Type A botulinum neurotoxin proteolytic activity: development of competitive inhibitors and implications for substrate specificity at the S_1' binding subsite

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Abstract Type A botulinum neurotoxin (botox A) is a zinc metalloprotease that cleaves only one peptide bond in the synaptosomal protein, SNAP-25. Single-residue changes in a 17-residue substrate peptide were used to develop the first specific, competitive inhibitors of its proteolytic activity. Substrate analog peptides with P_4, P_3, P_2' or P_3' cysteine were readily hydrolyzed by the toxin, but those with P_1 or P_2 cysteine were not cleaved and were inhibitors. Peptides with either D- or L-cysteine as the N-terminus, followed by the last six residues of the substrate, were the most effective inhibitors, each with a K_i value of 2 μM. Elimination of the cysteine sulfhydryl group yielded much less effective inhibitors, suggesting that inhibition was primarily due to binding of the active-site zinc by the sulfhydryl group. Botox A displayed an unusual requirement for arginine as the P_1' inhibitor residue, demonstrating that the S_1' binding subsite of botox A is dissimilar to those of most other zinc metalloproteases. This characteristic is an important element in shaping the substrate specificity of botox A.

Key words: Botulinum neurotoxin; Inhibitor; Protease

1. Introduction

The anaerobic, spore-forming bacteria *Clostridium botulinum* and *Clostridium tetani* produce the most potent neurotoxins known [1]. To date, one serotype of tetanus neurotoxin and seven different serotypes of botulinum neurotoxin (botox), types A–G, have been described [2,3]. The botulinum neurotoxins inhibit the release of acetylcholine from presynaptic nerve terminals at neuromuscular junctions, causing flaccid paralysis, while tetanus neurotoxin undergoes retrograde transport to the central nervous system, where it causes spastic paralysis by preventing transmitter release from inhibitory neurons [2].

The clostridial neurotoxins share a common molecular architecture, in that each is first synthesized as a single polypeptide of 150 kDa, which is then cleaved by endogenous protease(s) to give the dichain structure consisting of a light chain (50 kDa, containing the original N-terminus) linked to a heavy chain (100 kDa) by a single disulfide bond. Sites for receptor binding, transport, and primary immunological determinants are thought to be located on the heavy chains [4,5]. The light chains are zinc metalloproteases, highly specific for certain neuronal proteins. Hydrolysis of these proteins, catalyzed by botox or tetanus neurotoxin light chains, blocks neurotransmitter release [6–9].

Human botulism is a relatively rare occurrence. Nonetheless, there is great interest in research on the botulinum neurotoxins, not only because they are useful probes of neuromuscular function [10], but also because they have proven to be remarkably effective drugs for treating a wide variety of muscle dysfunctions in humans [11,12]. Therefore, elucidation of the catalytic properties of the botulinum neurotoxins and the development of inhibitors to reverse or modulate toxicity are important issues. Toward these goals, our report describes the first specific, competitive inhibitors of botox A proteolytic activity, and provides information on mechanisms that form the basis for the unique substrate specificity of botox A.

2. Materials and methods

2.1. Materials

Botox A was purchased from List Biological Laboratories, Campbell, CA, and from the Food Research Institute, Madison, WI. In both cases, the product was exclusively the dichain form of the toxin, and appeared to be of high purity (>95%), as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. For peptide synthesis, derivatives of D-amino acids were purchased from Bachem, King of Prussia, PA. All other reagents and chemicals were from Perkin Elmer-Applied Biosystems, Foster City, CA.

2.2. Peptide synthesis

The peptide synthesizer was a Model 431A from Perkin Elmer-Applied Biosystems, Foster City, CA. We used Fmoc chemistry and protocols supplied by the manufacturer. Purification was by reverse-phase liquid chromatography. All peptides were N-terminal acetylated, and had carboxamide as the C-terminus. Structures were confirmed by mass spectrometry, and by sequencing non-acetylated aliquots on a Model 494 protein sequencer (Perkin Elmer-Applied Biosystems, Foster City, CA). Each amino acid was the usual L form, unless specified as the D enantiomer.

