Substrate residues N-terminal to the cleavage site of botulinum type B neurotoxin play a role in determining the specificity of its endopeptidase activity

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Abstract ***Clostridium botulinum*** **type B neurotoxin is a highly specific zinc-endopeptidase which cleaves vesicle-associated membrane protein (VAMP/synaptobrevin), a critical component of the vesicle docking/fusion mechanism. In this study, substrate residues flanking the N-terminal side of the cleavage site are shown to play a key role in enzyme substrate recognition. Two aspartate residues in this region are identified as critical determinants of the neurotoxin's specificity. These findings are discussed in relation to the mechanism by which botulinum type B neurotoxin cleaves its substrate.**

Key words: Botulinum; Neurotoxin; VAMP; Synaptobrevin; Zinc-endopeptidase; Peptide substrate

1. Introduction

The clostridial neurotoxins include tetanus toxin and the seven antigenically different botulinum neurotoxins, all of which exert their action by blocking the calcium-mediated release of neurotransmitters [1,2]. The botulinum neurotoxins act on the peripheral nervous system where they inhibit the release of acetylcholine at the neuromuscular junction, an action which results in widespread paralysis and ultimately the syndrome botulism. In their activated forms, the clostridial neurotoxins consist of two subunits: a heavy chain (100 kDa) and a light chain (50 kDa), linked by a disulphide bridge. Present data suggest that the heavy chain primarily mediates toxin binding and internalisation while the light chain exerts its intracellular action. All of the botulinum neurotoxins have been shown to mediate this intracellular action via highly specific zinc-endopeptidase activities contained within the light subunit of each toxin [3]. Collectively, the clostridial neurotoxins are known to act on various isoforms of just three different proteins which form part of a putative vesicle docking/fusion mechanism [4]. Botulinum types B, D, F and G cleave vesicle-associated membrane protein (VAMP or synaptobrevin), at different sites [5-9]. Botulinum toxins types A and E cleave the protein SNAP-25 at distinct sites close to the C-terminus [6,10,11] and botulinum type C_1 toxin cleaves both syntaxin and SNAP-25. Tetanus toxin acts to cleave VAMP/synaptobrevin at an identical site to that of botulinum type B toxin [5]. The clostridial neurotoxins thus represent some of the most specific zinc-proteases so far characterised.

The mechanism of enzyme substrate recognition is presently unclear. Studies using both synthetic peptides and recombinant fragments derived from the target proteins of the toxins have shown that only relatively large peptide fragments (>30 residues) are substrates for the toxin zinc-endopeptidases [12,13]. In the case of botulinum neurotoxin type B (BoNT/B) only VAMP peptide substrates of >30 residues were cleaved by the toxin [12]. Even larger substrates (>50 residues) are required by tetanus toxin and botulinum type A neurotoxin. Surprisingly, in a study of the peptide substrate specificity of BoNT/B only one VAMP residue at the cleavage site (Phe77) was found to be critical to the endopeptidase activity of the toxin [14]. These data suggest that determinants other than the cleavage site sequence are required to account for the neurotoxin's highly specific proteolytic activity. In the present study, peptide substrates were used to determine the role of residues flanking the cleavage site of BoNT/B. Negatively charged substrate residues on the N-terminal side of the cleavage site are shown to be critical to the endopeptidase activity of BoNT/B.

2. Materials and methods

2.1. Purification of botulinum type B neurotoxin

Clostridium botulinum type B neurotoxin obtained from the Okra strain was purified by a modification [15] of the process described for botulinum type F neurotoxin [16]. Purified BoNT/B was dialysed against 0.05 M HEPES buffer pH 7.4 containing 0.15 M NaCl and stored at −80°C. Reduced and alkylated BoNT/B was prepared by the following method. BoNT/B (3 mg ml^{-1}) was incubated in 0.05 M HEPES pH 7.4 buffer containing 0.15 M NaCl and 10 mM dithiothreitol for 30 min at 22°C. Iodoacetamide was added to a final concentration of 50 mM and the mixture incubated for 1 h at 4°C. The alkylated toxin was then dialysed against 0.05 M HEPES pH 7.4 buffer containing 0.15 M NaCl and stored at −80°C.

2.2. Synthesis of peptides

Peptides were synthesised on 4-(2′,4′-dimethyl-Fmoc-aminomethyl)-phenoxy resin (Novabiochem) with an automated, solid-phase peptide synthesiser (model 431A, Applied Biosystems Inc, Foster City, CA) using Perkin-Elmer FastMoc chemistry. Peptides were purified by reverse-phase high pressure liquid chromatography on C8 columns (2.2×25 cm, Vydac), and characterised as described previously [12].

