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Crystallization and preliminary X-ray analysis of a thiol-activated cytolysin

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Abstract We present the first reported crystallization of a member of the thiol-activated family of protein toxins. Perfringolysin O, a virulence factor of *Clostridium perfringens*, has been crystallized in two different forms by the hanging drop vapor diffusion method. In one form the toxin crystallizes with PEG 20000 in the orthorhombic space group $C222_1$ with cell dimensions of $a = 47.8$ Å, $b = 182.0$ Å and $c = 175.5$ Å and the crystals diffract to beyond 2.5 Å resolution. In the second form the toxin crystallizes in a large variety of organic solvents including malt whisky. This crystal form belongs to the orthorhombic space group $P222_1$ with unit cell dimensions $a = 47.1$ Å, $b = 166.1$ Å and $c = 214.0$ Å and with diffraction observed to 2.4 Å resolution.

Key words: Perfringolysin O; Thiol-activated cytolysin; Pore-forming toxin; Crystallization; X-ray diffraction

1. Introduction

Perfringolysin O (PFO; theta toxin) belongs to a large family of membrane-damaging toxins which are produced by four genera of Gram-positive bacteria [1]. To date, more than 20 members have been characterized. This family is generally referred to as the oxygen-labile or thiol-activated cytolysins because biochemical modification of a key cysteine residue causes toxin inactivation, although the cysteine residue itself is not essential for toxin function [2,3]. The toxins share a common mode of action. In the first step the toxin interacts with the target cell membrane via cholesterol which acts as a receptor. The thiol-activated cytolysins are the only known bacterial toxins that absolutely require cholesterol for their activity. The toxin then oligomerizes and partitions into the membrane. It is not yet clear whether oligomerization occurs before, during or after membrane insertion. The resultant membrane-bound oligomers lead to membrane damage and eventual cell lysis [1]. The oligomers can be visualized as arcs and rings on membranes with an electron microscope [4]. The thiol-activated cytolysins are utilized by a wide variety of Gram-positive pathogens which exhibit distinct pathogenic traits. It is likely that each of the toxins have diverged to some extent to facilitate the pathogenic process of each pathogen.

PFO is a virulence factor of *Clostridium perfringens* which causes a number of human diseases including gas gangrene, food poisoning, necrotizing enterocolitis and enteritis necroticans [5,6]. The toxin is secreted as a single chain polypeptide with a molecular weight of 53 kDa. The gene for PFO has been cloned and expressed in *E. coli* [7]. The nucleotide sequence-derived primary structure shows that PFO consists of 500 amino acid residues (unpublished results) [8]. The protein is water-soluble and it does not possess a hydrophobic sequence long enough to span a membrane. There is nothing in its primary structure to explain how the toxin can insert into eukaryotic membranes to form ion channels. The pairwise sequence identity between members of the thiol-activated cytolysin family is high (40–70%) suggesting they will all have very similar three-dimensional structures [1].

We have initiated a crystallographic study of PFO as part of an on-going study to understand, at the molecular level, how proteins insert into or pass through biological membranes [9,10].

2. Material and methods

2.1. Cloning, expression and purification

The region of the *PFO* gene encoding residues Lys^{29} to the end of the protein was fused into the *Bam*HI and *Eco*RI sites of the plasmid vector pTrcHisA (termed pRT10) (Invitrogen). This fusion removed the signal peptide coding region from the *PFO* gene and fused the beginning of the coding region of the secreted form of PFO to the vector sequence which fused a peptide with the sequence of MGGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGS to Lys^{29}. Since the signal peptide was replaced, this fusion protein was expressed intracellularly in *E. coli*. The fusion peptide contained a polyhistidine region which facilitated the rapid purification of the protein using a nickel chelate resin (described below). Most of the fusion peptide was removed by cleavage of the fusion protein with enterokinase which cleaves on the carboxy terminal side of the DDDDK sequence. The sequence DRWGS remains attached to the amino terminus of purified PFO after enterokinase cleavage.

Growth of *E. coli* strain JM109 containing the vector pRT10 was carried out in the following way for maximum yield of toxin. Two 10 l carboys containing 8 l of terrific broth [11] were pre-equilibrated at 37°C and then inoculated 1:33 with an overnight culture of *E. coli* JM109 containing pRT10. Ampicillin was maintained at 100 μg/ml in both the inoculum culture and carboys. The large cultures were grown to an optical density of 1 at 600 nm and then induced with 1 mM IPTG for an additional 4 h. The cultures were continuously aereated during the growth of the organism by pumping air into the culture. The cells were then separated from the media by centrifugation. The cells were lysed by two passages through a French press (20000 psi) and the cell debris was removed by centrifugation at $47000 \times g$ for 30 min at 5°C. The supernatant (containing the fusion protein) was pumped onto a 2.5×20 cm column packed with metal chelate Sepharose (Pharmacia) at 2 ml/min. The column was washed with 100 ml of

