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Purification, crystallization and preliminary X-ray analysis of two crystal forms of ribonuclease Sa3

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RNase Sa3 produced by *Streptomyces aureofaciens* strain CCM 3239 belongs to the T1 family of microbial ribonucleases. It is closely related both to RNase Sa, studied in detail earlier, and to RNase Sa2 produced by the same microorganism. The most important property of RNase Sa3 is the relatively high cytotoxic activity, which was not observed for RNase Sa and Sa2. Recombinant RNase Sa3 was overexpressed in *Escherichia coli* and purified to high homogeneity. The hanging-drop vapour-diffusion method was used for crystallization. The two crystal forms are trigonal $P3_121$ and tetragonal $P4_12_12$, with unit-cell parameters $a = b = 64.7$, $c = 69.6$ Å, $\gamma = 120^\circ$ and $a = b = 34.0$, $c = 147.2$ Å, respectively. They diffract to 2.0 and to 1.7 Å resolution, respectively, using synchrotron radiation. The asymmetric units of crystal forms I and II contain one molecule of the enzyme, which corresponds to $V_M = 3.8$ Å^3 Da^{-1} with a solvent content of 68% and $V_M = 1.9$ Å^3 Da^{-1} with a solvent content of 37%, respectively.

1. Introduction

S. aureofaciens, a Gram-positive filamentous bacterium, is used in industry for the production of chlortetracycline. The bacteria synthesizes and secretes into the growth medium an extracellular ribonuclease, RNase Sa. Isolation of the enzyme from its natural producer was very tedious and we therefore aimed at obtaining its gene. Using an oligonucleotide probe derived from the RNase Sa amino-acid sequence, two new ribonuclease genes coding for RNase Sa2 and RNase Sa3 were surprisingly found in *S. aureofaciens* strains R8/26 (Nazarov *et al.*, 1991) and CCM 3239 (Homerova *et al.*, 1992), respectively. RNase Sa, Sa2 and Sa3 (Sa ribonucleases) belong to the family of microbial ribonucleases represented by RNase T1 (Ševčík *et al.*, 1990). Sa ribonucleases cleave single-stranded RNA specifically at the 3′-side of guanosine nucleotides. In spite of the expected similarity in tertiary structures and enzymatic properties, there are significant differences in their physicochemical behaviour, for example, pI values, thermal stability, conformational stability against urea and guanidine hydrochloride denaturation (Pace *et al.*, 1998), and in cytotoxic activity.

Some microorganisms protect themselves against the toxic effect of their ribonuclease by producing specific inhibitors. The first isolated and cloned microbial ribonuclease inhibitor was barstar, produced by *Bacillus amyloliquefaciens* (Smeaton & Elliot, 1967). Barstar inhibits barnase, a ribonuclease from the same organism, by sterically blocking its active site (Guillet *et al.*, 1993; Buckle *et al.*, 1994). Barstar inhibits not only barnase ($K_D = 10^{-14}$ *M*) but also RNase Sa (10^{-10} *M*), Sa2 (10^{-10} *M*) and Sa3 (10^{-12} *M*) (Hartley, 1993; Hartley *et al.*, 1996). This property was used for a contemporary expression of the genes of Sa ribonucleases with the gene of barstar, which allowed production of recombinant enzymes in high amounts (Hartley *et al.*, 1996; Hebert *et al.*, 1997). Expression of Sa ribonuclease genes alone failed owing to the high toxicity of the enzymes for the host cells. A specific ribonuclease inhibitor produced by *S. aureofaciens* was also isolated, cloned and expressed (Krajčíková *et al.*, 1998).

The best characterized streptomycete ribonuclease is RNase Sa. It is a small protein, containing 96 amino-acid residues with a molecular weight of 10 540 Da. RNase Sa has been the object of extended structural studies. The crystal structure of this enzyme was determined at various resolutions including atomic (Ševčík *et al.*, 1996). RNase Sa belongs to the $\alpha + \beta$ family of proteins with a three-turn α-helix packed against a three-stranded antiparallel β-sheet to form the hydrophobic core. The molecule contains one disulfide bond formed by Cys7 and Cys96. The structures of complexes of RNase Sa with the mononucleotides guanosine-2′-monophosphate, guanosine-3′-monophosphate and guanosine-2′,3′-cyclophosphorothioate were used for structural confirmation of the mechanism of catalytic reaction (Ševčík *et al.*, 1991; Ševčík,

Hill *et al.*, 1993; Ševčík, Zegers *et al.*, 1993). The structure of the complex of RNase Sa with barstar revealed the mechanism of inhibition and contributed to a better understanding of protein–protein recognition (Ševčík *et al.*, 1998). RNase Sa is also used as a model protein to study the conformational stability of globular proteins, with the aim of estimating the contribution of single hydrogen bond to the free energy (Pace *et al.*, 1998).

