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Crystallization and preliminary crystallographic analysis of a haloalkane dehalogenase, DbjA, from *Bradyrhizobium japonicum* USDA110

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Haloalkane dehalogenases are key enzymes for the degradation of halogenated aliphatic pollutants. The haloalkane dehalogenase DbjA constitutes a novel substrate-specificity class with high catalytic activity for β-methylated haloalkanes. In order to reveal the mechanism of its substrate specificity, DbjA has been crystallized using the hanging-drop vapour-diffusion method. The best crystals were obtained using the microseeding technique with a reservoir solution consisting of 17–19.5%(*w*/*v*) PEG 4000, 0.2 *M* calcium acetate and 0.1 *M* Tris–HCl pH 7.7–8.0. The space group of the DbjA crystal is $P2_12_12$, with unit-cell parameters a = 212.9, b = 117.8, c = 55.8 Å. The crystal diffracts to 1.75 Å resolution.

1. Introduction

Haloalkane dehalogenases (EC 3.8.1.5) are key enzymes for the microbial degradation of halogenated aliphatic compounds that occur as soil pollutants (Janssen *et al.*, 1994; Oberg, 2002; Ballschmiter, 2003; Janssen, 2004). The enzymes catalyze the hydrolysis of a carbon–halogen bond in halogenated substrates, producing a corresponding alcohol, a halide ion and a proton. To date, the crystal structures of three haloalkane dehalogenases have been determined: those of DhlA from *Xanthobacter autorophicus* GJ10 (Verschueren *et al.*, 1993), DhaA from *Rhodococcus rhodochrous* NCIMB 13064 (Newman *et al.*, 1999) and LinB from *Sphingobium japonicum* UT26 (Marek *et al.*, 2000). These crystal structures revealed that the three enzymes essentially adopt the same fold and are composed of two domains: the main and cap domains. The active site of the enzyme is in an occluded cavity located between the two domains.

In order to design novel haloalkane dehalogenases that can catalyze the hydrolysis of recalcitrant environmental pollutants, intensive protein-engineering studies have been carried out on haloalkane dehalogenases. Random mutagenesis of DhaA (Bosma *et al.*, 2002) and site-directed mutagenesis of LinB (Chaloupkova *et al.*, 2003) are the only examples that have successfully changed the substrate specificity of the haloalkane dehalogenase. However, since changing the substrate specificity using protein-engineering techniques remains difficult, the isolation and characterization of new haloalkane dehalogenases are still necessary in order to develop enzymes with unique substrate specificity.

In the 1990s, haloalkane dehalogenases were thought to only be present in soil bacteria that colonize contaminated environments (Fetzner & Lingens, 1994). Recent progress in the genome-sequence analyses of various bacteria, however, has revealed that more than 20 bacteria that colonize unpolluted environments have open reading frames (ORFs) encoding putative dehalogenases (Jesenska *et al.*, 2002). These ORF products should be useful as a genetic source to isolate new haloalkane dehalogenases. We focused on the genome sequence of *Bradyrhizobium japonicum* USDA110 (Kaneko *et al.*, 2002*a*,*b*) and succeeded in isolating a haloalkane dehalogenase with unique substrate specificity. The haloalkane dehalogenase was named DbjA (Sato *et al.*, 2005). DbjA has 25% identity to DhlA, 49% identity to DhaA and 41% identity to LinB. DbjA has typical haloalkane dehalogenase activity and a high catalytic activity for β-methylated haloalkanes (Sato *et al.*, 2005). It is of note that

β-methylated haloalkanes are scarcely hydrolyzed by most haloalkane dehalogenases, probably owing to the steric hindrance of the branched methyl group. A sequence comparison between DbjA and other haloalkane dehalogenases has suggested that an 11-amino-acid insertion between the main and cap domains of DbjA produces a unique active-site structure that results in the unique substrate specificity of DbjA (Sato *et al.*, 2005). To confirm this hypothesis on the basis of the three-dimensional structure of DbjA, we initiated crystal structure analysis of the enzyme. Here, we report the crystallization of DbjA.

2. Methods and results

2.1. Protein expression and purification

Escherichia coli BL21 cells containing the plasmid pYBJA2, which encoded His-tagged DbjA (Sato *et al.*, 2005), were cultured in Luria broth (LB) medium (Maniatis *et al.*, 1982). The DbjA construct codes for residues 1–310 of DbjA with its C-terminus fused to an affinity tag with sequence AHHHHHH (where A is residue 310 of the DbjA sequence). Expression of DbjA was induced with 0.5 m*M* isopropyl β-D-thiogalactopyranoside (IPTG) when the culture reached an OD_{660} of 0.6. After 4 h induction, the cells were harvested, washed twice with 50 m*M* potassium phosphate buffer pH 7.5 containing 10%(*v*/*v*) glycerol and resuspended in the same buffer. The cells were disrupted by sonication (VP-30S, Taitec). Cell debris and larger particles were removed by centrifugation at 20 000*g* for 30 min. The supernatant obtained was used for purification.

