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Crystallization and preliminary X-ray analysis of the human-specific toxin intermedilysin

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Intermedilysin is a human-specific toxin from *Streptococcus intermedius*, which is part of normal human oral flora. The bacterium is an opportunistic pathogen with a tendency for deep-seated infection in the brain and liver. Intermedilysin belongs to the cholesterol-dependent cytolysin (CDCs) family of toxins, which have been identified in several different bacteria including the serious human pathogens *S. pneumoniae* and *Clostridium perfringens*. Intermedilysin, however, is the only member that shows exclusive specificity for human cells. The toxin has a couple of non-conservative amino-acid substitutions in a tryptophan-rich region of the molecule (Cys to Ala and Trp to Pro), the most conserved region amongst the CDCs. Mutations in this region are known to render other CDCs inactive. In order to investigate the structure–function relationships of the unusual features of intermedilysin, which will help us to understand the molecular mechanism of the toxin family in general, recombinant intermedilysin has been crystallized. The crystals belong to an orthorhombic space group and contain two molecules per asymmetric unit. Diffraction data were collected to 2.3 Å using synchrotron radiation.

1. Introduction

Intermedilysin (ILY) was identified as a cytolytic factor of *Streptococcus intermedius*, one of the *S. milleri* group known to cause purulent infections in the mouth and internal organs, specifically in the brain and liver (Nagamune *et al.*, 1996). Higher ILY production levels by *S. intermedius* isolated from deep-seated infections (brain and liver) compared with that from normal sites (oral cavity) point to the toxin's significant role in infections involving this species (Nagamune *et al.*, 2000). Infections with *S. intermedius* in the brain can lead to abscess formation and has been linked to meningitis (Khatib *et al.*, 2000). In addition, ILY is capable of interfering with the immune system at sublytic concentrations (Macey *et al.*, 2001). Based on its significant sequence similarity to pneumolysin, ILY was assigned to the cholesterol-dependent cytolysin (CDC) family and indeed shows functional characteristics of the members of this family, such as haemolytic activity towards erythrocytes which can be inhibited by cholesterol. In contrast to other CDCs, however, ILY is only haemolytic towards human erythrocytes and is not effective towards erythrocytes from other animal species. Although ILY was inhibited by cholesterol, it required a two orders of magnitude higher concentration of cholesterol for inhibition than other CDCs. Since cholesterol cannot account for the exclusive specificity of human cells exhibited by ILY, a putative protein receptor has been implicated and human erythrocytes treated with trypsin were indeed less susceptible to lysis by ILY than intact erythrocytes (Nagamune *et al.*, 1996).

The CDC family includes over 20 members from various Gram-positive bacteria, among which are *S. pneumoniae*, *Clostridium perfringens*, *Listeria monocytogenes*, *C. botulinum*, *Bacillus cereus*, *Arcanobacteirum pyogenes* and *S. canis* (Billington *et al.*, 2000; Heuck *et al.*, 2001; Palmer, 2001; Tweten *et al.*, 2001). In addition to sharing 30–70% pairwise sequence identity, CDCs have many common functional features. They exist as soluble monomers ranging in molecular weight from 47 to 60 kDa, but can oligomerize and form pores in eukaryotic cells. The pores comprise 30–50 monomers and insert into the membrane, forming channels 240–300 Å in diameter. The pores result in cell lysis, providing nutrients and disrupting physical barriers for the pathogen.

The structure of a typical representative of the CDC family, perfringolysin O (PFO) from *C. perfringens*, has been determined previously (Rossjohn *et al.*, 1997) and is the only structure of a CDC known so far, although the crystallization of pneumolysin has been reported (Kelly & Jedrzejas, 2000). PFO, which shares about 40% sequence identity with ILY, is an elongated molecule comprised of four domains rich in β-strands. Multiple independent fluorescence studies determined that two regions in

