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Crystallization and preliminary X-ray diffraction analysis of a functional form of pneumolysin, a virulence factor from *Streptococcus pneumoniae*

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Pneumolysin is a virulence factor from *Streptococcus pneumoniae*, a Gram-positive bacterial pathogen which causes human infections with a severe impact on mortality and morbidity worldwide. The enzyme belongs to a group of cholesterol-dependent cytolysins and interacts with its cholesterol receptor on target cells, leading to pneumolysin insertion into target-cell membranes and subsequently to pore formation and cell lysis. Pneumolysin has been over-expressed, purified and crystallized for X-ray diffraction studies. Crystals have been obtained in the presence of cholesterol in an effort to produce a three-dimensional structure of pneumolysin in its fully functional form with the enzyme bound to its activator. This is the first report of the crystallization of a cholesterol-dependent cytolysin in the presence of bound cholesterol. The vapor-diffusion method using ammonium sulfate as a precipitation agent was used to grow crystals in the presence of *n*-octyl-β-D-glucopyranoside and phosphatidylcholine. Crystals of this 53 kDa molecule complexed with cholesterol diffracted X-rays to 3.3 Å. The crystal unit cell has parameters $a = b = 191.45$, $c = 66.16$ Å, $\alpha = \beta = 90.0$, $\gamma = 120^\circ$ and belongs to the trigonal space group $P3$. The determination of the three-dimensional structure of this pneumococcal cytolysin is in progress.

1. Introduction

Pneumolysin (PLY) is an important virulence factor from the pathogenic bacterium *S. pneumoniae*. The pneumococcus is responsible for many serious diseases such as bacterial meningitis, pneumonia and otitis media which cause significant mortality and morbidity of human populations worldwide. Various pneumococcal proteins allow efficient propagation of this bacterium within the host, including pneumolysin (PLY), hyaluronate lyase (SpnHL; Jedrzejas, Chantalat *et al.*, 1998; Jedrzejas, Mewbourne *et al.*, 1998; Li *et al.*, 2000) and pneumococcal surface protein A (PspA; Jedrzejas *et al.*, 2000). PLY is a 53 kDa protein contained within the cytoplasm of *S. pneumoniae* which upon release is toxic to target host cells. PLY is therefore essential to the efficient spread of pneumococcal infections by disrupting the physical barriers to free circulation within the tissues of the host (Paton *et al.*, 1993).

PLY is a member of a group of proteins from Gram-positive bacteria referred to as the cholesterol-dependent cytolysins (CDCs) which were known historically as thiol-activated cytolysins. CDCs are related in structure and function and are believed to operate *via* similar modes of action. In solution, the native toxin exists as soluble monomer and dimer populations (Gilbert *et al.*, 1998). However, as is the case in PLY, various properties of the toxin change upon binding to its cholesterol receptor in the membrane of the target host cell. Several studies have shown that an 11-amino-acid region, known as the Trp-rich loop, near the C-terminus of the molecule is involved in cholesterol and membrane binding (Nakamura *et al.*, 1995; Rossjohn *et al.*, 1998; Jacobs *et al.*, 1999). Binding to cholesterol at the Trp-rich loop activates the protein and initiates a conformational shift within the molecule, allowing insertion into the hydrophobic environment of the lipid bilayer followed immediately by oligomerization into large pores. These pores, consisting of 30–50 individual toxin units, create a large transmembrane channel 350–450 Å in diameter (Rubins & Janoff, 1998), resulting in rapid lysis of the target cell. Throughout this sequence of events leading to pore formation, PLY must undergo a series of conformational shifts resulting in a change from soluble monomer/dimer to membrane-embedded oligomer (Nakamura *et al.*, 1995; Rossjohn *et al.*, 1997, 1998; Shepard *et al.*, 1998; Gilbert *et al.*, 1999; Jacobs *et al.*, 1999; Shatursky *et al.*, 1999).

Cholesterol is absolutely essential for the toxicity of all members of the CDC family. We have recently shown using circular dichroism,

analytical velocity and equilibrium ultracentrifugation and fluorescence studies that structural changes occur in PLY after binding to cholesterol but before membrane insertion and pore formation (Kelly & Jedrzejas, 2000). Evidence for a pre-pore transition state in PLY suggests that similar mechanisms may be at work in other CDCs. This PLY form with a bound cholesterol receptor may provide detailed information regarding the structural changes that occur within the molecule in the moments before its insertion into the lipid bilayer and subsequent oligomerization.

