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Expression, purification, crystallization and preliminary X-ray analysis of rat ecto-ADP-ribosyltransferase 2 (ART2.2)

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ADP-ribosyltransferases catalyze the transfer of the ADP-ribose moiety from NAD^+ onto proteins and other targets. These enzymes have been found in prokaryotes and in vertebrates; a eukaryotic enzyme structure is not yet known. The enzyme from *Rattus norvegicus* was expressed in the *Escherichia coli* periplasm at a level of about 0.2 mg per litre of culture, purified and crystallized. Native data sets were collected to 2.0 Å resolution. A self-rotation function revealed a local twofold axis in crystal form *A* and a Patterson function showed a translational relationship in form *B*. Form *C* contains only one molecule in the asymmetric unit.

1. Introduction

ADP-ribosyltransferases (ART) transfer the ADP-ribose moiety of NAD^+ onto specific targets while releasing nicotinamide (Althaus *et al.*, 1985; Aktories, 1991; Koch-Nolte & Haag, 1997). The targets are mostly Arg, Cys, Asn or Glu residues of proteins. ADP-ribosylation is reversible and often profoundly affects protein function. Other targets are DNA, free amino acids, ADP-ribose itself and water. The enzyme group attracted interest as being bacterial toxins that, after being translocated into mammalian cells, ADP-ribosylate and inactivate key proteins such as G proteins, actin, ras, rho and elongation factor 2 (Aktories, 1991; Rappuoli & Montecucco, 1997). The suspicion that these toxins mimic the function of mammalian homologues was nurtured by the discovery of ART activities in uninfected tissues. This initiated a search that revealed a small family of extracellular mammalian enzymes, including the GPI-anchored membrane proteins, ecto-ART1 through ecto-ART4 and the secretory ecto-ART5 (Koch-Nolte & Haag, 1997; Okazaki & Moss, 1998).

To date, the structures of eight bacterial ART toxins and of the distantly related nuclear poly(ADP-ribose)polymerase (PARP) from chicken are known (Rappuoli & Montecucco, 1997; Ruf *et al.*, 1998; Han *et al.*, 2001), but no structure of a mammalian ecto-ART has been determined. Although the bacterial toxins and the mammalian ecto-ARTs exhibit less than 10% amino-acid identity, detailed sequence/structure analyses made it very likely that they form one family (Domenighini & Rappuoli, 1996; Koch-Nolte *et al.*, 1996; Bazan & Koch-Nolte, 1997; Pallen *et al.*, 2001).

The physiological functions of mammalian ecto-ARTs are still ill-defined. Mouse ART1 and ART2 ADP-ribosylate extracellular proteins: for instance, integrins and growth factors (Zolkiewska & Moss, 1995; Okamoto *et al.*, 1998; Saxty *et al.*, 2001). *R. norvegicus* ART2.2 displays NAD^+ glycohydrolase and auto-ADP-ribosylation activities (Haag *et al.*, 1995; Maehama *et al.*, 1995) and gains arginine transferase activity by the exchange Q187E (Hara *et al.,* 1996; Karsten *et al.*, 1997; Maehama & Katada, 1997). We focused on rat ART2.2 because it could be produced in the periplasm of *E. coli* and because it is the only non-glycosylated mammalian ecto-ART (Koch *et al.*, 1990; Koch-Nolte & Haag, 1997). The structure of this ART will provide a new starting point for functional studies.

2. Materials and methods

2.1. Expression and purification

In order to produce ART2.2, we tested expression systems based on insect cells (Sf9, Hi5), on mammalian cells (CHO, HEK) and on *E. coli*. Of these, only periplasmic *E. coli* expression was successful. The mammalian signal peptide (residues 1–20) was replaced by that of *E. coli* OmpA. Furthermore, the GPI-anchor signal sequences were deleted by placing a stop codon behind Ser226.

The resulting gene, here called ART2.2, was cloned into plasmid pASK60 (Biometra) and expressed in *E. coli* NM522 cells. Freshly transformed *E. coli* NM522 cells were grown

on agar plates. A single colony was picked and used to inoculate 10 ml Luria–Bertani broth supplemented with 50 µg ml^{-1} carbenicillin and 2 m*M* nicotinamide (LBCN broth). When the OD_{600} reached 0.7, the cells were sedimented, resuspended in 2 ml fresh LBCN broth and used to inoculate 2 × 400 ml LBCN broth. After 2 h of constant shaking at 310 K, the temperature was lowered to 293 K and the culture was grown for an additional 14 h. All further steps were performed at 277 K.

