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Crystallization and preliminary X-ray diffraction analyses of pseudechetoxin and pseudecin, two snake-venom cysteine-rich secretory proteins that target cyclic nucleotide-gated ion channels

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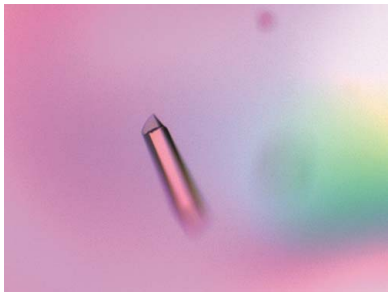


Cyclic nucleotide-gated (CNG) ion channels play pivotal roles in sensory transduction of retinal and olfactory neurons. The elapid snake toxins pseudechetoxin (PsTx) and pseudecin (Pdc) are the only known protein blockers of CNG channels. These toxins are structurally classified as cysteine-rich secretory proteins and exhibit structural features that are quite distinct from those of other known small peptidic channel blockers. This article describes the crystallization and preliminary X-ray diffraction analyses of these toxins. Crystals of PsTx belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 60.30$, $b = 61.59$, $c = 251.69$ Å, and diffraction data were collected to 2.25 Å resolution. Crystals of Pdc also belonged to space group $P2_12_12_1$, with similar unit-cell parameters $a = 60.71$, $b = 61.67$, $c = 251.22$ Å, and diffraction data were collected to 1.90 Å resolution.

1. Introduction

Cyclic nucleotide-gated (CNG) ion channels are non-selective cation channels gated by the direct binding of cyclic nucleotides to alter the membrane potential of the cell in response to stimulus-induced changes in the intracellular concentration of cyclic nucleotides (for reviews, see Kaupp & Seifert, 2002; Matulef & Zagotta, 2003). CNG channels were first discovered in the sensory epithelium of the visual and olfactory systems and are now known to play critical roles in sensory transduction in retinal photoreceptors and olfactory neurons. To date, CNG channels have been found in tissues throughout the body, such as the brain, heart, kidneys, liver and intestines, suggesting undefined physiological functions of these channels in addition to their function in the visual and olfactory systems.

Peptide toxins that target ion channels have been widely used in neurophysiological studies and play crucial roles in the study of the structures and functions of several ion channels. A first potent protein toxin that targets CNG channels has been isolated and cloned from the venom of the Australian king brown snake (*Pseudechis australis*) and designated pseudechetoxin (PsTx; Brown *et al.*, 1999; Yamazaki, Brown *et al.*, 2002). In contrast to well known small-peptide toxins targeting ion channels (<9 kDa), PsTx is relatively large in molecular weight (23.7 kDa) and is classified as a cysteine-rich secretory protein (CRISP), which are proteins that are thought to function in gamete fusion and immune system in mammals (for reviews, see Yamazaki & Morita, 2004; Yamazaki, Koike *et al.*, 2002; Yamazaki *et al.*, 2003). It has been shown that PsTx blocks the currents of the olfactory and retinal CNG channels by binding to the pore turret with high affinity (Brown *et al.*, 1999, 2003). Recently, a homologous toxin named pseudecin (Pdc) that also targets CNG channels has been isolated and cloned from the venom of the red-bellied black snake (*P. porphyriacus*; Yamazaki, Brown *et al.*, 2002). The primary structure of Pdc is quite similar to that of PsTx, with 97% identity, and differs at only seven residues. Interestingly, patch-clamp studies have emerged that show large differences in affinity between PsTx and Pdc for CNG channels; PsTx blocks both olfactory and retinal channels with a 15–30-fold higher affinity than Pdc (Yamazaki, Brown *et al.*, 2002).

