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Crystallization and preliminary X-ray diffraction studies of the water-soluble state of the pore-forming toxin sticholysin II from the sea anemone *Stichodactyla helianthus*

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Sticholysin II (StnII) is a potent cytolytic protein produced by the sea anemone *Stichodactyla helianthus*. StnII belongs to the actinoporin family, a group of proteins which are characterized by their ability to spontaneously interact with biological membranes. The cytolytic character of these proteins is currently explained in terms of a molecular mechanism involving the formation of transmembrane pores. StnII has been crystallized using the hanging-drop vapour-diffusion method at 291 K. Diffraction-quality crystals have unit-cell parameters $a = 32.30$, $b = 119.73$, $c = 43.42$ Å, $\beta = 90.04^\circ$ and belong to the monoclinic space group $P2_1$. Diffraction data to a resolution of 1.71 Å were collected at synchrotron facilities.

1. Introduction

Sea anemones (order Actiniaria) produce a large number of toxic polypeptides which are usually classified into two main groups according to their molecular weight: low-molecular-weight (5–8 kDa) neurotoxic peptides (Castañeda *et al.*, 1995) and ∼20 kDa cytolysins (Bernheimer & Avigad, 1976; Tejuca *et al.*, 1996; de los Ríos *et al.*, 1998; Anderluh & Macek, 2002). Proteins belonging to this last group, globally known as actinoporins (Kem, 1988), share many molecular characteristics: they are basic polypeptides (pI values ranging from 8 to 12) with a high chemical stability and a high affinity towards the lipid sphingomyelin (Bernheimer, 1990; Harvey, 1990; Macek, 1992; Anderluh & Macek, 2002). In addition, a high degree of amino-acid sequence similarity has been found between these cytolysins (Simpson *et al.*, 1990; Belmonte *et al.*, 1994; Anderluh & Macek, 2002). These proteins are highly cytotoxic and lytic to a variety of cells (Kem, 1988; Bernheimer, 1990; Norton, 1991; Turk, 1991), which is currently explained in terms of a colloid-osmotic type mechanism involving the formation of membrane pores for small solutes (Macek *et al.*, 1994). Studies performed with model membranes also support this hypothesis: membrane permeabilization would proceed through the formation of oligomeric transmembrane pores (Tejuca *et al.*, 1996; de los Ríos *et al.*, 1998). In this sense, kinetic analyses of the permeabilization process of liposomes induced by StnII revealed the existence of protein–protein interactions, which is consistent with a process of oligomerization prior to the leakage itself (de los Ríos *et al.*, 1998). This conclusion has also been proposed for equinatoxin II, which would form cation-selective channels composed of several monomers (Zorec *et al.*, 1990; Belmonte *et al.*, 1993; Macek *et al.*, 1995). Additionally, it has been shown that StnII behaves in solution as an associating monomer–tetramer system (de los Ríos *et al.*, 1999).

Pore-forming toxins (PFTs) have been revealed to be valuable experimental systems for exploring structural and mechanistic issues concerning protein–membrane interactions (Heuck *et al.*, 2001). In this regard, a number of bacterial toxins have been studied in detail (for reviews, see Gouaux, 1997; Heuck *et al.*, 2001): α-haemolysin and leukocidin from *Staphylococcus aureus* (Prevost *et al.*, 2001), colicins from *Escherichia coli* (Stroud *et al.*, 1998; Lakey & Slatin, 2001), aerolysin from *Aeromonas hydrophila* (Fivaz *et al.*, 2001*a*,*b*) and perfringolysin O from *Clostridium prefringens* (Billington *et al.*, 2000; Palmer, 2001). In contrast to this situation, eukaryotic PFTs are poorly understood from a structural viewpoint, the actinoporin family being one of the main focuses of attention. According to the results obtained by CD and FTIR spectroscopy, β-structure would be the predominant regular secondary structure in all these proteins (Belmonte *et al.*, 1994; Menestrina *et al.*, 1999; Poklar *et al.*, 1999; Mancheño *et al.*, 2001). These results are in agreement with the first three-dimensional structure reported for a member of the actinoporin family, the structure of the soluble form of equinatoxin II (EqtII) from the sea anemone *Actinia equina* (Athanasiadis *et al.*, 2001). The structure of EqtII is characterized by a β-sandwich core with six β-strands per sheet, each one being flanked by a short α-helix. Regarding the

membrane-bound state of actinoporins, FTIR-spectroscopy studies revealed small changes in the secondary-structure contents of EqnII upon membrane interaction (Menestrina *et al.*, 1999). Interestingly, EqnII and StnII show partially folded states which may help in thermodynamically understanding the conversion from a water-soluble state to a membrane-bound state (Mancheño *et al.*, 2001; Poklar *et al.*, 1997). Additionally, electron-microscopy analysis of two-dimensional crystals of StnII on lipid monolayers has revealed the existence of tetrameric motifs with a topology clearly resembling a pore (Martín-Benito *et al.*, 2000).

