Biochemical and Biophysical Research Communications 381 (2009) 50–53

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Crystal structure of a catalytically active, non-toxic endopeptidase derivative of *Clostridium botulinum* toxin A

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ARTICLE INFO

*Article history:*
Received 30 January 2009
Available online 8 February 2009

*Keywords:*
Botulinum neurotoxin
Protein engineering
Crystal structure
Therapeutics
Fusion

ABSTRACT

Botulinum neurotoxins (BoNTs) modulate cholinergic nerve terminals to result in neurotransmitter blockade. BoNTs consists of catalytic (LC), translocation (Hn) and cell-binding domains (Hc). The binding function of the Hc domain is essential for BoNTs to bind the neuronal cell membrane, therefore, removal of the Hc domain results in a product that retains the endopeptidase activity of the LC but is non-toxic. Thus, a molecule consisting of LC and Hn domains of BoNTs, termed LHn, is a suitable molecule for engineering novel therapeutics. The structure of LHA at 2.6 Å reported here provides an understanding of the structural implications and challenges of engineering therapeutic molecules that combine functional properties of LHn of BoNTs with specific ligand partners to target different cell types.

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Toxins from *Clostridium botulinum* species are feared for bio-terrorism and are the causative agent of the rare neuroparalytic illness botulism. There exist seven distinct serotypes (A–G) of botulinum neurotoxins (BoNTs) that affect humans and other species to varying degrees. Once inside the neuronal cell, BoNT blocks the release of neurotransmitters leading to paralysis which, without medical intervention, can lead to death. Despite their potent toxicity, various preparations of BoNTs are available commercially as therapeutic and cosmetic agents. The long-term effect and weakening of muscles by BoNTs is harnessed for use in the treatment of muscular overactivity at safe doses. For a general overview see refs [1,2].

BoNTs are synthesized as a single polypeptide chain (150 kDa), which is post-translationally cleaved into a di-chain molecule composed of light chain (LC) (~50 kDa) and heavy chain (HC) (~100 kDa). LC is the catalytic domain and a zinc-endopeptidase, while HC is further divided into two sub-domains of equal molecular mass called the translocation domain (Hn) and the membrane binding domain (Hc). LC and HC are linked by a single disulphide bridge and several intra-molecular hydrogen bonds. The substrate access to the catalytic zinc site is prevented by a "belt" region from the translocation domain. This is a common feature of all BoNT serotypes. On binding to the nerve terminals BoNTs are endocytosed into a vesicle, where at the acidic environment conformational changes occur and the LC is translocated into the cytosol. It has been proposed that Hn mediates the transfer of LC into the cytosol through the membrane by forming a pore. Inhibition of neurotransmission takes place by proteolysis of one of the soluble *N*-ethyl-maleimide-sensitive fusion protein attachment receptor (SNARE) proteins. These proteins are essential mediators of the secretion mechanism and consist of the vesicle associated membrane protein (VAMP) or synaptobrevin, the pre-synaptic membrane proteins- SNAP-25 (synaptosomal associated protein of 25 kDa) and syntaxin.

LHA is a non-toxic fragment of BoNT/A. It consists of only the LC and Hn of the whole toxin, hence it is incapable of binding extracellularly to cell membranes, but retains its full catalytic activity [3]. LHA was originally prepared by trypsinization of whole BoNT/A [4]. However, recombinant LHA has subsequently been achieved by Chaddock et al. [3].

LHA was proposed as a component in the design of a range of therapeutic molecules for the treatment of pain, control of secretion from multiple cell types and other muscular disorders. By coupling the LHA molecule to a ligand that selectively binds to a specific target cell that plays a role in the maintenance of a chronic disease state, it is proposed that the retargeted LC activity will result in inhibition of secretion from said cell, thereby leading to alle-

*Abbreviations:* BoNT(/N), botulinum neurotoxin (/serotypes A–G); HC, heavy chain of BoNT; Hn, translocation domain of BoNT; LC, light chain of BoNT; LHA, light chain and translocation domain of BoNT/A; LHn, light chain and translocation domain of BoNT serotypes; SNAP-25, synaptosome-associated protein of 25kDa; SNARE, soluble *N*-ethyl-maleimide-sensitive factor attachment protein receptor; VAMP, vesicle associated membrane protein; GFP, green fluorescent protein

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doi:10.1016/j.bbrc.2009.02.003

viation of symptoms and improvement in quality of life. As proof-of-principle, retargeting of LHA in fusion with other cell binding proteins such as *Erythrina cristagalli* lectin [5], wheat germ agglutinin [6], and nerve growth factor [7] have been successfully tested in vitro and in vivo models.

