J. Gen. Appl. Microbiol., **52**, 1–8 (2006)

Mini-Review

Botulinum neurotoxins: Perspective on their existence and as polyproteins harboring viral proteases

Bibhuti R. DasGupta*

Department of Food Microbiology and Toxicology, University of Wisconsin-Madison, 1925 Willow Drive, Madison Wisconsin 53706, USA

(Received July 13, 2005; Accepted November 15, 2005)

Key Words——*Clostridium botulinum*; *Clostridium tetani*; neurotoxin; polyprotein; viral protease

The set of serologically distinguishable (very little to no cross-reactivity) proteins known as botulinum neurotoxin (serotypes A–G) and tetanus neurotoxin (NT) have apparent similar structures and similar structure-function relationships (Humeau et al., 2000; Schiavo et al., 2000). These proteins do not appear to serve any physiological function for the producer anaerobic bacteria *Clostridium botulinum*, *C. baratii*, *C. butyricum*, *C. argentinense* and *C. tetani*, nor to be essential for their survival (Allen et al., 1999; Minton, 1995). Are the NTs vestiges or relics of evolution? Or is the ancestral NT gene (Collins and East, 1998; Henderson et al., 1997; Popoff and Marvaud, 1999) still evolving to acquire a "useful existence"? The NT genes, seemingly superfluous for the survival and reproduction of *C. botulinum*, express the most toxic poison in nature that does not kill any cell. This conundrum is considered with some conjectures, viewed from the published structural and functional properties of the 150-kDa proteins. Assessment of the literature provided rationale to propose a consideration of the NT as a polyprotein harboring viral protease (the ~50-kDa light chain) and to provide a perspective on the evolution of the NT into the present state where 8 NTs (botulinum plus tetanus) cleave 3 neuronal and 1 non-neuronal proteins at 7 sites.

Clostridial NTs are polyproteins harboring viral metalloproteinases—a proposal: Each NT synthesized as a ~150-kDa single chain protein shapes into three clearly visible (X-ray diffraction of crystals) structural domains each of ~50 kDa (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000). Proteolytic cleavage(s) at the two junctions between the three structural-functional domains is a facile process as is their ensuing separation(s) (Prabakaran et al., 2001, and refs. therein). The selective and relative susceptibilities of these junctional segments indicate that these segments are mere hinges, as is evident from the 3-dimensional structures of type A and B botulinum NTs, acting as purely provisional links between the three structurally independent domains. Each of the three ~50-kDa domains of the ~150-kDa NTs performs its individual function without the presence of the other two. The ~50-kDa C-terminal domain (the binding domain) mediates NT's binding to the receptors on the presynaptic membrane of neuromuscular junctions. The adjacent 50-kDa domain forms channels in the endosomal membrane (channel former or transmembrane domain) and promotes insertion/passage of the

* Address reprint requests to: Dr. Bibhuti R. DasGupta, Department of Food Microbiology and Toxicology, University of Wisconsin-Madison, 1925 Willow Drive, Madison, Wisconsin 53706, USA.

Tel: 608–263–6806 Fax: 608–263–1114

E-mail: brdasgup@wisc.edu

Abbreviations: NT, neurotoxin; L-chain, light chain; H-chain, heavy chain; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP-25, synaptosome associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; CNS, central nervous system; NMJ, neuromuscular junction.

50-kDa N-terminal domain (the light or L-chain)—an endoprotease that selectively cleaves one or two of the SNARE complex (the synaptic vesicle docking/fusion complex) proteins and disrupts neurotransmitter exocytosis (reviewed in Humeau et al., 2000; Schiavo et al., 2000). The contiguous transmembrane and binding domain (together ~100 kDa) is a heavy or H-chain. These and additional considerations noted below also support the proposal that clostridial NTs be regarded as polyproteins (one gene encoding multiple proteins with separate functions) instead of classical multidomain proteins.

