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Crystallization and preliminary X-ray studies of TON_1713 from *Thermococcus onnurineus* NA1, a putative member of the haloacid dehalogenase superfamily

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The haloacid dehalogenase (HAD) protein superfamily is one of the largest enzyme families and shows hydrolytic activity towards diverse substrates. Structural analyses of enzymes belonging to the HAD family are required to elucidate the molecular basis underlying their broad substrate specificity and reaction mechanism. For this purpose, TON_1713, a hypothetical protein from *Thermococcus onnurineus* that is a member of the HAD superfamily, was expressed in *Escherichia coli*, purified and crystallized at 295 K using 1.6 *M* magnesium sulfate as a precipitant. X-ray diffraction data were collected to 1.8 Å resolution using a synchrotron-radiation source. The crystals belong to the triclinic space group *P*1, with unit-cell parameters $a = 52.5$, $b = 65.8$, $c = 203.4$ Å, $\alpha = 71.1$, $\beta = 79.9$, $\gamma = 74.3^\circ$.

1. Introduction

The haloacid dehalogenase (HAD) superfamily is one of the largest enzyme families and ite members are found in most organisms (Koonin & Tatusov, 1994; Burroughs *et al.*, 2006). The known functions of HAD-family proteins include phosphatase (CO—P cleavage), dehalogenase (C—Cl cleavage), phosphonatase (C—P cleavage) and β-phosphoglucomutase (CO—P cleavage and intramolecular phosphoryl transfer) activities (Allen & Dunaway-Mariano, 2004). Therefore, HAD enzymes act on a diverse set of substrates including nucleotides, sugar phosphates and halogenated compounds (Allen & Dunaway-Mariano, 2004). The enzymes in this family show relatively low sequence identity, ranging from 15 to 30%, but share a unique HAD-like fold characterized by a three-layered α/β sandwich consisting of repeating β-α units. The HAD superfamily can be further classified into 33 major families based on structure-based sequence alignment (Burroughs *et al.*, 2006). Although the proteins in this family act on diverse substrates, the biological functions of the majority of the proteins have not been clearly identified (Burroughs *et al.*, 2006). However, it is assumed that HAD proteins play a role in detoxification or metabolic pathways since the biochemical activities of some known HAD proteins involve the removal of a halogen and the transfer of a phosphate group (Fetzner & Lingens, 1994). In this regard, the proteins in this family have potential application in bioremediation or bioprocessing (Fetzner & Lingens, 1994; Janssen *et al.*, 2001) and the development of an enzyme with novel substrates has also been explored (Glasner *et al.*, 2006).

Structural and biochemical studies of various members of the HAD superfamily have suggested the evolution of the proteins in this family to act on a wide range of substrates and have provided an atomic basis for their substrate specificities. However, more intensive biochemical and structural studies of HAD-family proteins are needed in order to understand their biological roles, to elucidate the evolutionary aspects of their diverse modes of substrate specificity and to explore the potential application of HAD proteins in the development of a novel hydrolytic enzyme. We cloned a gene, TON_1713, that encodes a hypothetical protein from *Thermococcus onnurineus* NA1, a bacterium isolated from a deep-sea hydrothermal vent area of the PACMANUS field (Bae *et al.*, 2006). Since

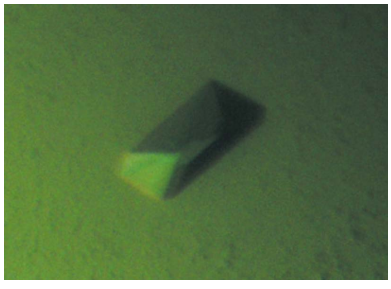

Table 1
Data-collection and processing statistics.

Values in parentheses are for the last shell.

Space group	*P*1
Unit-cell parameters (Å, °)	a = 52.5, b = 65.8, c = 203.4, α = 71.1, β = 79.9, γ = 74.3
Resolution range (Å)	50–1.8 (1.86–1.80)
No. of unique reflections	102852 (10237)
Redundancy	1.9 (1.5)
Data completeness (%)	94.8 (94.3)
$R_{\rm merge}$† (%)	3.3 (23.0)
$I/\sigma(I)$	13.9 (2.4)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$.

