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Crystal Structure of *Clostridium botulinum* Neurotoxin Serotype B

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Introduction

The toxigenic strains of *Clostridium botulinum* produce seven serologically distinct types of neurotoxins labeled A - G (EC 3.4.24.69), while *Clostridium tetani* produces tetanus neurotoxin (EC 3.4.24.68). Botulinum and tetanus neurotoxins (BoNTs and TeNT) are produced as single inactive chains of molecular mass of approximately 150 kDa. Most of these neurotoxins are released after being cleaved into two chains, a heavy chain (H) of 100 kDa and a light chain (L) of 50 kDa held together by an interchain disulfide bond, by tissue proteinases. BoNT/E is released as a single chain but cleaved by host proteinases [1]. *Clostridium botulinum* neurotoxins are extremely poisonous proteins with their LD_{50} for humans in the range of 0.1 - 1 ng kg^{-1} [2]. Botulinum neurotoxins are responsible for neuroparalytic syndromes of botulism characterized by serious neurological disorders and flaccid paralysis. BoNTs block the release of acetylcholine at the neuromuscular junction causing flaccid paralysis while TeNT blocks the release of neurotransmitters like glycine and γ-aminobutyric acid (GABA) in the inhibitory interneurons of the spinal cord resulting in spastic paralysis. In spite of different clinical symptoms, their aetiological agents intoxicate neuronal cells in the same way and these toxins have similar structural organization [3].

Both BoNTs and TeNT are classified as AB (Activating - Binding) proteins since they are characterized by similar functional and domain structures. In the case of neurotoxins the heavy chain forms the B (binding) domain while the L chain serves as the A (activating domain) [4]. The H chain can be cleaved into two chains by papain digestion; the C-terminal chain, H_C, is the binding domain and the N-terminal domain, H_N, the translocation domain. This three-domain organization is similar to that of diphtheria toxin (DT) and *Pseudomonas aeriginosa* exotoxin A (ETA) [5, 6]. However, the effect of these toxins on target cells is different. While in most cases toxins cause cell death, only the exocytosis is blocked by neurotoxins [7]. The structural motif of these toxins is different from other AB proteins like Cholera toxin where the B domain comprises an oligomer [8, 9]. Even though the general intoxication may be similar the details are different, with each class having a specific mode. The

intoxication by neurotoxins is proposed to be a four-step process [7, 10-12]: 1. cell binding, 2. internalization, 3. translocation into cytosol and 4. enzymatic modification of a cytosolic target.

These neurotoxins have been classified as zinc endopeptidases because of the presence of a conserved zinc binding motif, HExxH, approximately in the middle of the light chian [13, 14]. The inhibition of exocytosis by neurotoxins in cytosol has been identified as a zinc-dependent specific proteolysis of components in the neuroexocytosis apparatus. They act specifically on protein components of the same neuroexocytosis apparatus present in cytosol. Each neurotoxin attacks a specific target component: BoNT/B, D, F and G specifically cleave the vesicle-associated membrane protein (VAMP, also called synaptobrevin); BoNT/A and E cleave a synaptosomal associated protein of 25 kDa (SNAP-25) by specific hydrolysis, and BoNT/C cleaves syntaxin [15-20]. Söllner *et al.* have shown that these proteins together form part of a complex responsible for mediating vesicle docking and fusion [21]. The importance of Zn in the catalysis has been established by the fact that chelation of Zn or use of zinc inhibitors blocks this intoxication process. BoNT/A is being used and approved by the Food and Drug Administration to treat various neuromuscular disorders like strabismus, torticollis and blepharospasm because of it specificity in inhibiting neurotransmitter release at neuromuscular junctions [22]. However, for therapeutics, botulinum neurotoxin in complex with their associated proteins (NAPs) is being used. Recently, the use of BoNT/B for similar purposes is being investigated.

In spite of the availability of a large body of chemical, biological, and pathogenic information for these neurotoxins, the most vital information, *viz.*, three-dimensional structures, has started to appear only recently. The three-dimensional structures of BoNT/A [23], BoNT/B [24], BoNT/B light chain [25], and the C fragment of TeNT [26] have helped us understand the structure-function relationships in clostridial neurotoxins. Here we present the crystal structure of *C. botulinum* neurotoxin serotype B. Since these neurotoxins bind first to the large negatively-charged surface of the presynaptic membrane which consists of polysialogangliosides and other acidic lipids [10], the crystal structure of BoNT/B in complex with sialyllactose which partly mimics the sugar group of a ganglioside is also presented. These structures have helped us to map the active site and binding site.

Description of the structure

The BoNT/B molecule has three distinct structural domains, each one corresponding to one of the three steps of the mechanism of toxification, *viz.*, binding, translocation and catalytic activity. They are arranged sequentially in a linear fashion with the translocation domain in the middle. Accordingly, there are no interactions between the binding and catalytic domains. The catalytic domain has a compact globular structure and belongs to the α/β classification. The translocation domain is mainly α helical. The binding domain is made up of two sub-domains (Fig. 1). The sequence of BoNT/B along with its secondary structural elements is presented in Table 1. Even though the primary sequence starts with the catalytic domain, the domain structures are discussed below starting from the C-terminal end in the sequential mode of action of the toxin.