Recombinant RNase Sa3 consists of 99 amino-acid residues, with two cysteines in positions 10 and 99 which form a disulfide bond. The molecular weight of RNase Sa3 is 11 044 Da. The sequence similarity of the three enzymes is rather low: Sa2 and Sa3 have 56 and 69% identity with RNase Sa, respectively. Active-site amino-acid residues are conserved in all three Sa ribonucleases. Ribonucleases Sa and Sa2 are acidic enzymes with isoelectric points pI = 3.5 and 5.3, respectively; RNase Sa3 is a basic enzyme with pI = 7.2 (Hebert *et al.*, 1997). It is important to note that RNase Sa3 has a relatively high cytotoxic activity comparable with that of onconase, which was not observed for either RNase T1 (from *Aspergillus oryzae*) or other streptomycete RNases (Reines, unpublished results).

In this paper, we report the purification, crystallization and preliminary X-ray analysis of two crystal forms of recombinant RNase Sa3 from *S. aureofaciens*.

2. Experimental

2.1. Protein purification

RNase Sa3 was expressed in *E. coli* DH5α using the plasmid pMT 1100 (Hartley *et al.*, 1996). Cells were grown at 310 K on a rich medium according to Shirley & Laurents (1990). Overproduction of the enzyme was induced at $A_{600} = 0.5$ by adding isopropyl-β-D-thiogalactoside (IPTG) to 0.1 m*M* concentration. 500 ml cultures were grown in 2 l flasks and harvested after 16 h.

To release the enzyme from periplasmic space the medium was cooled to 273–275 K and concentrated acetic acid was added under constant stirring to a final concentration of 5%. The sample was centrifuged at 6000*g* for 30 min and the supernatant was collected. The supernatant was diluted four times with sodium acetate buffer pH 4.0 (buffer *A*) and loaded onto a 1.8 × 10 cm column of Whatman phosphocellulose equilibrated in this buffer. RNase Sa3 bound to phosphocellulose was released by 2.0 *M* NaCl in buffer *A*. Collected active fractions of a total volume of about 60 ml were applied onto a 4.2 × 117 cm column of Sephadex G-50 equilibrated in buffer *A* and eluted with the same buffer. Fractions containing ribonuclease activity were pooled (∼270 ml) and loaded onto a 1.8 × 15 cm phosphocellulose column equilibrated in buffer *A* and washed with 100 ml of this buffer. RNase Sa3 was eluted with a 400 ml gradient of 0–1.0 *M* NaCl in buffer *A*. Active fractions were collected (∼60 ml) and loaded onto a 4.2 × 117 cm column of Sephadex G-50 equilibrated with 50 m*M* ammonium bicarbonate and eluted with this buffer. Fractions containing enzyme were pooled, freeze-dried and stored at 253 K.

2.2. Crystallization

Crystals were grown in two forms by the hanging-drop vapour-diffusion method at room temperature.

2.2.1. Crystal form I. Initial crystallization conditions were found using the sparse-matrix set of crystallizing agents (Jancarik & Kim, 1991). The conditions, namely pH, type of buffers and concentration of precipitants, were modified to obtain the optimum results. Lyophilized RNase Sa3 was dissolved in 0.1 *M* Tris–HCl buffer pH 8.2 to a concentration of 15 mg ml^{-1}. 1.6 *M* $LiSO_4$ and 0.1 *M* HEPES pH 7.6 were used as the well solution. Crystallization drops of 4 µl volume were prepared by mixing equal volumes of protein and well solutions. The dimensions of the crystals were about 0.5 × 0.15 × 0.15 mm.

2.2.2. Crystal form II. RNase Sa3 was dissolved in 40 m*M* sodium acetate and 4 m*M* calcium acetate buffer adjusted to pH 4.2 with acetic acid. The concentration of the enzyme was 20 mg ml^{-1}. MPD at a concentration of 10% was used as a well solution. Drops of 6 µl volume were prepared by mixing the protein and well solutions in equivalent amounts. The largest crystals reached dimensions of about 1.5 × 0.8 × 0.8 mm.