domain 3 undergo an α to β conformational change, forming two β-hairpins that contribute to the formation of a β-barrel that spans the membrane (Shepard *et al.*, 1998; Shatursky *et al.*, 1999). Cholesterol has for a long time been considered to be a receptor for the CDCs, being absolutely required for lytic activity. However, new experimental evidence suggests that the major role of cholesterol is to trigger the conversion of the prepore to pore, rather than being responsible for initial recognition and binding (Giddings *et al.*, 2003). A highly conserved tryptophan-rich region of 11 amino acids located near the C-termini of CDCs has been implicated in cholesterol and membrane binding (Alouf, 2000). CDCs are known to be inactivated by thiol-modifying reagents and were previously referred to as thiol-activating toxins (Alouf, 2000; Palmer, 2001). A conserved cysteine in the tryptophan-rich region is responsible for this feature. However, this cysteine is not essential for toxin function, as mutation of this residue does not affect the lytic activity. Interestingly, ILY has an alanine in the equivalent position and as a consequence is not sensitive to thiol-modifying reagents (Nagamune *et al.*, 1996). Three highly conserved tryptophan residues in the tryptophan-rich region have been found to be absolutely essential for function, as mutations of any of them inactivate CDCs (reviewed in Alouf, 2000). Surprisingly, one of these tryptophans is substituted by a proline residue in ILY.

We have crystallized ILY, the human-specific CDC, in order to understand the structure–function relationships of the special features possessed by this atypical member of the CDC family. We believe the structure of ILY will provide us with new clues as to how CDCs are converted from soluble to membrane-bound oligomeric forms, what structural features might govern the exclusive specificity for human cells displayed by ILY and the role of the unusual substitutions in the highly conserved tryptophan-rich region of ILY.

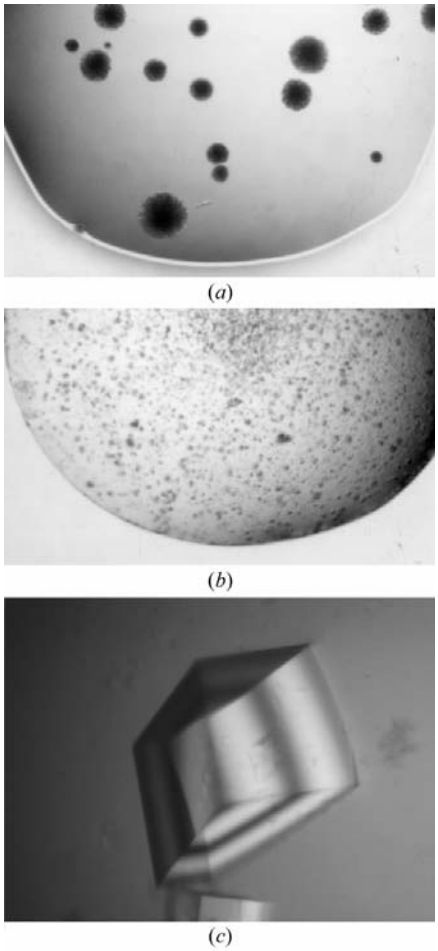

Figure 1
(*a*) and (*b*) Two types of crystalline-like objects observed in the initial screens where PEG was used as a precipitant; (*c*) ILY crystals grown in 100 m*M* MES pH 6.5, 14–18%(*w*/*v*) PEG 8000 after the protein was dialyzed against 20 m*M* HEPES pH 7.5 and 0.3 *M* Na_2SO_4. The maximum dimensions are 0.5 × 0.5 × 0.3 mm.

2. Experimental procedures and results

2.1. Expression and purification

ILY was cloned from *S. intermedius* and expressed in *Escherichia coli* as recently described (Giddings *et al.*, 2003). *E. coli* containing the recombinant plasmid was grown in 8 l of Terrific Broth medium (Becton-Dickinson, Sparks, MD, USA) at 310 K until an optical density of 1.0 at 600 nm was reached. Expression of ILY was then induced by adding IPTG to a final concentration of 500 µ*M* and continuing growth under the same conditions for 2 h. The bacterial cells were harvested by centrifugation, suspended in buffer *A* (10 m*M* MES pH 6.5, 150 m*M* NaCl) to 50 ml and lysed by passage through an EmulsiFlex-C5 cell disrupter (Avestin, Ottawa, Canada) at 103.5 MPa. The cell debris was removed by centrifugation at 15 000*g* for 20 min. The supernatant was applied to a cobalt metal-chelating Sepharose (2.5 × 10 cm) column (Amersham Biosciences, NJ, USA). The column was washed with buffer *A* until the optical density at 280 nm was less than 0.05 and ILY was then eluted from this column using buffer *A* containing 300 m*M* imidazole. The fractions containing ILY were pooled, diluted fivefold with buffer *A* and loaded onto a HP Sepharose S (2.5 × 20 cm) column (Amersham Biosciences, NJ, USA). ILY was eluted with a continuous gradient (total volume of 315 ml) from 150 to 500 m*M* NaCl in buffer *B* (10 m*M* MES pH 6.5). The His tag at the N-terminus of ILY was retained. The purified ILY stock solution at a concentration of about 1 mg ml^{-1} in 10 m*M* MES pH 6.5, 1 m*M* EDTA, 300 m*M* NaCl and 10% glycerol was stored at 253 K.