Purification was carried out at 277 K. The supernatant solution was applied onto an Ni column (2.6 × 30 cm, Chelating Sepharose Fast Flow, GE Healthcare) equilibrated with 20 m*M* potassium phosphate buffer pH 7.5, 0.5 *M* sodium chloride, 10%(*v*/*v*) glycerol and 125 m*M* imidazole (buffer *A*). The protein was eluted with a linear gradient of imidazole from 125 to 500 m*M* in buffer *A*. The fractions containing DbjA were pooled and dialyzed against 0.1 *M* glycine–NaOH pH 8.6 containing 10%(*v*/*v*) glycerol. The dialyzed enzyme was concentrated to 35 mg ml^{-1} using Amicon Ultra and Centricon (Millipore) for crystallization experiments.

2.2. Crystallization

Crystallization was performed using the hanging-drop vapour-diffusion method at 293 K. A droplet was prepared by mixing equal volumes (1 µl) of protein solution and reservoir solution. Each droplet was placed over 500 µl reservoir solution. Crystal Screens I and II (Hampton Research) and conditions similar to those used to crystallize LinB (Smatanová *et al.*, 1999) were used for the initial screening of crystallization conditions. Small crystals appeared under conditions similar to those used to crystallize LinB: (i) 20–25%(*w*/*v*) PEG 4000, 0.2 *M* magnesium chloride, 0.1 *M* Tris–HCl pH 8.3–8.6, (ii) 20–25%(*w*/*v*) PEG 4000, 0.2 *M* calcium acetate, 0.1 *M* Tris–HCl pH 7.8–8.1, (iii) 20–25%(*w*/*v*) PEG 6000, 0.2 *M* magnesium chloride, 0.1 *M* Tris–HCl pH 7.8–8.1 and (iv) 20–25%(*w*/*v*) PEG 6000, 0.2 *M* calcium acetate, 0.1 *M* Tris–HCl pH 8.3–8.6. The crystals that grew in 18–20%(*w*/*v*) PEG 4000, 0.2 *M* calcium acetate and 0.1 *M* Tris–HCl pH 7.7–8.1 diffracted to 2.60 Å resolution, although they grew as twinned crystals. To obtain single crystals, the crystallization condition was optimized using a microseeding technique (Stura & Wilson, 1992). A seed-stock solution was prepared using the twinned crystal. The crystal was crushed in 50 µl precipitant solution and the solution was diluted tenfold. This solution was used as a seed-stock solution. A small amount of the seed-stock solution was transferred using a Crystal Probe (Hampton Research) into droplets prepared by mixing equal volumes (1 µl) of protein solution and reservoir solution. The best crystal appeared in two weeks from droplets containing 1 µl enzyme (15–25 mg ml^{-1}) and 1 µl reservoir solution (17.0–19.5% PEG 4000, 0.2 *M* calcium acetate and 0.1 *M* Tris–HCl pH 7.7–8.0) together with microseeds. The obtained crystal was rectangular in shape, with typical dimensions of 0.40 × 0.12 × 0.10 mm (Fig. 1), and diffracted to 1.75 Å resolution.

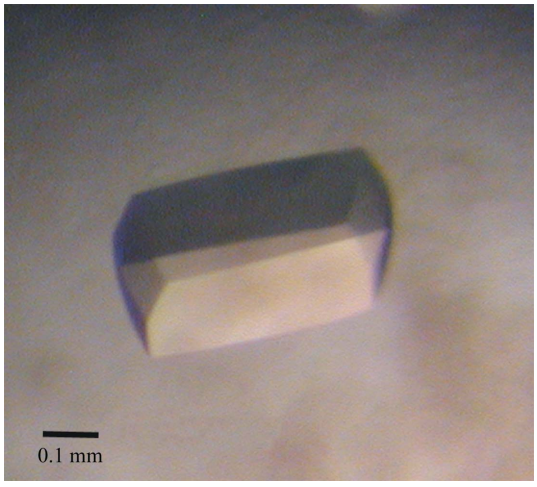

Figure 1
Crystal of DbjA.

Table 1
Diffraction data-collection statistics for DbjA.

Values in parentheses are for the highest resolution shell.

