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# Crystallization and preliminary X-ray analysis of tetanus neurotoxin C fragment

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## Abstract

Two crystal forms of recombinant tetanus neurotoxin C fragment have been obtained. The C fragment corresponds to the C-terminal 451 amino-acid residues of tetanus neurotoxin and is the subunit responsible for receptor binding by the toxin. Both forms belong to space group $P2_12_12_1$. Form I has unit-cell dimensions of $a = 71.3$, $b = 79.7$, $c = 94.0$ Å and produces thin plate crystals. Form II has unit-cell dimensions of $a = 67.4$, $b = 79.7$, $c = 91.1$ Å and produces thick rod-shaped crystals. Diffraction data to 2.6 Å have been collected from form II crystals.

## 1. Introduction

Tetanus neurotoxin (TeNT) is the sole causal agent of the pathological condition known as tetanus, or colloquially as 'lockjaw'. TeNT is secreted from mature *Clostridium tetani* (see Montecucco & Schiavo, 1993, 1994; Ahnert-Hilger & Bigalke, 1995; Halpern & Neale, 1995, for reviews). The spores of this bacteria are ubiquitous (Bytchenko, 1981; Schwartz & Rodelsperger, 1990), but they require anaerobic conditions, such as are found in necrotic tissue resulting from a puncture wound, in order to mature (Kefer, 1992; Popoff, 1995). The clinical symptom of tetanus is spastic paralysis, with the facial muscles often the first to be affected. TeNT causes this paralysis by acting within the central nervous system (CNS), interrupting the pre-synaptical release of the inhibitory neurotransmitters $\gamma$-aminobutyric acid (GABA) and glycine. TeNT prevents the exocytosis of these neurotransmitters by specifically cleaving vesicle-associated membrane protein (VAMP)/synaptobrevin, which is a constituent of a complex proposed to mediate the cellular event of vesicle docking and fusion to a target membrane (Schiavo *et al.*, 1992; Bennett & Scheller, 1993). TeNT first enters motoneurons at the neuromuscular junction by endocytosis (Montecucco, 1986; Halpern & Loftus, 1993), and then undergoes retrograde axonal transport and trans-synaptical transport to enter the inhibitory neurons of the CNS (Price *et al.*, 1975; Evinger & Erichsen, 1986). Under some conditions, such as high toxin dose, TeNT may also act upon VAMP/synaptobrevin within the peripheral motoneurons, preventing the exocytosis of excitatory neurotransmitter acetylcholine and thus resulting in flaccid paralysis (Habermann *et al.*, 1980). TeNT is related to the botulinum neurotoxins (Lebeda & Olson, 1994; Minton, 1995), with a major distinguishing property between them being the differing preferential sites within the nervous system of their respective catalytic activities.

TeNT is synthesized as a single 1315 amino-acid residue chain (Eisel *et al.*, 1986; Fairweather & Lyness, 1986). This chain is then cleaved to form a dichain linked by a single disulfide bond. The light chain is composed of the N-terminal 457 residues and contains the zinc protease catalytic site. The heavy chain is composed of the remaining 858 residues. Upon papain degradation of this heavy chain, two fragments are obtained. The 407-residue N-terminal fragment from the heavy chain ($H_N$) has been implicated in the translocation of the light chain through the vesicle membrane and into the neuronal cytosol (Hoch *et al.*, 1985; Montal *et al.*, 1992). The 451-residue C-terminal fragment of the heavy chain ($H_C$, traditionally termed the C fragment) retains the properties of binding to peripheral motoneuron pre-synaptic membrane and retrograde axonal and trans-synaptical transport to the CNS (Bizzini *et al.*, 1977; Halpern & Neale, 1995).

There have been no previously reported near-atomic resolution three-dimensional structures for any of the clostridial neurotoxins, or for any of their subunits. The crystallization of $H_C$ has been reported (Anderson *et al.*, 1993), but the crystals obtained were noted to diffract X-rays poorly beyond 7 Å. These crystals were also obtained under different conditions and were a different crystal form from those reported here. The crystallization of the entire botulinum neurotoxin serotype A has also been reported (Stevens *et al.*, 1991).

## 2. Materials and methods

Recombinant $H_C$ ($rH_C$) was purchased from the Boehringer Mannheim Corporation. $rH_C$ possesses the same properties as the fragment obtained from papain degradation of native TeNT, the only difference being the addition of an N-terminal methionine to the recombinant protein. The purity of the protein sample was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Additionally, the N-terminal sequence was also determined in order to assure that it agreed with that of $H_C$. The lyophilized protein was reconstituted with 0.1 *M* $NaHCO_3$ at pH 7.8 to a protein concentration of 5 or 10 mg ml$^{-1}$. This solution was then dialyzed against 0.1 *M* $NaHCO_3$ at pH 7.8 for 1 d at 277 K to ensure that the protein was fully dissolved. The buffer in the protein solution was then exchanged by dialysis against 0.01 *M* Tris–HCl, pH 7.8 for 1 d at 277 K.