We have recently determined the structure of a homologous CRISP from the venom of *Trimeresurus flavoviridis*, triflin (62% amino-acid sequence identity to PsTx and 61% to Pdc), which blocks

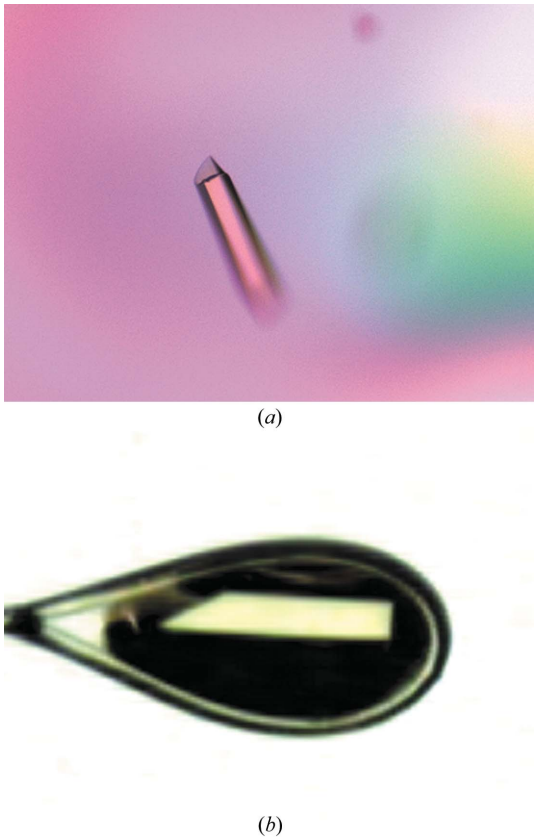
voltage-gated Ca^{2+} channels, and revealed that the protein is composed of an N-terminal domain (~160 residues) that has a fold similar to the group 1 plant pathogenesis-related proteins and a cysteine-rich C-terminal domain (CRD; Shikamoto *et al.*, 2005).

Here, we report the crystallization and preliminary X-ray crystallographic analyses of PsTx and Pdc. The tertiary structural information from these CRISPs may be a clue to the identification of the regions of these proteins that contact CNG channels and of the active sites of other snake-venom CRISPs. Furthermore, as the only known protein blockers of CNG channels, structural information on PsTx and Pdc could be useful to study the structure of the external face of these channels.

2. Materials and methods

2.1. Crystallization

PsTx and Pdc were purified from the lyophilized venoms and venom glands of *P. australis* and *P. porphyriacus*, respectively, as described previously (Yamasaki, Brown *et al.*, 2002). The protein solutions were dialyzed against 50 m*M* Tris–HCl buffer pH 8.0 containing 0.15 *M* NaCl and concentrated to 10 mg ml^{-1} by centrifugal ultrafiltration. Screening for initial crystallization conditions was conducted for Pdc using Hampton Research Crystal Screen and Crystal Screen 2 (Jancarik & Kim, 1991; Cudney *et al.*, 1994) by the microbatch method (Chayen *et al.*, 1990) at 293 K. Typically, 0.5 µl protein solution was mixed with an equal volume of precipitant reagent under Al's oil (Hampton Research). The initial crystallization condition found was then refined to produce crystals suitable for X-ray diffraction analysis. The optimum crystallization condition for PsTx was then established based on the condition for Pdc by changing the parameters.



(*a*)

(*b*)

Figure 1
Crystals of (*a*) Pdc from *P. porphyriacus* (approximate dimensions 0.1 × 0.1 × 0.5 mm) and (*b*) PsTx from *P. australis* (approximate dimensions 0.1 × 0.1 × 0.5 mm).

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

	PsTx	Pdc
Wavelength (Å)	1.00 (NW12, PF-AR)	1.00 (BL6B, PF)
Resolution (Å)	50–2.25 (2.33–2.25)	50–1.90 (1.97–1.90)
Temperature (K)	100	100
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 60.30, b = 61.59, c = 251.69	a = 60.71, b = 61.67, c = 251.22
R_{merge}† (%)	7.4 (25.3)	10.9 (31.5)
Completeness (%)	92.6 (72.2)	98.5 (100.0)
Redundancy	6.7 (6.1)	6.2 (7.2)
$I/\sigma(I)$	6.1 (3.5)	4.7 (2.1)
No. of observed reflections	283328	496582
No. of unique reflections	42215	75498

† $R_{merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity value of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of $I(h)$ for all i measurements.