We have crystallized the functional form of PLY in the presence of a cholesterol activator as part of an ongoing effort to understand the structure–function relationship as well as the mechanism of action and virulence of the cholesterol-dependent cytolysins. The structure of PLY complexed with cholesterol is likely to reveal the changes in secondary and tertiary structure that occur in the transition between the non-functional aqueous form and the membrane- and cholesterol receptor-bound form. This structure may lead to a more complete understanding of membrane-pore formation and of the precise mechanism of the structural changes that occur in the cholesterol-dependent pneumococcal cytolysin currently under debate.

2. Experimental procedures

2.1. Materials

Cholesterol used in this study was in its water-soluble form, polyoxyethanyl-cholesteryl sebacate (Proksch & Bonderman, 1978) and was purchased from Sigma Chemicals. Phosphatidylcholine was from Avanti Polar Lipids, Inc. All other chemicals were purchased either from Fisher Scientific or Sigma Chemicals.

2.2. Overexpression, purification and activity assay

Overexpression and purification of pneumolysin was accomplished as reported previously (Kelly & Jedrzejas, 2000). The activity assay followed the procedure reported by Paton *et al.* (1983). Hemolytic units (HU) were determined visually and were expressed as the reciprocal of the dilution at which 50% lysis of the target cells was observed.

2.3. Crystallization

For the crystallization trials, pneumolysin in 10 m*M* Tris–HCl buffer pH 7.4, 2 m*M* EDTA, 1 m*M* DTT and 150 m*M* NaCl (buffer *A*) was concentrated to 10 mg ml^{-1} using an Ultrafree-15 centrifugal filter (Millipore). Crystallization trials of purified pneumolysin and of PLY complexed with 10–50 m*M* cholesterol were performed using the vapor-diffusion hanging-drop method in 24-well Linbro culture plates at 295 K (McPherson, 1999; Ducruix & Giegé, 1992). The PLY–cholesterol complex was incubated for at least 1 d at room temperature before starting crystallization trials. Crystallization drops were prepared by mixing 0.5–1.0 µl of 5 mg ml^{-1} PLY or PLY–cholesterol complex in buffer *A* with 0.5–1.0 µl of the reservoir solution and were equilibrated against 1 ml of the reservoir solution. An initial crystallization screening was performed with Hampton Research crystallization kits I and II (Jancarik & Kim, 1991) and revealed small crystals with ammonium sulfate as the precipitating agent for the complex protein sample with cholesterol only. The condition was optimized by fine screening of the amounts of ammonium sulfate and cholesterol, the enzyme concentration, the buffers used and their pH, as well as additives such as 2-propanol and others. Addition of the detergent *n*-octyl-β-D-glucopyranoside to 1%(*w*/*v*) and phosphatidylcholine to 0.5%(*w*/*v*) improved crystallization.

2.4. X-ray diffraction

Complexed PLY–cholesterol crystals were cryoprotected by briefly soaking them in a solution of 23–25% glycerol, 2.5 *M* ammonium sulfate, 5% 2-propanol, 50 m*M* cholesterol and 50 m*M* sodium cacodylate buffer pH 6.5 and were flash-frozen at 103 K in a nitrogen flow using a Cryostream Cooler (Oxford Cryosystems). Diffraction data were collected using synchrotron X-ray radiation with a wavelength of 1.1 Å at beamline X25 at the Brookhaven National Laboratory/National Synchrotron Light Source. The diffraction oscillation images were processed with the *HKL* package (Otwinowski & Minor, 1997).

2.5. Other methods

Electrophoresis was carried out under reducing conditions in an 11% polyacrylamide gel using the buffer system described by Laemmli (1970) and a Mini Protein III gel system (Bio-Rad). The gels were stained with Coomassie Blue. The enzyme concentration was determined from the UV absorption at 280 nm using a molar extinction coefficient calculated based on the PLY amino-acid sequence data (Pace *et al.*, 1995).

