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CRITICAL ASPECTS OF BACTERIAL PROTEIN TOXINS

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INTRODUCTION

Bacterial protein toxins could be classified as the most dangerous group of agents faced by the human population as well as by other groups of biological systems. Consideration of the potency of toxins makes them dreadful. For example, based on the lowest minimal lethal dose consideration, botulinum neurotoxin produced by the anaerobic bacteria *Clostridium botulinum*, tops the list of known toxins of both proteinaceous and non-proteinaceous nature with a mouse lethal dose of 0.3 ng, which on molar basis is 300, 2 million, 5 million and 100 million fold lower than diphtheria, cholera, cobrotoxin, and sodium cyanide, respectively (Middlebrook, 1989). In addition, there are other important factors contributing to the dangerous nature of bacterial toxins. One such factor is the ubiquitous nature of bacteria that makes toxins' access to an unlimited target hosts easily permissible. Second, the invisible nature of bacteria to naked eye leaves only the option of preventive measures rather than avoidance, unlike in cases of most animal and plant toxins. Lastly, the short duplication time of bacteria provides them with ample opportunities to go through genetic evolution to produce bacterial strains adapted for the adversarial environmental conditions. Such trait perhaps also allows them to develop resistance to antibiotics. Short duplication time also helps bacteria to spread extensively and faster to cover a wide area of target population.

The purpose of toxin production by bacteria is a question that is not easy to settle. A dogmatic view most widely accepted in the absence of any evidence otherwise relates to three main features: First, it is a simple case of parasitism that provides bacteria with a forceful tool to invade its host with a degree of likely success. Second, faced with physiological need of every organism for survival, the toxin provides bacteria with a weapon to survive in adverse conditions of either other bacterial competition such as the case of colicins produced by a strain of *E. coli*, or against the odd of being destroyed by immune system of the host as in the case of most toxins for the animals and humans. Finally, these factors of parasitism and survival lead to the goal of proliferation and reproduction, and the ultimate goal of every living being. Despite the acceptance of the above dogmatic view on the possible

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purpose of the production of toxins, it is still an open question. Could toxins be produced by a sheer chance of genetic alteration or metabolic byproduct? Do the toxins play any critical physiological role(s) in the life of bacterial cell itself? Adequate experimental data are not available to answer these questions; in most cases they have not been attempted. It might be the last frontier of the bacterial protein toxin research, partly because the most pressing issues of bacterial protein toxins relate to the harmful effects of such toxins to other organisms, and most recently to the use of the specific potency of many toxins for therapeutic purposes.

Although bacterial toxins are produced by a variety of bacterial strains varying in morphology and physiology, most bacterial toxins can be classified basically in two categories based on their site of action. One, a group of protein toxins with extracellular target of action. This group includes cytolysins that target plasma membrane as the site of their action. Two, a group of toxins that have intracellular target of action, and includes toxins that range from being cytotoxins such as diphtheria to neurotoxins such as botulinum and tetanus.

Among the toxins acting on the plasma membrane are membrane pore forming hemolysins such as *Staphylococcus aureus* α-toxin (Walker et al., 1992), aerolysins produced by certain *Aeromonas* species (Buckley, 1992; Hirono et al., 1992), colicins produced by *E. coli* (Cramer et al., 1990; Pattus et al., 1990), thiol-activated cholesterol-binding toxins such as streptolysin O produced by *Streptococcus pyogenes* (Alouf et al., 1991; Sekiya et al., 1993), and several others that will not be listed in detail in this chapter (interested readers are referred to a detail account by Menestrina et al., 1994). Another group of toxins acting on plasma membrane are *Staphylococcus aureus* δ-lysin and streptolysin S from *Streptococcus pyogenes* (Bernheimer and Rudy, 1986; Freer et al., 1984; Thiaudiere et al., 1991), which act as detergents on the membrane in their mode of toxic action. Additionally, there are several toxins that have enzymatic activities to act on membrane components resulting in the cytolysis. These enzymatic activities include phospholipase A2 of *Vibrio parahemolyticus* (Shinoda et al., 1991), phospholipase C of α-toxin from *Clostridium perfringens* (Mollby, 1978), sphingomyelinase of *Bacillus cereus* (Tomita et al., 1991), phospholipase D of *Bacillus subtilis* (Fehrenbach and Jurgens, 1991), and cholesterol oxidase of *Brevibacters spp.* (Fehrenbach and Jurgens, 1991).

Another group of toxins that are so far known not to act beyond plasma membrane are staphylococcal enterotoxins and heat shock syndrome toxins produced *by Staphylococcus aureus*. These toxins act as superantigens (Marrack and Kapler, 1990), and have binding sites for major histocompatibility complex II on macrophage cell membrane as well as for the T-cell antigen receptor. Upon binding with T-cells, these toxins evoke massive release of cytokines which become harmful in such a large amount.

In case of toxins that have intracellular targets for their toxic action, they must first cross the membrane barrier to reach their targets. These toxins have a common macrostructure that reflects their common mode of action. Toxins with intracellular targets are made of two types of subunits, B and E. The B subunit generally helps the toxin anchor to the cell surface through receptor and membrane lipid groups, and assists in the translocation of the E subunit inside the cell. E subunit generally possesses specific enzymatic activity for modification of an intracellular target. This group of toxins includes some of the most commonly known toxins such as diphtheria, cholera, pertussis, tetanus and botulinum. Even though the bacteria producing these toxins are widely varied, the enzymatic activities of some of these toxins are same. For example, diphtheria, cholera and pertussis, and variety of other toxins have ADP-ribosyltransferase activity (Althaus and Richter, 1987; Ward, 1987) although substrates recognized by these toxins are totally different. Botulinum and tetanus neurotoxins have protease activity and recognize various components of neuronal exocytosis machinery (Montecucco and Schiavo, 1993; 1994).

Irrespective of the type of bacterial toxin, a common feature remains the membrane, either as the site of action or a barrier to cross. Membrane being a non-polar barrier to toxins

which are water soluble, interaction between toxins and membranes have remained a very intriguing problem for researchers. Toxins such as staphylococcal enterotoxins perhaps only interact with the surface of the membrane, and such process is physically compatible given the presence of protein receptors and lipid polar head groups. However, for other toxins which either form pores in membranes to cause toxicity (e.g., hemolysins) or form pores to translocate themselves to reach the intracellular target, question remains as to how a water soluble protein integrates itself into a non-polar lipid bilayer. Whereas there are individual traits attributable to individual toxic proteins, and a general feature of these toxins appears to be the existence of transmembrane and amphiphilic structure in the polypeptide chains (Singh and Be, 1992). Additionally, the polypeptide structures of toxins are flexible for conformational adaptation once in contact with the surface of membrane or are sensitive to environmental conditions such as low pH of the endosomes. Nature of conformational changes in toxins include not only secondary and tertiary structural foldings but also quaternary structure in several cases.

TOXINS WITH PLASMA MEMBRANE AS TARGET OF ACTION

As an example of the interaction of a toxin that targets plasma membrane for its toxic action, features of staphylococcal α-toxin are briefly discussed to elaborate the relevant points. Staphylococcal α-toxin is hemolytic toxin that is secreted as a single water soluble 33 kDa protein (Jiang et al., 1991; Walker et al., 1992).

Staphylococcal α-toxin is a 293 amino acid protein that seems to consist of two domains (N- and C-domains) that are separated by a glycine-rich loop (residues 119-143) (Gray and Kahoe, 1984). The loop is surface accessible in the monomeric form but becomes inaccessible in the hexameric form (Tobkes et al., 1985). The toxin is a β-sheet dominated protein with 5 % α-helix, 57 % β-sheets, 12% β-turns and 28 % random coils (Tobkes et al., 1985).