2.3. Assay of botox A proteolytic activity

Initial hydrolysis rates were determined as described previously [13,14]. Briefly, assays contained 20 mM HEPES buffer, pH 7.3, 5 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.20 mM $ZnCl_2$, and 1 μg/ml botox A (approximately 7 nM). The substrate peptide consisted of residues 187–203 of SNAP-25 [13–15]. The sequence of this peptide was: (*N*(α)-acetyl)-SNKTRIDEANQRATKML-(carboxamide).

Botox A catalyzed the hydrolysis of this peptide between residues 11 (glutamine) and 12 (arginine), corresponding to residues 197 and 198 of SNAP-25 [13]. Substrate concentrations were 0.7–1.0 mM.

2.4. Peptide nomenclature

For the 17-residue peptides shown in Table 1, their nomenclature is based on the SNAP-25 substrate peptide. For example, in peptide E8C, the glutamic acid residue that normally occupies position 8 in the substrate is replaced by cysteine. For the short peptides, it was

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Abbreviations: Botox, *Clostridium botulinum* neurotoxin (the subsequent capital letter indicates the serotype)

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Table 1
Seventeen-residue substrates and inhibitors of type A botulinum neurotoxin

Peptide	Partial sequence							K_m or K_i (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (nM^{-1} s^{-1})
	P_4 8	P_3 9	P_2 10	P_1 11	P_1' 12	P_2' 13	P_3' 14			
SNAP	E	A	N	Q	R	A	T	1.7	47	28
E8C	C	A	N	Q	R	A	T	0.59	23	38
A9C	E	C	N	Q	R	A	T	1.5	7.2	4.8
N10C	E	A	C	Q	R	A	T	0.22		
N10D-C	E	A	D-C	Q	R	A	T	0.65		
Q11C	E	A	N	C	R	A	T	0.11		
Q11D-C	E	A	N	D-C	R	A	T	0.0040		
R12C	E	A	N	Q	C	A	T	2.0		
A13C	E	A	N	Q	R	C	T	0.40	4.6	11
T14C	E	A	N	Q	R	A	C	0.89	31	35

[a]Kinetic constants for the SNAP-25 substrate peptide, shown here for comparative purposes, are taken from [14].

more convenient to assign each a number, in order of listing in Table 2.

2.5. Determinations of kinetic constants

Kinetic constants were obtained from plots of initial rate vs. seven different substrate concentrations in the presence and absence of inhibitor, and from Dixon plots [16] using fixed substrate and seven different concentrations of inhibitor. Results were calculated with non-linear regression analyses using the program Enzfitter (Biosoft, Cambridge, UK). In all cases, values were determined in triplicate and averaged to give the reported result. Standard deviations were always less than ±20%. In the text, mention is also made of estimated K_i values for certain weak inhibitors. These have not yet been ascertained to the degree of accuracy described above, but are nonetheless useful for comparative purposes.

3. Results and discussion

3.1. Characteristics of the inhibition

Peptides classified as effective inhibitors were not hydrolyzed in our assay, and had binding affinities higher than that of the native-sequence substrate peptide. The possibility that some inhibitors might be slowly hydrolyzed under more extreme conditions does not appreciably affect the findings described in this report.

Each of the inhibitors described herein displayed competitive kinetics because they increased the apparent K_m of the substrate, but had little or no effect on k_{cat} [16]. Preincubating botox A with an inhibitor for up to 30 min before adding substrate did not affect the extent of inhibition. Furthermore, dilution of the inhibitor during the assay, while maintaining substrate concentration, gave an immediate increase in reaction rate. These findings show that equilibrium binding between inhibitor and enzyme was achieved during the assay and was reversible. Finally, no significant changes in kinetic constants were found when the concentration of zinc was varied between 0.05 and 0.3 mM.