Binding domain: The binding domain comprises two distinct domains corresponding to the N- and C-terminal halves (B_N and B_C). B_N contains two seven-stranded anti-parallel β-sheets sandwiched together and resemble a jelly roll motif. This domain is very similar to the fold observed in legume lectins. Helix α24 runs almost parallel to the length of this β sandwich and connects B_N to B_C, and α22 is almost perpendicular to the sandwich and connects the binding domain to the translocation domain. One of the β-sheets has a concave surface and is exposed to the solvent region while the other with a convex surface is packed against the translocation domain. In addition to a six-stranded β-barrel, the second sub-domain (B_C) contains a β-trefoil motif formed by β strands β47, β48, β52, β55, β58 and β59. The β-trefoil is at the bottom of the molecule and is exposed to the solvent while the six-stranded β-barrel is closer to the β-sandwich of the B_N domain. The side chains from the hairpin bends at the bottom of the β-sandwich make van der Waals and hydrogen bond contacts with the B_C domain. The whole binding domain is tilted away from the central translocation domain and makes minimal interaction with it. The structure of the binding domain is very similar to the C-fragment of tetanus toxin (TeNT-C) and the binding domain of BoNT/A [23, 26], except for the conformation of long loops connecting β strands. Also, the interaction between the sub-domains in BoNT/B is weaker than in BoNT/A or TeNT-C. Even

though the overall sequence homology is poor for all clostridial neurotoxins in the C-terminal half of the binding domain, it was suggested that they would all adopt the same fold with the differences in sequences accounted for by the extended loop regions [26]. The interface between B_N and B_C domains is filled with aromatic side chains.

Translocation domain: Once toxins are bound to the membranes, a temperature- and energy-dependent process internalizes them. Neurotoxins have to cross the hydrophobic barrier of the vesicle membrane to attack their targets, the components of the neuroexocytosis apparatus, residing in the cytosol. This is common to all bacterial toxins with intracellular targets and is the least understood step in the process. It has been proposed that the acidification of the vesicle lumen by a proton-pumping ATPase leads to conformational changes in the toxin. The acidic conformation then exposes a hydrophobic area of the toxin molecule, creates an ion channel in the membrane and inserts the L chain into cytosol [2, 4, 10]. However, it is still not clear whether the L chain first unfolds to enter the ion channel or pore and then refolds within the cytosol.

The translocation domain consists of two long α-helical region, each about 105 Å in length forming coiled-coil helices. Because of this coiled-coil nature, the helices have kinks or breaks in the helical structure and hence the two long helices are split into four helices each of length approximately 50 Å. The core of the translocation domain consists of a four-helical bundle (α12, α13, α17 and α19) on one end and a three-helical bundle (α16, α20 and α21) on the other (Fig. 1). The three-helical bundle resembles the translocation region in other toxins like Colicin Ia [27]. The putative translocation membrane region as predicted by the TMAP program lies in the region 639 - 667 [28]. This region adopts an extended conformation and spans the molecule from the top to the middle of the molecule. The only α helix in this region is from 638 - 645 which is in the middle part of the molecule. Channel-forming regions in the heavy chain have also been predicted on the basis of hydrophobic moments [29]. This region and the region immediately preceding it have about ten charged residues, most of which form strong hydrogen bonds through their side chains to adjacent residues either in the binding domain

or in the translocation domain. It is apparent from this structure that this region is flexible and can change its conformation under favorable conditions. At low acidic pH, these side chains are expected to be protonated, thereby disturbing hydrogen bonding interactions which would further allow changes in conformation. Further, if sequence upstream from 600 is considered (toward N terminus), there are 5 more negatively-charged residues which will also be affected by a change in pH. The structure of the region from 550 to 672 has both flexible regions and helices. The flexible regions should help in accommodating any change in the conformation.

In both BoNT/B and BoNT/A determined at pH 6.0 and 7.0, respectively, this region has a very flexible conformation - neither an extended nor a helical conformation. At this stage it is difficult to speculate whether a further conformational change will takes place at lower pH, since structures at lower pH are not known. There is a long loop formed by residues 481-532 that wraps around the catalytic domain and is aptly called the belt. Even though in the primary sequence this loop region is part of the translocation domain, in three dimensions it forms part of the catalytic domain. The conformation and position of the loop seem to play an important role in the catalytic action of the toxin.

Catalytic domain: In all BoNTs and TeNT, the L chain, responsible for the catalytic activity, contains a zinc-binding HExxH motif in the middle of the chain. Accordingly, the L chain has been identified as a zinc endopeptidase. The L chain is released into the cytosol and attacks a specific target. Physicochemical measurements have shown that clostridial neurotoxins contain one zinc atom per molecule of toxin (except for BoNT/C which contains two zinc atoms [30]), bound to the L chain. As suggested by chemical modification and mutagenetic studies, two histidines and one glutamic acid provide ligands to zinc, similar to that in thermolysin [13, 31]. The fourth coordination is provided by a water molecule and is responsible for the hydrolysis of a peptide bond of the substrates.