2.3. Data collection and processing

X-ray diffraction data from crystal form I were collected at 100 K and data from crystal form II were collected at room temperature on the EMBL beamlines X31 and BW7A, respectively, at the DORIS storage ring, DESY, Hamburg, Germany with a MAR Research imaging-plate scanner. The well solution enriched by 22% glucose was used as a cryoprotectant. Both data sets were processed with the program *DENZO* (Otwinowski & Minor, 1997).

Table 1
Data-collection parameters.

Values in parentheses refer to the outermost resolution shell.

	Crystal form I	Crystal form II
Site	EMBL, Hamburg	EMBL, Hamburg
Source	DORIS, X31	DORIS, BW7A
Wavelength (Å)	1.1	0.9
Temperature (K)	100	293
Resolution range (Å)	29.6–2.0 (2.02–2.00)	20–1.7 (1.71–1.70)
Unique reflections	11779	10355
Completeness (%)	100 (99.5)	100 (100)
$R_{\rm merge}$† (%)	6.0 (30.6)	3.6 (11.9)
$I/\sigma(I)$	31.6 (5.8)	47.6 (14.0)

† $R_{\rm merge} = \sum_i |I_i - \langle I\rangle| / \sum \langle I\rangle$, where I is an individual intensity measurement and $\langle I\rangle$ is the average intensity for this reflection with summation over all data.

3. Results and discussion

High-purity RNase Sa3 was used for crystallization trials. The purity of RNase Sa3 was represented by a single band on SDS–PAGE (Laemmli, 1970). The ratio of the absorbance maximum and minimum in the UV region was $A_{278/252} = 3.0$.

X-ray data from single crystals of forms I and II were collected to 2.0 and 1.7 Å, respectively. Crystals of form I belong to the trigonal space group $P3_121$, with unit-cell parameters $a = b = 64.7$, $c = 69.6$ Å, $\gamma = 120^\circ$. Crystals of form II belong to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = b = 34.0$, $c = 147.2$ Å. A summary of data collection and processing is given in Table 1. The unit-cell dimensions, molecular weight of the protein (11 044 Da) and space group gave $V_{\rm M}$ values of 3.8 and 1.9 Å^3 Da^{-1} for crystal forms I and II, respectively. The asymmetric unit of both crystal forms contains one molecule of RNase Sa3, with solvent contents of 68 and 37%, respectively.

Both the $V_{\rm M}$ and solvent content of crystal form I are slightly outside the expected range (Matthews, 1968); however, molecular-replacement calculations performed with *AMoRe* (Navaza & Saludjian, 1997) using RNase Sa (PDB code 1rgg) as a search model have clearly shown that there is only one molecule of the protein in the asymmetric unit. The orientation of the molecule was given by the rotation function, with the top peak having peak-height/σ value of 16.1 with next highest peaks of 9.4 and 9.3. This orientation resulted in a solution to the translation function giving an initial model with correlation coefficient 47.7 and an R factor of 46.9%. Ten cycles of *REFMAC* refinement (Murshudov *et al.*, 1997) improved the

model, giving R = 33.9% and R_{free} = 40.3%. Building in the RNase Sa3 amino-acid sequence followed by ten refinement cycles gave R = 24.2% and R_{free} = 32.8%. The same procedure with data from crystal form II gave R = 22.8% and R_{free} = 30.8%. Refinement of both RNase Sa3 structures is under way.

The high solvent content in crystal form I is not typical for protein crystals; however, similar values have been frequently observed, for example, crystals of oxygen-evolving photosystem II with a solvent content of 66% (Shen & Kamiya, 2000), rotavirus protein VP6 with a solvent content of 70% (Petitpas *et al.*, 1998), the complex of tRNAAsp with aspartyl-tRNA synthetase with a solvent content of 75% (Briand *et al.*, 1998), FNR-like domain of the sulfite reductase flavoprotein subunit with a solvent content of 75% (Gruez *et al.*, 1998), glutathione S-transferase with a solvent content of 75% (Rossjohn *et al.*, 1997) and glucosamine-6-phosphate deaminase with a solvent content of 78% (Horjales *et al.*, 1999).

The structure of RNase Sa3 will contribute to an understanding of the differences in catalytic properties between the three Sa ribonucleases and will enable us to compare the structures solved at room and cryogenic temperatures. Special attention will be paid to understanding the structural basis of RNase Sa3 cytotoxic activity. This information will be used for mutation of the amino-acid sequence with the aim of modifying RNase Sa3 cytotoxic activity and inducing these changes in other Sa ribonucleases.

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