3.2. Substrates and inhibitors of botox A proteolytic activity

Because it is a zinc metalloprotease, specific inhibition of botox A enzymatic activity might result from incorporation of a sulfhydryl group into the substrate as a potential zinc-binding ligand. Sulfhydryl compounds such as 2-mercaptoethanol, dithiothreitol, and cysteine inhibited botox A, but in a non-competitive manner and only at relatively high concentrations (>10 mM; data not shown). Not only the presence of a sulfhydryl group but also its placement with respect to other structural elements are equally critical factors that influence the binding affinities of metalloprotease inhibitors [17]. Therefore, we synthesized a series of botox A substrate analogs, with cysteine substituted for the native-sequence residues at the P_4 through P_3' sites. At some locations, we also tested D-cysteine. The structures and kinetic constants are shown in Table 1. The tested peptides can be categorized as either substrates, or inhibitors, or as neither a substrate nor an effective inhibitor.

In the first category, substrate analogs with cysteine at the P_4, P_3, P_2', or P_3' sites were hydrolyzed by botox A. Compared to the native-sequence substrate, peptides E8C and T14C had lower K_m values but similar catalytic constants. In contrast, peptides A9C and A13C were hydrolyzed at relatively slow rates. However, this was not the result of weak binding to botox A, because the K_m of A9C was essentially the same as that of the SNAP-25 substrate peptide, while that for A13C was significantly lower. Overall, there was no correlation between binding affinity and rate of hydrolysis, suggesting that substrate discrimination at the S_4, S_3, S_2', and S_3' binding subsites of botox A occurs at the catalytic step.

In the second category, replacing the P_2 asparagine with cysteine gave an inhibitor, N10C, that bound more tightly to botox A than either the substrate or the corresponding alanine analog described in our earlier work (14). However, using D-cysteine proved less effective at this location, giving an inhibitor with a lower binding affinity, compared to the L enantiomer.

When P_1 glutamine in the substrate was replaced with cysteine, a difference in effectiveness between the L and D enantiomers was again found, but this time the situation was op-

Table 2
Short peptide inhibitors of botox A proteolytic activity

Peptide	Sequence								K_i (nM)
	P_2	P_1	P_1'	P_2'					
1	C	Q	R	A	T	K	M	L	0.19
2	D-C	Q	R	A	T	K	M	L	0.14
3		C	R	A	T	K	M	L	0.0019
4		D-C	R	A	T	K	M	L	0.0018
5	N	C	R	A	T	K	M	L	0.50
6	A	C	R	A	T	K	M	L	0.15
7	N	D-C	R	A	T	K	M	L	0.15
8	A	D-C	R	A	T	K	M	L	0.026
9	C	D-C	R	A	T	K	M	L	0.11
10		C	D-R	A	T	K	M	L	0.41

posite to that described above for the P_2 site. While peptide Q11C was a good inhibitor, Q11D-C was significantly better, with a K_i of 4.0 μM, more than 10-fold lower than Q11C. This was not simply the result of placing an amino acid with the D configuration at the P_1 site, because we found that peptide Q11D-Q was a relatively weak inhibitor (estimated $K_i > 2$ mM).

The results described above show that 17-residue substrate analogs with L-cysteine at the P_2 site or with D-cysteine at the P_1 site were relatively good inhibitors, particularly the latter peptide. In contrast, replacing the P_1' arginine with cysteine yielded a peptide that was neither a substrate nor a good inhibitor. The K_i value, 2.0 mM, indicated relatively weak binding to botox A.

3.3. Short peptide inhibitors of botox A proteolytic activity.

Based on the information in Table 1, it was clear that further development of peptides with potential zinc-binding ligands in the P_1 and/or P_2 positions were most likely to afford good inhibition of botox A proteolytic activity. Peptides with cysteine at the other tested locations (i.e. E8C or A13C) might also function as inhibitors of the hydrolysis of the native-sequence substrate, but because they are also substrates, they would be consumed during the reaction. Therefore we focused on the P_1 and P_2 sites, and investigated the effects of eliminating most of the other residues from the N-terminal area of the substrate. The results are shown in Table 2. None of the peptides was hydrolyzed by botox A under our assay conditions.