3. Results and discussion

3.1. Overexpression and purification

The pure recombinant pneumolysin from *Escherichia coli* harboring plasmid pJCP20 (Paton *et al.*, 1986) was obtained by overexpression in LB media and purified using a four-column purification process utilizing, in sequential order, DEAE–Sepharose anion exchanger, phenyl–Sepharose High Performance hydrophobic column, Superdex 75 size-exclusion column and finally high-resolution anion-exchanger Mono-Q (Amersham Pharmacia Biotech) as previously described (Kelly & Jedrzejas, 2000). This procedure yielded a purified protein which was hemolytically active and migrated as a single band on a polyacrylamide gel, with a molecular weight of ~53 kDa (Fig. 1). The final pneumolysin sample had a specific activity greater than 500 000 HU mg^{-1}.

3.2. Crystallization

Crystals were grown using the vapor-diffusion method (McPherson, 1999). Initially, small rectangular block- or plate-shaped crystals of the PLY–cholesterol complex grew within several weeks at 295 K using 2.0 *M* ammonium sulfate, 3% propanol at pH 6.5. Upon refinement of the crystallization conditions (see §2), diffraction-quality rectangular block crystals (0.3 × 0.15 × 0.15 mm) were obtained

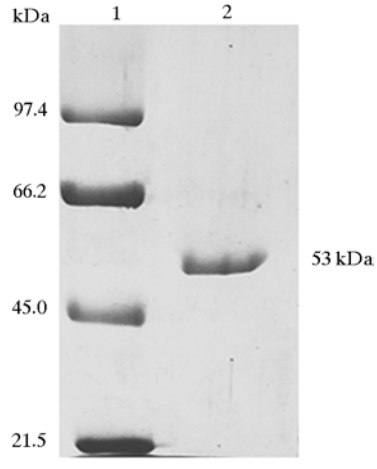

Figure 1
SDS–PAGE of pneumolysin was performed using an 11% polyacrylamide gel and stained with Coomassie Blue using the methodology developed by Laemmli (1970) as described in §2. From the left, the samples are 1, low-range molecular-weight standard; 2, final purified PLY sample.

Table 1
X-ray diffraction data statistics for methionyl PLY–cholesterol crystals.

The data set was collected at 103 K using the X25 beamline at the Brookhaven National Laboratory/National Synchrotron Light Source at a wavelength of 1.10 Å and the Brandeis-4k CCD detector.

Resolution shell (Å)	No. of unique reflections	R_{merge}†	$I/\sigma(I)$	Completeness (%)
50.00–7.10	4051	0.057	18.4	98.0
7.10–5.64	4116	0.130	8.4	98.7
5.64–4.93	3756	0.137	7.4	90.3
4.93–4.48	3338	0.140	7.3	80.8
4.48–4.16	3124	0.161	6.3	75.1
4.16–3.91	3040	0.202	4.8	73.6
3.91–3.72	3051	0.246	3.7	73.6
3.72–3.55	2984	0.265	3.1	71.8
3.55–3.42	3051	0.340	2.2	72.9
3.42–3.30	2730	0.413	1.8	67.3
50.00–3.30	33241	0.146	5.5	80.3

† $R_{merge} = \sum I - \langle I \rangle / \sum I$, where I is the intensity of an individual measurement and $\langle I \rangle$ is the average intensity from multiple observations.

within several days at 295 K (Fig. 2); the presence of cholesterol was absolutely essential for the crystal growth. Optimized crystallization conditions contained 1.9–2.0 *M* ammonium sulfate, 3% propanol, 0.5% *n*-octyl-β-D-glucopyranoside (BOG) detergent, 0.25% phosphatidylcholine and 100 m*M* sodium cacodylate buffer at pH 6.5. The optimum concentration for the enzyme was 5 mg ml^{-1} and was 10–50 m*M* for the cholesterol activator. The final crystals diffracted X-rays from a synchrotron source to beyond 3.0 Å resolution (Figs. 2 and 3).

The strict requirement for a cholesterol molecule in the crystallization process shows that cholesterol causes changes in pneumolysin, possibly structural, that facilitate the crystallization process. The cholesterol binding to PLY was confirmed in solution by fluorescence spectroscopy (Kelly & Jedrzejas, 2000). The amount of water-soluble cholesterol required to fully saturate the binding to the Ply receptor at 1 mg ml^{-1} protein concentration in buffer *A* was found to be ~10 m*M*. This concentration agrees with the minimal water-soluble cholesterol concentration required for the crystal growth. The receptor requirement for PLY crystal growth is in contrast to the behavior of the perfringolysin O and other CDCs, which were crystallized without the membrane receptor (Feil *et al.*, 1996). To our knowledge, this is the first CDC crystallized in the presence of cholesterol substrate bound to the CDC molecule.