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Abbreviations: Bicine, *N,N*-bis[2-hydroxyethyl]-glycine; DTT, 1,4-dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; MES, 2-[*N*-morpholino]-ethanesulfonic acid; MPD, 2-methyl-3,4-pentanediol; PEG, polyethylene glycol; PFO, perfringolysin O; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris-HCl, 2-amino-2-hydroxymethylpropane-1,3-diol hydrochloride

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10 mM MES, pH 6.5, with 150 mM NaCl and 50 mM imidazole. The fusion protein was eluted with the same buffer but using 300 mM imidazole instead of 50 mM imidazole. The fusion protein was applied directly to a column (3.0×20 cm) packed with HP Superose SP and eluted with a linear gradient of 0–80 mM NaCl in 10 mM MES, pH 6.5. The fusion protein was found to be pure at this point and was dialyzed against 10 mM Tris, pH 7.5, containing 1 mM $CaCl_2$. The fusion protein was then digested with enterokinase at a ratio of 5 U/100 μg of fusion protein for 24 h at room temperature. After digestion the amino terminal peptide and any undigested toxin was removed by repeating the HP Sepharose SP chromatography. The PFO was then dialyzed into 10 mM Tris, pH 8.0, containing 20% glycerol.

2.2. Crystallization

In preparation for crystallization, the protein was dialyzed overnight against 10 mM Tris-HCl at pH 8.0 and 1 mM DTT. Crystallization was performed by the hanging drop vapor diffusion method [12] using 24-well tissue culture plates. A 2 μl droplet containing 7.5 mg/ml protein was mixed with an equal volume of reservoir solution (as described below). Each well contained 1 ml of reservoir solution. Initial crystallization trials were performed using a screen similar to the one described by Jancarik and Kim [13] and with the crystallization reagent kit, Crystal Screen II, from Hampton Research (California, USA). The trials were carried out at a constant temperature at both 4°C and 22°C. For optimal crystal growth it was later found that the total volume of the hanging drop needed to be increased to 10 μl and the protein concentration increased to between 15 and 20 mg/ml.

2.3. Data collection

The X-ray diffraction data for crystal form A were collected on the beamline 6A2 at the synchrotron radiation source of the Photon Factory (Tsukuba, Japan). The wavelength was set to 1.0 Å and the data were measured at room temperature. The data were collected with image plates using the rotation method with 1.5° oscillations. X-ray diffraction data for crystal form B were collected using a MARresearch imaging plate detector on a Rigaku RU-200 rotating anode generator, with graphite-monochromated Cu$K\alpha$ radiation (Fig. 1). The data were collected at 15°C by the rotation method with oscillations of 1.0°. Determination of unit cell parameters, autoindexing and integration of reflection intensities were performed using DENZO [14] and the data scaled with SCALEPACK [14].

3. Results and discussion

Our initial crystallization screens resulted in the appearance of two different crystal morphologies which we refer to as crystal forms A and B. For crystal form A, crystals were initially observed after 1 day in 10% (w/v) PEG 20000, 2% (v/v) dioxane and 100 mM bicine buffer at pH 9.0. The best crystals were grown at 22°C. Without the presence of dioxane, the resultant crystals were very small and heavily twinned. The best crystals grow as thick plates in 6% (w/v) PEG 20000, 100 mM bicine buffer at pH 8.7 and 2% (v/v) dioxane. The crystals achieve maximum size (up to 0.75×0.6×0.1 mm) within 2 weeks. A native X-ray diffraction data set was collected using the synchrotron radiation source of the Photon Factory (Tsukuba, Japan). We were able to collect a 89% complete data set to 2.7 Å resolution off a single crystal with an R_{merge} of 8.1% (39.2% for the outer shell 2.8 to 2.7 Å). The autoindexing procedure of DENZO [14] indicated that the crystals belong to the orthorhombic crystal system, with unit cell dimensions of $a=48.8$ Å, $b=182.0$ Å and $c=175.5$ Å. Analysis of the various data, including a search for systematic absences, showed the data were consistent with the space group $C222_1$. The unit cell volume is consistent with there being two monomers in the asymmetric unit, yielding a specific volume of 1.9 $Å^3$/Da, a value which falls within the normal range observed for protein crystals [15]. There are no significant peaks in the native Patterson or self-rotation function map. A preliminary low-resolution electron density map based on four heavy atom derivatives demonstrates there is only one monomer in the asymmetric unit.