TON_1713 shows high sequence homology to known representative members of the HAD family, it was expected that TON_1713 also belonged to the HAD-enzyme superfamily. Of the HAD proteins for which structures and functions have been determined, TON_1713 shared the highest sequence identity with L-2-haloacid dehalogenase from *Pseudomonas* sp. YL (Hisano *et al.*, 1996; Li *et al.*, 1998) and DhlB from *Xanthobacter autotrophicus* GJ10 (Ridder *et al.*, 1997, 1999), with 16 and 17% identity, respectively. Therefore, TON_1713 seemed to be a novel HAD-family member that might show hydrolytic activity against various novel substrates. In this regard, we were interested in determining the crystal structure of TON_1713 and identifying its biochemical activity in order to understand the structure–function relationship and substrate specificity of TON_1713 as well as to obtain a more general understanding of the diverse modes of substrate specificity in the HAD-family proteins. To this end, we overexpressed, purified and crystallized TON_1713 from *T. onnurineus*.

2. Experimental

2.1. Cloning, overexpression and purification

The entire coding sequence of TON_1713, flanked by *Nde*I and *Sal*I sites, was amplified by PCR using genomic DNA from *T. onnurineus* NA1 and the following primers: sense, 5′-CGA-CCCGG**CATATG**CTTGTGCTCGTTGATCTCGAC-3′, and antisense, 5′-CTCCACAT**GTCGAC**CGCTTTAGCTCGCTTTTCATG-CTC-3′. The bold sequences in the sense and antisense primers indicate the *Nde*I and *Sal*I sites, respectively. The amplified DNA fragments were digested with *Nde*I and *Sal*I and then ligated into *Nde*I/*Sal*I-digested pET-24a(+) (Novagen Inc., Wisconsin, USA). The ligated fragments were transformed into *Escherichia coli* DH5α. Candidates with the correct construct were selected by restriction-enzyme digestion and the nucleotide sequence of the clones was confirmed by sequencing. For the expression of recombinant TON_1713, an *E. coli* Rosetta(DE3)pLysS strain transformed with the pET-24a(+)-TON_1713 plasmid was grown at 310 K in LB containing 50 µg ml^{-1} chloramphenicol and kanamycin to an optical density of 0.6 at 600 nm. Overexpression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside and cells were cultured at 303 K for 6 h. The cells were harvested by centrifugation at 6000*g* for 20 min and resuspended in 50 m*M* Tris–HCl buffer pH 8.0, 0.5 *M* KCl, 10% glycerol. The cells were then disrupted by sonication and centrifuged at 20 000*g* for 1 h. The resulting supernatant was applied onto a Talon metal-affinity column (BD Biosciences Clontech, California, USA) and washed with 10 m*M* imidazole (Sigma, Missouri, USA) in 50 m*M* Tris–HCl buffer pH 8.0, 0.5 *M* KCl and 10% glycerol. TON_1713 was eluted with 300 m*M* imidazole in the same buffer. The pooled fractions were loaded onto a Superdex 200 10/300 GL column (Amersham Biosciences, New Jersey, USA) equilibrated with 50 m*M* Tris–HCl buffer pH 8.0 and 0.15 *M* NaCl using an ÄKTA FPLC system (Amersham Biosciences, New Jersey, USA). Fractions containing TON_1713 were pooled and concentrated to 5 mg ml^{-1} using Centricon YM-10 (Millipore, Massachusetts, USA). The protein concentration was determined using the Bradford assay and protein purity was examined by SDS–PAGE.

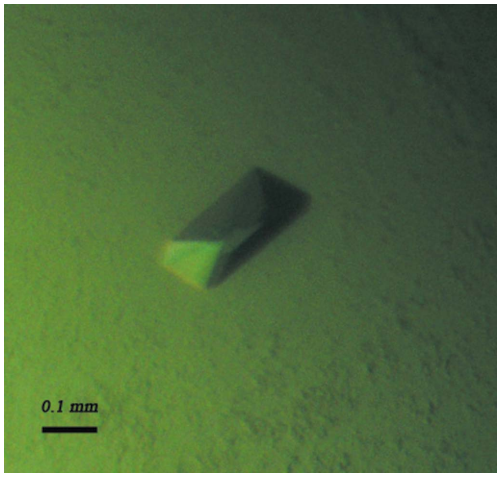

Figure 1
A crystal of TON_1713 protein grown in a solution consisting of 0.1 *M* MES buffer pH 6.5, 1.2 *M* magnesium sulfate. Crystal dimensions are about 0.1 × 0.1 × 0.3 mm.