2.2. Crystallization

For initial crystallization trials, ILY was dialyzed against 20 m*M* MES pH 6.5, 1 m*M* EDTA and concentrated to about 10 mg ml^{-1}. The initial screens produced some crystalline-like objects in conditions containing PEG as a precipitant (Figs. 1*a* and 1*b*). However, we noticed a small amount of precipitation appearing during dialysis. We therefore decided to try different dialysis conditions; for example, including NaCl at concentrations of 0.1 or 1 *M* in the dialysis buffer. Further screening did not produce crystals. Interestingly, ILY activity has been reported to be lost after ion-exchange chromotography in a low ion concentration buffer and the effect of different ions on stability of ILY was investigated (Nagamune *et al.*, 1996). The order of the stabilization effect of cations followed the series $Na^+ > K^+ > NH_4^+$ and among anions sulfate was found to be the most effective. Improvement of crystal quality by manipulation of the protein-storage buffer rather than alteration of crystallization conditions has been reported by others (Schubert *et al.*, 2001). We therefore dialyzed ILY against either 20 m*M* HEPES pH 7.0 and 0.3 *M* sodium formate as in Schubert *et al.* (2001) or 20 m*M* HEPES pH 7.0 and 0.3 *M* sodium sulfate. ILY was then concentrated to about 10 mg ml^{-1}. Crystals of 0.1 × 0.1 × 0.2 mm in size were obtained from an ILY sample dialyzed against 20 m*M* HEPES pH 7.0 and 0.3 *M* Na_2SO_4 in two of the screening conditions where the precipitant was either PEG 4000 or PEG 8000. We have also observed crystals when the protein was dialyzed against 20 m*M* HEPES pH 7.0 and 0.3 *M* sodium formate, but they were small and needle-like. Izit (Hampton Research, CA, USA) and crystal violet (BioRad Laboratories Pty Ltd, New South Wales, Australia) were used to establish whether the crystals were protein crystals. Surprisingly, the crystals stained with crystal violet but remained clear when soaked using Izit. Diffraction experiments confirmed that the crystals were protein crystals. They diffracted in-house to about 3.3 Å resolution. The crystals decayed after prolonged

exposure to X-rays and flash-freezing was required in order to acquire a complete data set.

The optimal crystallization conditions were found to be 100 m*M* MES pH 6.5, 14–18%(*w*/*v*) PEG 8000. The crystals grew at 295 K using the hanging-drop vapour-diffusion method, reaching a maximal size of 0.5 × 0.5 × 0.3 mm after one week (Fig. 1*c*). Although ILY was dialyzed against 20 m*M* HEPES pH 7.0 and 0.3 *M* Na_2SO_4 prior to crystallization, it was imperative that the reservoir solution did not contain salt for successful crystallization.