In considering the phylogeny and common ancestry of the genes of the 150-kDa clostridial NTs it was noted that the different domains of the NTs evolved at *different rates* (Henderson et al., 1997; Popoff and Marvaud, 1999) and "by the assembly and exchange of small gene segments" (Johnson and Bradshaw, 2001). The gene(s) encoding the single ancestral form of all the clostridial NTs may have originated not in *C. botulinum* and *C. tetani* but probably "in an ancestor of a recognized *C. botulinum* organism" (Collins and East, 1998). Bacteriophages clearly encode botulinum NT types C and D, and other clostridial NT genes are located in chromosome and plasmid (Brüggemann et al., 2003; Eklund et al., 1989; and reviewed in Johnson and Bradshaw, 2001; Popoff and Marvaud, 1999). But it cannot be ruled out unequivocally that the genes of botulinum NT types A, B, E and F are not associated with phages (Johnson, 1997). Therefore it appears not unreasonable to propose that the protease domain of the clostridial NTs, originally a viral protease, became fused with two other domains (the H-chain) and now exists as part of a polyprotein. This fusion brought about two developments: i) The payload (the protease) became coupled to a delivery vehicle with aiming ability which (two-thirds of the 150-kDa NT) becomes, as believed, a *throwaway* piece after the protease reaches the target neuronal cytosol. ii) The protease with autoproteolysis activity (Ahmed et al., 2001, 2003; DasGupta et al., 2005) became refractory to self-digestion by the presence of the H-chain. The probability of this imagined scenario of the past is demonstrable: the ~50-kDa protease domain by itself and also separately the protease domain fused to its adjacent neighbor the ~50-kDa transmembrane (translocation) domain, both recombinant products (the ~50- and ~100-kDa, respectively), are proteolytically active (Ahmed et al., 2001; Chaddock et al., 2002).

The junction of the protease domain (L-chain) and the N-terminal of the ~100-kDa H-chain, flanked by a conserved disulfide bridge has the following two features (Fig. 3 in Lacy and Stevens, 1999) that hint a conjecture: i) The amino acid residues spanning this disulfide bridge range from 8 to 23 among the seven botulinum NT serotypes and 27 in tetanus NT; alignment of their sequences shows gaps and hardly any homology. ii) In contrast to this are the residues neighboring the two outsides of this bridge; the 27–26 residues upstream of the conserved Cys (e.g. in type A Cys 429) among the L-chains have notable homology as do the 53 residues (in type A; fewer in others) downstream of the conserved Cys (e.g., in type A Cys 453) among the H-chains. Therefore does the highly heterologous bridged section (8 to 27 residues) represent remnants of various "trials" in the coupling of the L-chain with the H-chain? Did both originate independently?

Viral proteases are known to cleave cellular proteins (Kräusslich and Wimmer, 1988; Poorman et al., 1991) and to be autocatalytic, and autocatalysis (and alternatively a second protease) releases the protease domain from the precursor polyprotein format and/or the polyprotein processing can occur in heterologous cells. All of these features are now apparent in the clostridial NT proteases. A virus encoded metalloproteinase had not been identified when a review article (Dougherty and Semler, 1993) was written although serine, cysteine (or thiol) and aspartic (or acidic) proteases were known. Since then at least three virus-encoded metalloproteinases (including the Zn binding site HEXXH in enhancin) have been discovered (Lepore et al., 1996; Liu et al., 2000; Peng et al., 1999; Suzuki et al., 1999). To this growing list can now be added the phage-encoded proteinase of clostridial polyprotein NT, given the proposed new recognition.

It is also worth speculating whether the viral protease (the L-chain of the NT), expressed only in a few anaerobic bacterial species, when placed in the neuronal cytosol, an alien and remote milieu, is resistant to protein degradation by virtue of the N-end rule, i.e., because of the presence of Met or Pro at the amino terminus and absence of Lys at position 15 or 17 (discussed in Gottesman and Maurizi, 1992). All clostridial NTs have Pro or Met at their N-terminus except botulinum type NT D, which has Thr, and none have Lys at the indicated positions. This perceived resistance agrees with two experimental results; the NT type B

survives in the presence of endogenous aminopeptidase (Millonig, 1956) and the NT type A is resistant to exogenous aminopeptidases (Simpson et al., 2005).

When the three domains of the clostridial NT were just becoming apparent Simpson (1986) had surmised that "the clostridial NTs may be proteins encoded by nucleic acid that at one time served as a template for virus domains, but which through mutations and other modifications now serve as a template for toxin domains." To the above ideas we like to add the observation that lectins from *Triticum vulgaris* and *Limax flavus* (one of plant and the other of animal origin) competitively antagonize actions of clostridial NTs (Bakry et al., 1991) by binding to the specific receptors of the neurotoxic polyprotein. Hence this conjecture: did genes of such lectins (they function as adhesion molecules; Sharon, 1987) get incorporated and code for the NT's receptor binding domain? The idea is based on i) known interkingdom mobilization of DNA, e.g. transport and incorporation of a bacterial (*Agrobacterium tumefaciens*) DNA to the plant genome (Burns, 2003), and ii) well known proteins of similar function in widely different organisms (the plant protease papain and the mammalian lysosomal proteases cathepsin B and H have a high degree of sequence identity; hen egg-white lysozyme and bacteriophage T4 lysozyme show no similarities in amino acid sequence; Neurath, 1984, 1985). This proposal that the clostridial NTs are polyproteins and are of viral origin—in which the three independently functional domains are synthesized in equimolar amounts—provides a conceptual model by consolidating various biochemical and biophysical data (such as the nucleotide sequences encoding the NTs, their complete amino acid sequences, their phylogenetic relationships, 3-D structures, protease-, channel formation- and receptor binding activities) rather than calling the toxin/neurotoxin protein merely by a different name. This link—between virus, bacteria and blockage of fusion of vesicles to membranes in eukaryotes—may provide a stimulus for further thinking and research in newer areas.