The elucidation of the 3D structure of LHA to confirm its native state and to aid in designing suitable ligands as delivery vehicle has become important. Here, we present details of the first crystal structure of a novel LHn non-toxic fragment from BoNT/A at 2.6 Å resolution. We have also performed SNAP-25 cleavage assay to confirm the catalytic activity, and have assessed the stability of LHA. These results have provided the 'structural basis' for the LHA protein and should lead the way forward for the design of a new class of therapeutic molecules.

## Materials and methods

*LHA cloning, expression, purification and activation.* The synthetic gene encoding 877 amino acids of LHA was cloned into modified pMAL-c2x vector (NEB, UK) with a Factor Xa cleavable N-terminal 6 x His-tag and transformed into *Escherichia coli* BL21 expression cells. Another Factor Xa cleavage site (IEGR) was engineered between the LC and Hn domain to mimic the native protein activation. The cultures were grown in a 2 l conical flasks at 220 rpm, 37 °C until the $OD_{600}$ reads 0.5–0.6. The cultures were induced with 1 mM IPTG at 16 °C and harvested after 20 h. The cell pellets were stored at −80 °C until further use.

Cells were resuspended in 50 mM HEPES, pH 7.2, 0.2 M NaCl (buffer A) and lysed using a homogeniser (Constant Systems Ltd). Lysate was centrifuged for 45 min at 12,000 rpm. Soluble fraction was loaded onto a $Ni^{2+}$-charged chelating sepharose column (GE Healthcare). LHA eluted at 100 mM imidazole (dissolved in buffer A) and dialysed overnight at 4 °C against buffer A. Activation of purified LHA is achieved by Factor Xa (BioLabs) treatment. The cleaved fusion protein was loaded onto a Ni-charged chelating sepharose equilibrated with buffer A. The flow-through was collected and supplemented with ammonium sulphate to 1 M. Sample was loaded onto a phenyl sepharose column (GE Healthcare) equilibrated with 1 M ammonium sulphate in buffer A. LHA eluted at 0.7 and 0.6 M and dialysed overnight against buffer A at 4 °C. The sample was finally concentrated using Vivaspin 50000 MWCO concentrator to 5 mg/mL. All concentrations were determined by $A_{280}$ and stored at −20 °C.

*SNAP-25 cleavage assay.* LHA (1.5 mg/ml) was incubated in assay buffer (50 mM HEPES pH 7.2, 20 μM $ZnCl_2$, 1 μg/μl BSA, 10 mM DTT) and incubated at 37 °C for 30 min. SNAP-25-GFP (4 μM) (recombinant in-house substrate) was then added to the reduced enzyme and incubated for 4 hours at 37 °C. Reactions were stopped by adding 2× reducing sample buffer. Samples were then analysed by SDS–PAGE.

*X-ray crystallography.* Crystals were grown in 15% sucrose, 0.1 M Tris–acetate pH 8.5, 1.7 M ammonium sulphate. Diffraction data were collected at the Diamond Light Source, UK, beamlines IO2 and IO3 in the presence of 25% glycerol as cryoprotectant. A complete dataset to 2.6 Å was collected from a single crystal. The data were processed and scaled in orthorhombic space group $P2_12_12_1$ using HKL2000 [8] suite (Table 1). Initial phases were obtained by molecular replacement using MOLREP [9] with the coordinates of a homology based model of the LHA fragment. Crystallographic refinement was carried out using CNS suite version 1.2 [10] and model fitting were done using COOT version 0.4.1 [11]. The structure was validated using PROCHECK [12] and MOLPROBITY [13]. Figures were drawn with PyMOL[§] (DeLano Scientific LLC).

