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Processing of A-form ssDNA by cryptic RNase H fold exonuclease PF2046

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ABSTRACT

RNase H fold protein PF2046 of *Pyrococcus furiosus* is a 3′-5′ ssDNA exonuclease that cleaves after the second nucleotide from the 3′ end of ssDNA and prefers poly-dT over poly-dA as a substrate. In our crystal structure of PF2046 complexed with an oligonucleotide of four thymidine nucleotides (dT_4), PF2046 accommodates dT_4 tightly in a groove and imposes steric hindrance on dT_4 mainly by Phe220 such that dT_4 assumes the A-form. As poly-dA prefer B-form due to the stereochemical restrictions, the A-form ssDNA binding by PF2046 should disfavor the processing of poly-dA. Phe220 variants display reduced activity toward poly-dA and the A-form appears to be a prerequisite for the processing by PF2046.

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1. Introduction

Pyrococcus furiosus is a hyperthermophilic archaeon and can grow optimally at 98 °C and tolerate temperature of 103 °C [1]. Owing to resistance to heat and radiation, *P. furiosus* is an attractive experimental model for DNA repair research. One study showed that *P. furiosus* can tolerate ionizing radiation because of an unknown DNA repair system [2]. In an effort to elucidate the mechanism of this robust DNA repair system, ssDNA-specific nucleases have been screened for these unusual properties, and thus PF2046 was identified as an ssDNA-specific exonuclease [3]. The structure of PF2046 has been determined previously and found to contain an RNase H fold [4], which was not inferred from its amino acid sequence. Because of this homology to the RNase H family of proteins, PF2046 was predicted to function as a ribonuclease. Structural analysis, however, did not lead to functional annotation and the function of PF2046 was identified later in the aforementioned study involving the search for DNA repair enzymes in *P. furiosus* [3]. In that study, the authors were looking for ssDNA-specific nucleases in a *P. furiosus* genomic library expressed in *Escherichia coli* and found PF2046 to have a 3′-5′ ssDNA-specific exonuclease activity. PF2046 has unique substrate specificity: it prefers a series of dT over a series of dC or dA. Furthermore, PF2046 shows an unusual catalytic mechanism, namely, it removes dinucleotide units from the 3′ end. Recently, structures of PhoExo I (PDB ID: 4YOX), a PF2046 homolog from *Pyrococcus abyssi* have been reported to explain the structural basis of two-nucleotide removal [5].

The RNase H fold is defined as three layers—α/β/α structure with a five-strand β-sheet—the second strand being antiparallel to the rest, according to the SCOP database [6]. RNase H is classified into three classes depending on the amino acid sequence homology [7]. RNases HI and HIII are Mg^{2+} dependent and cleave only the RNA strand of a RNA/DNA hybrid duplex. RNase HII is catalytically less efficient and nicks the 5′ side of a ribonucleotide present in DNA. Compared to RNase HI, RNase HII contains additional helices at the C terminus, which is involved in binding to a substrate [8]. RNase H containing proteins have other domains relevant for functions [9] and reaction mechanism follows two-metal ion catalysis [10]. In addition to simple ribonucleases, an RNase H fold is also found in the functional domain of larger proteins driving various cellular processes such as DNA replication, DNA repair, and post-transcriptional modifications involved in RNA interference [RNAi]

Abbreviations: dT_4, an oligo of four thymidine deoxynucleotides.

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[11]. Phylogenetic analyses suggest that during evolution, RNase H proteins diverged to be readily detected by amino acid sequence homology; unexpected proteins with an RNase H fold are still being reported to take part in novel cellular processes. The best example is the RNase H fold proteins participating in RNAi, such as the argonaute protein of *P. furiosus* [12]. Its cryptic RNase H fold, also known as the Piwi domain, can bind double-stranded RNA and cleave complementary viral mRNA, thus implementing RNAi.

To understand the structural basis for the preference of PF2046 of a stretche of dTs over dAs, our aim was to determine the crystal structure of PF2046 complexed with poly-dT. The structure revealed that all three protomers of PF2046 bind ssDNA dT_4. Oligos bind mainly via interaction with sugar backbone atoms, and the N3 atom is hydrogen-bonded to the main-chain carbonyl group of Thr53, thus explaining the preference for thymidines over cytosine residues. The binding pocket for the 3′ nucleotide is flanked by Pro19 and Phe220, which are applying van der Waals strain to bases and give rise to the A-form DNA, which may pose further restriction on substrate binding.