Clostridial NTs—a perspective on the present and past of the protease and its substrates: First the substrates: Many biochemical reactions in the evolutionary development became segregated (compartmentalized), particularly in eukaryotic cells, by membrane bound organelles (e.g., endosomes, lysosomes); vesicles formed and began to deliver their loaded "cargo" (by precise trafficking, targeting and docking) to an acceptor organelle following their fusion. The proteins participating in the above processes now appear in two families rab/ypt (in yeast) GTPases (an earlier idea) and SNAREs (v- and t-SNAREs located on vesicle and target membrane, respectively) beside "a variety of additional peripheral membrane proteins" (Pelham, 2001). Neurotransmission (a specialized form of exocytosis; Bock et al., 2001; Fukuda et al., 2000; Rizzoli and Betz, 2003) is preceded by fusion of synaptic vesicles with presynaptic membrane governed by the assembly of a bundle of four helices of which one is from the v-SNARE (VAMP-2), one from syntaxin 1A and two helices from SNAP-25 (both t-SNAREs). The SNARE family proteins essentially conserved through phylogeny from yeast to man, testify that "multicellular organisms do not have an inherently more complex secretory pathway and that a set of core SNARE is sufficient to mediate most intracellular vesicle fusion events" (Bock et al., 2001). Of the above mentioned SNAREs only three are known to be cleaved by the clostridial NT proteases. This background elicits the following questions and considerations:

Cleavage of any one of the three SNARE proteins "serves the purpose" to block fusion of vesicles to membranes, yet the ancestral protease evolved into 8 serotypes (botulinum plus tetanus) with the ability to cut all 3 proteins at 7 different sites (Humeau et al., 2000). If there was only one susceptible protein, development of a protease resistant isomer would defeat the NT protease, for example, VAMP-1 of rats and chickens are not cleaved by tetanus or botulinum NT type B (but is cut by NT types F, D, G) and SNAP-25 of leech are resistant to NT type A (but cleaved by NT types C and E) and syntaxin 4 (but not syntaxin 2 and 3) of rat is refractory to NT type C (Humeau et al., 2000). So was this contingency countered by expanding the repertoire of substrates to the three targets?

Conversely did the NT-susceptible organisms develop, to protect their vesicle fusion system, clostridial protease resistant isomers of VAMP, SNAP-25 and syntaxin, opting for single point mutations at the cleavage sites as noted and cataloged in Humeau et al. (2000)? The rates of growth and evolution of the susceptible higher organisms being slower than the NT producing microbes left them with partial successes in acquiring refractive peptide bonds but ultimately vulnerable, for no animal species is known to be resistant to all the clostridial NT proteases (Humeau et al.,

2000; Montecucco and Schiavo, 1995).

Does the ~150-kDa clostridial NT protein(s), carrier of a ~50-kDa protease that cuts only v- and t-SNAREs to disrupt their helical bundle formation, provide any metabolic benefit to the toxigenic bacteria? Or did the NT(s) evolve just to impair fusion of neurotransmitter loaded vesicles to presynaptic membrane? Or is it a member of a family of clostridial proteases (conjectured above and below) that evolved to block exocytosis (thus neurotransmission) and cause paralysis leading to death; in other words, did the polyproteins come into being specifically to kill? If so α-neurexins appear theoretically an excellent target for the NT's lethality. This is a family of evolutionarily conserved presynaptic transmembrane proteins (the larger portion is extracellular, and shorter is intracellular) that "grips" the cognate neurolignin on the postsynaptic membrane and is essential for Ca^{2+}-triggered neurotransmitter release and essential for survival (Littleton and Sheng, 2003; Missler et al., 2003). The large extracellular region of the α-neurexin (for a schematic model see Fig. 1 in Littleton and Sheng, 2003) could be conjectured cleavable by a NT protease (or its variant) under any of the three circumstances by being accessible to the i) extracellular 150-kDa NT (prior to internalization by endocytosis) while bound to its receptors on the presynaptic membrane or ii) the ~50-kDa L-chain released in an extracellular milieu from the ~100-kDa H-chain following rupture of the interchain –S–S– bridge (Bhattacharya et al., 1988) or following probable autoproteolytic cleavage outside the –S–S– bridge (DasGupta et al., 2005), or iii) the ~50-kDa protease (the conjectured viral protease before it became fused to the transmembrane and receptor domains; noted above). Whether the α-neurexins have determinants of NT cleavage (binding and cleavage sites) is not yet known. Their absence may suggest success in fending off the NT's attack.