The toxin causes lysis of erythrocytes by damaging their membranes in the following three steps:

1. Binding of the native α-toxin to the cell membrane. Binding to cells is assumed to involve a protein receptor on susceptible cells (Hildebrand et al., 1991) although no such receptor has been isolated and characterized.
2. Conformational changes in the protein is followed by oligomerization of the toxin to form amphiphilic hexameric complex generating a transmembrane channel. The channel activity results in the leakage of ions. Conformational changes are believed to occur upon interaction with the receptor and membrane lipids. Such structural change does not seem to be significant at the secondary structure level (Table 1; Tobkes et al., 1985). It has been suggested that tertiary structural alteration is significantly involved in the interaction of the toxin with membrane (Forti and Menestrina, 1989; Tobkes et al., 1985; Walker et al., 1992). There has not been a direct analysis of the tertiary structure of the toxin to demonstrate the point. However, based on results of differential proteolytic susceptibility (Tobkes et al., 1985; Walker et al., 1992), it is indirectly shown that the tertiary structure of the toxin changes. The structural changes partly turn the protein inside out that results in the formation of hexamer structure-a amphiphilic structure that has its hydrophilic domain lining the channel for the leakage of ions.
3. Osmotic shock resulting from the leakage of ions through the pore formed by the toxin that leads to the lysis of the cell.

Table 1. Secondary structure contents of staphylococcal α-toxin (Tobkes et al., 1985)

Toxin	α-helix(%)	β-sheet(%)	β-turn(%)	random coil(%)
Monomer	5	57	11	27
Hexamer	7	52	16	27
Monomer in deoxycholate	5	55	12	28
Hexamer in deoxycholate	10	62	9	19

While oligomeric structure of toxin is believed to be formed on the membrane surface, it can also be demonstrated *in vitro* under several conditions including in the presence of deoxycholate (Bhakdi et al., 1981). Deoxycholate, however, does not seem to change the back bone secondary structure (Table 1). Deletion mutant studies (Walker et al., 1992) have suggested that removal of 2 to 22 amino acid residues from N-terminus of the toxin results in the loss of lytic activity although it still forms hexameric structure both on red blood cell membrane and in deoxycholate. Mutants with missing 3 or 5 amino acids from the C-terminus fail to form significant amount of the hexameric structure, but retain hemolytic activity, albeit very low. These results in combination with proteolytic accessibility of different mutant compared with the wild type toxin have lead to the following model (Fig. 1) for the molecular mode of staphylococcal α-toxin action. According to this model, staphylococcal α-toxin first binds to the target cells as a monomer, which is transformed into a non-lytic oligomeric intermediate before formation of the lytic pore. While the C-terminal residues are apparently important for the formation of oligomers, N-terminal residues seem to be critical for the formation of the lytic pore of the oligomer.

TOXINS WITH INTRACELLULAR TARGET OF ACTION

Among the toxins with intracellular targets, we will focus mostly on the botulinum neurotoxins although some references will be made to tetanus neurotoxin because of the structural and functional relationships between tetanus and botulinum neurotoxins. While

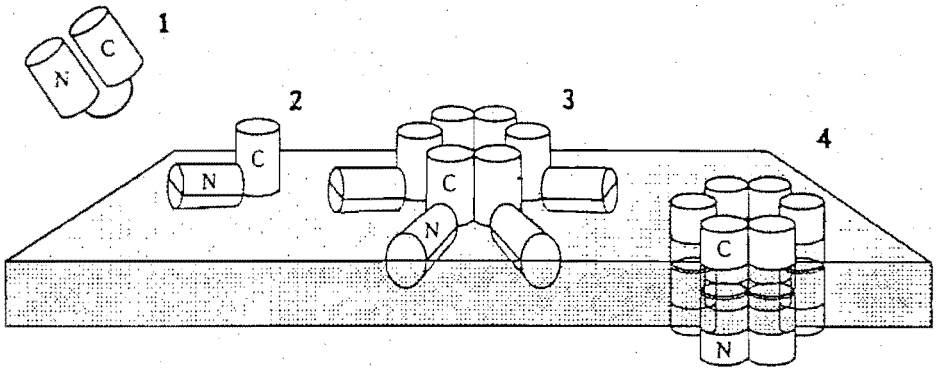

Figure 1. Schematic representation of the steps involved in the binding and membrane pore formation by *Staphylococcus aureus* α-toxin (adapted from Walker et al., 1992). (1) Monomeric form of water soluble α-toxin consists of a N-terminal domain and a C-terminal domain that are separated by a glycine-rich loop. (2) The toxin binds to membrane in its monomeric form. (3) A non-lytic oligomer is formed consisting of up to six subunits. (4) The subunits then penetrate further to form the lytic pore.

greater research progress has been made with other toxins in this group such as diphtheria, cholera, etc., botulinum and tetanus neurotoxins provide a unique set of proteins with respect to their mode of action in terms of their potency, specificity, involvement of metals in their biological function, and the intracellular targets of their action.

Similarities between Botulinum and Tetanus Neurotoxins and Implications

Botulinum and tetanus neurotoxins have several common features including over 30% sequence homology (Binz et al., 1990a; Eisel et al., 1986; Fairweather and Lyness, 1986; Whelan et al., 1992b), and antagonistic effects of heterologous protein fragments (Simpson, 1984). Both the neurotoxins have similar secondary structural contents (Singh et al., 1990), and both have the so called 'catalytic domain' on the C-terminal domains of their respective light chains (Singh, 1990). Tetanus is capable of causing botulism symptoms at high concentrations (Matsuda et al., 1982), and blocks the release of acetylcholine from the presynaptic membranes just like botulinum neurotoxins. Therefore, it is tempting to assume a similar mechanism of action for botulinum and tetanus neurotoxins at the molecular level. Indeed, experimental studies have revealed many similarities between the two neurotoxins including the membrane channel formation by the N-terminal fragments of their respective heavy chains and blockage of the neurotransmitter release from cultured cells (Bittner et al., 1989) and identical cleavage site of type B botulinum and tetanus neurotoxins' proteolytic activity against synaptobrevin-2 (Montecucco and Schiavo, 1993). Thus, the results related to the botulinum neurotoxins are relevant to the tetanus neurotoxin, and vice versa.

Disease and the Organism

Botulism, the deadly food poisoning disease is caused by the growth of various strains of *Clostridium botulinum* in food. The organism produces a large polypeptide (neurotoxin) which is the most toxic protein known to the human kind. Seven serotypes of botulinum neurotoxins produced by different strains of *C. botulinum* have been characterized, and serotypes A, B and E are known to cause botulism in humans. Ingestion of food contaminated with the neurotoxin causes flaccid muscle paralysis that can result in patients' death. Wound botulism has also been reported where the organism can grow in the wounds, and produces the neurotoxin that causes paralysis.

Infant botulism has been observed in babies of 3-35 weeks where ingestion of *C. botulinum* spores leads to the organisms' growth and production of the neurotoxin in the intestine (Miduara and Arnon, 1976; Pickett et al., 1976). Recently, non-botulinum clostridial species, such as *Clostridium butyricum* and *C. barati* have been found to be responsible for human botulism cases (McCrosckey et al., 1988; Arnon, 1992). In addition, botulism has been held responsible for several sudden infant death syndrome (SIDS) cases (Arnon, 1992).

