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Crystallization and preliminary crystallographic analysis of type 1 RNase H from the hyperthermophilic archaeon *Sulfolobus tokodaii 7*

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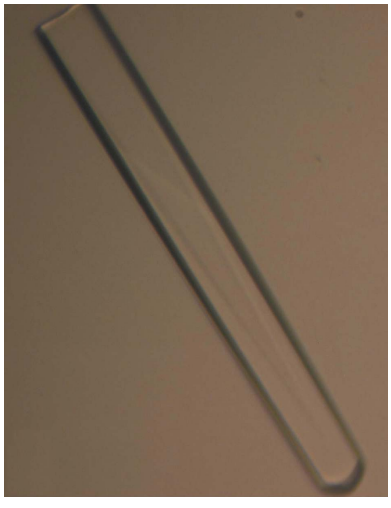

Crystallization and preliminary crystallographic studies of type 1 RNase H from the hyperthermophilic archaeon *Sulfolobus tokodaii* 7 were performed. A crystal was grown at 277 K by the sitting-drop vapour-diffusion method. Native X-ray diffraction data were collected to 1.5 Å resolution using synchrotron radiation from station BL41XU at SPring-8. The crystal belongs to space group $P4_3$, with unit-cell parameters $a = b = 39.21$, $c = 91.15$ Å. Assuming the presence of one molecule in the asymmetric unit, the Matthews coefficient V_M was calculated to be 2.1 Å^3 Da^{-1} and the solvent content was 40.5%. The structure of a selenomethionine Sto-RNase HI mutant obtained using a MAD data set is currently being analysed.

1. Introduction

Ribonuclease H (RNase H) is an enzyme that specifically hydrolyzes the RNA moiety of DNA/RNA hybrids in the presence of divalent metal ions (Crouch & Dirksen, 1982). The enzyme is present in all three kingdoms of living organisms, including retroviruses. RNase H has been suggested to be involved in DNA replication, repair and/or transcription (Kogoma & Foster, 1998; Murante *et al.*, 1998; Qiu *et al.*, 1999; Rydberg & Game, 2002; Broccoli *et al.*, 2004). The most intriguing physiological function of RNase H is the removal of RNA primers from Okazaki fragments during lagging-strand DNA synthesis and processing of R loops to modulate replication initiation and restore DNA topology. RNase H is also an essential component of the pharmacology of DNA-like antisense drugs (Wu *et al.*, 2004). Moreover, RNase H is localized in the C-terminal domain of human immunodeficiency virus reverse transcriptase (HIV-RT), which is responsible for the conversion of the HIV single-stranded RNA genome into double-stranded DNA (Hughes *et al.*, 1998). During the reverse-transcription process, the RNase H activity is required for the proliferation of HIV at several steps including RNA-strand transfer during the synthesis of DNA minus and plus strands, for the generation of the polypurine tract primer that initiates DNA plus-strand synthesis and for the removal of the minus- and plus-strand primers. Therefore, RNase H is regarded as one of the therapeutic targets for AIDS.

According to differences in amino-acid sequence, RNases H are classified into two major families: the type 1 and type 2 RNases H (Ohtani *et al.*, 1999, 2004*b*). Type 1 enzymes include bacterial RNases HI, archaeal RNases HI, eukaryotic RNases H1 and retroviral RNases H (RNase H domains of RT). Type 2 enzymes include bacterial RNases HII and HIII, archaeal RNases HII and eukaryotic RNases H2. Crystal structures are available for bacterial RNases HI (Katayanagi *et al.*, 1990; Yang *et al.*, 1990; Ishikawa *et al.*, 1993; Nowotny *et al.*, 2005), retroviral RNases H (Davies *et al.*, 1991; Kohlstaedt *et al.*, 1992), archaeal RNases HII (Lai *et al.*, 2000; Muroya *et al.*, 2001; Chapados *et al.*, 2001) and bacterial RNase HIII (Chon *et al.*, 2006), but not for archaeal RNases HI and eukaryotic RNases H. These structures share a main-chain fold and steric configuration of the active-site residues, suggesting that their catalytic mechanisms are basically identical. Based on the cocrystal structures of RNase H with an RNA/DNA substrate and Mg^{2+} (Nowotny *et al.*, 2005) and with reaction intermediate and product (Nowotny & Yang, 2006), two-

