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Crystallization and preliminary X-ray crystallographic analysis of RNase HIII from *Bacillus subtilis*

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The genome of *Bacillus subtilis* contains three different genes encoding RNase H homologs: RNases HI, HII and HIII. RNase HIII from *B. subtilis* degrades RNA in RNA–DNA hybrids in an Mg^{2+}-dependent manner like *Escherichia coli* RNase HI. However, they belong to different classes; the former belongs to the 'class II' or 'large' RNase H family, while the latter belongs to the 'class I' or 'small' RNase H family. RNase HIII of *B. subtilis* has been overexpressed in *E. coli* and crystallized at 296 K using sodium formate as a precipitant. The native X-ray diffraction data have been collected to 2.8 Å resolution using synchrotron radiation. The crystals are hexagonal, belonging to the space group $P6_1$, with unit-cell parameters $a = b = 86.89$, $c = 214.49$ Å, $\alpha = \beta = 90.0$, $\gamma = 120.0^\circ$. A self-rotation function calculation indicated the presence of two monomers of the recombinant RNase HIII in the crystallographic asymmetric unit, giving a V_M of 3.43 Å^3 Da^{-1} and a solvent content of 64.2%.

1. Introduction

The RNase H family of enzymes degrade RNA in RNA–DNA hybrids in a divalent cation-dependent manner (Crouch & Dirksen, 1982). RNase H activity has been found in various organisms ranging from prokaryotes to eukaryotes (Crouch, 1990). Among them, *E. coli* RNase HI has been extensively studied. It is composed of 155 amino-acid residues and Mg^{2+} is required for its activity (Kanaya & Crouch, 1983; Crouch, 1990; Hostomsky *et al.*, 1993; Kanaya & Ikehara, 1995). The second RNase H (RNase HII) of *E. coli* was discovered on the basis of its ability to support growth of *E. coli* strains defective in RNase HI (Itaya, 1990). It exhibits much lower RNase H activity than RNase HI and is Mn^{2+}-dependent (Itaya, 1990). RNases HI from prokaryotes have been referred to as the 'small' or 'class I' RNases H, while RNases HII from prokaryotes and archaea as well as RNases HI from mammals and yeast have been referred to as the 'large' or 'class II' RNases H (Lai *et al.*, 2000). Crystal structures are available for four members of the 'class I' RNases H (Katayanagi *et al.*, 1990; Yang *et al.*, 1990; Ishikawa *et al.*, 1993; Mueser *et al.*, 1996; Davies *et al.*, 1991), whereas only one crystal structure has been reported for a 'class II' RNase H (Lai *et al.*, 2000).

Database searches indicated that the genome of the Gram-positive bacterium *B. subtilis* contains three different genes encoding RNase H homologs (Itaya *et al.*, 1999; Ohtani *et al.*, 1999). The *ypdQ* gene encodes an RNase HI homolog with 132 amino-acid residues; however, the *ypdQ* gene product showed no RNase H activity (Ohtani *et al.*, 1999). The other two genes, *rnhB* (*rnh*) and *rnhC* (*ysgB*), showing no significant sequence similarity to the *ypdQ* gene, have sequence similarities to the *E. coli* RNase HII gene and to each other (Itaya *et al.*, 1999; Ohtani *et al.*, 1999). However, there exist significant differences in the biochemical properties of their gene products. An RNase HII homolog encoded by the *rnhB* gene is composed of 255 amino-acid residues and shows a preference for Mn^{2+}, with wide specificity for the cleavage site. On the other hand, the *rnhC* gene product, named RNase HIII, is composed of 313 amino-acid residues and exhibits Mg^{2+}-dependent RNase H activity, with higher specific activity and stricter specificity for the cleavage site (Ohtani *et al.*, 1999). Mutational analysis indicated that simultaneous inactivation of both *rnhB* and *rnhC* genes renders *B. subtilis* unable to grow, suggesting essential roles for these RNase H genes in cell viability (Itaya *et al.*, 1999). It was proposed that the Mg^{2+}-dependent RNase HIII of *B. subtilis*, which may have evolved from the Mn^{2+}-dependent RNase HII, functions as a substitute for RNase HI in *B. subtilis* (Ohtani *et al.*, 1999).

In order to expand the structural information on the different members of the RNase H family, we have initiated a structural study on RNase HIII from *B. subtilis*. Here, we report the crystallization conditions and preliminary X-ray crystallographic data.