The catalytic domain comprises a mixture of α helices and β sheets and strands (Fig. 1). This forms a compact globular-like protein structure. The active site zinc is found deep inside a large open cavity which has a high negative electrostatic potential. This zinc is coordinated by two histidine residues (229 and 233) and a glutamate residue (267). In this structure the fourth coordination is

supplied by an oxygen atom of a sulfate ion which is present in this structure. The protein was supplied as a precipitate in 60% ammonium sulfate which was removed by dialysis before the crystallization trials. It is proposed that the water molecule bound to the zinc ion was displaced by the sulfate ion when the protein was precipitated and remains tightly bound even after dialysis. However, since phosphate buffer was used throughout the protein purification, we cannot completely rule out the possibility of a phosphate ion in this position from crystallographic studies alone. The presence of a sulfate or phosphate ion at the catalytic site has also been reported in the crystal structures of diphtheria repressor, carbonic anhydrase IV, and *Streptomyces griseus* aminopeptidase [32-34]. The presence of a sulfate or phosphate ion also raises some interesting possibilities - could they be used to inhibit the toxin? We have also determined the crystal structure of BoNT/B where the sulfate ion was completely removed before crystallization. In this structure the fourth coordination for the zinc ion is through a water molecule which is believed to be nucleophilic.

Figure 2 shows the zinc coordination in BoNT/B. Zinc is coordinated by protein atoms Nε2 of His 229, Nε2 of His 233, and Oε1 of Glu 267. The fourth coordination is O of the nucleophilic water molecule. The coordination distances are 2.25, 2.20, 2.28 and 2.62 Å, respectively. This arrangement is very similar to that in thermolysin [31], where the HExxH motif is formed by residues 142 to 146. However, the distance between the nucleophilic water and the catalytic zinc ion in BoNT/B is much larger than in thermolysin (1.88 Å). Also, in thermolysin the nucleophilic water has two hydrogen bonds to Oε1 and Oε2 of residue Glu 143 (corresponding to Glu 230 in BoNT/B). It is argued that the nucleophilicity of this water is enhanced because of these hydrogen bonds in addition to the oxygen being coordinated to the zinc ion. A similar mechanism is also proposed for BoNT/A [35]. In BoNT/B the situation is different, at least in the native structure. The coordination distance of the nucleophilic water to the zinc ion is 2.62 Å, much larger than in thermolysin. Also, the distance between the nucleophilic water and Oε1 or Oε2 of Glu 230 is more than 3.6 Å, outside the hydrogen bonding distance. Under these circumstances it is not clear how the water could be in an activated state.

However, these distances are reported to be 2.2 and 2.4 Å, respectively in BoNT/B-LC [25]. The difference between BoNT/B and BoNT/B-LC could be due to the differing pH. Maybe the mode of catalytic action is different in BoNT/B than in thermolysin. There is also very little similarity between the secondary structures of the two proteins in this site. The active site cavity is 15 x 24 x 25 $Å^3$ in dimension with a very wide mouth and an opening on the other side which is partly shielded by Phe 271. Modeling studies and inhibitor studies have shown that this cavity is big enough to accommodate medium sized organic molecules. Our recent results (unpublished) with inhibitor binding studies support this hypothesis. Modeling studies based on our polyalanine model of the catalytic domain with 7-N-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin have shown that the cavity is big enough to accommodate this molecule (M. Adler, Meeting of the Interagency Botulism Research Coordinating Committee, Orlando, 1999).

Comparison of BoNT/A and B

Overall the three-dimensional structures of BoNT/A and B are similar. Individual domains fold similarly and the C-alpha chains superpose with a root mean square deviation (rmsd) of less than 2.0 Å. The rmsd's between the binding, translocation, and the catalytic domains of BoNT/A and B are 1.43, 1.56, and 1.43 Å, respectively for about 80 - 85% matched residues. However, when the molecules are considered as a whole, only 49% of the residues match with a rmsd of 2.06 Å, indicating that the association of the three domains may be slightly different in the two molecules. Especially, the orientation of the binding domain with respect to the translocation domain is different. In BoNT/B the binding domain makes a smaller tilt to the translocation domain than in BoNT/A. Also the orientation of the belt region and its position with respect to the zinc site is different. While the catalytic and translocation domains (except for the belt region) superpose fairly well, the binding domains, especially the C-terminal halves, do not. These differences may be due to the fact that the pH of crystallization was different.

In BoNT/A there is a disulfide bridge between Cys 1234 and Cys 1279 in the C terminal domain (B_C). This disulfide bond is absent in BoNT/B even though sequence alignment shows cysteines aligned at these positions [36]. The distance between the two Sγ's is about 10 Å. Of the ten cysteine residues present in the molecule only Cys 436 and Cys 445 form a disulfide bond. All others except Cys 70 have an accessible surface area of zero suggesting they are all buried. Cys 70 has a non-zero value and it is exposed to the solvent as predicted from biochemical studies [37].

