doi:10.1016/j.jmb.2006.09.050 J. Mol. Biol. (2007) 365, 123–134

JMB

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The H_C Fragment of Tetanus Toxin forms Stable, Concentration-dependent Dimers *via* an Intermolecular Disulphide Bond

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Protein oligomerisation is a prerequisite for the toxicity of a number of bacterial toxins. Examples include the pore-forming cytotoxin streptolysin O, which oligomerises to form large pores in the membrane and the protective antigen of anthrax toxin, where a heptameric complex is essential for the delivery of lethal factor and edema factor to the cell cytosol. Binding of the clostridial neurotoxins to receptors on neuronal cells is well characterised, but little is known regarding the quaternary structure of these toxins and the role of oligomerisation in the intoxication process. We have investigated the oligomerisation of the receptor binding domain (H_C) of tetanus toxin, which retains the binding and trafficking properties of the full-length toxin. Electrophoresis, size exclusion chromatography and mass spectrometry were used to demonstrate that H_C undergoes concentration-dependent oligomerisation in solution. Reducing agents were found to affect H_C oligomerisation and, using mutagenesis, Cys869 was shown to be essential for this process. Furthermore, the oligomeric state and quaternary structure of H_C in solution was assessed using synchrotron small-angle X-ray scattering. *Ab initio* shape analysis and rigid body modelling coupled with mutagenesis data allowed the construction of an unequivocal model of dimeric H_C in solution. We propose a possible mechanism for H_C oligomerisation and discuss how this may relate to toxicity.

Keywords: tetanus toxin; oligomerisation; ganglioside binding

Introduction

Tetanus neurotoxin (TeNT) and the seven serotypes of botulinum neurotoxin (BoNT/A-G), compose the family of clostridial neurotoxins (CNTs).[1] CNTs are proteins of approximately 150 kDa consisting of a 50 kDa light (L) chain, containing an active site for proteolysis, which is disulphide-bonded to a 100 kDa heavy (H) chain. The H chain is composed of a translocation (H_N) and a binding (H_C) domain, each of approximately 50 kDa.[2] During an infection, TeNT is released from the bacterium *Clostridium tetani* into the tissue fluid where the toxin circulates until binding to motorneurons.[3] TeNT binds initially to complex gangliosides of the 1b series, most notably GT1b and GD1b, tri or di-sialogalactosyl biantennary gangliosides which are expressed at high density on neuronal tissue. This binding is mediated solely by the H_C fragment, and specifically by the 25 kDa carboxy-terminal domain, termed H_CC.[4,5] Upon binding to sensitive cells, TeNT is endocytosed and trafficked in a retrograde fashion to the central nervous system where uptake by inhibitory interneurons occurs.

Abbreviations used: TeNT, tetanus neurotoxin; CNT, clostridial neurotoxin; SEC, size exclusion chromatography; MALLS, multi-angle laser light scattering; ESI-ToF, electrospray ionisation time-of-flight; SAXS, small angle X-ray scattering.

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Following acidification of vesicles, the L chain is translocated into the cell cytosol, where it enzymatically cleaves synaptobrevin II, preventing the release of the inhibitory neurotransmitters.

Binding to gangliosides in lipid rafts[6] is proposed to concentrate the toxin and bring it into close proximity to a putative protein receptor, although none has been unambiguously identified.[7] An attractive modification of this hypothesis is that binding to polysialogangliosides may occur outside the lipid micro-domain, but that lateral movement of the protein into a lipid rich environment may facilitate interaction with both alternative polysialogangliosides and protein receptors.[8] This two-stage binding would drastically increase the local concentration of the toxin on the membrane such that lower affinity binding to protein receptors would be facilitated. This local concentration of the toxin could also facilitate oligomer formation, which would in turn increase uptake of the toxin by endocytosis.