2. Experimental

2.1. Protein expression and purification

The gene encoding *B. subtilis* RNase HIII was amplified by the polymerase chain reaction using the genomic DNA as template. The amplified DNA was inserted into the *Nde*I/*Xho*I-digested expression vector pET-22b. The enzyme was highly overexpressed in soluble form in B834(DE3) cells upon induction with 0.5 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) at 310 K. Cells were grown in Luria–Bertani medium for 4 h after IPTG induction and were harvested by centrifugation at 4200*g* (6000 rev min^{-1}, Sorvall GSA rotor) for 10 min at 277 K. The cell pellet was re-suspended in ice-cold lysis buffer (20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2 m*M* $MgCl_2$, 1 m*M* β-mercaptoethanol) and then homogenized with an ultrasonic processor. The crude cell extract was centrifuged at 36 000*g* (18 000 rev min^{-1}, Hanil Supra 21K rotor) for 50 min at 277 K and the recombinant protein was purified by three chromatographic steps. First, the supernatant was loaded onto an SP-Sepharose column (Amersham–Pharmacia), which was previously equilibrated with buffer *A* (20 m*M* Tris pH 8.0, 2 m*M* $MgCl_2$, 1 m*M* β-mercaptoethanol), and the column was eluted with a linear gradient of 0–1.0 *M* NaCl. The fractions containing RNase HIII were diluted with buffer *A* to lower the NaCl concentration to 100 m*M*. They were then loaded onto a Mono S column (Amersham–Pharmacia) equilibrated with buffer *A* and the column was eluted with a linear gradient of 0–1.0 *M* NaCl. Final purification was achieved by gel filtration on a HiLoad XK 16 Superdex 200 prep-grade column (Amersham–Pharmacia), which was previously equilibrated with buffer *A* containing 100 m*M* NaCl. The purified RNase HIII was homogeneous as judged by polyacrylamide gel electrophoresis in the presence of 0.1%(*w*/*v*) sodium dodecyl sulfate (Laemmli, 1970). This procedure yielded approximately 67 mg of homogeneous RNase HIII from a 5 l culture. The protein solution was concentrated using a YM10 membrane (Amicon) to about 20 mg ml^{-1}. The protein concentration was estimated by measuring the absorbance at 280 nm, employing a correspondence of 1 mg ml^{-1} concentration to an A_{280} of 0.55 for the 1 cm path length (SWISS-PROT; http://www.expasy.ch/).

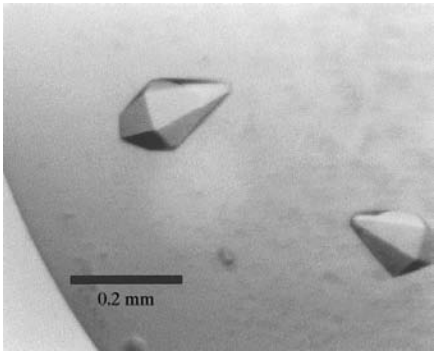

Figure 1
Hexagonal bipyramidal crystals of RNase HIII from *B. subtilis*. Approximate dimensions are 0.25 × 0.1 × 0.1 mm.

2.2. Crystallization

Crystallization was achieved by the hanging-drop vapour-diffusion method at 296 K using 24-well tissue-culture plates (Hampton Research). A hanging drop was prepared by mixing equal volumes (2 µl each) of the protein solution and the reservoir solution. The protein concentration was 20 mg ml^{-1} before mixing with the reservoir solution. Each hanging drop was placed over 1.0 ml of reservoir solution.

2.3. X-ray diffraction experiment

A crystal of RNase HIII was transferred to a solution of 3.8 *M* sodium formate, 100 m*M* NaCl, 100 m*M* Tris pH 7.6, 13 m*M* potassium dichromate, 1 m*M* $MgCl_2$, 1 m*M* β-mercaptoethanol and 30%(*v*/*v*) glycerol in six steps before being flash-frozen. In each step, the crystal in the drop was equilibrated against the new condition for 5 min and the glycerol concentration was increased by 5%. The native X-ray diffraction data were collected at 100 K with an ADSC Quantum 4R CCD detector at the BL-6A experimental station of the Photon Factory, Tsukuba, Japan (Sakabe, 1991). The wavelength of the synchrotron X-rays was 1.000 Å. The crystal was rotated through a total of 100°, with a 1.0° oscillation range per frame. The raw data were processed and scaled using the program *MOSFLM* (Leslie, 1992).

3. Results

Bipyramidal-shaped crystals were obtained with reservoir solution consisting of 100 m*M* Tris–HCl pH 7.6–7.8, 3.6–3.8 *M* sodium formate and 13 m*M* potassium dichromate. The crystals appeared under these conditions after about one week and grew to maximum dimensions of 0.25 × 0.15 × 0.15 mm within three months (Fig. 1).

The crystals diffracted to about 3.3 Å using conventional X-rays but were radiation-sensitive at room temperature. Therefore, the diffraction data were collected at 100 K using synchrotron radiation. A total of 373 179 measured reflections were merged into 22 549 unique reflections with an $R_{\rm merge}$ (on intensity) of 7.8%. The merged data set is 99.9% complete to 2.8 Å. The crystals belong to the hexagonal space group $P6_1$, with unit-cell parameters $a = b = 86.89$ (6), $c = 214.49$ (11) Å, $\alpha = \beta = 90.0$, $\gamma = 120.0°$, where estimated standard deviations are given in parentheses. Table 1 summarizes the data-collection statistics. The self-rotation function calculation indicated the presence of two copies of the recombinant RNase HIII molecule in the crystallographic asymmetric unit, giving a crystal volume per unit protein mass ($V_{\rm M}$) of 3.43 Å^3 Da^{-1} and a solvent content of 64.2%. These values are within the frequently observed ranges for protein crystals (Matthews, 1968). Two heavy-atom derivatives have been found and determination of the three-dimensional structure by multiple isomorphous replacement is in progress.

Table 1
Synchrotron data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.95–2.80 Å).

X-ray wavelength (Å)	1.000
Temperature (K)	100
Space group	$P6_1$
Unit-cell parameters (Å,°)	$a = b = 86.89$, $c = 214.49$, $\alpha = \beta = 90.0$, $\gamma = 120.0$
Resolution range (Å)	43.7–2.80
No. of measured reflections	373179
No. of unique reflections	22549
$R_{\rm merge}$† (%)	7.8 (37.3)
Data completeness (%)	99.9 (99.9)
Average $I/\sigma(I)$	8.1 (2.0)

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

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