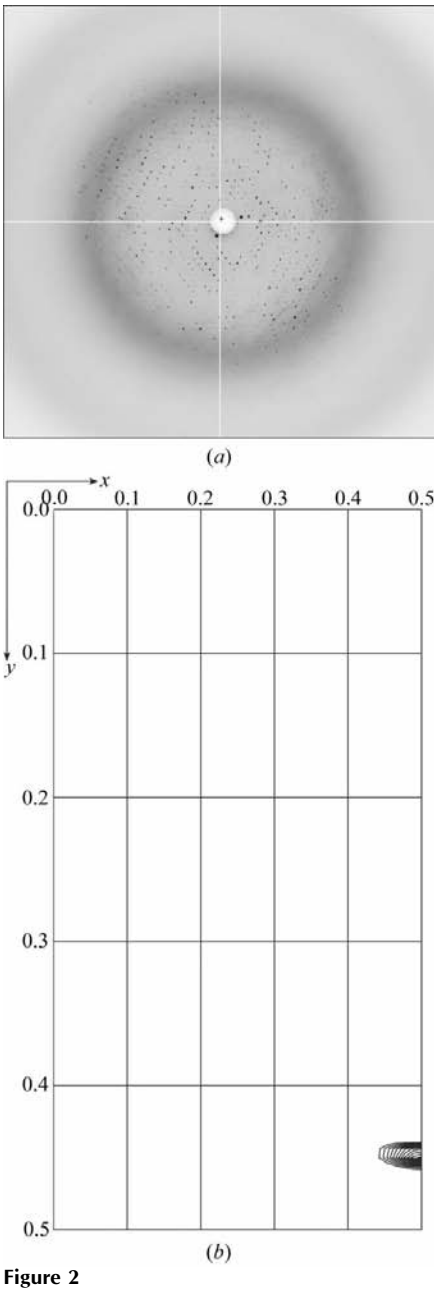

Figure 2
(*a*) X-ray diffraction pattern from a crystal of ILY using synchrotron radiation. (*b*) Native Patterson map calculated from 30 to 4 Å resolution, section $z = 0.5$.

Table 1
Diffraction data statistics.

Values in parentheses indicate data in the highest resolution shell (2.38–2.30 Å).

Space group	$P2_12_12$
Wavelength (Å)	0.9
Resolution range (Å)	30.0–2.3
Total observations	451968
Unique reflections	69506
Completeness (%)	95.5 (75.7)
Mean $I/\sigma(I)$	36.5 (2.2)
$R_{\rm merge}$ (%)	5.2 (70.2)

2.3. Data collection and preliminary X-ray analysis

Crystals were flash-frozen after stepwise transfer into the final cryobuffer. The cryoprotectant MPD was added in steps of 5%(*v*/*v*) up to 20%(*v*/*v*) to stabilizing buffer containing 100 m*M* MES pH 6.5, 0.1 *M* Na_2SO_4, 20%(*w*/*v*) PEG 8000. Because the crystals were relatively large, the soaking time in each step had to be about 20 min to ensure that diffraction quality was preserved. A complete X-ray data set has been collected on an ADSC Q4 detector on BioCARS beamline 14-BM-C at the Advanced Photon Source (Chicago, USA). The data set extended to 2.3 Å resolution. It was noted that the diffraction was anisotropic, with weaker diffraction along *a** (Fig. 2*a*). The crystals belong to space group $P2_12_12$, with unit-cell parameters a = 88.5, b = 173.6, c = 105.1 Å. The intensity measurements for odd reflections along the $h00$ and $0k0$ were all less than 3σ, consistent with the presence of two screw axes. The data were indexed and scaled with the *HKL* package (Otwinowski & Minor, 1997) and the statistics are shown in Table 1. The calculated Matthews coefficient ($V_{\rm M}$) of 3.45 Å^3 Da^{-1} suggested the presence of two molecules in the asymmetric unit and a solvent content of about 60% (Matthews, 1968). A self-rotation function was calculated using the programs *AMoRe* (Navaza, 1994) and *MOLREP* (Vagin & Teplyakov, 2000) at different resolution ranges. No significant peak corresponding to non-crystallographic symmetry was found, suggesting the possibility that the non-crystallographic symmetry axis might be parallel to one of the crystallographic axes. A native Patterson map calculated in the resolution range 30–4 Å revealed a significant peak over 25σ at $x = 0.5$, $y = 0.44$, $z = 0.5$, thus confirming that there are indeed two molecules per asymmetric unit (Fig. 2*b*). Our attempts to determine the structure by molecular replacement using different programs and different search models including PFO, separate domains of PFO or a model of ILY that we created based on the PFO crystal structure were unsuccessful. We have now prepared putative heavy-atom derivatives as the basis for solving the ILY structure by multiple isomorphous replacement.

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