Several lines of evidence do in fact suggest that the CNTs form oligomeric structures. Investigation into the pore forming abilities of BoNT/C at low pH has suggested that an aggregation step is involved in channel formation.[9] The observed dose-dependent increase in conductance by BoNT/C pore formation was not consistent with a pore being formed by a single toxin molecule. Instead, the data indicated that oligomeric forms of the toxin were involved in this process.[9] Furthermore, electron cryo-microscopy has allowed the direct visualisation of membrane pores formed by BoNT/B, with four molecules comprising each channel.[10] This, taken together with the demonstration that ganglioside can cross-link TeNT H_C molecules,[11] could lead to concentration-dependent oligomerisation of TeNT at the cell surface which may aid endocytosis. In support of this theory is the observation that TeNT, BoNT/A, /B and /E have been found to oligomerise in solution using cross-linking studies and native PAGE.[12] Furthermore, oligomerisation of BoNT/A is concentration-dependent with the equilibrium shifting from the dimeric to the monomeric form below 50 nM.[13]

Structural studies on the H_C fragment of TeNT show a monomer with one molecule in the asymmetric unit under the crystallisation conditions used.[14–16] TeNT H_C has also been crystallised in the presence of an analogue of ganglioside GT1b, demonstrating the cross-linking of two H_C molecules at two distinct saccharide-binding sites by one molecule of ganglioside.[11] Two crystal forms of the H_C–receptor complex were obtained. One contains one molecule in the asymmetric and the other contains two molecules in the asymmetric unit. Notably, there are no observed intermolecular interactions between H_C molecules observed in the latter form.

All CNTs contain several cysteine residues within their amino acid sequence. An inter-chain disulphide bond between the L and H chains is conserved in all CNTs and is necessary for neurotoxicity.[17] The positions of the remaining cysteine residues are in general not conserved between the CNTs, although all serotypes do contain cysteine residues within their H_C domains. In TeNT (Figure 1), four cysteine residues are present within the H_C fragment, and two of these, Cys869 and Cys1093, can form an intramolecular disulphide bond.

Here, we characterise different oligomeric states of wild-type TeNT H_C (H_CWT) and cysteine replacement mutants, and we determine the stability and nature of the associated intermolecular interaction. Furthermore, we propose a model for the protein–protein interactions and discuss the relevance of oligomerisation to ganglioside-mediated receptor binding.

Results

Mutagenesis of TeNT H_C

Under non-reducing conditions, purified recombinant TeNT H_C protein (H_CWT) was found to migrate through SDS–PAGE as a series of protein bands ranging in size from 44 – >200 kDa (Figure 2).

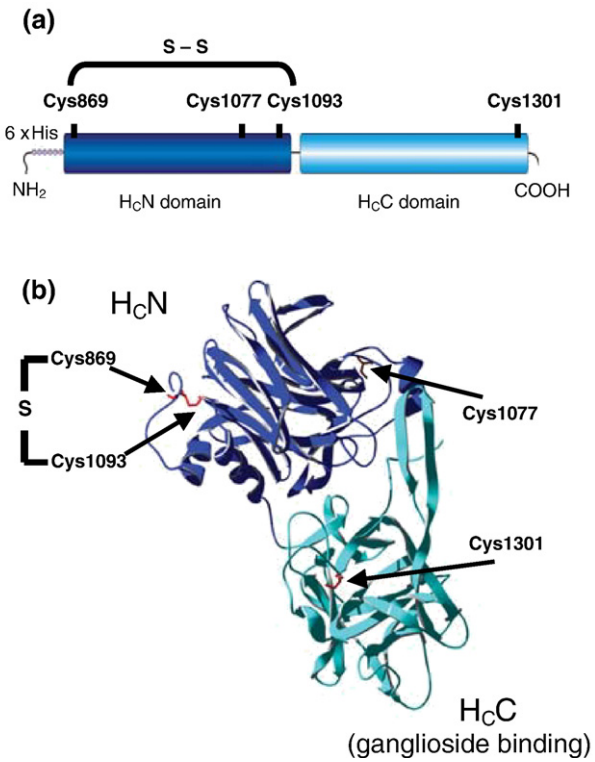

Figure 1. Location of cysteine residues in the TeNT H_C protein. (a) Line drawing of TeNT H_C showing positions of cysteine residues. (b) Ribbon structure of TeNT H_C, showing the two protein domains: H_CC and H_CN.[11] The four cysteine residues are shown in green. Cys869 and Cys1093 form an intramolecular disulphide bond in the N-terminal domain of the protein. The structure was displayed using Swiss PDB viewer and the image rendered using POV-Ray.