Crystallization was performed *via* the vapor-diffusion method. Hanging drops were primarily used, but sitting drops were also successfully employed. The crystallization droplet contained a mixture of $rH_C$ in 0.01 *M* Tris–HCl, pH 7.8, and the reservoir solution. The reservoir solution was composed of 14–18%(*w*/*v*) polyethylene glycol (PEG) 4000, 0.1 *M* imidazole, and 0.05 *M* $MgCl_2$ at a pH of 6.5. NaCl or $NaCH_3CO_2$ could be substituted for the $MgCl_2$. $rH_C$ crystals did grow in the absence of salt in the reservoir solution, but the presence of the salt improved crystal quality and the reproducibility of the crystal-

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lization. Two different crystal forms grew under similar conditions. The ratio of the volume of the protein solution to the volume of the reservoir solution initially contained within the crystallization droplet, along with the initial concentration of the protein solution, determined which one of the two crystal forms was obtained. Equal volumes of protein solution (initially at 5 mg ml$^{-1}$ of $rH_C$) to reservoir solution favored the growth of thin plate crystals (form I). A ratio of three volumes of reservoir solution to one volume of protein solution whose initial concentration was 10 mg ml$^{-1}$ in $rH_C$ favored the growth of thick rod-shaped crystals (form II). Crystals of both forms grew within 1–2 weeks at room temperature. The maximum size of the plate crystals was approximately 0.5 × 0.3 × 0.05 mm and that of the rod crystals was approximately 0.8 × 0.2 × 0.2 mm.

Single-crystal X-ray diffraction data were collected using a Siemens X100 multiwire area detector at room temperature. A Rigaku RU200 rotating-anode generator operated at 42 kV and 65 mA produced Cu $K\alpha$ X-rays which were filtered with a nickel foil. The focal spot was 0.2 × 2.0 mm and a double focusing Frank's mirror system was used to provide a highly focused beam. The crystal-to-detector distance was 120 mm and 0.25° oscillation data frames were collected. Data collection was conducted by the software of Blum (Blum *et al.*, 1987). The data were processed using *XENGEN* (Howard *et al.*, 1987) through to the calculation of the integrated intensities, and then the locally modified programs of Weissman (Weissman, 1982) were employed for scaling and reducing the data to a set of unique reflections.

## 3. Results and discussion

The initial conditions under which $rH_C$ was found to crystallize were determined following a screening of precipitant type and concentration, pH, buffer type and concentration, and protein concentration in hanging-drop vapor-diffusion crystallization experiments. Crystals were obtained from a number of the first conditions screened, but these crystals appeared only after several months following the setup of the screens. The successful crystallization experiments had PEG 4000 as the precipitant and imidazole as the buffer. These crystals were extremely thin plates which tended to grow in clusters. Even though these crystals did diffract X-rays, they were not suitable for high-quality X-ray diffraction experiments. Thus, the screening for crystallization conditions continued, both fine screens about the conditions which produced the poor quality plate crystals and coarser screens of novel conditions. None of the screens which significantly differed from the initial successful crystallization conditions produced crystals.

However, the addition of trace amounts of a salt (*i.e.* $MgCl_2$, NaCl or $NaCH_3CO_2$) accompanied by slight adjustments of the protein, buffer, and precipitant concentrations and of the pH to the initially successful crystallization conditions yielded the form I plate crystals which were suitable for the collection of diffraction data. These crystals are orthorhombic, with the space group being $P2_12_12_1$. The unit-cell dimensions are $a$ = 71.3, $b$ = 79.7, $c$ = 94.0 Å. The Matthews coefficient is 2.67 Å$^3$ Da$^{-1}$ when the contents of one asymmetric unit is assumed to be a single $rH_C$ molecule weighing 50 kDa. This translates to an estimated solvent content for the crystal of 51%. If two $rH_C$ molecules were assumed to make up the asymmetric unit, then the Matthews coefficient (Matthews, 1968) would be 1.33 Å$^3$ Da$^{-1}$, which is outside of the accepted range, as this would represent a crystal with an estimated solvent content of only 6%.

The form I crystals were not particularly strong diffractors of X-rays, most likely due to their thinness. Nonetheless, a usable data set was compiled by merging data collected from three crystals (Fig. 1*a*). A total of 51 564 observed reflections were scaled and reduced to yield a data set containing 12 355 unique reflections with $I \geq 2\sigma(I)$, where $I$ is a reflection's integrated intensity. The $R_{merge}$ was 9.67%. The data were 89.3% complete to 3.0 Å resolution and 81.9% complete to 2.75 Å resolution, with the data in the shell 3.0–2.75 Å being 56.4% complete. A limited number of reflections were observed past 2.75 Å resolution.

