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Crystallization and preliminary X-ray diffraction analysis of cytotoxic ribonucleases from bullfrog *Rana catesbeiana*

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RC-RNases are ribonucleases from *Rana catesbeiana* oocytes with pyrimidine–guanine sequence specificity. They also possess cell cytotoxicity and lectin activity. Protein crystals of three RC-RNase isozymes, RC-RNase 3, RC-RNase 4 and RC-RNase 6, were grown in various crystal systems under different conditions. Crystals of RC-RNase3 belong to the orthorhombic $C222_1$ space group, with unit-cell parameters a = 66.66, b = 97.38, c = 85.74 Å. Crystals of RC-RNase 4 belong to the trigonal space group $P3_1$ or $P3_2$, with unit-cell parameters $a = b$ = 32.22, c = 92.12 Å. Crystals of RC-RNase 6 complexed with cytidylyl 2′-5′ guanosine belong to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b$ = 61.80, c = 65.96 Å.

1. Introduction

Ribonucleases are ubiquitous proteins that have been found widely in various living organisms. This class of enzymes has been suggested to participate in RNA metabolism and gene-expression regulation (D'Alessio, 1993). Bovine pancreatic ribonuclease (RNase A) is the most extensively studied endoribonuclease and serves as a model in studying the fundamental aspects of protein function and structure (Eftink & Biltonen, 1987). Several proteins with known biological functions have been found to also possess intrinsic ribonucleolytic activities. Some examples are angiogenin (Shapiro *et al.*, 1986), eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) (Gleich *et al.*, 1986; Gullberg *et al.*, 1986). These proteins also share sequence homology with the RNase A superfamily.

Conversely, several frog ribonucleases have been found to possess cytotoxicity toward tumour cells and lectin activity to sialic acid-rich glycoproteins (Irie *et al.*, 1998). Onconase isolated from *Rana pipiens* oocytes is currently being evaluated in phase III clinical trials for tumour therapy (Ardelt *et al.*, 1991; Youle & D'Alessio, 1997). RC-RNases from the oocytes of *R. catesbeiana* are also cytotoxic to several tumour-cell lines (Liao *et al.*, 1996 2000; Huang *et al.*, 1998). Two sialic acid-binding lectins isolated from the eggs of *R. catesbeiana* (SBL-C) and *R. japonica* (SBL-J) belong to the ribonuclease superfamily (Nitta *et al.*, 1987). They possess agglutination activity for various human and animal tumour cells (Okabe *et al.*, 1991; Nitta *et al.*, 1994). The primary sequence of RC-RNase from the oocytes of *R. catesbeiana* is identical to that of SBL-C (Titani *et al.*, 1987; Liao *et al.*, 1996, 2000).

In addition to their cytotoxicity, these frog ribonucleases have many unique properties. The catalytic residues on frog ribonucleases and RNase A are identical. However, the substrate specificity of most frog ribonucleases is pyrimidine–guanine, while the mammalian RNases prefer pyrimidine–adenine sequences. Frog ribonucleases are resistant to ribonuclease inhibitor from human placenta and the location of the fourth disulfide bridge differs from that of mammals. Lastly, the N-terminal pyroglutamate is crucial for catalysis and cytotoxicity (Irie *et al.*, 1998).

Recently, a group of frog ribonucleases (RC-RNase, RC-RNase 2, RC-RNase 3, RC-RNase 4 and RC-RNase 6) were isolated from *R. catesbeiana* oocytes and shown to be cytotoxic to tumour cells (Liao *et al.*, 2000). Interestingly, their cytotoxicities are not closely correlated with their enzymatic specific activities. For instance, RC-RNase 3, RC-RNase 4 and RC-RNase 6 have lower enzymatic activity than other RC-RNases but have similar cytotoxicity to tumour cells. These results imply that residues participating in antitumour activity might differ from those involved in enzyme catalysis. To elucidate the antitumour mechanism and their potential application in tumour therapy, we have undertaken the task of determining the structures of these ribonucleases. We report here the crystallization and preliminary X-ray diffraction analysis of three oocytic ribonucleases RC-RNase 3, RC-RNase 4 and RC-RNase 6.

2. Materials and methods

Ribonucleases from bullfrog oocytes were purified as described by Liao *et al.* (2000). Crystallization trials were set up by the

hanging-drop vapour-diffusion method (McPherson, 1982). Protein samples were equilibrated against a 500 µl reservoir solution in Linbro plates. Protein drops were prepared by combining equal volumes of reservoir solution containing different buffers with protein concentrations in the range 15–30 mg ml^{-1}. Initial crystallization conditions were obtained using Hampton Research Crystal Screen kits (Hampton Research, California, USA) and then further optimized to obtain diffraction-quality crystals.

X-ray diffraction data was collected on a Rigaku R-AXIS IV image-plate detector using Cu $K\alpha$ radiation from a Rigaku RU-300 rotating-anode X-ray generator operated at 50 kV and 80 mA. Data on RC-RNase 3 and 4 crystals were collected under cryogenic conditions. The crystals were flash-frozen at 110 K in a stream of cold nitrogen gas. The programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) were used for the X-ray diffraction data processing and analysis.

Table 1
Summary of X-ray diffraction data.