The Neurotoxin

Botulinum neurotoxins (seven serotypes, A-G) are relatively large water soluble proteins (150 kDa) produced by the *Clostridium botulinum*. Each protein has two polypeptide chains (a 100 kDa heavy chain and a 50 kDa light chain) linked through a disulfide bond (Fig. 2). In the proposed mode of action of botulinum and tetanus neurotoxins (Simpson, 1986, 1989), the C-terminal half of the heavy chain binds to the nerve membrane leading to internalization of the neurotoxin in the nerve cell through endocytosis. Subsequently, the pH of the endosome is lowered causing the heavy chain to get integrated in the membrane

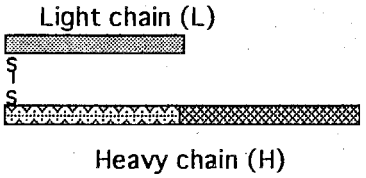

Figure 2. Schematic diagram of botulinum neurotoxin showing its light and heavy chains. The two different domains of the heavy chain shaded with different patterns indicate the N-terminal and C-terminal halves (about 50 kDa each). These two domains are believed to play different functional roles during the intoxication process. The light chain has been shown to contain the toxic site.

through the N-terminal half of the heavy chain for the membrane channel formation. The whole neurotoxin or a part (light chain) of it is translocated through this channel into the cytoplasm of the nerve cell where it blocks the neurotransmitter release.

The primary sequences of types B, C1, D, E, F and G have already been published (Binz et al., 1990a; 1990b; 1990c; Campbell et al., 1993; Hauser et al., 1990; East et al., 1992; Thompson et al., 1990; Whelan et al., 1992a; 1992b). While knowledge of primary structures has been very useful for the comparison of botulinum and tetanus neurotoxins, they had not provided any obvious clues to its mechanism of toxic action until recently. Several reports have appeared (Wright et al., 1992; Schiavo et al., 1992a, 1992b; 1992c; 1993a; 1993b; Fujii et al., 1992) which strongly suggest botulinum and tetanus neurotoxins to be zinc proteases. One significant observation made from the sequence data had been the existence of 3 histidine residues on a short stretch of the light chain that may be involved in the toxic site of the neurotoxin (Binz et al., 1990a). Recent studies have provided evidence that botulinum and tetanus neurotoxins bind to zinc not only in a stoichiometric ratio (Wright et al., 1992; Schiavo et al., 1992c) but that zinc-protease activity is part of the toxic activity of the neurotoxins (Schiavo et al., 1992a). Because of the structural and functional similarities between botulinum and tetanus neurotoxins, results of botulinum or tetanus are commonly used to understand each other's mode of action (Simpson, 1989a).

The neurotoxin appears to have a highly ordered polypeptide folding with predominantly ß sheet structure (approximately 50%) although a significant amount of α-helical structures (20-30%) is present (Table 2; Singh and DasGupta, 1989a; 1989b; 1990). Similar back bone secondary structure has been observed for the tetanus neurotoxin also (Singh et al., 1990). An observation made during the structural analysis of the neurotoxin and its toxoid was that the secondary structural content remained unchanged during the toxoiding (Singh and DasGupta, 1989c). This is interesting because toxoiding with formaldehyde only destroys the toxicity while retaining the immunogenic properties, indicating that the toxic site is perhaps not just made of a sequential site (contiguous stretch of a polypeptide segment), but rather a site that is composed of several polypeptide segments brought together by tertiary structural folding. Accordingly, tertiary structure was found to change considerably upon toxoiding (Singh and DasGupta, 1989c).

Mode of Action

The mode of action of the botulinum neurotoxin is not well understood at the molecular level. Based on some experimental evidence and analogies with other dichain

Table 2. Secondary structure estimation of botulinum and tetanus neurotoxins as analyzed by circular dichroism under physiological pH conditions (Singh et al., 1990; Singh and DasGupta, 1989b)

Neurotoxin	α-helix(%)	β-sheets(%)	β-turns(%)	Random coils(%)
Type A botulinum	21	44	5	30
Tetanus	20	51	0	30

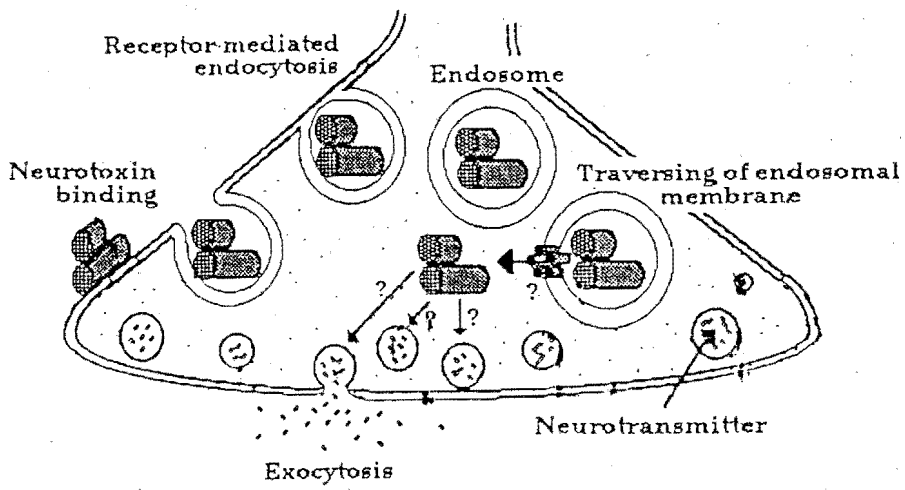

Figure 3. Schematic representation of the steps involved in the binding, internalization and intracellular activity of the botulinum neurotoxins. The steps with question mark are intensive areas of current research.

toxins such as diphtheria, cholera and *Pseudomonas* exotoxin A, a working model has been proposed (Simpson, 1981; 1986). Three major steps (Fig. 3) involved are:

(i) Extracellular Step involves the binding of the neurotoxin to presynaptic membranes through the C-terminal half of the heavy chain. The C-terminal half of the heavy chain binds to the gangliosides on the presynaptic membrane (Simpson, 1981; 1986). According to the double receptor model (Montecucco, 1986), the C-terminal domain of the neurotoxin binds first with the ganglioside, altering the protein structure and making it compatible for binding with a protein receptor. Structural changes in botulinum and tetanus neurotoxins upon binding with gangliosides (or detergent) were experimentally observed (Lazarovici et al., 1987; Singh et al., 1991). These changes could also be involved in exposing transmembrane and surface-seeking peptide domains. Recently, several reports have appeared suggesting existence of different protein receptors for different serotypes of botulinum neurotoxin and of tetanus neurotoxin (Li and Singh, 1995; Nishiki et al., 1993; 1994; Schengrund et al., 1992; Schiavo et al., 1991). However, no confirmed involvement of these putative receptors has been demonstrated in the neurotoxin-mediated toxicity of the neuronal cells.

(ii) Internalization and Translocation. Upon binding, the neurotoxin is internalized through endocytosis (Fig. 3). Inside the cell, the pH of the endosome is lowered to 4-5, which leads to the formation of a membrane channel by the N-terminal half of the heavy chain. This channel helps translocate the whole or a part of the neurotoxin into the cytoplasm.