Peptides containing an intramolecular disulphide bridge were prepared as follows. Peptide (2.5 mg) was dissolved in water to a concentration of 0.1 mg ml^{-1} and the pH adjusted to 7.0 with 1 M NH_4OH. Potassium ferricyanide (2 mg ml^{-1}) was then added dropwise until a pale yellow colour was observed and the reaction stirred for 1 h at 22°C. The resulting oxidised peptide was then purified and characterised by FAB mass spectrometry as described [12]. The number of free thiol groups was determined by reaction with 3 mM dithio-

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Abbreviations: BoNT/B, botulinum type B neurotoxin; VAMP, vesicle-associated membrane protein

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Table 1
Influence of charged residues on the cleavage of VAMP peptides by BoNT/B

VAMP peptide sequence	Cleavage rate (nmole s^{-1})	% Control
(a) Influence of substitutions to negatively charged residues (X=^{78}ETSAAKLKRKYWWKNLK^{94})		
^{60}LSELDDRADALQAGASQ↓F^{77}-X	24.5 ± 1.5	100%
LSSLDDRADALQAGASQF-X	27.2 ± 0.7	111%
LSLSSLSDRADALQAGASQF-X	9.8 ± 1.5	40%
LSLSSLSSRADALQAGASQF-X	0.8 ± 0.05	3.3%
LSLSELSSRADALQAGASQF-X	0.9 ± 0.2	3.7%
LSRADALQAGASQF-X	<0.5	<3%
LSLSELDDRPDALQAGASQF-X	17.4 ± 2.5	72%
(b) Influence of substitutions to positively charged residues (X= ^{60}LSELDDRADALQAGAS^{75})		
X-^{76}Q↓FETSAAKLKRKYWWKNLK^{94}	27.3 ± 2.5	100%
X-QFETSAAKLKRKYWWSNLS	24.8 ± 2.1	91%
X-QFETSAAKLSSKYWWKNLK	9.0 ± 0.8	33%
X-QFETSAASLSRKYWWKNLK	7.6 ± 0.5	27.6%
X-QFETSAAKLSYWWKNLK	5.5 ± 0.4	20.2%

Substituted residues are shown underlined. The cleavage site for BoNT/B is indicated by the arrow. A putative motif region (residues 62–70) is shown as bold characters in the first sequence.

nitrobenzoate in 0.1 M sodium phosphate buffer pH 7.3 containing 0.1 mM EDTA. Molar extinction coefficients of 14150 M^{-1} cm^{-1} (412 nm) and 12315 M^{-1} cm^{-1} (280 nm) were used for the nitrothiobenzoate ion and the peptides respectively.

2.3. Cleavage of VAMP peptides by BoNT/B

Peptides (0.31 mM) in 50 mM HEPES buffer pH 7.2 containing 10 mM $ZnCl_2$ and 10 mM 2-mercaptoethanol were incubated at 37°C for 20 min. BoNT/B was diluted with 50 mM HEPES buffer pH 7.2 containing 10 mM dithiothreitol and 20 mM $ZnCl_2$ and incubated at 37°C for 20 min. In the case of the cleavage of disulphide-loop peptides, 2-mercaptoethanol and dithiothreitol were omitted from the incubation mixtures. Reactions were initiated by addition of 10 ml of toxin solution to the 250 ml peptide mixture and then incubated at 37°C for 30 min. Proteolysis were stopped by the addition of 0.5 ml of 0.5% (by vol) trifluoroacetic acid. Final concentrations of BoNT/B were between 3 and 100 nM and were chosen so that less than 15% of the total peptide was cleaved during the reaction time. The extent of peptide cleavage was estimated from HPLC analysis of reaction mixtures on C8 columns (4.6×45 mm, Ultrasphere, Beckman) as described [12]. Initial rates of peptide cleavage were estimated from the integral analysis of the two fragment peak areas as well as that of the parent peak. Concentrations of peptide solutions were estimated from absorbance of the tryptophan and tyrosine residues at 280 nm. All determinations of cleavage rates were performed in triplicate.

3. Results

The endopeptidase activity of BoNT/B was studied with either VAMP(60–94) or VAMP(62–91) synthetic peptides derived from the sequence of human VAMP isoform-2. These peptides are cleaved by BoNT/B at the Gln76-Phe77 bond, consistent with in vivo observations and at the same rate as larger fragments of VAMP, making them convenient model substrates with which to study the endopeptidase activity of BoNT/B [12,14].