*Stability assay.* Purified LHA sample was dialysed in buffer A and 100 mM Tris acetate pH 8.5, 1.5 M ammonium sulphate, 15% glycerol. Samples were then diluted to 1.5 mg/ml in corresponding buffer, aliquoted and stored at −20, +4 and +25 °C until the day of testing. All samples were loaded in duplicate (2 μg) on non-reduced and reduced gels (4–12% Bis–Tris, Invitrogen). The percentage purity of LHA was determined by densitometry (GeneTools, Synoptics Ltd).

**Table 1**
X-ray data collection and refinement statistics.

| | |
|---|---|
| *(A) Data collection statistics* | |
| Space Group | $P2_12_12_1$ |
| Number of molecules/asymmetric unit | 2 |
| Cell dimensions | $a$ = 78.4, $b$ = 157.0, $c$ = 211.7 Å ; $\alpha = \beta = \gamma = 90^o$ |
| Resolution range (Å) | 50–2.59 |
| $R_{symm}$[a] (%) | 10.9 (55.3) |
| I/σI (outer shell) | 11.5 (2.0) |
| Completeness (outer shell) % | 97.1 (98.4) |
| Total no. of reflections | 528,420 |
| Unique no. of reflections | 82,260 |
| Redundancy | 1.9 (1.8) |
| Wilson B-factor ($Å^2$) | 50.8 |
| *(B) Refinement statistics* | |
| Resolution range (Å) | 50–2.59 |
| $R_{cryst}$[b] (%) | 21.2 |
| $R_{free}$[c] (%) | 25.3 |
| Number of non-H atoms | |
| Protein | 13,740 |
| Ligand | 2 Zinc, 3 Chloride, 1 Acetate, 4 Sulphate, and 5 Glycerol |
| Water molecules | 309 |
| Average temperature factor (B-factor) ($Å^2$) | 41.0 |
| RMSD in bond lengths (Å) | 0.009 |
| RMSD in bond angles (°) | 1.3 |

[a] $R_{symm} = \Sigma_h \Sigma_i |I(h) - I_i(h)|/\Sigma_h \Sigma_i I_i(h)$, where $I_i(h)$ and I(h) are the ith and the mean measurements of the intensity of reflection h, respectively.

[b] $R_{cryst} = \Sigma_h |F_o - F_c| / \Sigma_h F_o$, where $F_o$ and $F_c$ are the observed and calculated structure factor amplitudes of reflection h, respectively.

[c] $R_{free}$ is equal to $R_{cryst}$ for a randomly selected 5.0% subset of reflections not used in the refinement.

## Results and discussion

### *First crystal structure of LHA*

The structure of LHA using X-ray diffraction studies has been determined at 2.6 Å resolution. The structure was refined to a final $R_{free}$ = 25.3%, and $R_{cryst}$ = 21.2% (Table 1, Fig. 1A), with 98.6% of amino acids in allowed region and 1.4% in the generously allowed region of Ramachandran plot. The two molecules in the crystallographic asymmetric unit are tightly packed against each other in a dimeric arrangement. The interactions are stabilised by 15 potential H-bonds and 152 van der Waals contacts. However, the interactions between the two molecules could be due to crystal packing and may not be an inherent property of LHA. The buried surface area between the two molecules is 377 $Å^2$ compared to the total surface area of 21802 $Å^2$. Engineered Factor Xa site for LC activation was confirmed both by SDS–PAGE analysis and in the crystal structure, there is a clear evidence of break in electron density between LC and Hn (Fig. 1C). Furthermore, ordered electron density allowed model building of the full-length LC C-terminus and the disulphide bridge (Cys 430–Cys 454) between LC and Hn are stabilised through an anti-parallel β-sheet arrangement.

The overall structure of LHA (Fig. 1A) resembles BoNT/A without the binding domain, and superposition of LHA on BoNT/A gives an overall root mean square deviation of 0.85 Å for 830 $C^{\alpha}$-atoms (Fig. 1B). This proves that the absence of binding domain in LHA did not produce any major conformational change compared with the structure of full length BoNT/A. The LC is characterised by a