Peptides 1 and 2 in Table 2 are truncated versions of N10C and N10D-C. The K_i values for peptides 1 and 2 were lower than those for the corresponding full-length inhibitors. Furthermore, using L- or D-cysteine proved to be almost equally effective, in contrast to the situation for N10C and N10D-C, where the former had a lower K_i than the latter. Therefore, eliminating the first nine residues not only enhanced inhibitor binding, but also eliminated the difference in binding seen with the two cysteine enantiomers in the full-length peptides.

A similar lack of discrimination between the L or D enantiomers was found when cysteine was placed at the P_1 position, and all 10 residues on the N-terminal side were eliminated (peptides 3 and 4 in Table 2). K_i values for peptides 3 and 4 were identical, and were the lowest of any of the tested inhibitors. Furthermore, as noted above for peptides 1 and 2, placing cysteine at the N-terminus eliminated the difference in inhibition observed in the 17-residue substrate analog peptides, when either L- or D-cysteine was present at the P_1 site (compare K_i values of peptides Q11C and Q11D-C in Table 1, with 3 and 4 in Table 2).

Several other peptides, not shown in Table 2, were synthesized to test the specific contribution of the P_1 sulfhydryl group to the binding affinity of a short-peptide inhibitor. In the first of these, the P_1 cysteine of peptide 3 was replaced with aspartic acid, also a potential zinc ligand. This inhibitor had a K_i of 0.60 mM, indicating substantially lower binding affinity with respect to peptide 3. Similarly, peptides with glutamine, serine or alanine as the P_1 substituent were even less effective inhibitors, with K_i values of 2.5, 1.4, and 2.5 mM, respectively.

The S_1 subsite of botox A resembles that of many other zinc metalloproteases, including botox B, in that several different side chains can be accommodated without loss of cleavage [14,18]. However, the findings summarized in Tables 1 and 2 show that substituting cysteine for the usual P_1 substrate residue (glutamine) produced peptides that were not hydrolyzed by botox A under our assay conditions, but were good inhibitors, with K_i values of 2–4 μM. Their greatly increased binding affinity, compared to the native sequence substrate, might result from more favorable binding interactions at the S_1 subsite of the toxin, and/or from binding of the catalytically important zinc ion by the sulfhydryl group of cysteine. Two lines of evidence strongly favor the latter possibility. First, the nearly 400-fold increase in binding affinity obtained by changing the usual P_1 glutamine to D-cysteine is unlikely to result solely from tighter binding to the S_1 subsite, because it is clear that the S_1 subsite of botox A is relatively non-specific with respect to binding, and changes of this magnitude were not seen with several other P_1 residue substitutions [14]. Second, it is equally clear that the sulfhydryl group of cysteine, a potential zinc ligand, is the major contributor to the binding affinities of these inhibitors, because replacement or elimination of the sulfhydryl group yielded peptides with considerably higher K_i values. Based on these observations, we propose that the strong binding of peptides with P_1 cysteine is due mainly to binding of the active-site zinc ion by the side-chain sulfhydryl group and not merely from binding of cysteine to the S_1 subsite of the toxin. Crystallographic studies on other metalloproteases, complexed with inhibitors containing P_1 zinc ligands, consistently show that high inhibitor affinity is derived mainly from binding to the active site zinc [17,19].

Peptides 5–9 in Table 2 demonstrate the effects, on K_i values, of placing a residue on the N-terminal side of the P_1 cysteine. Surprisingly, adding asparagine (the usual P_2 substituent in the substrate), alanine, or cysteine led to markedly lower binding affinities, compared to peptides 3 and 4. However, this effect was less pronounced when D-cysteine was the P_1 residue. We saw the highest increase in K_i, relative to peptides 3 and 4, when P_1 was L-cysteine and P_2 was asparagine (peptide 5). Finally, the incorporation of a second cysteine residue (peptide 9) did not result in enhanced binding affinity but had the opposite effect, and to a greater degree than did addition of alanine, when P_1 was D-cysteine. Apparently the sulfhydryl group of cysteine must be able to rotate about the α carbon into a particular orientation to bind the active-site zinc. When rotational freedom is restricted by the presence of other residues on the amino-terminal side of the P_1 site, the D configuration at the α carbon of cysteine presents the sulfhydryl group in a more favorable orientation for zinc binding, compared to the L enantiomer.