3.1. Effect of substitution of charged substrate residues on the cleavage rate by BoNT/B

The distribution of charged residues within the VAMP(60–94) peptide substrate is such that the N-terminal region consists largely of negatively charged residues while the C-terminal region is predominantly positively charged. Table 1 illustrates the effect of neutralisation of these charged residues on the cleavage rate of BoNT/B. Substitution of Glu62 with a serine residue, a neutral but hydrophilic residue, had no effect on the cleavage rate. Similar changes to either Asp64 or Asp65 reduced the cleavage rate to approximately 30–40% of the control value. Substitution or removal of both the Asp64 and Asp65 virtually abolished cleavage of the peptide by BoNT/B. Since Asp64,65 lie in a peptide sequence predicted to adopt an α-helical structure [17], the effect of disrupting such secondary structure was investigated by inserting a proline residues in this region. Substitution of Ala67 with a proline residue did not significantly reduce the cleavage rate by BoNT/B (Table 1a).

Analogous substitutions to the positively charged residues to the C-terminal side of the cleavage site were also studied. Replacement of both Lys91 and Lys94 with serine did not significantly affect the cleavage rate by BoNT/B. Substitution of various combinations of the cluster of the positively charged residues between Lys83 and Lys87, however, reduced the cleavage rate by BoNT/B to 20–30% of control values (Table 1b).

3.2. Effect of deletion of substrate residues on the cleavage rate by BoNT/B

The above data suggests that residues N-terminal to the cleavage site of BoNT/B play a key role in enzyme substrate recognition. To assess the importance of relative position of these residues with respect to the cleavage site the effect of the addition and deletion of amino acid residues was studied. Deletion of either Ala69 or Ala72 greatly reduced the cleavage rate by BoNT/B (Table 2a). In control experiments in which Ala69 was substituted for a cysteine residue the cleavage rate was not significantly reduced compared to control values illustrating that the properties of the amino acid side chain are not critical to substrate recognition (result not shown). Addition of an alanine residue at position 72 was found to completely abolish peptide cleavage by BoNT/B (Table 2a). Analogous experiments were performed to investigate the role of deleting residues in the peptide sequence flanking the C-terminal side of the cleavage site. In contrast to the above, deletion of either Ala82 or Leu85 were both found to result in a slight increase in the peptide cleavage rate by BoNT/B (Table 2b).

Table 2
Influence of residue deletions/additions on the cleavage of VAMP peptides by BoNT/B

VAMP peptide sequence	Cleavage rate (nmole s^{-1})	% Control
(a) Deletions/additions to the N-terminal region (X=^{78}ETSAAKLKRKYWWKNLK^{94})		
^{62}ELDDRADALQAGASQ↓F^{77}-X	23.4 ± 1.5	100%
ELDDRAD–LQAGASQF-X	2.9 ± 0.3	12.3%
ELDDRADALQ–GASQF-X	<0.5	<2%
ELDDRADALQAAGASQF-X	<0.5	<2%
(b) Deletions to the C-terminal region (X= ^{62}ELDDRADALQAGAS^{75})		
X-^{76}Q↓FETSAAKLKRKYWWKNLK^{94}	13.4 ± 1.6	100%
X-QFETSA–KLKRKYWWKNLK	15.5 ± 1.9	116%
X-QFETSAAK–KRKYWWKNLK	17.2 ± 2.1	128%

Amino acid deletions are indicated by a hyphen; additions are shown underlined. The cleavage site for BoNT/B is indicated by the arrow. Concentrations of BoNT/B were 10 nM in a and 3.3 nM in b.

3.3. Cleavage of disulphide-loop VAMP peptides by BoNT/B

To investigate the influence of substrate conformation on the cleavage rate by BoNT/B, VAMP(60–91) peptides containing an intra-chain disulphide bridge were produced (Table 3). Disulphide bridges were introduced into peptides by oxidation of dilute solutions of peptide with potassium ferricyanide. Mass spectrometry of the oxidised peptides showed the purified products to be 2 mass units smaller than the corresponding reduced peptide illustrating the successful formation of intra-chain disulphide bridges. In addition, no free thiol groups were detectable in these peptides. To avoid the use of thiol reagents in the reaction mixtures, BoNT/B which has been reduced and alkylated was used for this part of the study. BoNT/B treated in this way was found to retain approximately 70% of its original activity. A VAMP peptide containing a cysteine bridge between positions 69 and 84 was poorly cleaved by BoNT/B while no detectable cleavage was observed with a peptide containing a cysteine bridge between positions 67 and 84. In control experiments, both peptides were cleaved rapidly prior to oxidation. In the case of VAMP (Cys69,84) treatment of the oxidised peptide with dithiothreitol recovered more than 60% of the cleavage rate of the non-oxidised control.