2.2. Crystallization

Purified TON_1713 protein was further concentrated to 15 mg ml^{-1} using a Vivaspin with a 10 kDa membrane cutoff (Sartorius, Göttingen, Germany). All crystallization trials were carried out at 195 K by the microbatch method using commercially available screening kits from Hampton Research (California, USA) and Emerald BioSystems (Washington, USA). Each drop was prepared by mixing 1 µl reservoir solution and 1 µl protein solution and was covered by a thin layer of AI's oil in Nunc MiniTrays (Nalgen Nunc International, New York, USA). The crystallization conditions were further optimized by changing various parameters. Diffraction-quality crystals were obtained using precipitant solution containing 100 m*M* MES buffer pH 6.5 and 1.6 *M* magnesium sulfate (Fig. 1).

2.3. X-ray data collection and analysis

A single crystal was transferred to a cryoprotectant solution containing 50 m*M* MES buffer pH 6.5, 1.6 *M* $MgSO_4$ and 25%(*v*/*v*) glycerol prior to flash-freezing in a cold nitrogen stream. Initial X-ray diffraction experiments were performed at the home source, Photon Factory, Japan and Pohang Light Source, Republic of Korea. Native data were collected at 100 K on beamline NW12 of Photon Factory, Japan and recorded on an ADSC Q4R CCD detector. The wavelength of the synchrotron radiation was 1.0000 Å and the distance between the crystal and the detector was 240 mm. A total of 360 images were collected with 1° oscillation range and 2 s exposure per frame. Diffraction data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

The gene encoding TON_1713, a HAD-superfamily protein from a hyperthermophilic archeon, was cloned. The protein was over-expressed and purified for structural studies with an approximate

yield of 10 mg homogenous protein from 1 l culture. The molecular weight of truncated TON_1713 was about 28.0 kDa according to SDS–PAGE, which is in agreement with the calculated molecular weight of 28.6 kDa. In the initial screen, TON_1713 crystals were grown in a cluster form using magnesium sulfate as precipitant within 6 d. By varying the concentration of magnesium sulfate and the pH, a single crystal of TON_1713 was finally obtained in a drop containing 1 µl protein solution mixed with 1 µl reservoir solution (0.1 *M* MES buffer pH 6.5, 1.2 *M* magnesium sulfate). The TON_1713 crystals grew to dimensions of about 0.1 × 0.1 × 0.3 mm within 7 d (Fig. 1). X-ray diffraction data were collected to 1.8 Å resolution from a crystal at 100 K using synchrotron radiation, with an $R_{\rm merge}$ (on intensity) of 3.3% and a completeness of 94.8% (Table 1). TON_1713 crystals belong to the triclinic space group *P*1, with unit-cell parameters a = 52.5, b = 65.8, c = 203.4 Å, α = 71.1, β = 79.9, γ = 74.3°. Based on the molecular weight of 28.6 kDa and assuming the presence of eight molecules in the crystallographic asymmetric unit, the values of the crystal-packing parameter $V_{\rm M}$ and solvent content are 2.72 Å^3 Da^{-1} and 54.7%, respectively, which fall within the range commonly observed for protein crystals (Matthews, 1968). Table 1 summarizes the data-collection and processing statistics. Multi-wavelength anomalous dispersion methods will be used to obtain phase information. In summary, we have expressed, purified and crystallized TON_1713 protein from *T. onnurineus* NA1. The preliminary crystallographic analyses necessary to determine the structure of TON_1713 were also performed. In combination with biochemical analyses, the crystal structure of TON_1713 will provide structural insights into substrate specificity and reaction mechanism. Furthermore, these data may provide essential information to deepen our understanding of the structure–function relationship of the HAD superfamily of enzymes.

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