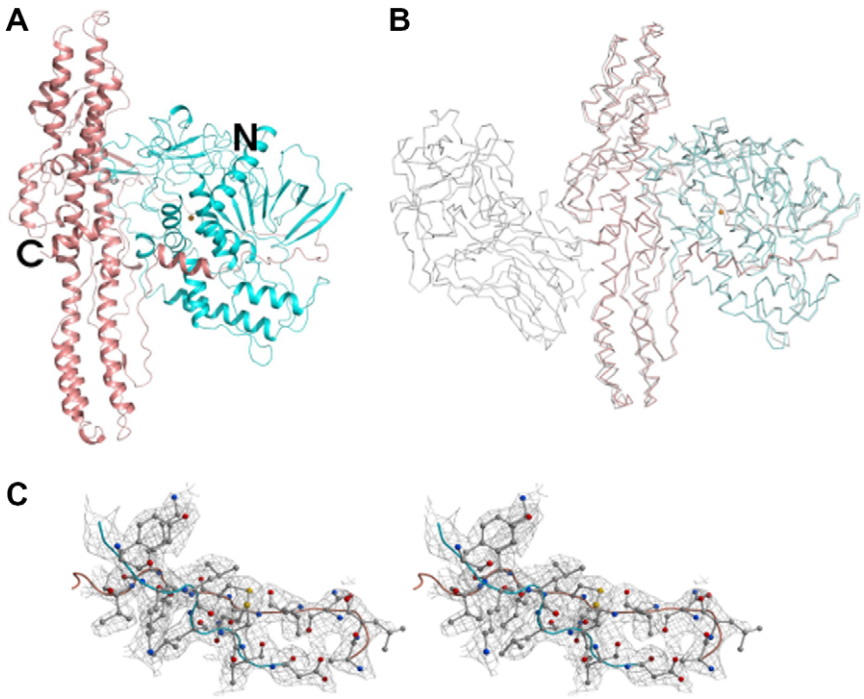


**Fig. 1.** (A) Ribbon diagram representation of LHA structure. Zinc ion shown as orange sphere. (B) Superposition of LHA (LC-cyan; Hn-salmon) on BoNT/A (grey) (PDB code 3BTA). (C) Electron density showing the engineered peptide break between LC and Hn. $2F_O$–$F_C$ map contoured at 1.0 σ. All the residues are shown in grey and ball-and-stick model. Disulphide link between the LC and Hn domain are shown and sulphur atoms are coloured yellow.

mixture of α-helix and β-strands and has the conserved zinc binding motif HEXXH. The Hn consists of three long anti-parallel α-helices of length ~105 Å. A small region of Hn called as 'belt' wraps LC and prevents access to the catalytic site. At least two roles are proposed for the belt, one as a chaperone for the catalytic domain [14] and the other as a regulatory element in membrane interaction [15].

*Domain components of engineered LHA*

Requirement of optimal length for a functional LHA is critical in LHA engineering. Deletion analysis carried out by Kurazono et al. [16], showed that removal of 10 amino acids at amino terminal and 57 amino acids from the carboxy terminal of LC abolished the toxicity even in the presence of Hn domain. Hence, full length catalytic domain is a requisite. A construct upto Ser 877 was selected to be an optimal length for Hn domain ending at the linker between Hn and Hc.

*The light chain (LC)*

The LC from all serotypes exhibit high degree of structural similarity. The catalytic zinc site is conserved in all serotypes and zinc is essential for LC's enzymatic activity. In the engineered LHA molecule, the zinc ion is tetrahedrally coordinated to His 223, His 227, Glu 262 and a water molecule involving Glu 224, as observed in LC structures of BoNT/A.

*The translocation domain (Hn)*

The non-toxic Hn domain is required after endocytosis of the toxin for translocation of the LC within the cytosol [17] where it is released and cleaves its substrate, thereby blocking neurotransmitter release. Hn domain's accessible surface area is 6038 $Å^2$ compared to its total area of 19992 $Å^2$ excluding the belt. The absence of the binding domain did not cause any major conformational change in the translocation domain. Hn normally interacts with Hc through eight potential hydrogen bonds and some 160 van der Waals' contacts (calculated using holotoxin structure, PDB ID 3BTA) around the 650 loop and residues Pro 801, Lys 805, Glu 808 and Ser 845. Superposition with the holotoxin model shows a change in the position of loop 642–650 and in particular with Tyr 648 which would form a hydrogen bond with Hc at His 886. Hn stability could be explained by strong inter-helical interactions not influenced by the new solvent accessibility.