Features of the substrates of the proteases are: i) Neuronal proteins cleaved in vivo (VAMP/synaptobrevin, syntaxin and SNAP-25) result in profound consequences (Humeau et al., 2000; Schiavo et al., 2000). ii) A protein (cellubrevin) found in *non-neuronal* cells is cleaved; consequences of the cleavages are becoming known (see below). iii) The light chain of the NT is autoproteolyzed (Ahmed et al., 2001, 2003; DasGupta et al., 2005), implications of which are not yet clear. In addition a speculated feature is a protein (hypothetical) that is cleaved in vivo but the effect is benign and/or manifestation of the cleavage consequence is not acute (see below, e.g., Rab3A). Cellubrevin, a homologue of VAMP-2 (synaptobrevin) distributed in a wide range of tissues and not expressed in neurons (Proux-Gillardeaux et al., 2005b; Yamasaki et al., 1994) has several important biological functions (e.g., recycle transferrin receptors which are inhibited on cleavage of cellubrevin) in the constitutive exocytosis of vesicles collateral to regulated pathways with triggered exocytosis (Galli et al., 1994). It is cleaved like VAMP-2 by tetanus and botulinum NT types B, D, F and G (Humeau et al., 2000). Recently Proux-Gillardeaux et al. (2005a) demonstrated cleavage of cellubrevin by tetanus NT "significantly reduced the speed of migrating epithelial cells . . . enhanced the adhesion of epithelial cells to collagen, laminin, fibroactin, and E-cadherin; altered spreading on collagen and impaired the recycling of β-1 integrins." Cellubrevin is a good substrate for five clostridial NT proteases (in vitro and transfected cells), so is it probable that under special circumstances (yet to be recognized) some tissue(s) might let in a clostridial NT allowing proteolysis of cellubrevin that might produce unrecognized manifestations in humans and animals. Are some of the actions of the clostridial NTs, other than blockage of neurotransmitter release (cited in DasGupta, 1993; Humeau et al., 2000) relevant in these considerations?

Thoughts on the protease: The NT proteases cleave VAMP and SNAP-25 at 4 and 3 different peptide bonds, respectively. Does this indicate additional contingency against cleavage of a bond that would not impair vesicle fusion? Thus the question, is there (was there but now lost?) a protease that cleaves a bond in one of the SNARE proteins that does not impair the vesicle fusion and hence is not (or not acutely) neurotoxic and thus remains unrecognized (see below, Autism)?

In considering redundancy, precision and success Radman (2001) noted "The apparent perfection of organisms and the accuracy of biological processes . . ." where "survival . . . is the ultimate virtue . . . life's structures do not emerge by fully deterministic design, and . . . a precise, single shot [arrow] would often miss a target of uncertain position, whereas successive, imprecise firing will eventually lead to a hit." Is this the "strategy" that developed in clostridial NTs; five proteases (tetanus and botulinum NT types B, D, F, G) target one protein (VAMP/synaptobrevin), three (botulinum NT types A, C and E) target another protein

SNAP-25 and syntaxin targeting (cleavage) remained limited to only one protease (botulinum type C NT)?

Membranes keep the NT protease secluded from its specific substrates and the two become interactive only after the mediation of the receptor binding and translocation domains. So, before the ~50-kDa protease became part of the 150-kDa NT polyprotein (by fusing with the two domains as proposed above) was (were) its substrate(s) different (from now) in not being shielded by a membrane (as conjectured above about α-neurexins)? Before compartmentalization set in were the substrates (in the earlier evolutionary form) accessible to the proteases? Are their vestiges still lingering? In the past did the NT protease(s) arrive on the scene before or after the SNAREs or did they (the "predator and prey") develop together? Existence of a protease without a substrate is unlikely. Did the protease acquire the exquisite substrate specificity in an unlikely single evolutionary leap or by gradualism ("trial and error") in substrate specificity along with the evolution of compartmentalization (noted above)? *Before* and *after* the NT protease(s) became selective for only the three SNARE proteins as their prey were the proteases also designed with other substrate specificities, to cleave other proteins involved in organelle trafficking besides VAMP-2, syntaxin 1A and SNAP-25? Several other SNAREs have been identified (e.g., in yeast 21 different ones) that have multiple functions and/or locations (Fukuda et al., 2000; Lewis and Pelham, 2002), so are such other proteins participating (known and presumed) in lipid bilayer fusion but are not cleaved? Two syntaxins PEN1 and ROR2 conserved in plants (angiosperms) function in their resistance to pathogens; vesicles appear to deliver antimicrobial cargo at the entry site of a pathogen (Collins et al., 2003). These two SNARE protein homologues, like rat syntaxin 4, do not have the site Lys-Ala that is cleaved in syntaxin 1A/1B by type C botulinum NT. Given the right set of conditions, could entry of the viral protease (the 50-kDa protein) into a plant cleave a SNARE protein and also breach the "SNARE-protein-mediated disease resistance at the plant cell wall" promoting the plants' demise and death? Are there remnants of the "trial and error" scenario still around or are these hypothetical variant proteases of antiquity lost for lack of biochemical fitness?