	Data I	Data II
Unit-cell parameters (Å)	a = 213.3, b = 117.7, c = 55.8	a = 212.9, b = 117.8, c = 55.8
Space group	$P2_12_12$	$P2_12_12$
V_M (Å^3 Da^{-1})	2.4	2.4
Solvent content (%)	49	49
No. of subunits in ASU	4	4
Resolution (Å)	30–2.60 (2.74–2.60)	50–1.75 (1.81–1.75)
Observations	146217	825534
Unique reflections	37471 (4963)	134673 (12666)
R_{merge}	0.092 (0.352)	0.041 (0.175)
Completeness (%)	85.3 (85.3)	95.2 (90.7)
$I/\sigma(I)$	7.8 (2.1)	26.3 (7.4)
Redundancy	3.9 (3.9)	6.1 (5.6)

2.3. Data collection and preliminary crystallographic analysis

Preliminary diffraction data were collected from a twinned DbjA crystal at 100 K using an R-AXIS IV^{++} imaging-plate system mounted on a Rigaku rotating-anode X-ray generator (FR-D) operated at 50 kV and 60 mA. Cryoprotectant was not used in this particular data collection. These diffraction data, data I, were processed and scaled at 2.6 Å resolution using the programs *MOSFLM* (Leslie, 1992) and *SCALA*, respectively, from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994; Table 1). The space group of the crystal was $P2_12_12$, with unit-cell parameters a = 213.3, b = 117.7, c = 55.8 Å. Assuming the presence of four molecules in the asymmetric unit, the V_M value (Matthews, 1968) was calculated as 2.4 Å^3 Da^{-1}, which corresponds to a solvent content of 49%.

The crystal structure was determined using the molecular-replacement (MR) method. The MR calculation was performed using *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) using data I in the

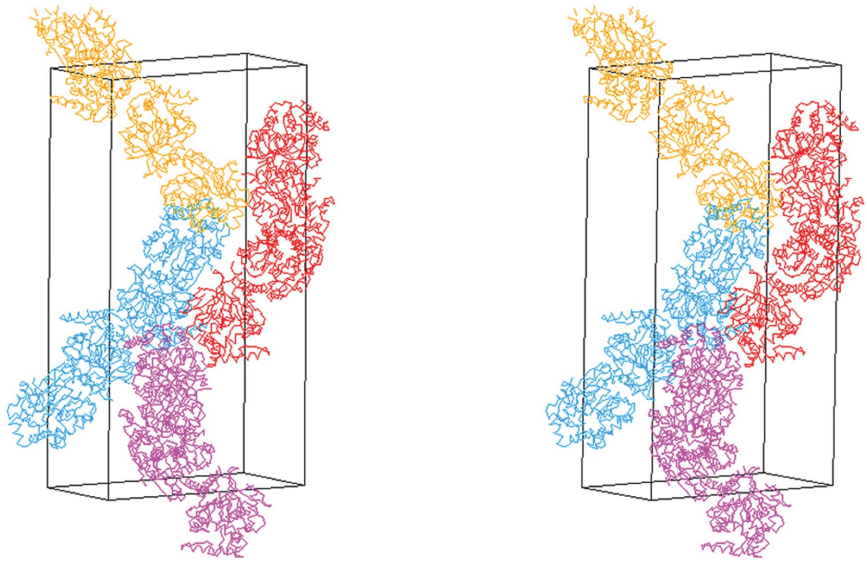

Figure 2
The packing of four DbjA molecules in the orthorhombic unit cell (stereoview). This figure was prepared using the program *MolViewX* (Smith, 2004).

resolution range 30.00–3.10 Å. The subunit structure of DhaA (PDB code 1bn6; Newman *et al.*, 1999) was used as a search model. Four molecules were found in the asymmetric unit (Fig. 2), which was consistent with the calculated V_M value. The *R* factor and correlation coefficient obtained from the MR calculation were 0.51 and 0.36, respectively.

A high-resolution data set, data II, was collected at NW12 of PF-AR (Tsukuba) using an ADSC Quantum 210 CCD camera. The DbjA crystal, which was grown using the microseeding technique, was soaked in a cryoprotectant solution [25%(*w*/*v*) PEG 4000, 20%(*w*/*v*) sucrose, 10%(*w*/*v*) PEG 400, 0.2 *M* calcium acetate and 0.1 *M* Tris–HCl pH 7.8] for 5 s, mounted on a cryo-loop (Hampton Research) and flash-frozen in a nitrogen stream at 100 K. The collected data were processed and scaled using the program package *HKL*-2000 (Otwinowski & Minor, 1997), which showed that the crystal was nearly isomorphous to the twinned crystal used in the preliminary data collection (Table 1). Data-collection statistics for data II are shown in Table 1. Crystallographic refinement with data II at 1.75 Å resolution is in progress.

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