metal-ion catalysis has been proposed for RNase H, which is used by many other nucleases and DNA and RNA polymerases (Yang *et al.*, 2006). Because high-resolution structures of RNase H often show that only one metal ion binds to the active site of the apoenzyme (Kanaya, 1998; Goedken & Marqusee, 2001; Tsunaka *et al.*, 2005), two-metal-ion coordination is substrate-dependent.

So far, two archaeal RNases HI have been biochemically characterized. They are the RNases HI from *Halobacterium* sp. NRC-1 (Halo-RNase HI; Ohtani *et al.*, 2004*a*) and *Sulfolobus tokodaii* 7 (Sto-RNase HI; Ohtani *et al.*, 2004*b*). These enzymes lack a basic protrusion which has been shown to be important for substrate binding (Haruki *et al.*, 1997) and cleave the substrate at the RNA–DNA junction. Interestingly, Sto-RNase HI exhibits RNase H* activity, which degrades the RNA strand of the RNA–RNA duplex. Thus, archaeal RNases HI are more closely related to retroviral RNases H than bacterial and eukaryotic type 1 RNases H in primary structure and enzymatic properties. Retroviral RNases H lack a basic protrusion (Davies *et al.*, 1991), cleave RNA–DNA junctions (Gotte *et al.*, 1999) and exhibit RNase H* activity (Hughes *et al.*, 1998). However, archaeal RNases H do not have a domain corresponding to the polymerase domain of RT, which functions as a substitute for the basic protrusion. In addition, archaeal RNases H lack a conserved histidine residue which acts as a proton pump in the catalytic reaction of type 1 RNases H (Oda *et al.*, 1993). The following questions thus arise. Do archaeal RNases H possess a unique substrate-binding site? Does another amino-acid residue function as a substitute of conserved His in archaeal RNases H? To answer these questions, it is necessary to determine the crystal structures of archaeal RNases H. The amino-acid sequence similarities between members of the archaeal RNases HI and any of the other type 1 RNases H for which crystal structures are available are too low to construct a three-dimensional model for archaeal RNases H. For example, Sto-RNase HI shows amino-acid sequence identities of 20.1% to *Escherichia coli* RNase HI, 15.4% to Bh-RNase HI and 14.7% to the RNase H domain of HIV-1 RT.

Here, we report the crystallization and preliminary X-ray crystallographic studies of Sto-RNase HI.

2. Experimental procedures

2.1. Overproduction and purification

The gene encoding Sto-RNase HI was amplified by PCR using primer 1 (5′-ACATTACTAC**CATATG**ATAATTGGTTATTTTGACGGT-3′) and primer 2 (5′-CTG**GAATTC**GGATCCTTACTAAGTTAATATTATACATCCTAT-3′), where the bases in bold represent an *Nde*I site for primer 1 and an *Eco*RI site for primer 2. The genomic DNA of *S. tokodaii* 7, which was prepared from a Sarkosyl lysate as described previously (Imanaka *et al.*, 1981), was used as a template. The resulting DNA fragment was digested with *Nde*I and *Eco*RI and ligated to the *Nde*I–*Eco*RI sites of plasmid pET-25b(+) (Novagen) to construct the plasmid for overproduction of Sto-RNase HI. PCR was performed in 25 cycles using a thermal cycler (Gene Amp PCR System 2400; Perkin–Elmer) and KOD DNA polymerase (Toyobo). The nucleotide sequence was confirmed with an ABI PRISM 310 Genetic Analyzer (Perkin–Elmer). All DNA oligomers for PCR were synthesized by Hokkaido System Science.