Cells from 10 l of culture (25 × 400 ml) were harvested, combined and subjected to osmolytic shock using lysis buffer [39 g sucrose, 25 mg Pefabloc SC (Fluka), 1.5 ml 0.5 *M* EDTA dissolved in 250 ml 0.1 *M* Tris–HCl pH 7.8]. After 60 min vigorous stirring, 250 ml water was added and stirring was continued for another 60 min. The lysate was clarified by centrifugation (48 000*g*, 60 min), dialyzed against buffer *A* (0.1 *M* Tris–HCl pH 7.5) and applied at 5 ml min^{-1} onto a pre-equilibrated (buffer *A*) Source-30S column (Pharmacia). The column was washed and the protein was eluted using a gradient of 0–500 m*M* NaCl in buffer *A*. The ART2.2-containing fractions were concentrated to less than 1 ml (Centriprep, Amicon) and run at 0.6 ml min^{-1} through a HighLoad16/60 Superdex-75 column using 150 m*M* NaCl in buffer *A*.

Table 1
Data-collection statistics.

All data were collected at 100 K using Cu $K\alpha$ radiation. Values in parentheses refer to the last shell. The molecular weight of mature ART2.2 is 26 028 Da.

Data	Crystal form *A*	Crystal form *B*	Crystal form *C*
Space group	$P2_1$	$P2_12_12_1$	$P3_221$
Unit-cell parameters			
a (Å)	46.1	69.6	81.4
b (Å)	85.9	77.3	81.4
c (Å)	57.7	86.5	77.5
β (°)	95.1		
Molecules per asymmetric unit	2	2	1
V_M (Å^3 Da^{-1})	2.23	2.23	2.85
Resolution range (Å)	26–2.5	33–2.0	28–2.1
Observations	84259	126778	138463
Unique reflections	15600 (2273)	30925 (4218)	18816 (2673)
Completeness (%)	99.9 (99.9)	99.1 (99.1)	99.2 (99.2)
Multiplicity	5.4 (5.3)	4.1 (4.0)	7.4 (7.4)
Average $I/\sigma(I)$	12.1 (4.9)	8.1 (3.8)	10.1 (4.4)
$R_{sym}(I)$ (%)	5.7 (15.5)	6.3 (17.7)	5.5 (16.7)

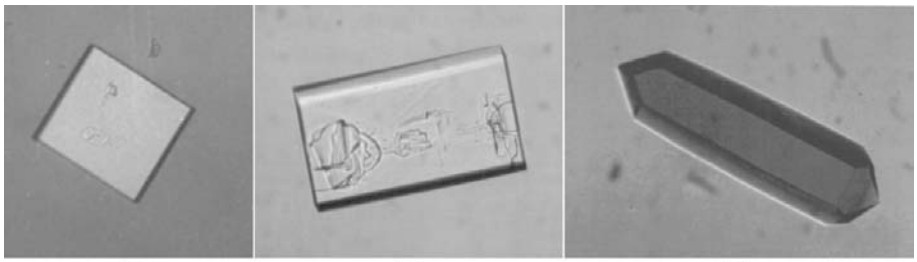

Figure 1
Crystal forms of ART2.2. (*a*) Monoclinic crystal form *A* with dimensions of 250 × 250 × 100 µm. (*b*) Orthorhombic crystal form *B* with dimensions of 400 × 250 × 100 µm. (*c*) Trigonal crystal form *C* with dimensions of 300 × 100 × 100 µm.