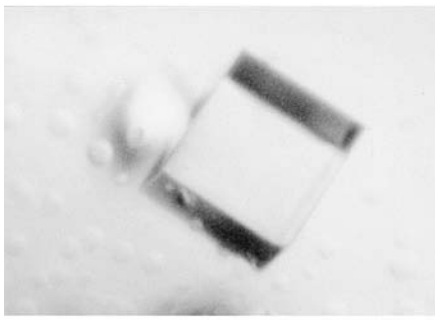
Values in parentheses refer to the outermost shell, 1.76–1.70 Å for RC-RNase 3, 2.18–2.10 Å for RC-RNase 4 and 2.14–2.10 Å for RC-RNase 6.

	RC-RNase 3	RC-RNase 4	RC-RNase 6–2′-5′ CpG complex
Crystal system	Orthorhombic	Trigonal	Tetragonal
Space group	$C222_1$	$P3_1$ or $P3_2$	$P4_12_12$ or $P4_32_12$
Unit-cell parameters			
a (Å)	66.66	32.22	61.80
b (Å)	97.38	32.32	61.80
c (Å)	85.74	92.12	65.96
Resolution (Å)	1.7	2.1	2.1
Total No. of reflections	196518	23346	53962
No. unique reflections	30476	6297	7776
Redundancy	6.45	3.71	6.94
Completeness	98.0 (94.8)	99.8 (99.2)	98.6 (98.2)
$I/\sigma\langle I\rangle$	39.56 (6.43)	25.17 (4.79)	17.50 (3.45)
$R_{\rm merge}$† (%)	5.0 (32.6)	4.9 (30.9)	7.5 (45.2)

† $R_{\rm merge}(I) = \sum_h \sum_i |I_i - I|/\sum_h \sum_i I$, where I is the mean intensity of the i observation of reflection h.

(*a*)

(*b*)



(*c*)

Figure 1
Photographs showing crystals of bullfrog ribonucleases: (*a*) RC-RNase 3 orthorhombic crystal, (*b*) RC-RNase 4 trigonal crystal and (*c*) RC-RNase 6 tetragonal crystal.

3. Results

3.1. Crystals of RC-RNase 3

RC-RNase 3 was concentrated to 30 mg ml^{-1} in 20 m*M* HEPES pH 8.0 and 50 m*M* sodium chloride. Crystals of RC-RNase 3 were obtained at room temperature in a solution containing 0.1 *M* sodium acetate pH 5.0, 3.2 *M* ammonium sulfate and 5% glycerol. Small crystals appeared within 1 d and grew to maximum dimensions of 0.6 × 0.4 × 0.2 mm (Fig. 1*a*) within one week. The crystals diffract to beyond 1.7 Å resolution and belong to a *C*-centered orthorhombic space group, with unit-cell parameters a = 66.66, b = 97.38, c = 85.74 Å (Table 1). The systematic absences (00l except for $l = 2n$) indicate that the space group is $C222_1$. Assuming two molecules per asymmetric unit, $V_{\rm M}$ (Matthews, 1968) was calculated to be 2.93 Å^3 Da^{-1} and the solvent content was estimated to be 58%.

3.2. Crystals of RC-RNase 4

Crystallization drops containing RC-RNase 4 at 20 mg ml^{-1} in phosphate-buffered saline (PBS) pH 7.3 were equilibrated against 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* sodium acetate and 25.5% PEG 4000 at room temperature. Crystals appeared overnight and reached final dimensions of 0.5 × 0.15 × 0.15 mm (Fig. 1*b*) within one week. These crystals diffracted to 2.1 Å resolution and belong to a trigonal crystal system, with unit-cell parameters $a = b = 32.22$, $c = 92.12$ Å (Table 1). Examination of systematic absences allowed the identification of the space group $P3_1$ or its enantiomorph $P3_2$. These crystals have one molecule per asymmetric unit, with a calculated $V_{\rm M}$ (Matthews, 1968) of 2.29 Å^3 Da^{-1}. The solvent content is 46%.

3.3. Crystals of RC-RNase 6 in complex with cytidylyl 2′-5′ guanosine

RC-RNase 6 (15 mg ml^{-1}) in 20 m*M* HEPES pH 8.0, 30 m*M* sodium chloride and 12.6 m*M* cytidylyl 2′-5′ guanosine was equilibrated against 3.3 *M* ammonium sulfate and 1.6% PEG 400 at room temperature. After 2–3 d, rectangular-shaped crystals surrounded by droplets of phase separation were observed. They grew to maximum dimensions of 0.1 × 0.1 × 0.15 mm (Fig. 1*c*). The crystals were found to have unit-cell parameters $a = b$ = 61.80, c = 65.96 Å (Table 1). The systematic absences (00l except for $l = 4n$; h00 except for $h = 2n$) and Laue symmetry of the diffraction pattern (4/*mmm*) indicate that the space group is $P4_12_12$ or $P4_32_12$. These crystals diffract to a 2.1 Å resolution and contain one molecule per asymmetric unit. The value of $V_{\rm M}$ (Matthews, 1968) is calculated to be 2.59 Å^3 Da^{-1} and the solvent content is estimated to be 53%.

The three-dimensional structures of several ribonucleases and their complexes have been solved by X-ray crystallographic and NMR methods (D'Alessio & Riordan, 1997). However, there is only one NMR solution structure reported for the ribo-

nuclease of *R. catesbeiana* (Chang *et al.*, 1998). The structural basis of the functional differences among these ribonucleases from *R. catesbeiana* is not well understood. RC-RNase 3, 4 and 6 share high sequence homology but different catalytic activity and base specificity (Liao *et al.*, 2000). The structural basis for the functional differences observed among these *R. catesbeiana* ribonucleases has not been elucidated. High-resolution structural data are needed to clarify the functional differences among these ribonucleases. The structure determination of these proteins is under way.

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