Botulinum and tetanus neurotoxins, which are extensively soluble in water, are known to form efficient membrane channels at a low pH in artificial membranes (Hoch et al. 1985; Boquet and Duflot, 1982). Membrane channel formation by a water soluble protein is an intriguing phenomenon, because for water solubility, hydrophobic domains are needed on the surface of a protein whereas for membrane channel formation, adequate hydrophobic segments will be required for the interaction with non-polar membrane bilayer. A major question to be answered is how are the polypeptides integrated in the lipid bilayer. Are the hydrophobic segments of these neurotoxins "hidden" in aqueous medium which get exposed

at a low pH to interact with membrane? A screening of primary sequences of tetanus neurotoxin revealed one segment (H-650-681) in the heavy chain and one segment in the light chain (L-223-253) with sufficient hydrophobicity and a sequence length capable of spanning the lipid bilayer (Eisel et al., 1986). Similar observations were noted for type A botulinum neurotoxin, except no hydrophobic segment was observed in the light chain (Thompson et al., 1990). Hydrophobic protein segments can only indicate membrane interacting domains. A membrane interacting segment does not necessarily form a "channel" by itself, especially to translocate large proteins such as the neurotoxin or its light chain. Because of this function, it is likely that the channel will probably have an amphiphilic structure to interact with the non-polar lipid bilayer on one surface and with hydrophilic groups of the protein on the other. Membrane channel formed by water soluble proteins is a phenomenon which is not well understood. Examples are diphtheria, cholera, *Pseudomonas* exotoxin A, botulinum and tetanus (Jiang et al., 1989; Jinno et al., 1989; Papini et al., 1987a, 1987b). In order to remain water soluble, these proteins must contain adequate hydrophilic domains on the exterior of the protein. However, for membrane channel formation, hydrophobic segments of proteins are required to interact with the non-polar lipid bilayer.

To explain adequate interaction of botulinum and tetanus neurotoxin with lipid bilayer, we analyzed hydrophobicity and hydrophobic moment characteristics (Be et al., 1994; Doyle and Singh, 1993; Singh and Be, 1992) of both neurotoxins. The results indicate several polypeptide segments with properties that can characterize them as integral membrane segments or as surface-seeking segments (amphiphilic) (Fig. 4), which are compatible for interaction with lipid bilayer. Surface and membrane segments were identified in both light and heavy chains of the neurotoxin, suggesting a likely interaction of both the chains with the lipid bilayer.

A surface-seeking peptide domain has low average hydrophobicity because of the presence of the hydrophilic amino acid residues, but it has relatively high hydrophobic

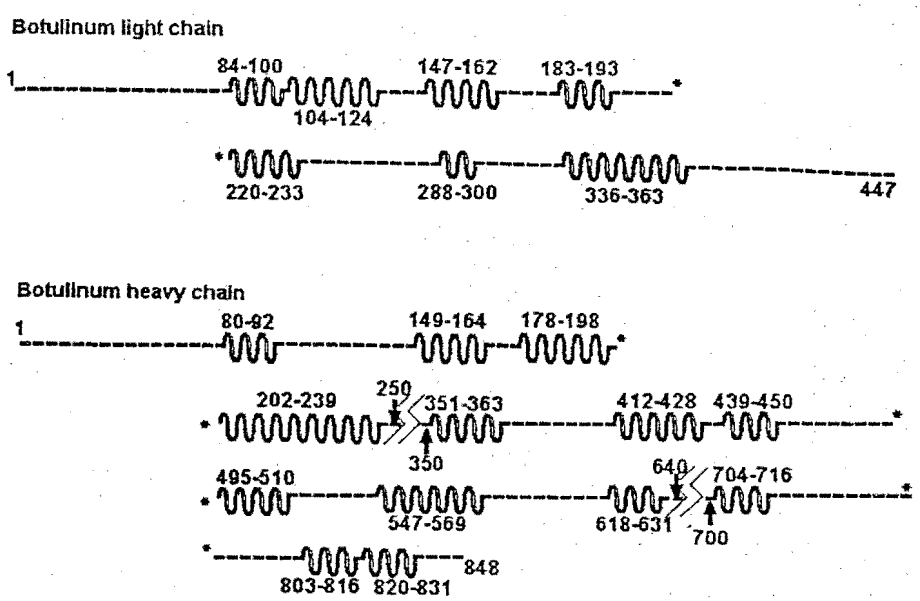

Figure 4. Cartoon drawing of the light and heavy chains of type A botulinum neurotoxin indicating amphiphilic (open helices) and transmembrane (solid helices) peptide segments. The numbers correspond to the amino acid sequence numbers in respective chains (see Fig. 2).

moment, i.e. amphiphilicity due to the topographical location of hydrophobic and hydrophilic residues. Therefore, a surface-seeking domain could interact with hydrophobic groups on one surface and with hydrophilic groups on the other. The presence of transmembrane and surface-seeking domains in the light chains of botulinum and tetanus neurotoxins is puzzling because light chain by itself has not been shown to have any membrane channel activity (Boquet and Duflot, 1982; Hoch et al., 1985), except for a recent report for the release of calcein (Kamata and Kozaki, 1994; F.-N. Fu and B. R. Singh, unpublished results). The role of transmembrane and surface-seeking segments of the light chain could be in (i) the interaction of light chain with the heavy chain; and/or in (ii) the direct interaction of light chain with lipid bilayer during its translocation. Light and heavy chains of type A botulinum neurotoxin have significant interactions with each other (Singh and DasGupta, 1989a). The role of hydrophobic surfaces of light chain in its translocation can be envisaged in the "cleft" model proposed by Montecucco and co-workers (Montecucco et al., 1989). According to this model, the heavy chain protects the hydrophilic groups of the light chain by combining with them and leaving the hydrophobic surface of the light chain to interact with the lipid bilayer. Experimental results of photo-crosslinking techniques have suggested light chains can enter the lipid bilayer at low pH (Montecucco et al., 1989).

Respective light and heavy chains of botulinum and tetanus neurotoxins have substantial similarities in the location and characteristics of membrane compatible peptide segments (Be et al., 1994), which is consistent with their common mode of biological activities. Based on our results, it may be possible to explain membrane channel formation by water soluble proteins such as botulinum or tetanus neurotoxins because they have sufficient numbers of transmembrane or amphiphilic (surface-seeking) polypeptide segments. It should also be pointed out that hydrophobicity calculations did not reveal any transmembrane segment in the botulinum light chain (Thompson et al., 1990). However, hydrophobic moment analysis (Be et al., 1994) resulted in the first observation of potential membrane interacting domains of botulinum light chain. The presence of transmembrane and amphiphilic segments is also consistent with experimental results which suggest direct light chain interaction with lipid bilayers (Montecucco, 1986). Analysis of hydrophobicity alone had revealed at least one segment in tetanus heavy chain (H-203-234) which although may be capable of traversing the membrane bilayer, but it may not be adequate to form a channel for the translocation of the light chain for two reasons: (i) the segment is too small to form one side of the whole channel structure; and (ii) it has strong hydrophobic residues that may not allow its interaction with the light chain for translocation of the latter. A channel activity for the translocation of light chain can be partly rationalized under the above conditions if we assume that the interaction between light and heavy chains are through hydrophobic domains, and that the neurotoxins exist as oligomers (at least a dimer) which has been suggested at least for the botulinum neurotoxin (Shone et al., 1985; Ledoux et al., 1994). In an oligomeric structure two or more heavy chains can be envisaged to form the channel, and their respective light chains can pass through the hydrophobic region of the heavy chain on one side and with the other light chain on the other.

A second explanation of membrane channel formation by botulinum and tetanus neurotoxins is as follows: since hydrophobic moment calculations have helped identify several membrane interacting hydrophobic domains both in light and heavy chains of both neurotoxins, more than one segment of the heavy chains can be allowed to integrate into the membrane bilayer. Because some of these segments are amphiphilic in nature, interaction with hydrophilic domains of light chain is possible during translocation of the latter. This should also allow interaction of the light chain directly with lipid bilayer which will be consistent with the photo-labeling experiments that suggest direct light chain interaction with lipid bilayer (Montecucco et al., 1988).

