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Structural analysis of the catalytic domain of tetanus neurotoxin

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Abstract

Clostridium neurotoxins, comprising the tetanus neurotoxin and the seven antigenically distinct botulinum neurotoxins (BoNT/A-G), are among the known most potent bacterial protein toxins to humans. Although they have similar function, sequences and three-dimensional structures, the substrate specificity and the selectivity of peptide bond cleavage are different and unique. Tetanus and botulinum type B neurotoxins enzymatically cleave the same substrate, vesicle-associated membrane protein, at the same peptide bond though the optimum length of substrate peptide required for cleavage by them is different. Here, we present the first experimentally determined three-dimensional structure of the catalytic domain of tetanus neurotoxin and analyze its active site. The structure provides insight into the active site of tetanus toxin's proteolytic activity and the importance of the nucleophilic water and the role of the zinc ion. The probable reason for different modes of binding of vesicle-associated membrane protein to botulinum neurotoxin type B and the tetanus toxin is discussed. The structure provides a basis for designing a novel recombinant vaccine or structure-based drugs for tetanus.
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1. Introduction

Tetanus neurotoxin (TeNT) produced by *Clostridium tetani* and the seven antigenically distinct botulinum neurotoxins (BoNT/A-G) produced by *Clostridium botulinum* together constitute the family of clostridial neurotoxins (CNTs) (Montecucco and Schiavo, 1995; Rawlings and Barrett, 1995). Tetanus neurotoxin acts on the central nervous system and inhibits the release of glycine and γ-aminobutyric acid causing spastic paralysis, tetanus (Galazka and Gasse, 1995). In contrast, botulinum neurotoxins (BoNTs) act on the peripheral nervous system and inhibit the release of acetylcholine at the neuromuscular junction, causing flaccid paralysis, botulism (Simpson, 1986). These proteins are of public health concern as paralysis by these toxins still takes hundreds of lives every year (Galazka and Gasse, 1995). They are also emerging as biowarfare threats.

CNTs consist of three functional domains: binding, translocation, and catalytic. CNTs bind to the neuronal cells via gangliosides and a second protein receptor and then are internalized. Their catalytic domain is translocated through the vesicle membrane into the cytosol where it attacks and cleaves one of the proteins forming the core of the synaptic vesicle fusion apparatus. CNTs synthesized as 150 kDa inactive single chain molecules are post-translationally modified either by endogenous or exogenous proteases into two polypeptide chains covalently linked by a disulfide bond: the C-terminal heavy chain (HC, 100 kDa)

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and the N-terminal light chain (LC, 50 kDa). The C-terminal heavy chain is responsible for the binding of the toxin to specific neuronal receptors and the translocation of the N-terminal LC, the catalytic domain, into the neuronal cytosol. The LCs of CNTs contain the zinc-binding motif HExxH + E and are accordingly zinc-dependent metalloproteinases causing toxicity (Eswaramoorthy et al., 2004; Fillippis et al., 1995; Kurazono et al., 1992; Schiavo et al., 1994; Simpson et al., 2001). Interestingly, though CNTs display high sequence homology, have similar functions and probably have similar structure, they are unique in that their target protein and the scissile bonds they cleave are different, a property very commonly shared by members of a given protease family. TeNT, BoNT/B, /D, /F, and /G cleave vesicle-associated membrane protein (VAMP) (Schiavo et al., 2000). While the cleavage site is different for D, F and G, it is the same for TeNT and BoNT/B, a unique case in CNTs. Similarly, BoNT/A, /E, and C cleave synaptosomal-associated protein 25 kDa (SNAP-25) at different peptide bonds (Binz et al., 1994; Schiavo et al., 1993; Vaidyanathan et al., 1999). BoNT/C is unique in that it also cleaves syntaxin.