4. Discussion

The clostridial neurotoxins are among the most specific proteases characterised. VAMP/synaptobrevin appears to be the only protein cleaved by BoNT/B which, like tetanus toxin, cleaves the protein at a single site between Gln76 and Phe77 [5]. The mechanism by which BoNT/B recognises its target protein is unclear. Previous studies have shown that the primary structure of the peptide in the vicinity of the cleavage site is insufficient to account for the specificity of the endopeptidase since modified sequences are still cleaved by BoNT/B [14]. One possibility is that BoNT/B requires a unique peptide conformation for cleavage. Another possible mechanism is that, in addition to the site of cleavage, there are one or more sub-sites or recognition sites where the substrate binds to the neurotoxin. Recently, it has been suggested that a nine residue motif (residues 62–70; see Table 1), of which there are multiple copies in VAMP, syntaxin and SNAP-25 acts as a common recognition site for the clostridial toxins [17]. Strong evidence in favour of such a mechanism is the observation that the endopeptidase activity of one neurotoxin serotype can be inhibited by the presence another toxin serotype which suggests a common recognition element [17]. Data presented in the present study support the hypothesis of a sub-site for substrate binding in the case of BoNT/B. Neutralisation of the negatively charged residues, Asp64 and 65, which lie within the putative motif region (Table 1), virtually abolished substrate cleavage by BoNT/B. These data may indicate a role for these charged residues in binding at a sub-site. If Asp64 and 65 do play a direct role in substrate binding then the distance between the motif region and the cleavage site should be critical. Consistent with this is the greatly reduced rates of cleavage observed with substrates in which either a residue deletion or addition had been made at Ala 69 or 72. In contrast, analogous deletions made on the C-terminal side of the cleavage site had no inhibitory effect on the cleavage rate. Collectively these data suggest a key role for substrate residues N-terminal to the cleavage site as determinants of the specificity of BoNT/B. This region of VAMP has also been identified as a conserved signal sequence which plays a role in the regulation of VAMP transport to synaptic vesicles [18,19].

Insertion of a proline residue within the motif region on VAMP peptide substrates only slightly reduced the rate of substrate cleavage. This observation is inconsistent with pre-

Table 3
Cleavage of disulphide-loop peptides by BoNT/B

VAMP peptide	Cleavage rate (nmole s^{-1})	% Control
VAMP(Cys69,84) reduced	23.4 ± 0.9	100%
VAMP(Cys69,84) oxidised	3.2 ± 0.3	4.4%
VAMP(Cys67,84) reduced	23.4 ± 0.9	100%
VAMP(Cys67,84) oxidised	<0.5	<2%

Cleavage reactions were performed using 100 nM alkylated BoNT/B and 0.3 mM peptide. Peptides were oxidised by treatment with potassium ferricyanide as described in Section 2. Peptide sequences were: VAMP(Cys69,84)= ^{62}LSELDDRADCLQAGASQFETSAAKCKRKYWWK^{91} VAMP(Cys67,84)=^{62}LSELDDRCDALQAGASQFETSAAKCKRKYWWK^{91}.

dictions of an α-helical structure for this region [17] and also suggests that any binding to the toxin within this region does not evoke the formation of such secondary structure.

While a model in which BoNT/B recognises its substrate via cleavage site and motif (sub-site) sequences could explain the unique specificity of the neurotoxin, this model is too simplistic to explain the available data. Previous studies have shown that a VAMP(55–82) peptide which contains both the motif and cleavage site is poorly cleaved by BoNT/B [12]. This observation clearly suggests that substrate residues flanking the C-terminus of the cleavage site also play some role in substrate recognition. The possibility that this region contains a second sub-site for substrate binding seems unlikely since amino acid deletions in this region had little effect on the rate of proteolysis. Neutralisation of the positively charged residues in this region did reduce the cleavage rate by BoNT/B, but to a much lesser extent than that observed with the negatively charged residues in the motif region. A more likely explanation is that the residues on the C-terminal side of the cleavage site are required to provide a specific substrate conformation which is essential for cleavage by BoNT/B. Constraining VAMP peptides by the introduction of disulphide bridges greatly reduced the rate proteolysis by BoNT/B which suggests that peptide conformation is an important factor. Further studies are clearly required to understand the factors determining the specificity of BoNT/B.

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