It is also possible that a combination of both modes suggested above is involved in the formation of membrane channel for the translocation botulinum neurotoxin. The main assumptions of the hypothesis are: (i) The presence of amphiphilic and transmembrane segments in the light and heavy chains as predicted by the hydrophobic moment analysis. (ii) Existence of botulinum neurotoxin in oligomeric form.

Tetanus neurotoxin has been extensively analyzed in the past (Robinson and Hash, 1982) for its molecular size using analytical ultracentrifugation, and it was reported that purified tetanus neurotoxin exists as a monomer (125-145 kDa), although "aggregation" was observed at certain concentrations. Moreover, to avoid "aggregation", Robinson and co-workers (Robinson and Hash, 1982) used their toxin preparations "immediately" after purification on a preparative scale electrophoresis which was carried out in 4 M urea. Urea is likely to facilitate disaggregatation of tetanus neurotoxin into the monomeric form.

Botulinum neurotoxin type A is also reported to form "aggregation" based on native gel electrophoresis band of 450-600 KDa (Shone et al., 1985; Ledoux et al., 1994). Whether or not the "aggregated" form is a natural form of the neurotoxins remains to be confirmed. We have carried out native gel electrophoresis of type A botulinum and tetanus neurotoxins (Ledoux et al., 1994), which indicates that the tetanus neurotoxin exists primarily as a dimer although a small fraction exists as trimer, whereas type A botulinum neurotoxin exists in the form of at least a trimer. Type A botulinum neurotoxin band is spread over a range of 450-600 kDa, but most of it appears as a trimer. This observation is consistent with those of shone et al. (Shone et al., 1985). Crystal forms of type A botulinum neurotoxin reported recently are also believed to exist as a dimer (Stevens et al., 1991). We have further confirmed the existent of oligomeric type A botulinum neurotoxin by chemical cross-linking experiments (Ledoux et al., 1994).

Chemical cross-linking experiments with both BS^3 and glutaraldehyde suggested the presence of oligomeric forms of type A botulinum neurotoxin corresponding to molecular weights of 249 and 366 kDa (Ledoux et al., 1994). Although both of these species do not correspond to exact dimer or trimer molecular weight, these are obviously distinct molecular species. A molecular weight species higher than a trimer was also observed at the top of the gel in the form of sharp bands (Ledoux et al., 1994).

An intriguing observation was made with a careful analysis of the amino acid sequences of the heavy and light chains of tetanus and botulinum neurotoxins (Singh et al., 1994). It suggested possible sites of association between monomeric neurotoxin molecules in the formation of oligomers. Specifically, leucine zipper-like structures have been located in the heavy chain of the tetanus neurotoxin, and in the heavy and light chains of the type A neurotoxin. The ideal leucine zipper possesses a leucine residue every seventh residue of a polypeptide chain having a helical structure. Aberrations from the ideal could include, for example, the substitution of leucine for a different residue, resulting in a leucine zipper-like structure. Possible structures of this type have been identified in the type A neurotoxin heavy and light chains, as well as in the tetanus heavy chain (Fig. 5). The type A botulinum heavy chain exhibits such a structure with leucine and isoleucine residues. The two exceptions (noted in the table) are residues which offset the "scheme" of the zipper structure by one residue. The substitution of an isoleucine residue for a leucine residue does not seem unreasonable, since the relative bulk of the side chains of each residue is comparable, and would allow the "packing" of these leucine-like structures to form a "zipper." An interesting possibility appears in the light chain of the type A neurotoxin, where a leucine zipper-like structure with a hydrophobic residue every seventh residue, with the exception of residue 270 (Aspartate) and 291 (Lysine). When these regions (in separate neurotoxin molecules) are placed in proximity and arranged in a parallel fashion (C-terminus to N-terminus, C-terminus to N-terminus), the positively-charged lysines and negatively-charged aspartate residues will repel. However, if these regions are placed in anti-parallel fashion (C-terminus

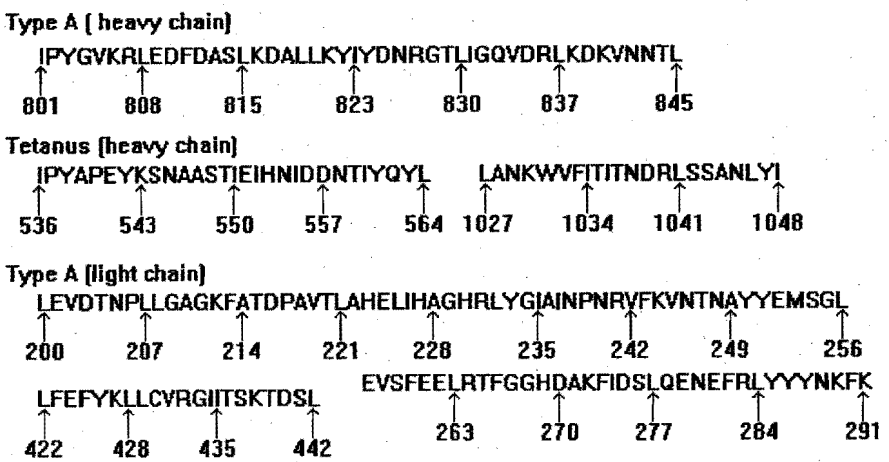

Figure 5. Primary amino acid sequence of type A botulinum neurotoxin and tetanus neurotoxin showing possible leucine zipper-like structures. Residues with asterisk(*) mark offset the zipper scheme by one residue.

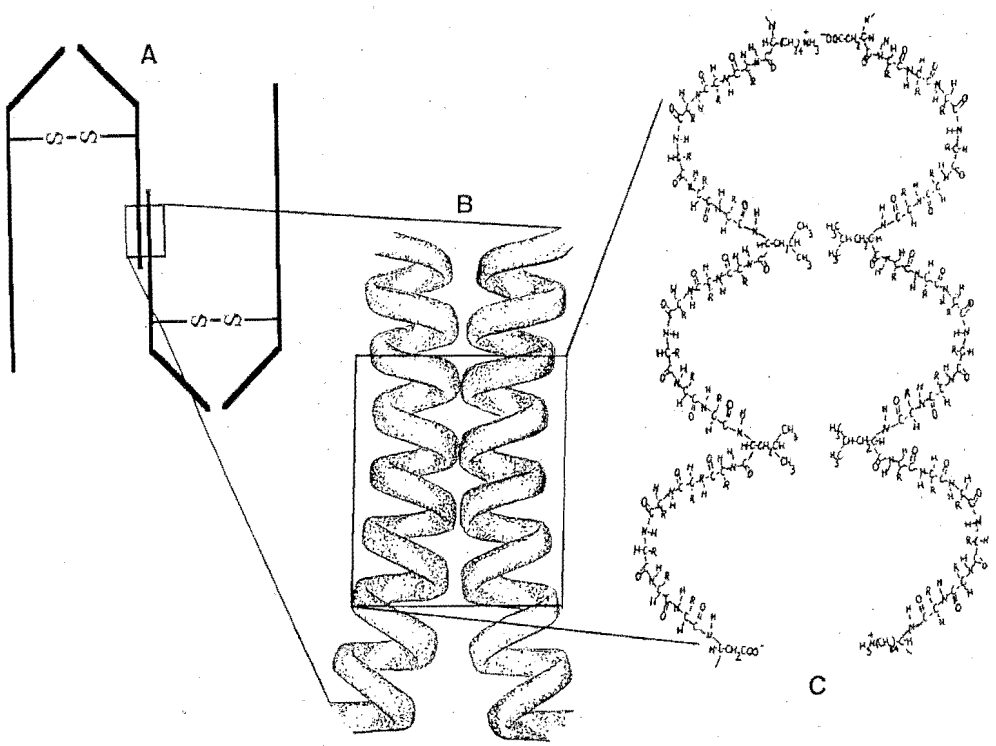

Figure 6. Schematic diagram depicting possible model for association between type A botulinum neurotoxin molecules involving leucine-zipper like structure. (A) Representation of an association between the light chains of the monomeric neurotoxin molecules for a dimer formation. (B) Representation of a helical structure which is assumed for the leucine-like structure. (C) Depiction of amino acid residues which may be in favorable contact. The amino acid sequence corresponds to the residues 270 to 291 on the light chain of type A botulinum neurotoxin (Fig. 5). The two sequences are represented antiparallel to depict favorable ionic contacts.