The X-ray crystallographic structure of StnII will reveal valuable information about the structural basis of pore formation for this particular cytolysin and, by extension, to the proteins belonging to the actinoporin family. This will provide new insights into the molecular basis of the cytotoxicity of these proteins and therefore provide additional interest in this currently attractive type of protein as a biotechnological and pharmaceutical tool (Avila *et al.*, 1988; Pederzolli *et al.*, 1995; Panchal *et al.*, 1996; Tejuca *et al.*, 1999).

2. Experimental

2.1. Sticholysin II purification

The cytotoxic protein StnII was purified from body extracts of the sea anemone *Stichodactyla helianthus* (purchased from Nayeco, Barcelona, Spain) as previously described (de los Ríos *et al.*, 1998, 1999; Mancheño *et al.*, 2001). The whole sea-anemone body was minced and homogenized. An aliquot of this homogenate (approximately 4 ml) was loaded onto a Sephadex G-50 chromatographic column (95 × 3 cm) previously equilibrated in 20 m*M* ammonium acetate pH 5.0. Fractions containing the highest haemolytic activity were pooled, dialyzed against double-distilled water (Milli-Q) and lyophilized. The lyophilized protein sample was dissolved in 0.1 *M* ammonium acetate pH 5.0 (starting buffer) and loaded onto a CM-cellulose CM-52 column (30 × 1.5 cm). Under these experimental conditions, StnII is a positively charged species and strongly interacts with this ion exchanger. Elution of the proteins was achieved with a 0.1–0.4 *M* ammonium acetate linear gradient after exhaustively washing the chromatographic column with starting buffer. This procedure renders two well resolved peaks, the more basic of which corresponds to StnII (the first peak corresponds to StnI). Fractions containing StnII were pooled, dialyzed against double-distilled water (Milli-Q) and lyophilized. All chromatographic steps were performed at 277 K.

StnII thus purified showed spectroscopic properties (fluorescence and near- and far-UV CD), amino-acid composition and amino-terminal sequence (the first 53 residues) identical to those previously described for this cytolysin (de los Ríos *et al.*, 1998, 1999; Mancheño *et al.*, 2001).

2.2. Crystallization

The StnII solution for crystallization experiments was prepared as follows: the lyophilized protein was dissolved in 20 m*M* Tris–HCl pH 7.0 and subsequently centrifuged at 10 000 rev min $^{-1}$ for 3 min. Protein concentration was determined from absorbance measurements employing an $E(0.1\%, 280\ \text{nm}, 1\ \text{cm})$ of 2.54 (de los Ríos *et al.*, 1998) on a Cintra 5 UV–VIS spectrophotometer (GBC Scientific Equipment) with 1 cm quartz cells. The protein solution was finally diluted to 10 mg ml^{-1} with the same buffer and stored at 277 K. The initial crystallization conditions were established using the sparse-matrix sampling technique (Jancarik & Kim, 1991) with the hanging-drop vapour-diffusion method at 291 K using Crystal Screens I and II (Hampton Research). Drops containing equal volumes (2 µl) of protein and reservoir solution were equilibrated against 500 µl of reservoir solution. A microcrystalline precipitate was obtained from 0.2 *M* lithium sulfate monohydrate, 0.1 *M* Tris–HCl pH 8.5, 30%(*w*/*v*) PEG 4000. Further optimization of these conditions to 0.2 *M* lithium sulfate monohydrate, 0.1 *M* Tris–HCl pH 7.5, 30%(*w*/*v*) PEG 4000 yielded crystals suitable for diffraction in 15–20 d. These crystals were systematically twinned thin plates (Fig. 1) that could not be further optimized with the employment of Additive Screens I, II or Detergent Screens (Hampton Research). These crystals were crushed and used for microseeding. However, the crystals that appeared in the seeded drops did not grow to a size sufficient for X-ray diffraction. Macroseeding was also attempted without positive results. Finally, successful plates for X-ray diffraction measurements were obtained after careful selection of clean small untwinned regions using Micro-Tools (Hampton Research).