The role of the belt region

The loop region 481-532 of the translocation domain that wraps around the catalytic domain seems to play a significant role in botulinum neurotoxins. The substrates for all neurotoxins are long polypeptides with separate binding and cleavage sites. The separation between these two sites is speculated to play a role in the specificity of the targets. We speculate that either the substrates take the position of the belt in the separated light chain or perhaps the belt region helps in forming interactions with the substrates, if the L chain is not separated. Once they are bound to the toxin, the cleavage site may protrude inside the active site cavity. In BoNT/A the active site is shielded from the environment by the belt region [23]. But it has a different disposition in BoNT/B than in BoNT/A (Fig. 2). In BoNT/A, the cavity is partially covered by the belt on looking down the cavity. This enables the zinc ion to be shielded from the environment. In BoNT/B, the belt region does not shield the zinc ion, thus making it completely accessible to inhibitor or substrate molecules. This difference in orientation of the belt region may be either due to the shorter length of the belt region in BoNT/A than in B and E or because of the different pH values at which BoNT/A and B were crystallized. In any case, this fact presents interesting possibilities in the design of inhibitor molecules for various botulinum neurotoxins. It also suggests that the belt region in type E may be more similar to type B than type A. Interestingly, all residues within a radius of 8 Å from zinc are identical in BoNT/A and B, except for five. However, as the radius increases, differences set in and the belt region gets involved at about 15 Å. This similarity suggests that

the difference in substrate specificity between the two molecules may not be due to the residues in the immediate vicinity of zinc site but may be a long-range effect especially near the mouth of the cavity.

Tyrosine phosphorylation and enhanced catalytic activity

It has been reported that non-receptor tyrosine kinase Src phosphorylates botulinum neurotoxins A, B, and E and tetanus neurotoxin. Tyrosine phosphorylation of serotypes A and E of *Clostridium botulinum* neurotoxin increases their catalytic activity in addition to increasing their thermal stability. The effect is also reversed when the toxins are dephosphorylated. This led to the postulation that the toxin may exist in phosphorylated form inside the neuron [38]. Tyr 372 which is in the vicinity of the active site is believed to be a proton donor to stabilize the leaving group after the scissile bond is cleaved [25, 35, 39]. However, in the case of BoNT/B, the distance between the zinc and the OH of Tyr 372 is more than 6.6 Å and the orientation seems not to be conducive for a proton donation. If Tyr 372 is phosphorylated, in addition to its pK value changing from phenolic to phosphoric value, the required OH for donating a proton comes much closer to zinc. A hypothetical model with Tyr 372 phosphorylated brings the OH group of the phosphate group within 4.5 Å (figure not shown). The enhancement of the catalytic activity may be due to this shorter distance.

Reduced form of BoNT/B

Reduction of the inter chain disulfide is a prerequisite for toxic activity and the rate-limiting step in toxicity [40]. The absence of the toxic activity in the unreduced state suggests that after reduction, the toxin undergoes some structural change in order for the substrate to come closer to the active site which is in a deep cavity. It is also believed that the light chain will separate from the heavy chain to enter the cytosol to attack the targets. However, in a recent structure determination in our laboratory where the protein was reduced with 10 mM DTT and crystallized in the presence of DTT, though the Sγ - Sγ distance is more than 3.1 Å, the two chains stay together in a compact form (unpublished results). Also, there is no significant difference in the main chain fold of the molecule. We believe that reduction of disulfide alone is not enough for the catalytic domain to separate. There could still be an unidentified process required to separate the catalytic domain.

Evidence for ganglioside binding

The first step in the intoxication process is binding of neurotoxins to the neuronal cell. This takes place *via* gangliosides available at the surface of the neuronal cell. However, to explain the high activity of this neurotoxin, a double receptor model - a low-affinity ganglioside and a high-affinity protein receptor - has been proposed. The toxins first bind to the neuronal cells *via* low affinity gangliosides and then move laterally to bind to the high-affinity receptor, a protein. Neurotoxins bind to di- and trisialogangliosides, especially to the 1b series. GD1b and GT1b have been shown to bind effectively to the neurotoxins. The structure of BoNT/B in complex with sialyllactose defines the binding site of sialyllactose to the C-fragment of BoNT/B. Sialyllactose partly mimics one branch of the sugar moiety of the GT1b and hence this structure provides a model for interaction of ganglioside to the neurotoxin. The results from our studies correlate with the mutagenic and biochemical studies.

The C-terminal half of the binding domain preserves the ganglioside binding property of the neurotoxin [41]. In the case of TeNT, the C-terminal residues (1281-1314) are sufficient for ganglioside binding and photoaffinity labeling occurs predominantly at His 1292 [42]. Also, in BoNT/A tryptophan fluorescent quenching is accompanied by ganglioside binding, suggesting that tryptophans are part of the ganglioside binding site. Sequence comparison of BoNTs and TeNT reveals that there are a number of conserved tryptophan residues at the C-terminus of all neurotoxins [43]. In TeNT, residues 1235-1294, containing tryptophan at 1288, are particularly critical for binding. This tryptophan corresponds to Trp 1261 in BoNT/B which is also exposed to solvent, making it more amenable for fluorescent quenching. This tryptophan is exposed in all neurotoxins for which three-dimensional structures are known. In BoNT/B this residue is located just before the N-terminus of α25. In view of all this, it was thought that the ganglioside-binding site may include this tryptophan and residues in this site. However, the crystal structure of the complex reveals significant differences in the interactions predicted by these methods. For example, it was suggested that the two lysines close to His 1292 in TeNT might bind to the negatively-charged carboxylate groups of sialic acids of GT1b [42], which suggests that three lysine residues and one arginine close to Glu 1265 of BoNT/B might bind to the negatively-charged