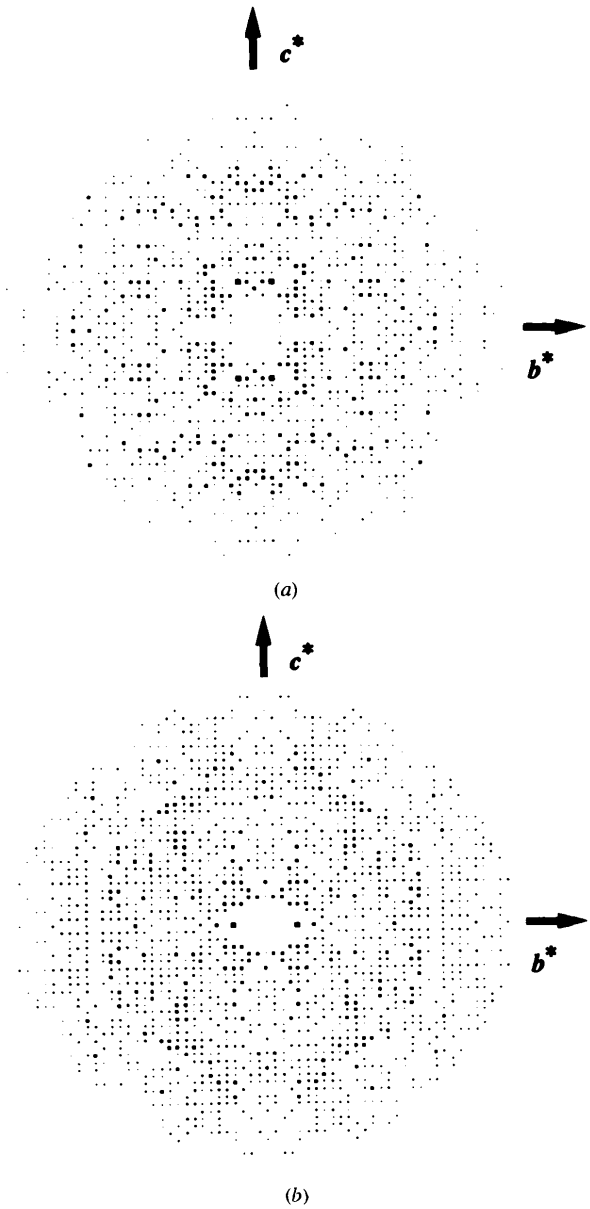


Fig. 1. Pseudo precession photographs generated by *PRECESS* (Furey & Swaminathan, 1998) from multiwire area detector data of the 0*kl* zone of (*a*) form I and of (*b*) form II crystals. Data up to 2.6 Å resolution are included in both.

The self-rotation function (Rossmann & Blow, 1962) was calculated using the diffraction data collected from the form I crystals. This did not reveal the presence of any non-crystallographic symmetry. This was as expected from the results of the Matthews coefficient calculations and the absence of any indications that $rH_C$ contains intramolecular symmetry.

The screening of crystallization conditions continued even after the form I crystals had been obtained. This was carried out in an effort to grow crystals even more suitable for a structure-determination study. Form II crystals were grown in conditions similar to those which yielded form I crystals except that the relative concentrations of the various components of the crystallization droplet had been altered. Form II crystals are also orthorhombic and the space group is $P2_12_12_1$. However, the unit-cell volume of form II ($4.89 \times 10^5$ Å$^3$) is smaller by 8.43% when compared with that of form I ($5.34 \times 10^5$ Å$^3$). The unit-cell dimensions for form II are $a$ = 67.4, $b$ = 79.7, $c$ = 91.1 Å, and so the $a$ and the $c$ axes have decreased while the $b$ axis has remained constant compared to the cell dimensions of form I. The Matthews coefficient for form II crystals is 2.45 Å$^3$ Da$^{-1}$ when the asymmetric unit is assumed to be composed of one $rH_C$ molecule, or an estimated solvent content of 47%. If the assumed contents of the asymmetric unit were two $rH_C$ molecules, then the Matthews coefficient would be 1.22 Å$^3$ Da$^{-1}$, which is outside of the accepted range, as this would represent a protein crystal containing little if any solvent.

Higher quality diffraction data were obtained from the form II crystals (Fig. 1*b*). This may be due to the larger size to which these crystals grew and the lower solvent content compared with the form I crystals. Data to approximately 2.6 Å resolution were collected from a single crystal using two data-collection passes. The number of observed reflections with $I \geq 1\sigma(I)$ was 47 673. These were reduced to 14 445 unique reflections, yielding an $R_{merge}$ of 7.63%. The data were 91.6% complete to 2.6 Å resolution, with the data in the shell 2.8–2.6 Å resolution being 71.2% complete. Diffraction from form II crystals has been observed up to 2.3 Å resolution, and this higher resolution data will be collected during the course of the structure determination. The self-rotation function was also calculated from data collected from form II crystals, and again no non-crystallographic symmetry was revealed.

In recent years there have been many important advances in the understanding of the actions of the very potent clostridial neurotoxins at the molecular level. However, no high-resolution three-dimensional models for any of the clostridial neurotoxins has yet been reported. Thus, the structure determination of $rH_C$ will proceed with a search for heavy-atom derivatives suitable for use in the isomorphous replacement method. The determination of the structure of $rH_C$ will provide insights into the recognition of and the binding to peripheral motoneurons by TeNT. These insights will also be applicable to the related BoNT's. In addition, detailed structural information of $rH_C$ may prove useful in exploiting the ability of $rH_C$ to transport other moieties specifically to the CNS, and in the development of novel vaccines for both TeNT and BoNT.

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