2. Materials and methods

2.1. Overexpression and purification of PF2046

PF2046 was amplified from genomic DNA of *P. furiosus* DSM 3638 and ligated into pET-21a (Novagen, USA) resulting in an ORF with one additional Met residue at the N terminus of the protein. The expression vector was introduced into *E. coli* BL21 (DE3) and grown in 1.0 L of the Luria-Bertani (LB) medium in a baffled Erlenmeyer flask at 37 °C until optical density at 600 nm reached 0.6. The gene expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.25 mM final concentration) and the cells were further grown at 18 °C for 20 h and harvested. The cell pellet was washed with phosphate buffered saline (PBS) and stored at −20 °C until use. The cell pellet was thawed in 30 mL of PBS and lysed by VCX-750 sonicator (Sonics & Materials, USA) at room temperature (a 1-s pulse after a 5-s pause for 30 min at 50% power output), and the lysate was cleared by centrifugation at 29,693*g* for 1 *h*. The absence of refrigeration during the sonication elevated the lysate temperature to ~60 °C, which caused aggregation of heat-labile *E. coli* proteins; the resultant supernatant contained mostly PF2046. Nucleic acids in this fraction were removed by polyethyleneimine precipitation (final concentration 0.1%, w/v) and by centrifugation at 29,693*g* for 1 *h*. PF2046 was purified by anion exchange chromatography (Q-Sepharose HP 5 mL, GE Healthcare, USA) in 20 mM Tris-HCl buffer pH 8 using a gradient from 0 to 1 M NaCl. At the final purification step, PF2046 was isolated by size exclusion chromatography (Superdex S200, GE Healthcare, USA); the column was equilibrated and subjected to elution with the buffer 20 mM Tris pH 8 containing 100 mM NaCl. PF2046 was concentrated to ~25 mg/mL and stored at −80 °C until use. The protein concentration was measured by the Bradford method (Bio-Rad, USA).

2.2. Crystallization and determination of the crystal structure

PF2046 was crystallized in the presence of an oligodeoxynucleotide consisting of nine thymidine deoxynucleotides (dT_9). dT_9 was purchased in the lyophilized form (Cosmogenetech, Korea) and used without further purification. Prior to crystallization, PF2046 (1.13 mM in 20 mM HEPES pH 7, 100 mM NaCl) and dT9 (2.5 mM in H_2O) were mixed in the volume ratio of 3:2, yielding the ~1:2.5 M ratio, and the mixture was incubated on ice for 30 min. Crystallization conditions for the PF2046-ssDNA complex were selected by means of the PEGRx Crystal Screen Kit (Hampton Research, USA). Sitting-drop crystal screening was performed in a 96-well Intelli-Plate (Art Robbins Instruments, USA), where 0.5 μl of the PF2046-ssDNA complex was mixed with an equal volume of crystallization buffer from the kit. The crystals in the plate shape appeared after two days in the buffer composed of 0.1 M sodium malonate pH 8.0, 0.1 M Tris-HCl pH 8.0, and 30% (w/v) polyethylene glycol 1000 (PEG1000).

An X-ray diffraction dataset was collected at beamline BL-1A at the Photon Factory (Tsukuba, Japan) at wavelength 1.0000 Å. The crystal was mounted on a nylon loop and chilled to 100 K by a stream of nitrogen gas to reduce radiation damage. Due to the high concentration of PEG1000, the use of cryogenic buffer to prevent ice formation was not necessary. Diffraction images were processed and scaled by means of Mosflm [13] and Scala [14] in the CCP4 software suite [15]. The crystal structure was determined by molecular replacement in the MolRep software [16] using PF2046 structure without the nucleotide [PDB ID: 4O8U] as a search model. Crystal structure was modeled and refined in software packages XtalView [17] and CNS [18] (See Table 1). DNA conformation was analyzed in 3DNA [19] version 2.1. The final model coordinates were deposited in Protein Data Bank (PDB ID: 5CHI).

2.3. The exonuclease assay

During the purification of PF2046 and its variants for exonuclease assays, buffers were supplemented with 5 mM $MgCl_2$. In the poly-dA, poly-dC or poly-dT oligonucleotide digestion assay, 30-mer oligos labeled at the 5′ end by fluorescein isothiocyanate were purchased from Cosmogenetech (Republic of Korea) and used for fluorescence-based detection on a blue-LED transilluminator (IO Rodeo, USA). For exonuclease assays, 10 μl of reaction mix was composed of 20 mM Tris pH 8.0, 100 mM NaCl, 2 μM oligonucleotide and 5 nM PF2046 or its variants. The reaction proceeded at 75 °C for 10 min and was stopped by adding 2 μl of 500 mM EDTA pH 7. All reaction products were loaded and analyzed by 10% polyacrylamide gel electrophoresis (PAGE) in TAE (Tris, acetate, EDTA, pH 8.0) buffer. For pH dependent exonuclease assays, Tris pH

Table 1
Data collection and refinement statistics.

PF2046-dT_4	
Data collection	
PDB ID	5CHI
Space group	$P2_1$
Cell dimensions	a = 69.30, b = 85.96, c = 69.02 [Å], β = 118.4°
Resolution	50–2.47
No. of reflections	162,455
Unique reflections	24,745
Redundancy	6.57
Completeness [%]	96.5 [80.8]
I/σI	12.54 [2.10]
Rmeasure	0.145 [0.776]
CC[1/2] [%]	99.6 [73.8]
Refinement	
Resolution	34.5–2.47
R-factor	0.1824
R_{free}	0.2278
RMSD	
Bonds	0.010
Angles	1.358
B-factors	
Overall	44.66
Protein	44.19
dT_4	58.44
Mg^{2+}	41.35
Water	37.51

RMSD: root mean square deviation, PDB: Protein Data Bank.

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