2.3. X-ray diffraction experiments

Preliminary diffraction data were collected in-house on a MAR Research

Table 1
Data-collection and processing statistics..

Values for the outermost shell (1.81–1.71 Å) are given in parentheses.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	32.30
b (Å)	119.73
c (Å)	43.42
β (°)	90.04
Resolution range (Å)	32.3–1.7
No. of measured reflections	68073
No. of unique reflections	31889
$R_{\rm merge}$ (%)	8.6 (15.1)
Completeness (%)	90.3 (90.3)
Average $I/\sigma(I)$	5.0 (4.6)

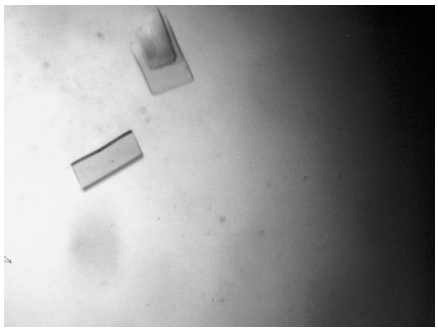

Figure 1
Crystals of StnII grown at 291 K in 0.2 *M* lithium sulfate monohydrate, 0.1 *M* Tris–HCl pH 7.5, 30%(*w*/*v*) PEG 4000.

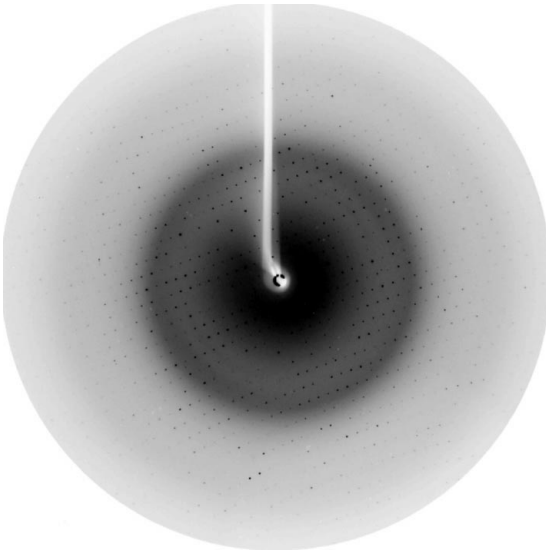

Figure 2
Diffraction pattern of StnII crystals using synchrotron radiation on beamline BM 14 at the ESRF. The edge of the plate corresponds to 1.70 Å resolution.

MAR345 image-plate detector with Cu $K\alpha$ X-rays generated by an Enraf–Nonius rotating-anode generator equipped with a double-mirror focusing system, operated at 40 kV and 90 mA. Initially, crystals were mounted in the mother liquor on a cryoloop and flash-cooled to 120 K in a stream of nitrogen. Optimization of the cryoprotectant solution to 0.1 *M* Tris–HCl pH 7.5, 30%(*w*/*v*) PEG 4000, 20%(*v*/*v*) glycerol yielded the best results. A native data set was finally collected using synchrotron-radiation sources at ESRF (Grenoble, France) on beamline BM 14 using a CCD detector. The crystal-to-detector distance was set to 100 mm. All data were processed and scaled using the programs *MOSFLM* (Leslie, 1994) and *SCALA* from the *CCP4* package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

StnII crystals diffract to 1.71 Å resolution (Fig. 2) and belong to the $P2_1$ monoclinic space group with a near-orthorhombic topology (unit-cell parameters a = 32.30, b = 119.73, c = 43.42 Å, β = 90.04°). These results are consistent with two monomers of StnII per asymmetric unit and a solvent content of 42%. Data-collection and processing statistics are summarized in Table 1. A total of 68 073 measured reflections were merged into 31 889 unique reflections with an $R_{\rm merge}$ of 8.6%. The merged data set was 90.3% complete to 1.71 Å resolution. Using the recently reported crystal structure of EqtII (Athanasiadis *et al.*, 2001) as a search model, phasing of diffraction data has been attempted with the molecular-replacement method using the program *AMoRe* (Navaza & Vernoslova, 1995). The lack of success may be a consequence of significant structural differences between EqtII and StnII. Hence, structure determination with the use of halides according to previously described methods (Dauter & Dauter, 1999; Dauter *et al.*, 2000) is now in progress.

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