carboxylate groups of sialic acids of GT1b, by analogy. There is a cleft between Trp 1261 and His 1240 and the sialic acid sits between these two and the sugar moiety makes several hydrogen bonds with the protein molecule. Sialic acid and the galactose make hydrogen-bonding contacts with Glu 1188, Glu 1189, His 1240 and Tyr 1262. Sialic acid also makes hydrophobic contacts with Trp 1261. However, the sialic acid does not make any contact with Lys 1267, Arg 1268, Lys 1269 or Glu 1265, as proposed for TeNT [42]. These residues, though close to Trp 1261 in primary sequence, are spatially far from this binding site. The closest contact the sialic acid makes with a lysine is with Lys 1264 at 6.4 Å. Only the terminal sialic acid and the adjacent galactose are making contacts with the protein molecule while glucose is protruding outside of the protein. Superposition of the three C-fragment models after least squares fit brings His 1252 (BoNT/A), His 1240 (BoNT/B) and His 1270 (TeNT) spatially in the same place. Similarly Glu 1202 (BoNT/A), Glu 1189 (BoNT/B) and Asp 1221 (TeNT) align both in sequence and space. Tryptophan and these residues form the same kind of pocket in all three structures. Since one branch of the sugar moiety of GT1b has a terminal sialic acid with the same 2→3 link with the galactose, we propose that this will provide a model for interaction for all neurotoxins. His 1292, the site of photoaffinity labeling in TeNT, lies on the other side of Trp 1288 relative to His 1240, creating a small pocket. However, preliminary studies with other branched sugar moieties show no density in this region in the difference density map which could accommodate the other sialic acid of GT1b. The site where sialyllactose binds is shown in Figure 1. Sialyllactose and the interacting protein residues form a nice lock and key arrangement.

As discussed above, the structure of the BoNT/B:silalyllactose complex provides a model for the interaction for the sugar moiety of ganglioside GT1b and the binding domain. The residues defining this site are common to all neurotoxins and hence the interaction of ganglioside sugar may be the same in other complexes also. Accordingly, this binding site offers itself a target for inactivated recombinant vaccine development. If the binding of neurotoxin to the cell could be prevented, the toxicity itself could be eliminated. Recombinant vaccine must be superior to the presently available experimental vaccine, a chemically inactivated toxin.

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Figure captions

1. Ribbons representation of BoNT/B molecule. The three functional domains are marked as binding, translocation and catalytic. The active site and its ligands are shown as ball and stick model in the catalytic domain and lie below the plane of the paper. The sialyllactose molecule is also shown and is above the plane of the paper. This figure was generated by MOLSCRIPT [44].
2. The C-alpha trace of the light chains of BoNT/A and BoNT/B are superposed with the belt region shown in worm drawing. Light and dark shades correspond to BoNT/A and BoNT/B, respectively. In BoNT/A, the zinc site is partly covered by the belt region. Zinc and its ligands of BoNT/B are shown in ball and stick model.

References

1. Sathyamurthy V, Dasgupta BR. Separation, purification, partial characterization and comparison of the heavy and light chains of botulinum neurotoxin types A, B and E. J. Biol. Chem. 1985;260:10461-10466.
2. Schiavo G, Rossetto O, Montecucco C. Clostridial neurotoxins as tools to investigate the molecular events of neurotransmitter release. Semin. Cell Biol. 1994;5:221-229.
3. Simpson LL. Molecular Pharmacology of botulinum toxin and tetanus toxin. Annu. Rev. Pharmacol. Toxicol. 1986;26:427-453.
4. Montecucco C, Papini E, Schiavo G. Bacterial protein toxins penetrate cells via a four-step mechanism. FEBS Lett. 1994;346:92-98.
5. Choe S, Bennett MJ, Fujii G, et al. The crystal structure of diphtheria toxin. Nature 1992;357:216-222.

6. Allured VS, Collier RJ, Carroll SF, McKay DB. Structure of exotoxin A of Pseudomonas aeruginosa at 3.0 Angstrom resolution. Proc. Natl. Acad. Sci. USA 1986;83:1320-1324.

7. Schantz EJ, Johnson EA. Properties and use of botulinum toxin and other microbial neurotoxins in medicine. Microbiol. Rev. 1992;56:80-99.

8. Sixma TK, Kalk KH, van Zanten BA, et al. Refined structure of Escherichia Coli heat - labile enterotoxin, a close relative of cholera toxin. J. Mol. Biol. 1993;230:890 - 918.

9. Stein PE, Boodhoo A, Armstrong GD, Cockle SA, Klein MH, Read RJ. The crystal structure of pertussis toxin. Structure 1994;2:45-57.

10. Menestrina G, Schiavo G, Montecucco C. Molecular mechanisms of action of bacterial protein toxins. Mol. Aspects Med. 1994;15:79-193.

11. Montecucco C, Schiavo G. Mechanism of action of tetanus and botulinum neurotoxins. Mol. Microbiol. 1994;13:1-9.

12. Oguma K, Fujinaga Y, Inoue K. Structure and Function of clostridium Botulinum toxins. Microbiol. Immunol. 1995;39:161-168.

13. Schiavo G, Rossetto O, Santucci A, Dasgupta BR, Montecucco C. Botulinum neurotoxins are zinc proteins. J. Biol. Chem. 1992;267:23479-27483.

14. Blasi J, Chapman ER, Link E, et al. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature 1992;365:160-163.

15. Schiavo G, Shone CC, Rossetto O, Alexander FCG, Montecucco C. Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. J. Biol. Chem. 1993;268:11516-11519.