Table 3. Change in the secondary structures of type A botulinum and tetanus neurotoxins with lowering of pH (Singh et al., 1990; 1991; Singh and DasGupta, 1989b)

pH	α-helix(%)	β-sheets(%)	β-turns(%)	random coils(%)
Botulinum				
7.2	21	44	5	30
5.5	29	45-49	0	22-26
Tetanus				
7.0	22	51	0	27
5.5	25	47	0	28

to N-terminus, N-terminus to C-terminus), the lysine and the aspartate residues could provide a favorable coulombic contact (Fig. 6). In addition to this structure, the light chain of type A also has a significantly long stretch of zipper-like structure with leucine and isoleucine between residues 200-291 (Fig. 5). The heavy chains of the tetanus and botulinum neurotoxins also seem to possess leucine zipper-like structures (Fig. 5). Type A botulinum heavy chain possesses such a structure between heavy chain residues 353 and 397, whereas tetanus heavy chain has them between heavy chain residues 536 and 564, and 1027 and 1048 (Fig. 5). The first possible leucine zipper-like region of tetanus heavy chain (residues 536-564, Fig. 5) possesses lysine and aspartate residues at position 543 and 557, respectively. When the polypeptides are placed antiparallel, the lysine and aspartate residue may come in favorable ionic contact. The tetanus heavy chain also has another sequence of leucine and isoleucine residues (residues 1027-1048, Fig. 5) with the potential to form a leucine zipper structure. These data suggested leucine zipper-like structures are possible sites of association among monomeric forms of the neurotoxin molecules in the formation of oligomeric forms of the neurotoxin. Since more than one of these regions exists per neurotoxin molecule, it is possible that these regions act together in causing an association between neurotoxin molecules.

The significance of the observations presented in this report pertain not only to the function of botulinum and tetanus neurotoxins at the molecular level, but also to many other water-soluble proteins such as colicin and other bacterial toxins (diphtheria, cholera, pertussis, etc.) that are believed to form membrane channels as part of their mode to gain entry into their target cells. In the case of botulinum and tetanus neurotoxins, the neurotoxins are known to have a common mode of action (Simpson, 1989a) which may have a basis in the common observation of the existence of oligomeric forms. There are significant differences between botulinum and tetanus neurotoxins in terms of primary sites and degree of toxicity which, in part, could be the result of differential oligomeric species observed for the neurotoxins.

If botulinum and tetanus neurotoxins exist as dimer or trimer/tetramer in aqueous solutions (Fig. 7), what could be the physiological role of such structures? The observation could be relevant to explain the behavior of botulinum neurotoxin with mouse phrenic hemidiaphragm at different concentrations (Bandyopadhyay, 1987; Maisey et al., 1988). Because both the neurotoxins exist in more than one oligomeric form, it is possible that these oligomeric forms are in equilibrium with each other (Fig. 7), and this equilibrium could be altered in different conditions such as in low pH and upon interaction with membranes. A model of oligomeric channel is shown in Figure 8 assuming a trimeric form of botulinum neurotoxin.

Low pH is apparently required for the strong channel formation activity of botulinum and tetanus neurotoxins and their heavy chains (Boquet and Duflot, 1982; Hoch et al., 1985). A pH of 5.0 or lower induces the channel formation. Two possible effects of a low pH are

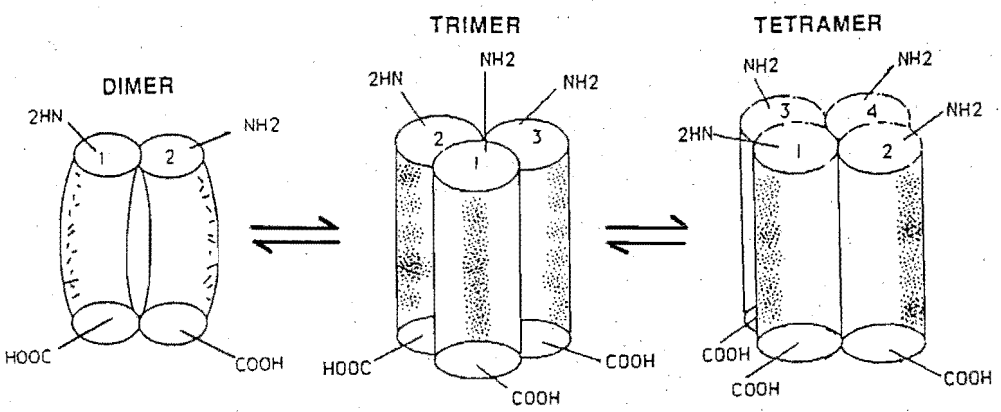

Figure 7. Schematic representation of oligomeric structure of type A botulinum and tetanus neurotoxins. Based on results in Ledoux et al. (1994) and modified after Singh (1993), it is assumed that botulinum neurotoxin exists as trimer and tetramer whereas tetanus neurotoxin exists as dimer and trimer. The arrows indicate possible interconversion between two oligomeric form. The shaded areas indicate the location of amphiphilic/transmembrane region of the monomeric units.

(i) neutralization of negative charges of the protein and/or (ii) conformational changes in the polypeptide folding which allow the integration of the neurotoxin into the membrane bilayer. It has been observed that low pH increases the surface hydrophobicity of tetanus neurotoxin as demonstrated by a labeled Triton-X-100 binding (Duflot and Boquet, 1982). Similar observations were made for other dichain toxins such as *Pseudomonas* exotoxin A (Idziorek et al., 1990). Based on our calculations, the potential membrane interacting domains of the neurotoxins are predicted at pH 7.0 as the hydrophobicity scale used is based on partition