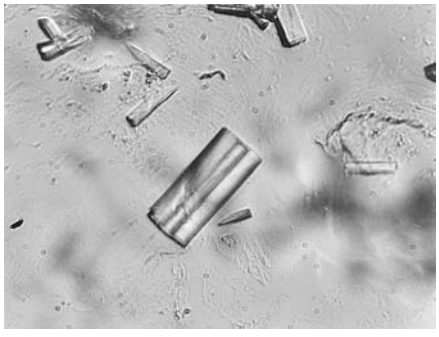

Figure 2
Crystals of the pneumolysin–cholesterol complex were grown in the presence of cholesterol as described in §2. The crystal size was 0.3 × 0.15 × 0.15 mm.

3.3. X-ray diffraction and analysis

The diffraction of crystals at room temperature led to their rapid decay upon X-ray exposure. Therefore, to minimize the decay in the X-ray beam, crystals were cryoprotected by briefly soaking them in a glycerol-based cryo-solution and were flash-frozen to 103 K as described in §2. Under these conditions the crystals still showed some diffraction decay, but increased resolution upon X-ray exposure was obtained compared with the room-temperature diffraction.

Analysis of the diffraction data for the PLY–cholesterol crystals using the *HKL* package (Otwinowski & Minor, 1997) indicated that the crystals belong to the hexagonal space group *P*3, with unit-cell parameters $a = b = 191.45$, $c = 66.16$ Å, $\alpha = \beta = 90.0$, $\gamma = 120^\circ$ (Table 1). A 3.3 Å resolution native data set was collected using synchrotron radiation and the diffraction data were processed and scaled with the *HKL* package (Table 1, Fig. 3; Otwinowski & Minor, 1997). A total of 77 844 observed reflections were processed and reduced to 33 241 unique reflections with an R_{merge} of 14.6%. The data was 80.3% complete, with an average $I/\sigma(I)$ of 5.5. The determination of the three-dimensional crystal structure by either molecular replacement (Rossmann, 1972) using the perfringolysin O model (Rossjohn *et al.*, 1997) and/or by the multiwavelength anomalous dispersion (MAD) method using a selenomethionyl derivative of PLY is currently in progress (Hendrickson & Ogata, 1996). Using molecular replacement, two Ply molecules were initially identified in the asymmetric unit. The resultant calculated phases and electron-density maps were of a very high quality, allowing improvement of the model as well as the specific identification of selected amino acids. The search for additional molecules and the structure solution of this CDC are currently in progress.

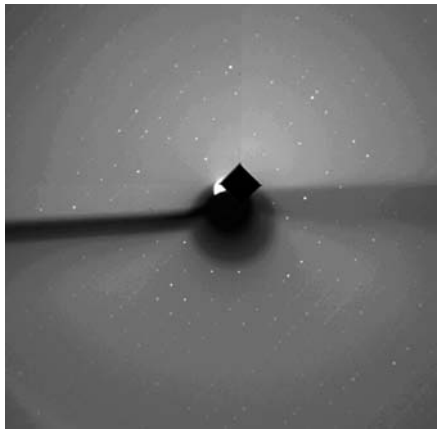

Figure 3
The X-ray diffraction image of the PLY–cholesterol crystals was acquired at 103 K using the X25 synchrotron X-ray beamline at the Brookhaven National Laboratory/National Synchrotron Light Source as described in §2.

3.4. Comment

Structural information obtained from the X-ray crystallographic studies of this PLY conformation will undoubtedly lead to a better understanding of this toxin and its interactions with the membrane receptor. This is likely to explain the structural changes that occur in PLY upon binding to the cell receptor, during target membrane insertion and pore formation, as well as the relationships between its structure and function (Nakamura *et al.*, 1995; Rossjohn *et al.*, 1998). Knowledge of the enzyme's structure is likely to allow the design of inhibitory compounds specific to PLY which might have therapeutic utility not only against pneumococci but perhaps also against other pathogenic bacteria possessing cholesterol-dependent cytolysins.

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