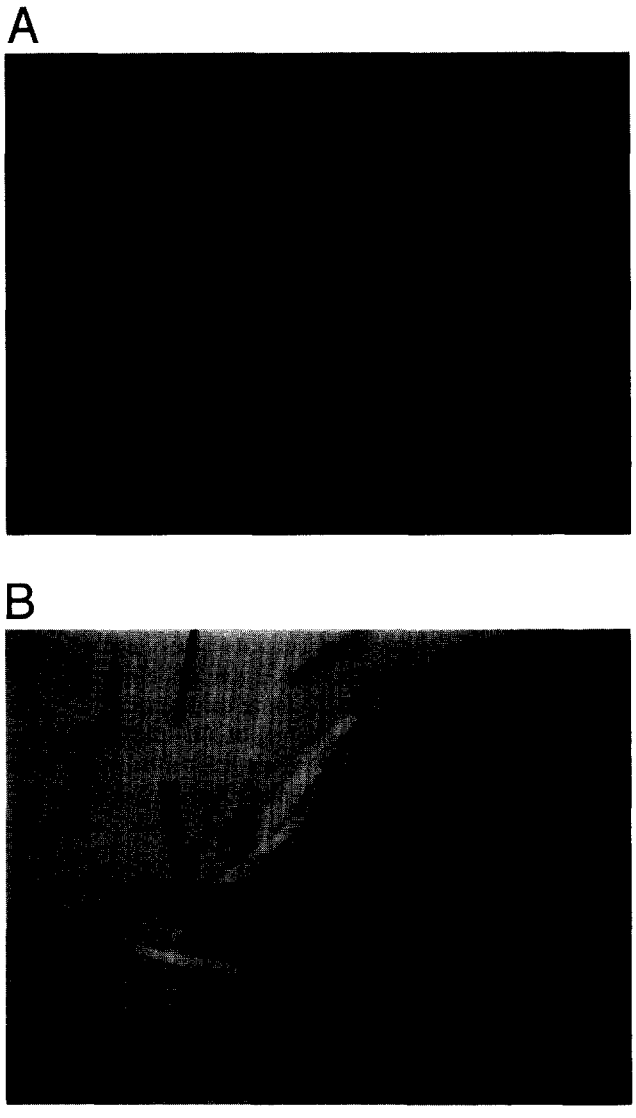

Fig. 1. Photograph of crystals of perfringolysin O. A: Crystal form A grown from PEG. The largest crystal is 0.6 mm in its longest dimension. B: Crystal form B grown from *t*-butanol. The largest crystal is 0.5 mm in its longest dimension. See the text for detailed crystallization conditions.

For crystal form B, crystals were initially observed after 1 day in 30% (v/v) *t*-butanol and 100 mM HEPES buffer at pH 7.5. In an attempt to improve the crystal size, the nature (*t*-butanol was replaced by ethanol, methanol, *n*-propanol, isopropanol, MPD and various combinations of the above) and concentration of the precipitant was varied. There was marginal difference in the results obtained by varying the organic solvent: indeed *t*-butanol could be replaced by pure malt whisky with ill-effect! Further research in this direction is planned! The best crystals grow as thin plates at 22°C in 20% (v/v) *t*-butanol, 100 mM HEPES buffer at pH 7.0. The crystals achieve maximum size (up to 0.5×0.2×0.04 mm) within 2 weeks. We were able to collect a 55% complete data set in-house to 4.5 Å resolution off a single crystal with an R_{merge} of 16.5% (28.7% for the outer shell 4.7 to

4.5 Å). The quality of the data set was poor due to a combination of high crystal mosaicity (> 1°), weak diffracting power and radiation damage. Nevertheless the data was sufficiently well-measured to indicate that the crystals belong to the orthorhombic crystal system, with unit cell dimensions of $a = 47.5$ Å, $b = 169.4$ Å and $c = 214.6$ Å. Analysis of the various data, including a search for systematic absences, showed the data were consistent with the space group $P222_1$. The unit cell volume is consistent with there being either three or four monomers in the asymmetric unit, yielding specific volumes of 2.7 Å^3/Da and 2.1 Å^3/Da, respectively, values which fall within the normal range observed for protein crystals [15]. We have recently discovered that crystals transferred into 15% MPD, HEPES buffer pH 7.0 before mounting are significantly less mosaic than crystals mounted directly from the crystallization drop. These crystals diffract to approximately 2.4 Å resolution using synchrotron radiation and hence represent a feasible crystal form for future structural studies. Because the organic solvent may mimic the membrane environment the toxin encounters on pore formation, we feel work on this crystal form is well worth pursuing.

We have chosen to pursue structural studies on crystal form A at this stage since these crystals are much easier to handle than crystal form B. We are planning to determine the structure of PFO by the standard method of multiple isomorphous replacement. We will then solve crystal form B by molecular replacement. If necessary we will make use of electron density map averaging between the crystal forms to aid in the map interpretation.

We have already obtained a low resolution (6 Å) electron density map of the toxin from crystal form A. Despite the high concentration of protein used in the crystallization trials, the toxin appears monomeric. The preliminary map shows the toxin to be an elongated molecule with similar dimensions to that visualised by electron microscopy [16]. This suggests we maybe able to construct the oligomeric membrane form of the toxin using a combination of the crystallographic and electron microscopic data.

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