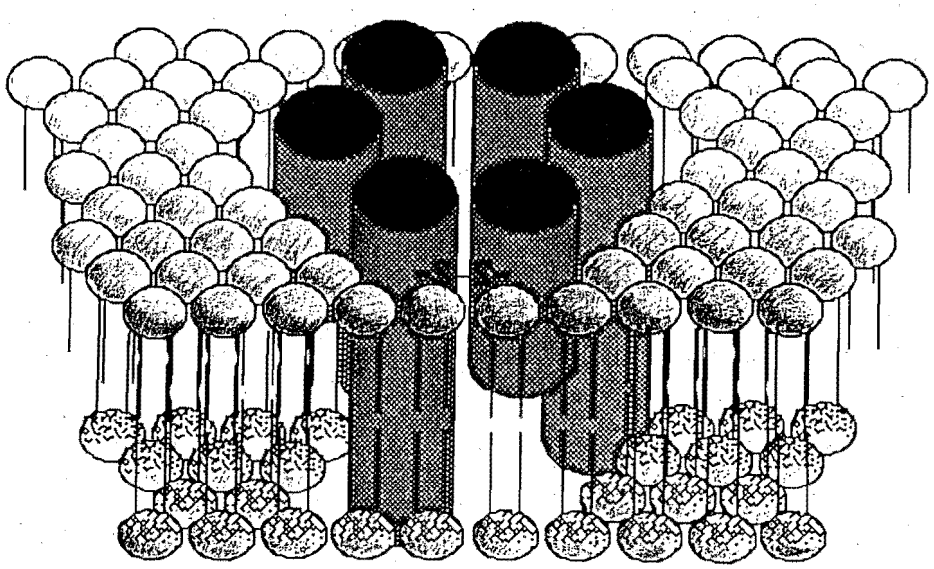

Figure 8. Schematic model of an oligomeric membrane channel structure by botulinum or tetanus neurotoxin. The model shows a trimer of the neurotoxin with smaller cylinders representing light chain whereas larger lobe representing heavy chain.

coefficients of amino acids between a non-polar solvent and water (Fauchere and Pliska, 1983). Therefore, it is likely that topographical changes are introduced by the low pH which could expose the membrane compatible domains of the neurotoxin. FT-IR studies of the tetanus neurotoxin did not suggest a significant gross conformational change with pH (Singh et al., 1990). However, there was a clear indication of conformational changes in certain segments of the polypeptide. Secondary structure of type A botulinum neurotoxin, on the other hand, seems to change significantly upon lowering the pH from 7.2 to 5.5 (Table 3; Singh et al., 1994). This observation will be consistent with the presence of membrane interacting domains even at pH 7.0. The low pH could perhaps refold the protein at the tertiary structural level without altering much of the secondary structure. Trp fluorescence quenching experiments have suggested a significant alteration in the tertiary structure of tetanus neurotoxin at low pH (B. R. Singh, unpublished results). Also, similar indications were observed from the differential scanning calorimetric analysis of type A botulinum at pH 7.0 and pH 4.0 (unpublished results).

(iii) Intracellular Step of the botulinum neurotoxin action is not well understood. It is known that the light chain subunit of the neurotoxin, once inside the nerve cells, is sufficient to block the neurotransmitter release (Bittner et al., 1989; Lomneth et al., 1991; Mochida et al., 1989; Stecher et al., 1989). The neurotoxin does not affect either biosynthesis or packaging of the acetylcholine in the nerve cell (cf. Simpson, 1989b). Recent experimental evidence has suggested that botulinum and tetanus neurotoxins are zinc-proteases, and the substrates for the different serotypes of botulinum neurotoxins and tetanus neurotoxin are the constitutive components of the secretory machinery (see Huttner, 1993). The substrate for the protease activity of the type B botulinum and tetanus neurotoxins has been identified as synaptobrevin-2, a 19 kDa protein present on synaptic vesicles (Schiavo et al., 1992a). It has now been established that synaptobrevein-2 also acts as intracellular substrate for the proteolytic activity of botulinum neurotoxin types D, F and G (Schiavo et al., 1993a; 1993b; Schiavo and Montecucco, 1994) although site of cleavage on the synaptobrevin-2 for each neurotoxin is different. Intracellular target for the proteolytic activity of types A and E has

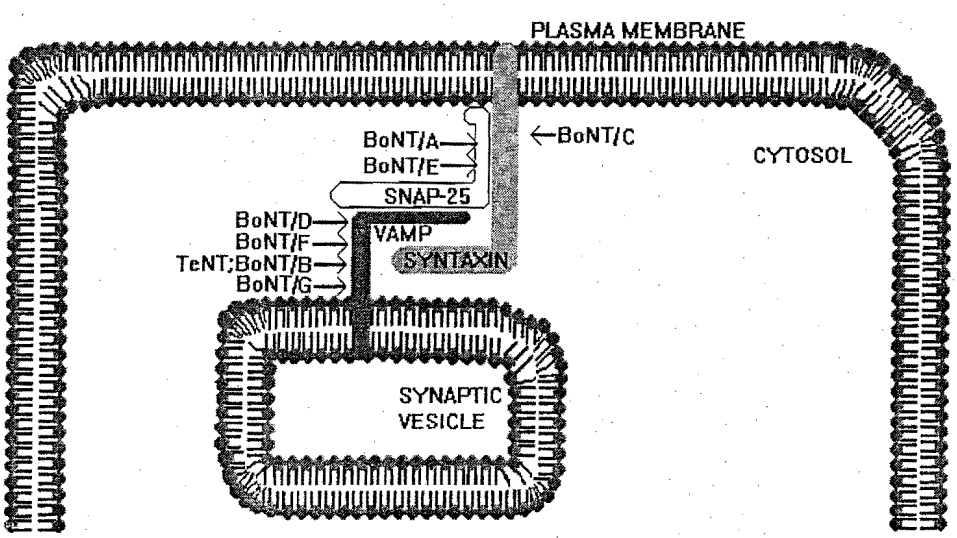

Figure 9. Schematic diagram of synaptic vesicle and presynaptic plasma membrane showing target substrates of the proteolytic activity of botulinum and tetanus neurotoxins.

been as SNAP-25 (a presynaptic plasma membrane protein) (Blasi et al., 1993a; Schiavo et al., 1993a). Again, the site of cleavage for the two neurotoxin is different on SNAP-25 (Schiavo et al., 1993c). Finally, the intracellular target for the type C1 botulinum neurotoxin has been identified as syntaxin (Blasi et al., 1993b). The targets identified, so far, as the substrates of botulinum and tetanus neurotoxins are the constitutive components of the secretory machinery (Huttner, 1993; Menestrina et al., 1994; Montecucco and Schiavo, 1993; Fig. 9) rather than any of the regulatory components.

An alternative mechanism of the involvement of the transglutaminase catalyzed immobilization of the synaptic vesicles have been proposed for tetanus-induced blockage of the neurotransmitter release (Facchiano et al., 1993). In the past, attempts have been made to demonstrate ADP-ribosyl transferase activity in botulinum neurotoxins (cf. Singh, 1990) without any success. The hypothesis of ADP-ribosyl transferase activity of botulinum neurotoxins derives from analogies with diphtheria, cholera and botulinum C2 toxins, all with similar extracellular mode of action (Simpson, 1989b).

The most recent experimental evidence (Fujii et al., 1992; Schiavo et al., 1992a; Wright et al., 1992; Blasi et al., 1993; Montecucco and Schiavo, 1993) clearly suggests that botulinum neurotoxins are metal-binding proteins, and the most likely metal ligand is zinc, although iron has also been suggested to bind to botulinum neurotoxins (Bhattacharyya and Sugiyama, 1989). The question remains as to what is the role of the metal binding to the neurotoxin and how is this protease activity related to the toxic activity of the neurotoxin? Based on current data, it appears that the toxic site of the neurotoxin consists of not only the binding site of the metal. This is likely because the metal binding site (HEXXH, where X is any amino acid residue) has been conserved in all the serotypes of the botulinum neurotoxins, but botulinum neurotoxins have different substrates for proteolysis (see Huttner 1993). Discovery of different substrates of different serotypes of botulinum neurotoxins poses further question for molecular basis of the substrate specificity of botulinum neurotoxins with nearly identical zinc-protease motif (Table 4).