16. Schiavo G, Benfenati F, Poulain B, et al. Tetanus and botulinum-B neurotoxins block neurotransmitter release by a proteolytic cleavage of synaptobrevin. Nature 1992;359:832-835.

17. Blasi J, Chapman ER, Yamasaki S, Binz T, Niemann H, Jahn R. Botulinum neurotoxin C blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. EMBO J. 1993;12:4821-4828.

18. Schiavo G, Santucci A, Dasgupta BR, et al. Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptid bonds. FEBS Lett. 1993;335:99-103.

19. Schiavo G, Rossetto O, Catsicas S, et al. Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D and E. J. Biol. Chem. 1993;268:23784-23787.

20. Schiavo G, Malizio C, Trimble WS, et al. Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. J. Biol. Chem. 1994;269:20213-20216.

21. Söllner T, Whiteheart SW, Brunner M, et al. SNAP receptors implicated in vesicle targeting and fusion. Nature 1993;362:318-324.

22. Johnson EA, Goodnough MC. Preparation and properties of botulinum toxin type A for medical use. In: Tsui JKC, Calne DB, eds. Handbook of dystonia. Newyork: Marcel Dekkar, Inc., 1995:347-365.

23. Lacy DB, Tepp W, Cohen AC, DasGupta BR, Stevens RC. Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nat. Struct. Biol. 1998;5:898-902.

24. Swaminathan S, Eswaramoorthy S. Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. Nat. Struct. Biol. 2000;7:693-699.

25. Hanson MA, Stevens RC. Cocrystal structure of synaptobrevin-II bound to botulinum neurotoxin type B at 2.0 A resolution. Nat. Struct. Biol. 2000;7:687-692.

26. Umland TC, Wingert LM, Swaminathan S, Furey WF, Schmidt JJ, Sax M. Structure of the receptor binding fragment H_C of tetanus neurotoxin. Nat. Struct. Biol. 1997;4:788-792.

27. Weiner M, Freymann D, Ghosh P, Stroud RM. Crystal structure of colicin Ia. Nature 1997;385:461-464.

28. Persson B, Argos P Topology prediction of membrane proteins. Protein Sci. 1996;5:363-371.

29. Lebeda FJ, Olson MA. Structural predictions of the channel-forming region of botulinum neurotoxin heavy chain. Toxicon 1995;33:559-567.

30. Schiavo G, Shone CC, Bennett MK, Scheller RH, Montecucco C. Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. J. Biol. Chem. 1995;270:10566-10570.

31. Holmen MA, Matthews BW. Structure of thermolysin refined at 1.6 A resolution. J. Mol. Biol. 1982;160:623-639.

32. Pohl E, Holmes RK, Hol WGJ. Motion of the DNA-binding domain with respect to the core of the diphtheria toxin repressor (DtxR) revealed in the crystal structures of apo- and holo-DtxR. J. Biol. Chem. 1998;273:22420-22427.

33. Stams T, Nair SK, Okuyama T, Waheed A, Sly WS, Christianson DW. Crystal structure of the secretory form of membrane-associated human carbonic anhydrase IV at 2.8-A resolution. Proc. Natl. Acad. Sci. USA 1996;93:13589-13594.

34. Greenblatt HM, Almog O, Maras B, et al. *Steptomyces griseus* aminopeptidase: X-ray crystallographic structure at 1.75 A resolution. Journal Molecular Biology 1997;265:620-636.

35. Li L, Binze T, Niemann H, Singh BR. Probing the mechanistic role of glutamate residues in the zinc-binding motif of type A botulinum neurotoxin light chain. Biochemistry 2000;39:2399-2405.

36. Antharavally BS, DasGupta BR. Covalent structure of botulinum neurotoxin type B; location of sulfhydryl groups and disulfide bridge and identification of C-termini of light and heavy chains. J. Protein Chem. 1998;17:407-415.

37. Antharavally B, Tepp W, DasGupta BR. Status of Cys residues in the covalent structure of botulinum neurotoxin types A, B, and E. J. Protein Chem. 1998;17:187-196.

38. Ferrer-Montiel AV, Canaves JM, DasGupta BR, Wilson MC, Montal M. Tyrosine phosphorylation modulates the activity of clostridial neurotoxins. J. Biol. Chem. 1996;271:18322-18325.

39. Schiavo G, Matteoli M, Montecucco C. Neurotoxins affecting neuroexocytosis. Physiol. Rev. 2000;80:717-766.

40. de Paiva A, Poulain B, Lawrence GW, Shone CC, Tauc L, Dolly JO. A role for the interchain disulfide or its participating thiols in the internalization of botulinum neurotoxin A revealed by a toxin derivative that binds to ecto-acceptors and inhibits transmitter release intracellularly. J. Biol. Chem. 1993;268:20838-20844.

41. Halpern JL, Loftus A. Characterization of the receptor-binding domain of tetanus toxin. Nature 1993;268:11188-11192.

42. Shapiro RS, Specht CD, Collins BE, Woods AS, Cotter RJ, Schnaar RL. Identification of a ganglioside recognition domain of tetanus toxin using a novel ganglioside photoaffinity ligand. J. Biol. Chem. 1997;272:30380-30386.

43. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673-4680.

44. Kraulis P. MOLSCRIPT: a program to produce both detailed and schematic plots of proteins. J. Appl. Crystallogr. 1991;24:946-950.