The ability of the contemporary protease (and the possible variants) to cleave other proteins involved in lipid bilayer fusion might not be readily apparent if the substrate protein is functionally redundant (e.g., SNARE Nyv1 of yeast; Pelham, 2001) or the consequence of cleavage is not acute; e.g. "Rab3A (present on synaptic vesicles) knock out mice are viable, but certain synapses in their brain fail to undergo the plasticity called long-term potentiating" (Bock et al., 2001). The case of Rab3A indicates a probable tangible medical benefit from a systematic analysis of the proteolytic susceptibility of other SNARE proteins to the clostridial NTs. Certain cases of autism, a non-fatal neurological disorder, were recently postulated to be caused by subacute chronic infection by clostridial species and tetanus NT (Bolte, 1998; Finegold et al., 2002). If this is correct, it follows that the familiar acute spastic or flaccid paralysis does not ensue because, perhaps, the NT protease acts at site(s) other than NMJ and CNS (the well recognized sites of action of the NT protease), by gaining entry in an as yet unknown way, on the familiar or other protein substrates.

Experimental pursuit of the above questions (aided by the emerging whole genome sequences from various organisms, including that of *C. tetani* and *C. botulinum*; Brüggemann et al., 2003) may be rewarding in constructing a biochemical history and in discovering a bioactive protein of practical use unimaginable now as were the therapeutic uses of botulinum NT before 1978.

The clostridial NTs developed eight different antigenic profiles (seven serotypes of botulinum NT and tetanus). Was it to evade immune defense? If so, in what host and where is the evidence? In doing so did the structural (epitopes) alterations bring about in the protease domain the array of substrate specificities, or was it the other way around, i.e., as the different protease substrate specificities developed (possible impelling force/pressure mentioned above) through structural changes antigenic profiles also changed in the other two domains? This enigma appears apparent in the immunoglobulin A1 (IgA1) endopeptidases produced by a variety of human pathogens such as *Neisseria gonorrhoeae*, *N. meningitidis*, and *Haemophilus influenzae* (Lomholt et al., 1992; Lorenzen et al., 1999). They cleave with high specificity a single peptide bond in the hinge region of human IgA1 but not IgA2. The protease type 1 IgA1 and the type 2 IgA1 cleave one of several -Pro-Ser- and -Pro-Thr- bonds, respectively. The autocatalytic sites inside the protease precursor molecule (like clostridial NTs) are the only known alternative substrates of the *Neisseria* and

Haemophilus IgA1 proteases. The Gram-negative bacterial IgA1 proteases are derived from a polyprotein precursor molecule. Although IgA1 protease gene of *N. meningitidis*, *N. gonorrhoeae* and *H. influenzae* suggest a common evolutionary origin, substantial heterogeneity exists, "even among the IgA1 protease secreted by members of a single species" and they differ in antigenicity.

The above considerations culminate in two simple issues; the first one we understand the other we don't know: An inanimate object, a virus, multiplies in a prokaryotic anaerobic bacteria, a byproduct protein specifically targets the highly evolved presynaptic junctions and disrupts electrophysiological function in situ. So what is eluding us in the simple biological connection between virus, anaerobic bacteria and presynapse—up the evolutionary ladder that presumably benefits any one of the three participant players?

Acknowledgments

Some of the above ideas developed during the experimental work that was funded by the US Army Medical Research and Development Command (DAMD 17-80-C-0100; 17-83-C-3034; 17-84-C-4245) and the National Institutes of Neurology and Communicative Disorders and Stroke [NS17742-01; NS17742-04; NS17742-07 (Javits Neuroscience Investigator award); NS24545-01 and NS25063-01]. Barbara Cochrane's meticulous retyping of the manuscript is appreciated.

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