There are two possible ways in which neurotoxins may be providing the observed differential substrate specificity. (i) The three dimensional structure of the light chain (the toxic subunit) of each neurotoxin inherently differ significantly. (ii) The metal binding to the light chain introduces differential conformation for the binding of each neurotoxin to a specific substrate. The toxic site of botulinum neurotoxin is likely to consist of more than one polypeptide domain, and metal binding may be causing the formation of the native toxic site. It is possible that while the metal binding sites are conserved, the other peptide segments of the toxic domain are not conserved among all the serotypes of botulinum neurotoxins, and the toxic site of the neurotoxin results from a specific folding of each neurotoxin polypeptide initiated by the metal binding.

A recent study (Foran et al., 1994) has provided some insight into the enzymatic properties of the protease activity of type B botulinum and tetanus neurotoxins. Several

Table 4. Primary amino acid sequence in the vicinity of zinc-protease motifs, substrates and cleavage sites of botulinum and tetanus neurotoxins

Neurotoxin	Amino Acid Squence																				Substrate	Peptide Bond Cleavage
Botulinum type A	D	P	A	V	T	L	A	H	E	L	I	H	A	G	H	R	N	Y	G	I	Snap-25	NQ-RA
Botulinum type B	D	P	A	L	I	L	M	H	E	L	I	H	V	L	H	G	L	Y	G	I	Snaptobrevin	SQ-FE
Botulinum type C1	D	P	I	L	I	L	M	H	E	L	N	H	A	M	H	N	Y	G	I		Syntaxin	?
Botulinum type D	D	P	V	I	A	L	M	H	E	L	T	H	S	L	H	Q	Y	G	I		Snaptobrevin	QK-LS
Botulinum type E	D	P	A	L	T	L	M	H	E	L	I	H	S	L	H	G	L	Y	G	A	Snap-25	DR-LM
Botulinum type F	D	P	A	I	S	L	A	H	E	L	I	H	A	L	H	G	L	Y	G	A	Snaptobrevin	DQ-K-L
Botulinum type G	D	P	A	L	T	L	M	H	E	L	I	H	V	L	H	G	L	Y	G		Snaptobrevin	SA-A-K
Tetanus	D	P	A	L	L	L	M	H	E	L	I	H	V	L	H	G	L	Y	G		Snaptobrevin	SQ-FE

Table 5. Kinetic properties of the protease activity of type B botulinum and tetanus neurotoxins with 62 amino acid long fragment of synaptobrevin (Foran et al., 1994)

Neurotoxin	Km (mM)	Vmax (nM s^{-1})	Kcat (s^{-1})
Tetanus	2.4	55	2.7
Botulinum	0.65	62	12.3

observations make the protease activity of these neurotoxins unique. First, known inhibitors of metalloproteases such as captopril and dithiothreitol were largely ineffective on the protease activity at their normal concentrations. Second, unusually large size of the substrate is required. A peptide fragment consisting of the cleavage site of the substrate, synaptobrevin, did not inhibit the protease activity of the neurotoxins, suggesting that perhaps substrate is recognized by multiple contacts between the substrate and the neurotoxin. Third, proteolytic activity of the neurotoxin was not inhibited at 1 mM Zn^{2+} ion concentration whereas other metalloproteases such as thermolysin are reduced at submicromolar concentrations of Zn^{2+} (Pangburn and Walsh, 1975). In addition, the kinetic studies of type B botulinum and tetanus neurotoxin suggested unusually large Km values (Table 5).

Treatment of the neurotoxin with metal chelators such as ethylenediamine tetraacetic acid (EDTA) inactivate most (80%) of the enzymatic activity, and large part (80%) of the lost activity can be restored with the addition of Zn^{2+} ions activity (Foran et al., 1994). Similar loss of toxic activity of type A botulinum neurotoxin was recently observed in our studies with the neurotransmitter release studies in PC12 cells (F. -N. Fu, R. Lomneth and B. R. Singh, unpublished data). However, the loss of activity was not restored with the replenishment with Zn^{2+} ions. The loss of activity at least in part seems to be caused by the change in the polypeptide folding (Fu and Singh, 1995).

It is also possible that substrates other than those already identified exist in the neuronal cells that are modified by botulinum neurotoxins either proteolytically or non-proteolytically. Two main reasons for such a possibility are as follows:

a. Irreversible impairment of the exocytosis by proteolytic degradation of constitutive components of secretory machinery does not explain Ca^{2+}-induced reversibility of the neurotransmitter release from type A botulinum and tetanus neurotoxins (Dreyer, 1989; Simpson, 1989b; Ashton et al., 1993).
b. Biochemical task of degradation of a constitutive component (e.g., synaptobrevin) on about 500,000 vesicles present in each nerve ending of each neuro-muscular junction (Tauc and Poulain, 1993) within a few minutes (mice death occurs within 30 min of injection of the neurotoxin) is perhaps very large even for an enzyme. This may be especially true for botulinum and tetanus neurotoxins as their enzymatic activity seems to be poor (Table 5; Foran et al., 1994). Furthermore, there are two zones of the vesicles within the neuronal cells. About 200 vesicles are in active zones at a time, and even if the neurotoxin can act upon that population for proteolysis, it may act on another target to simultaneously block the movement of the vesicles within the cell.

In addition, based on the electrophysiological data, the vesicular mode itself has been questioned for the exocytosis of neurotransmitters (Tauc and Poulain, 1993). Therefore, it is very important to identify other possible targets for the neurotoxins in addition to those already identified. It is likely that the neurotoxins act on the regulatory and constitutive components of the secretory machinery simultaneously. An evidence of this possibility is a report that tetanus neurotoxin activates transglutaminase activity which could cross-link vesicle synapsin 1 to actin to block the vesicular movement (Facchiano et al., 1993).

Interestingly, in almost all the reports coming out of main research group that initially demonstrated the proteolytic activity of the neurotoxin on certain neuronal substrates, it has been suggested that other substrates are likely to exist (Menestrina et al., 1994; Montecucco and Schiavo, 1993; 1994).

CONCLUDING REMARKS

Bacterial protein toxins although of varied origins to cover many classes of bacteria seem to have a common functional mode for their biological activity. Because of such commonality in function, their structural evolution points to one critical direction, which involves their ability to interact with membranes. All the known toxins are water soluble with potential for interaction with membrane. The water solubility permits their transportation to target cells through the body fluid. Initial toxin interaction with membrane involves polar head groups of lipid bialyer as well as specific association with protein receptors. In many cases, the initial association with membrane 'primes' toxins for their ultimate integration into the non-polar lipid bilayer.

Detailed molecular features as well as events leading to the integration of toxins into lipid bilayers is not clear at this point, and is a subject of intense current research. While the available knowledge of the structure of a handful toxins such as cholera, *Psudomonas* exotoxin A and porin, some common factors are visible, no toxic motif(s) has been identified yet. Availability of three dimensional structure of an adequate number of toxins is likely to reveal interesting structural features. Another area of needed research is the characterization of receptors for toxins. In most cases, cell surface receptors hold the key of toxin entry and potency. Receptor knowledge will not only help develop antidotes against toxins, but also could also help our understanding of toxins themselves.

Toxins that have intracellular targets are perhaps the most complex group of proteins. Such toxins are generally multi-subunit toxins. Although the subunits have their defined function, they still seem to have a well coordinated series of steps among themselves to express toxicity. Interaction among the toxin subunits and between toxin and its target are the areas of current and future research.

Bacterial toxin research is obviously warranted for public safety. However, their is also a silver lining about the toxins. Some of the toxins are currently being used as therapeutic drugs (Scott, 1989) while others are being projected to be altered for their use against such diseases as cancer. Thus, there is more than curiosity involved when it comes to research with protein toxins, especially bacterial protein toxins.

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