Table 1. The sequence of BoNT/B with the secondary structural elements marked below the sequence. E and H represent β strands and α helices as denoted in the line below the secondary structures. G and T represent 3/10 helices and turns, respectively.

```
  1    PVTINNFNYN DPIDNNNIIM MEPPFARGTG RYYKAFKITD RIWIIPERYT
              TT         EEE EE GGGTT     EEEEEETT TEEEE

                          β1                  β2       β3
 51    FGYKPEDFNK SSGIFNRDVC EYYDPDYLNT NDKKNIFLQT MIKLFNRIKS
       TT  GGGG          TT   EE  TTTT   HHHHHHHHHH HHHHHHHHTT

                         β4                  α1
101    KPLGEKLLEM IINGIPYLGD RRVPLEEFNT NIASVTVNKL ISNPGEVERK
        HHHHHHHHH HHH        TT  TTEE     TTTEEEEE EEEEE    E

            α2                      β5             β6
151    KGIFANLIIF GPGPVLNENE TIDIGIQNHF ASREGFGGIM QMKFCPEYVS
       EEEE  EEEE       TT  E EE  EETTEE GGGTT    E EEE    EEE

        β7   β8             β9  β10 β11          β12
201    VFNAAAAAAA AAAFNRRGYF SDPALILMHE LIHVLHGLYG IKVDDLPIVP
       EE  TT   T T       EE   HHHHHHHH HHHHHHHHTT

       β13                 β14         α3
251    NEKKFFMQST DAIQAEELYT FGGQDPSIIT PSTDKSIYDK VLQNFRGIVD
           TT         HHHHHH H TTGGGG   HHHHHHHHHH HHHHHHHHHH

                         α4                  α5
301    RLNKVLVCIS DPNININIYK NKFKDKYKFV EDSEGKYSID VESFDKLYKS
       HHHH  EE    TT  HHHHH HHHHHHTT E E TT  EE   HHHHHHHHHH

            β15             α6       β16      β17       α7
351    LMFGFTETNI AENYKIKTRA SYFSDSLPPV KIKNLLDNEI YTIEEGFNIS
       HHHT  HHHH HHHHT            EEEE EE  TT TTT   TTT T  G

                α8                      β18
401    NKDMEKEYRG QNKAINKQAY EEISKEHLAV YKIQMCKSV- --GICIDVDN
       GGT  GGGGG G TTT GGG  EE  GGG EE  EEEEE        EEEEEG

                          β19      β20 β21          β22
451    EDLFFIADKN SFSDDLSKNE RIEYNTQSNY IENDFPINEL ILDTDLISKI
       GG      GG G   GGG  E E                HHHH H

                         β23                  α9
501    ELPSENTESL TDFNVDVPVY EKQPAIKKIF TDENTIFQYL YSQTFPLDIR
            EE                 EEEEEEEE      HHHHH HTT   TT

           β24                     β25          α10
551    DISLTSSFDD ALLFSNKVYS FFSMDYIKTA NKVVEAGLFA GWVKQIVNDF
         EEE  HHH HHH TTEEE     HHHHHHH T    HHHHH HHHHHHHHHH

       β26  α11       β27       α12          α13
601    VIEANKSNTM DKIADISLIV PYIGLALNVG NETAKGNFEN AFEIAGASIL
       HHHHHGGG    GGGT      TTHHHHH  T TTTT   HHH HHHHHGGGGG

                              α14                 α15