Overproduction of Sto-RNase HI in *E. coli* mutant strain MIC2067(DE3), which lacks all functional RNases H (RNases HI and HII), was carried out as described previously (Ohtani *et al.*, 2004*b*). The cells were collected by centrifugation, suspended in buffer *A* (10 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA), disrupted in a French pressure cell and centrifuged at 30 000*g* for 30 min. The soluble fraction was loaded onto a HiTrap SP HP column (Pharmacia/GE Healthcare) equilibrated with buffer *A*. The protein was eluted from the column with a linear gradient of 0–0.5 *M* NaCl. The fractions containing Sto-RNase HI were combined, dialyzed against buffer *B* (10 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA) and loaded onto a HiTrap Heparin HP column (Pharmacia/GE Healthcare) equilibrated with buffer *B*. The protein was eluted from the column with a linear gradient of 0–0.5 *M* NaCl. The fractions containing Sto-RNase HI were combined, dialyzed against buffer *C* (5 m*M* sodium phosphate pH 6.8) and loaded onto a Bio-Scale CHT20-I column (Bio-Rad) equilibrated with buffer *C*. The protein was eluted from the column with a linear gradient of 5–400 m*M* sodium phosphate. The fractions containing Sto-RNase HI were combined, dialyzed against 10 m*M* Tris–HCl pH 8.0 and concentrated using a ultrafiltration system Centricon (Millipore) to about 17 mg ml^{-1}. All purification procedures were carried out at 277 K. The protein concentration was determined from UV absorption using a cell with an optical path length of 1 cm and an A_{280} value of 0.97 for 0.1% solution. The purity of the protein was confirmed by SDS–PAGE (Laemmli, 1970), followed by staining with Coomassie Brilliant Blue.

Selenomethionine-substituted enzyme was overproduced using methionine-auxotrophic *E. coli* strain B834 (DE3) pLysS in a defined medium (Hendrickson *et al.*, 1990). The purification procedures were the same as those for the native enzyme.

2.2. Crystallization

The crystallization conditions were initially screened using crystallization kits from Hampton Research (Crystal Screens I, II and Cryo) and Emerald Biostructures (Wizard I and II) with a TASCAL-1 semiautomatic protein crystallization system (Kentoku Industry Co. Ltd, Suita, Japan; Adachi *et al.*, 2004). The conditions were surveyed using the sitting-drop vapour-diffusion method at 277 and 293 K. Drops were prepared by mixing 1 µl each of protein solution and reservoir solution and were vapour-equilibrated against 100 µl reservoir solution. Native Sto-RNase HI crystals appeared after one week using Wizard I solution No. 5 (0.1 *M* CAPS pH 10.5, 30% PEG 400) at 277 K. The crystallization conditions were further optimized and single crystals suitable for X-ray diffraction analysis appeared when the drop was prepared by mixing 1.5 µl protein solution and 1 µl reservoir solution and vapour-equilibrated against 100 µl reservoir solution at 277 K.

The crystals of selenomethionine-substituted Sto-RNase HI were obtained under the same conditions as those of the native Sto-RNase HI.

2.3. Data collection

Crystals were mounted on a CryoLoop (Hampton Research) without adding cryoprotectant and then flash-frozen in a nitrogen-gas stream at 100 K. The native data set for Sto-RNase HI was collected at a wavelength of 1 Å and the multiple-wavelength anomalous diffraction (MAD) data set for a selenomethionine-substituted crystal was collected at wavelengths of 0.9796 Å (edge), 0.9793 Å (peak) and 0.973 Å (remote) on beamline BL41XU at SPring-8 (Hyogo, Japan). Diffraction images were indexed, integrated and scaled using the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

Table 1
Statistics of data collection.

Values in parentheses are for the highest resolution shell.