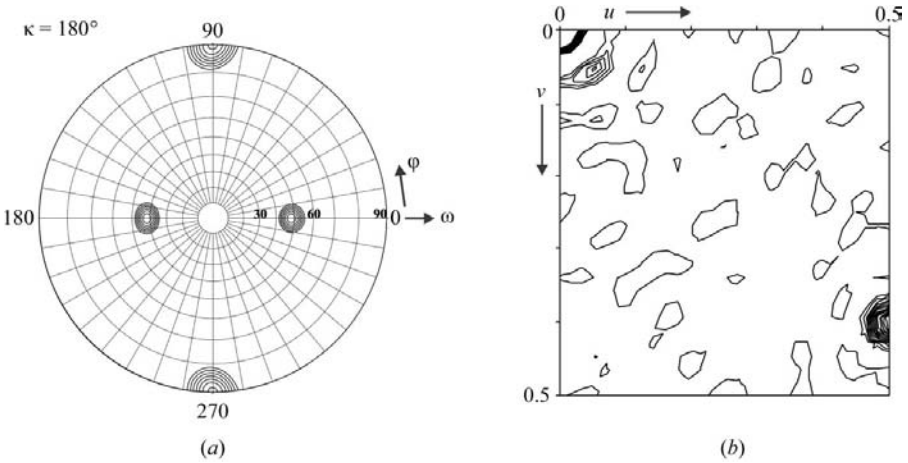

Figure 2
Non-crystallographic symmetry of ART2.2 crystals. (*a*) The self-rotation function of crystal form *A* was calculated using *POLARRFN* (Collaborative Computational Project, Number 4, 1994) in the resolution range 20–2.5 Å with an integration radius of 10 Å. The contours are at 2, 3, ..., 8σ. (*b*) The Patterson map of crystal form *B* was calculated using all data in the resolution range 20–4 Å. Contouring is at 2, 3, 4, ..., 16σ. The origin and the translation peak heights are 84 and 26σ, respectively.

2.2. Crystallization and data collection

Crystallization was performed at 293 K using hanging drops. The protein was concentrated to 8.8 mg ml^{-1} (Millipore) and subjected to Crystal Screens I and II (Hampton Research), mixing equal volumes (2 µl) of the protein and the well solutions. Small crystals appeared at 100 m*M* Tris–HCl pH 8.5, 200 m*M* Li_2SO_4, 30%(*w*/*v*) PEG 4000. Refinement resulted in good-quality crystals of two different morphologies with 100 m*M* Tris–HCl pH 8.3, 200 m*M* Li_2SO_4 and 22%(*w*/*v*) PEG 4000 in the well. For storage, the PEG 4000 concentration was increased to 32%(*w*/*v*).

Increasing the glycerol concentration in five steps, the crystals were transferred to a buffer containing 25%(*v*/*v*) glycerol, equilibrated for 2 min, mounted on cryoloops and then flash-frozen at 100 K using nitrogen gas (Cryostream, Oxford Cryosystems). Data were collected on an imaging plate (MAR Research) using graphite-filtered Cu $K\alpha$ radiation from a rotating-anode generator (model RU-200B, Rigaku) and processed using the programs *MOSFLM* (Leslie, 1992), *SCALA* and *TRUNCATE* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Expression of ART2.2 into the periplasm of *E. coli* NM522 cells secured the formation of the two potential disulfide bridges (Koch *et al.*, 1990). The periplasmic fraction was isolated by osmolysis and the protein was purified using cation-exchange and gel-permeation chromatography. The yields ranged between 0.1 and 0.3 mg of ART2.2 per litre of culture. The protein was identified by N-terminal sequencing, which yielded the correct first 12 amino-acid residues. Under the given crystallization conditions, we obtained three crystal forms suitable for X-ray diffraction analysis (Fig. 1). They grew within 3–21 d. Forms *A* and *B* had identical crystal habits and their packing parameters (Matthews, 1968) indicated two ART2.2 molecules per asymmetric unit (Table 1). In contrast, crystal

form *C* contained only one molecule in the asymmetric unit.

The addition of glycerol permitted data collection at 100 K. Crystal form *A* data were used for calculating a self-rotation function (Fig. 2*a*), which showed strong peaks at $(\omega, \varphi, \kappa) = (49, 0, 180^\circ)$ and $(42, 180, 180^\circ)$, indicating that the asymmetric unit contains two ART2.2 molecules related by a local twofold axis. Furthermore, a Patterson map of crystal form *B* (Fig. 2*b*) revealed a non-crystallographic translation described by the vector (0.5, 0.4, 0.0). The height of the corresponding peak amounts to 31% of that of the origin peak, indicating that the relationship is a rather exact translation. These analyses confirmed the assignment of two molecules per asymmetric unit for crystal forms *A* and *B* (Table 1). The structure analysis of crystal form *A* is in progress.

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