Placing cysteine at the P_2 substrate site yielded inhibitors with binding affinities lower than those of P_1 cysteine peptides, but higher than that of the substrate. In an earlier report, we found that substituting alanine or glutamine for the native-sequence P_2 asparagine, relatively conservative changes, caused large decreases in the rate of botox-A catalyzed hydrolysis but had little effect on substrate binding [14]. Clearly, the S_2 subsite of botox A displays a strong preference for the side chain of asparagine with respect to catalysis, but not for the initial binding step.

3.4. Specificity of botox A S_1' binding subsite for arginine

Peptide 10, the last entry in Table 2, illustrates the effect of replacing the P_1' arginine of peptide 3 with the corresponding D enantiomer. This change resulted in a 200-fold increase in

K_i, relative to peptide 3, indicating substantially lower binding affinity. In addition, we tested the importance of having arginine, in particular, at this location by replacing it with alanine and lysine (not shown in Table 2). The latter were relatively weak inhibitors, with estimated K_i values of 1–3 mM.

X-ray crystallography and other structural studies on many other zinc metalloproteases have consistently revealed a common feature: the S_1' binding subsites are deep, hydrophobic, and inaccessible to solvent [17,20]. In sharp contrast, we found a markedly different situation for the S_1' subsite of botox A. In all cases, substituting the native-sequence P_1' L-arginine with a wide range of other residues (including D-arginine and lysine) in substrates and inhibitors of botox A ([14], and this report) resulted in a loss of functionality. Our data show that this is due to the relatively low binding affinities of these peptides. Therefore, the strong requirement for L-arginine as the P_1' residue in substrates and inhibitors of botox A suggests that its S_1' binding subsite is hydrophilic and solvent-accessible. Furthermore, this distinction is likely to remain true for botox A even when it is compared to the other clostridial neurotoxins, including tetanus toxin, where none of these zinc metalloproteases have arginine as the P_1' residue in their physiological substrates. Indeed, only botox F has a hydrophilic residue, lysine, at that site in its substrate, synaptobrevin [3]. The other P_1' substituents range in decreasing order of hydrophobicity from isoleucine (botox E), to leucine (botox D), to phenylalanine (botox B and tetanus toxin), and finally to alanine (botox C and G) [3]. These observations suggest that, with the notable exception of botox A, the S_1' subsites of the other clostridial neurotoxins are typical of most zinc metalloproteases.

The weak binding of substrate analogs that have residues other than L-arginine as the P_1' substituent indicates that for botox A, substrate discrimination at the S_1' subsite occurs primarily at the initial binding step. Furthermore, we showed that amino acids at substrate sites *other* than P_1' could be replaced without loss of binding and/or functionality ([14], and this report). These findings suggest that the requirement for L-arginine as the P_1' residue, highly unusual among zinc metalloproteases, is the predominant element in shaping the substrate specificity of botox A. Furthermore, this requirement must be taken into account when designing botox A substrates and inhibitors.

Each of the clostridial neurotoxins requires a relatively large polypeptide substrate for efficient catalysis [3]. This property implies, at least superficially, that peptide-based inhibitors must also be of similarly high molecular weight. However, we show that short peptides (e.g. peptides 3 and 4 in Table 2) can bind to botox A with affinities considerably higher than that of the substrate peptide. This is explained by our findings that with the exception of the S_1' binding site, substrate discrimination by botox A occurs primarily at the catalytic stage and not at formation of the initial Michaelis-Menten complex ([14], and this report). Therefore, non-functionality as a substrate or inhibitor does not necessarily reflect weak binding, and short peptides can be good inhibitors of botox A if they contain two critical elements: a P_1 sulfhydryl group able to bind the active-site zinc, and a P_1' arginine to bind in the highly-specific S_1' subsite.

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