		SeMet MAD		
	Native	Edge	Peak	Remote
X-ray wavelength (Å)	1.0000	0.9796	0.9793	0.9730
Temperature (K)	100	100		
Space group	$P4_3$	$P4_3$		
Unit-cell parameters (Å)	$a = b = 39.21$, $c = 91.15$	$a = b = 38.72$, $c = 90.87$		
Resolution range (Å)	26.5–1.52 (1.57–1.52)	38.6–2.2 (2.28–2.2)		
No. of measured reflections	69362	45180	45203	44756
No. of unique reflections	21009	13349	13272	13252
$R_{\rm merge}$† (%)	4.7 (19.9)	8.8 (22.2)	8.9 (22.1)	8.7 (21.8)
Data completeness (%)	99.1 (93.9)	99.2 (94.5)	99.3 (95.6)	99.1 (95.0)
Average $I/\sigma(I)$	28.1 (3.8)	14.0 (2.6)	14.0 (2.7)	14.1 (2.6)

† $R_{\rm merge} = \sum |I_{hkl} - \langle I_{hkl}\rangle| / \sum I_{hkl}$, where I_{hkl} is the intensity measurement for the reflection with indices hkl and $\langle I_{hkl}\rangle$ is the mean intensity for multiply recorded reflections.

3. Results

Upon induction for overproduction, recombinant Sto-RNase HI accumulated in the cells in a soluble form and was purified to give a single band on SDS–PAGE with three column-chromatographic steps (data not shown). Its production level was approximately 15 mg per litre of culture and approximately 7 mg purified protein was obtained from 1 l of culture.

Recombinant Sto-RNase HI was successfully crystallized using the sitting-drop vapour-diffusion method. The crystals appeared after one week and grew to maximum dimensions of 0.4 × 0.05 × 0.05 mm after one month (Fig. 1). The crystals diffracted to 1.52 Å resolution. A total of 69 362 measured reflections were merged into 21 009 unique reflections with an $R_{\rm merge}$ of 4.7%. The crystals belong to the primitive tetragonal space group $P4_3$, with unit-cell parameters $a = b = 39.21$, $c = 91.15$ Å. The data-collection statistics are summarized in Table 1. Assuming the presence of one molecule in the asymmetric unit, the solvent content of the crystals was calculated to be 40.5%, corresponding to a Matthews coefficient $V_{\rm M}$ of 2.1 Å^3 Da^{-1}. These values are within the range frequently observed for protein crystals (Matthews, 1968), suggesting that this crystal is suitable for structural determination. A starting model was obtained by the MAD method from the data set of selenomethionine-substituted Sto-RNase HI, which was subsequently used to solve the structure of the native Sto-RNase HI. We are currently in the process of solving this structure.

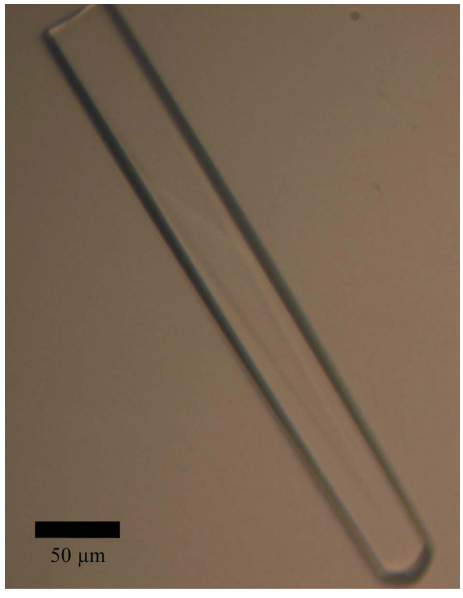

Figure 1
Crystals of Sto-RNase HI grown by the sitting-drop vapour-diffusion method. The approximate dimensions of the crystal are 0.4 × 0.05 × 0.05 mm.

The synchrotron-radiation experiments were performed at BL41XU at SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI; Proposal No. 2005B1766). We thank Dr A. Yamagishi for providing *S. tokodaii* 7. This work was supported in part by a Grant-in-Aid for the National Project on Protein Structural and Functional Analyses and by an Industrial Technology Research Grant Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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