

Abstract No. eswa741

Studies on Clostridial Neurotoxin: Inhibitor Complex

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Beamline(s): X12C and X25

Introduction: *Clostridium botulinum*, a Gram-positive spore-forming bacterium, produces seven serotypes (A-G) of neurotoxins (BoNTs) which are solely responsible for botulism causing flaccid paralysis and death^{1, 2}. These neurotoxins fall under the general category of AB toxins: promoter A (activating) is 50 kDa light chain and promoter B (binding) is 100 kDa heavy chain held together by a disulfide bond after proteolytic cleavage. The three-dimensional structures of BoNT/A and B reveal three structural domains corresponding to three functions, viz., binding, translocation and catalytic activity^{3, 4}. In all serotypes, the light chain contains a zinc-binding motif HExxH+E in their catalytic domain and their three-dimensional structures are expected to be similar in general because of significant sequence homology. The catalytic zinc is located in a deep cavity in the active site that has a wide opening for the substrate or inhibitor to enter the site.

Since chelation of zinc antagonizes the neuromuscular blocking properties of botulinum neurotoxins, specific zinc chelators or inhibitors of zinc metalloproteases are being evaluated. Recently, a coumarin derivative, Bis(5-amidino-2-benzimidazolyl)methane (BABIM), an effective zinc chelator, has been tested with BoNT/B light chain and its IC50 (inhibition constant) is in the range of 5 - 10 μ M⁵. Here we present the crystal structure of a protein-inhibitor complex of intact botulinum neurotoxin type B and BABIM determined at different duration of soaking to understand the mechanism of inhibition and the probable path of entry for the inhibitor. These structures provide a series of snapshots during the reaction and reveal a possible mechanism of binding and chelation of the zinc ion.

Methods and Materials: Crystals were obtained in MES buffer of pH 6.0 using PEG 6000 as precipitant. Crystals were soaked with 3mM BABIM concentration in the buffer suitable for cryo-conditions (100mM cacodylate buffer of pH 6.0 with 15% glycerol) for four time intervals of 2, 4, 12, 22 minutes. Data were collected for four crystals with different soak time as mentioned above at liquid nitrogen temperature. The Beamline X12C of the NSLS, Brookhaven National Laboratory with Brandeis CCD based B1 detector was used for data collection⁶. Data were reduced with DENZO/SCALEPACK⁷. Crystal structure of the wild type toxin (PDB ID 1EPW) was used as model for the refinement of these complexes. Structures of the different forms were determined by Molecular Replacement method using AMoRe⁸. The protein model was first refined using CNS⁹ until convergence. Electron density map ($|F_o - F_c|$) calculated with the refined protein model showed clear density for E267 at the other conformation and good additional density at two places accountable for BABIM molecule. The model was corrected and the inhibitor molecules added for further refinement.

Results: The botulinum neurotoxin molecule comprises three structural domains and are named as catalytic domain, translocation domain and binding domain according to their functions. The catalytic domain contains a metal binding motif, HExxH+E at the active site. The toxin is a metalloprotease and a Zn atom is present at the active site coordinated to H229, H233, E267 and a water molecule. The structures of protein-inhibitor complex show an alteration in the active site. The side-chain of E267 has moved to another orientation and its coordination to the Zn atom was replaced with a water molecule. When the Zn atom was studied carefully, it is found that the Zn atom occupancy decreased as the soaking time increased. At the fourth experiment the Zn occupancy was 0.5.

Two BABIM molecules were found in all the four structures though not at the active site. The inhibitor BABIM1 was bound at a cleft between the catalytic and translocation domain facing the active site. BABIM2 also binds between the same domains at a cleft facing the solvent. These two BABIM molecules partially occupy the binding site of the natural substrate of the protein. BABIM1 has a Zn atom coordinated to it through N3 and N3' atoms. The rearrangement of E267 side-chain and the Zn coordination to one of the BABIM molecules indicate that the inhibitor has interacted with the active site zinc though it was not observed directly. As the soaking time increased, it is found that the occupancy of the zinc ion bound to BABIM1 increased while the occupancy of the active site zinc decreased.

Conclusions: We believe that BABIM1 binds to the zinc ion and grabs it away from the active site and moves to a position where interactions for it to bind to the protein are more favorable. The mode of zinc binding to BABIM1 suggests that initially BABIM1 should have bound to the active site zinc via N3 and N3' of BABIM.

Acknowledgments: Research supported by the Chemical and Biological Non-proliferation Program - NN20 of the U.S. Department of Energy under Prime Contract No. DE-AC02-98CH10886 with Brookhaven National Laboratory.

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