*LHA stability and SNAP-25 cleavage assay*

LHA stability in storage buffer and crystallization condition was assessed at different temperatures by SDS–PAGE. Analysis by densitometry revealed that LHA-S877 is >95% pure after the final purification (Fig. 2A) and extremely stable even after 21 days in the storage conditions from −20 to 25 °C (Fig. 2B). LHA's stability is also not affected by high concentration of ammonium sulphate (1.5 M) from the crystallization condition. There was ~10% degradation observed after 36 days of incubation at 25 °C. The catalytic property of LHA was confirmed by the SNAP-25 cleavage assay. A SNAP-25-GFP substrate was engineered to monitor LHA proteolytic action resulting in two peptides of approximately 28 and 23 kDa, respectively. The stability assays confirm that LHA is a very stable molecule.

## Conclusion

From the protein structure point of view, novel engineered molecules provide a fascinating framework from which to study the structural implications of domain engineering. The data presented here have led to the solution of the first crystal structure for an $LH_N$ fragment of botulinum neurotoxin and does not show any noticeable structural difference between the parent neurotoxin and the recombinant fragment lacking the $H_C$ domain. With a growing interest in the use of the $LH_N$ fragment for therapeutic [5–7,18]

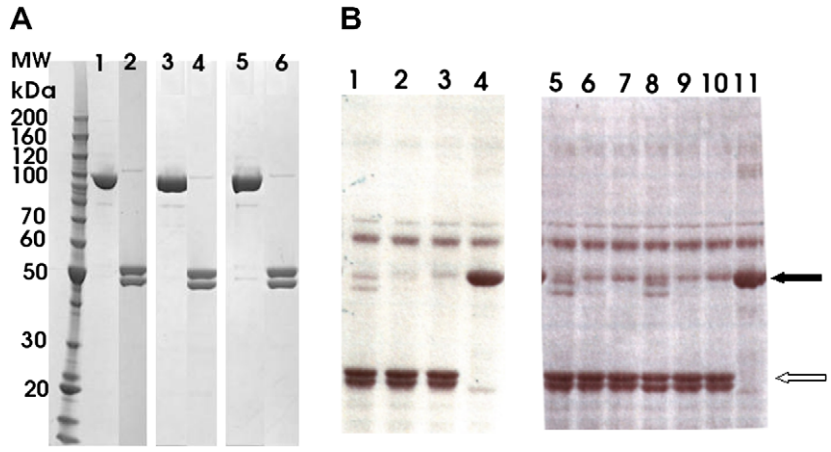


**Fig. 2.** (A) SDS–PAGE analysis of stability of LHA. Lanes 1, 3 and 5 – non-reduced samples. Lanes 2, 4 and 6 – reduced samples. Lanes 1, 2 on day 1, and 3, 4 on day 36, in buffer A. Lanes 5, 6 on day 36 in crystallization buffer. (B) SDS–PAGE analysis of SNAP-25 cleavage assay by LHA. GFP-SNAP-25 (black arrow) is ~50.7 kDa. LC cleaves GFP-SNAP-25 in two peptides of 28 and 23 kDa, respectively (white arrow). Lanes 1, 5, 8 – LHA (100 μg/ml), 2, 6, 9 – LHA (20 μg/ml), and 3,7, 10 – LHA (4 μg/ml). Lanes 4, 11 – negative control (BSA visible at ~70 kDa). On lanes 1, 5, 8 the two bands below the non-cleaved substrate correspond to the two LHA domains under reduced condition.

and vaccine [19,20] purposes, the structure presented here will facilitate the development of optimized proteins that will have greater applicability. LHA's structural integrity is unlikely to alter the property of any fusion proteins and provides additional degree of freedom for the design of future novel ligands which will have implications for BoNT 'engineering' in general.

## Acknowledgments

We thank the support of scientists-Liz Duke, Thomas Sorensen, Katherine McAuley, David Hall, James Sandy and Mark Williams at Diamond Light Source, UK. We also thank Keith Foster for scientific review of the manuscript. This work was supported by a post-graduate studentship to G.M. through a BBSRC-Syntaxin Limited (UK) CASE award. K.R.A. wishes to acknowledge the Royal Society (UK) for an Industry Fellowship. The atomic coordinates and the structure factors that have been deposited with the RCSB Protein Data Bank under accession codes 2W2D and R2W2DSF, respectively.

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