_3'$ are critically important for BoNT F substrate recognition near the cleavage site, while arginine at $P_3$, glutamate at $P_1$, and serine at $P_2'$ are less significant.

Earlier studies reported that many nonhydrolyzable substrate analogues are weak inhibitors of BoNTs A and B, indicating that the substitututions resulted in greatly diminished binding affinities (29, 31). Loss of binding was especially pronounced when arginine at $P_1'$ was changed in the BoNT A substrate. Although peptides containing $P_3$ and $P_1'$ substitutions were not cleaved by BoNT F, they bound to this enzyme with affinities similar to those of the native sequence substrates. On the basis of these findings, we concluded that for BoNT F, substrate discrimination near the cleavage side occurs mainly at the catalytic stage and to a lesser degree at the initial binding stage.

This report is the first to describe specific and highly potent inhibitors of BoNT F protease activity. The inhibitors were obtained by substituting cysteine, an amino acid containing a potential zinc ligand, for the native sequence glutamine 58 at $P_1$. Binding affinity was considerably higher when d-cysteine was present, compared to the l-isomer. Furthermore, strong binding to BoNT F required the presence of inhibitor residues on the N-terminal side of the d-cysteine at $P_1$, while including residues on the C-terminal side actually diminished the affinity. Apparently, adding the latter amino acids increased the entropy of the inhibitor without a significant increase in binding strength. This situation is the opposite of that for BoNT A inhibitors, where residues C-terminal to the zinc ligand, but not N-terminal, are required (35, 36). Similar results were described for inhibitors of tetanus toxin (44, 45) and for the high-affinity pseudotripeptide inhibitors of BoNT B (42), all of which are thought to occupy the $S_1$, $S_1'$, and $S_2'$ binding subsites on the toxins. Indeed, a requirement for inhibitor binding on the C-terminal side of the zinc ligand is a common feature among zinc metalloproteases. Examples include thermolysin (49), angiotensin-converting enzyme (49, 50), gelatinase A (51), human neutrophil collagenase (52), and matrix metalloproteinase 2 (53). Therefore, the BoNT F requirement for N-terminal inhibitor binding moieties stands in marked contrast to the binding properties of other zinc metalloproteases.

Residue replacement experiments showed that inhibition of BoNT F required the cysteine sulfhydryl group, which presumably binds to the BoNT F active site zinc. Furthermore, the d-configuration exhibited maximum affinity. d-Cysteine alone is a strong inhibitor of bovine carboxypeptidase A, binding to the active site zinc through a sulfur ligand (54). d-Cysteine was a weak inhibitor of BoNT F ($K_i > 0.5$ mM (data not shown)), and additional amino acids were needed (VAMP residues 22–57) for optimum inhibitor positioning in the BoNT F active site. Enhanced binding afforded by these residues is unlikely to be due to the presence of any secondary structure in the inhibitor before binding to BoNT F, because whole uncomplexed VAMP in solution is mostly random coil (55).

The best inhibitors of BoNT F exhibited very strong binding, with $K_i$ values of 1–2 nM (Table 4). Because assays contained excess zinc, these effects were not due to simple chelation of active site zinc by the sulphydryl group. Furthermore, inhibition was highly specific for BoNT F, compared to that for BoNTs A, B, and D. Tests against other zinc metalloproteases such as thermolysin have not yet been carried out, but strong inhibition is not expected because the most effective BoNT F inhibitors have no binding groups C-terminal to the zinc ligand.

In summary, we have defined the binding and catalytic requirements of BoNT F near the cleavage site by synthesizing and testing substrate analogues. On the basis of these findings and earlier work with other serotypes, we designed and synthesized the first specific, extremely potent inhibitors of BoNT F protease activity. Furthermore, among zinc metalloendoproteases described to date, the results presented herein indicated that BoNT F is unique with respect to its requirement for inhibitor-binding groups on the N-terminal side of the zinc ligand. Finally, the substrate and inhibitor characteristics described in this report suggest that BoNT F will have fewer highly structured binding subsites C-terminal to the hydrolysis site, compared to the N-terminal side.

**ACKNOWLEDGMENT**

The research described herein was conducted under research plan 02-4-3U-063. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. We thank Mr. Ernst Brueggemann and Dr. Harry Hines for analyses of peptides by mass spectrometry and Dr. Frank Lebeda for helpful review of the manuscript.

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