Chemically modified TeNT is the most used human vaccine and BoNTs are increasingly used in the therapy of human diseases caused by hyperfunction of cholinergic nerve terminals (Jankovic and Hallett, 1994). Chemically treated toxoid vaccine is available against BoNTs but no therapeutic treatments exist as of now. To develop an effective structure-based vaccine/inhibitor/antitoxin to treat tetanus victims, an understanding of the molecular mechanism at the atomic level, especially the peptidase activity by the catalytic domain, is a prerequisite. Though experimental three-dimensional structures are available for some of the CNTs and their functional fragments, no experimental structure is available for TeNT-LC except for a homology-based model (Agarwal et al., 2004a; Breidenbach and Brunger, 2004; Emsley et al., 2000; Hanson and Stevens, 2000; Lacy and Stevens, 1998; Rossetto et al., 2001; Segelke et al., 2004; Swaminathan and Eswaramoorthy, 2000; Umland et al., 1997). These structures helped to map the active site and the residues forming it. Although the active sites are similar, the specificity and selectivity of CNTs suggest that there must be additional factors that define the substrate specificity. Thus, it becomes important to understand the molecular structure of each of the CNTs at the atomic level to gain insight into their unique ability to cleave at specific scissile bonds within the same substrate and also among different substrates. A possible catalytic mechanism has been proposed for TeNT from the homology model (Rossetto et al., 2001). Extensive mutational studies have been carried out on TeNT (Li et al., 1994; McMahon et al., 1993; Yamasaki et al., 1994). Some of these mutants were proteolytically inactive indicating that they may have a direct role in the catalytic activity of the protease. However, there was no matching X-ray structural information available to interpret these results. The chemical characterization of the active site and of the residues involved in zinc binding of TeNT is important to understand the mechanism of the proteolytic activity of this novel family of metalloproteases (Montecucco and Schiavo, 1995; Schiavo et al., 1994) and may lead to the development of novel and safer recombinant vaccine produced outside of *C. tetani*. A crystal structure analysis provides a tremendous amount of insight into both the structure and the function of the protein. Although the crystallization of TeNT-LC has been reported, no experimental structure is available in literature (Tonello et al., 1994). Here, we present the first experimental three-dimensional structure of TeNT-LC and compare it with the crystal structures of available BoNT-LC structures.

2. Materials and methods

2.1. Expression and purification of his-tagged TeNT-LC protein

The procedure for expression and purification is as described in Agarwal et al. (2004b) except that ampicillin-containing medium was used. The protein was eluted from Ni-NTA agarose columns with increasing concentrations of imidazole buffer. Fractions of the eluate were analyzed by SDS-PAGE using 4–20% gels followed by staining with Coomassie blue. A ~52 kDa band corresponding to TeNT-LC reproducibly eluted in 50–100 mM imidazole fractions was obtained. Recovery of TeNT-LC was more than 4 mg/l of induced cell culture. At this stage, it is nearly 80% pure. TeNT-LC was further purified by gel filtration on a (2×20 in.) column of Superdex-75 using Akta FPLC which also helped in exchanging the buffer from phosphate to 20 mM HEPES, pH 7.2 + 200 mM NaCl. Peak fractions containing only pure TeNT-LC were pooled and concentrated to ~10.0 mg/ml using Centriprep YM-10.

2.2. Enzymatic activity of TeNT-LC

The proteolytic activity of TeNT-LC was assayed in vitro on its substrate VAMP which had an N-terminal GST tag. The assay was performed in a final volume of 20 μL [20 mM HEPES, pH 7.4, 2 mM DTT, 10 μM $Zn(CH_3COO)_2$] containing a 10–5000 nM concentration of LC and a 5 μM concentration of VAMP and the mixtures were incubated at 37 °C for 30 min. The reactions were stopped by adding 10 μl of 3× concentrated SDS-PAGE sample buffer. The extent of cleavage was then evaluated following SDS-PAGE (4–20% gels) by the appearance and intensity of a new band at ~30 kDa due to the cleavage of GST–VAMP at Gln76–Phe77 peptide bond of VAMP. A 1000 nM concentration of LC was required to cleave 50% of the substrate in 30 min.