651    LEFIPELLIP VVGAFLLESY INNKNKIIKT IDNALTKRNE KWSDMYGLIV
                     EE      TT       HH HHHHHHHHH HHHHHHHHH

                        β28                  α16
701    AQWLSTVNTQ FYTIKEGMYK ALNYQAQALE EIIKYRYNIY SEKEKSNINI
       HHHHHH HHH HHHHHHHHHH HHHHHHHHHH HHHHHHHHT   HHHHHT

                         α17                      α18
751    DFNDINSKLN EGINQAIDNI NNFINGCSVS YLMKKMIPLA VEKLLDFDNT
        HHHHHHHHH HHHHHHHHHH HHHHHHHHHH HHHHH HHHH HHHHHHHHH

            α19                          α20
801    LKKNLLNYID ENKLYLIGSA EYEKSKVNKA LKTIMPFDLS IYTNDTILIE
       HHHHHHHHH H HHHH TTT TTTHHHHHHH TT      GG GT   HHHHH

                                  α21                 α22
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 851  MFNKYNSEIL NNIILNLRYK DNNLIDLSGY GAKVEVYDGV ELNDKNQFKL
      HHHHHHT GG GG  EEEEE     EEE       EEEE TT  EE TT EEEE
                          β29      β30       β31      β32     β33
 901  TSSANSKIRV TQNQNIIFNS VFLDFSVSFW IRIPKYKNDG IQNYIHNEYT
         TT  EEE E  TT          EEEEEE EE     GGG  HHHHH EEE
              β34                    β35               α23
 951  IINCMKNNSG WKISIRGNRI IWTLIDINGK TKSVFFEYNI REDISEYINR
      EEEEEETTEE EEEEEETTEE EEEEE TT   EEEEEEE           TT
        β36       β37       β38              β39
1001  WFFVTITNNL NNAKIYINGK LESNTDIKDI REVIANGEII FKLDGDIDRT
       EEEEEEE    EEEEEETTE EEEEEE            EEE EEEEE   TT
         β40       β41       β42                β43
1051  QFIWMKYFSI FNTELSQSNI EERYKIQSYS EYLKDFWGNP LMYNKEYYMF
        EEEEEEEE E     HHHH HHHHHHHH       TT     EE  EEEEE
          β44              α24                        β45  β46
1101  NAGNKNSYIK LKKDSPVGEI LTRSKYNQNS KYINYRDLYI GEKFIIRRKS
      ETT TTEEEE E TT   EEE EE                      EEEEE
             β47       β48                          β49
1151  NSASIADDIV RKEDYIYLDF FNLNQEWRVY TYKYFKKEEE KLFLAPISDS
           EE   TT EEEEEE EETTEEEEEE EETT    EE E EEEE
          β50       β51       β52          β53    β54
1201  DEFYNTIQIK EYDEQPTYSC QLLFKKDEES TDEIGLIGIH RFYESGIVFE
      TTEE  EEEE           E EEEEE       EEEEEEEE EEEEE    E
       β55  β56             β57               β58
1251  EYKDYFCISK WYLKEVKRKP YNLKLGCNWQ FIPKDEGWTE
      EEEEEEEEET THHHHTT      TT TT EE EE   TT
        β59        α25              β60
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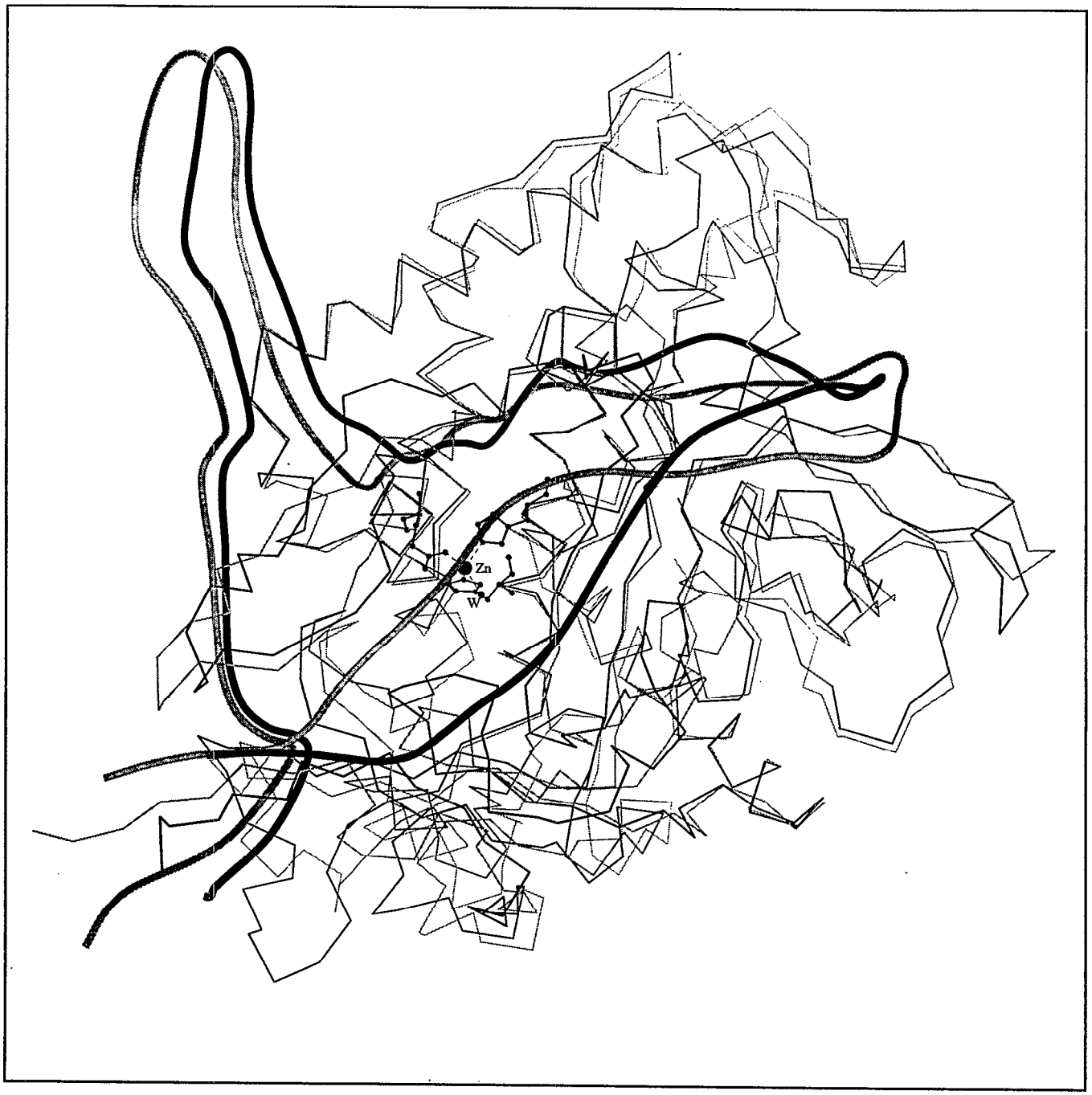
CATALYTIC TRANSLOCATION BINDING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