2.2. Diffraction experiment

X-ray diffraction experiments on PsTx and Pdc crystals were conducted at beamlines NW12 at PF-AR and BL6B at PF, Tsukuba, Japan, respectively. Rod-shaped crystals of PsTx and Pdc were mounted in nylon loops (Hampton Research) and flash-cooled in a nitrogen-gas stream at 100 K prior to data collection. The data for PsTx were collected at 100 K using an ADSC Quantum 210 CCD detector with a wavelength of 1.0 Å and 0.5° oscillation steps over a range of 180°. The data set was processed using *HKL*2000 (Otwinowski & Minor, 1997). Data collection for Pdc was conducted at 100 K using a Rigaku R-AXIS IV^{++} imaging-plate detector using a wavelength of 1.0 Å with 0.5° oscillation steps over a range of 180° and the data were processed with *CrystalClear* (Rigaku).

3. Results and discussion

Initial screening of the crystallization conditions for Pdc resulted in a large number of small crystals from condition No. 33 of Crystal Screen after 2–3 weeks. After refinement based on this condition, rod-shaped crystals with approximate dimensions of 0.1 × 0.1 × 0.5 mm were obtained within two weeks from droplets composed of equal volumes of protein solution (~10 mg ml^{-1}) and reservoir solution [3.6 *M* sodium formate and 10%(*w*/*v*) glycerol, 0.15 *M* NaCl and 50 m*M* Tris–HCl pH 8.0] by the sitting-drop vapour-diffusion method at 293 K (Fig. 1*a*). Diffraction data from the crystals were collected and processed to 1.9 Å resolution. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 60.71, b = 61.67, c = 251.22 Å. Assuming the presence of four monomers of Pdc in the asymmetric unit, the Matthews coefficient (Matthews, 1968) was calculated to be 2.3 Å^3 Da^{-1}, corresponding to a solvent content of 47.0%. The data-collection statistics are summarized in Table 1.

Crystals of PsTx were obtained by the sitting-drop vapour-diffusion method at 293 K (Fig. 1*b*) using a similar crystallization condition within two weeks using ~10 mg ml^{-1} protein solution and a reservoir solution consisting of 2.4 *M* sodium formate, 6.7%(*w*/*v*) glycerol, 0.15 *M* NaCl and 50 m*M* Tris–HCl pH 8.0. The diffraction data of the crystals were collected and processed to 2.25 Å resolution. The crystals belong to the orthorhombic space group $P2_12_12_1$, with

unit-cell parameters a = 60.30, b = 61.59, c = 251.69 Å, a V_M value of 2.3 Å^3 Da^{-1} and a solvent content of 47.0% estimated assuming the presence of four molecules in the asymmetric unit. These data showed that the crystals of Pdc and PsTx are isomorphous. The data-collection statistics are shown in Table 1.

The initial phases of the crystal structure of Pdc were obtained by the molecular-replacement method using the program *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). A search model for Pdc was constructed by homology modelling based on the coordinates of triflin (PDB code 1wvr; Shikamoto *et al.*, 2005) using *InsightII* 2000 (Accelrys). After rotation and translation calculations over the resolution range 20–4.0 Å, a clear solution was found with a correlation coefficient of 24.0% and an R factor of 55.8% which contained four molecules in the asymmetric unit. Cycles of rigid-body refinement using *REFMAC*5 (Murshudov *et al.*, 1997) from the *CCP*4 suite improved the R factors to R_{work} = 54.1% and R_{free} = 54.7%. The resulting electron-density maps of the N-terminal domain of each molecule were clearly interpretable, while those at the C-terminal CRD were ambiguous. This might suggest that the orientation of the CRD is different from that in triflin, which is important for its biological activity. Further refinement of the Pdc model is currently in progress. The structure determination and refinement of the PsTx model is also ongoing using the structure of Pdc as the starting model.

During the preparation of our manuscript, the crystal structure of another snake-venom CRISP, named stecrisp (59% amino-acid sequence identity to PsTx and 58% to Pdc), from the venom of *Trimeresurus stejnegeri* was reported (Guo *et al.*, 2005).

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