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Expression, purification and preliminary X-ray characterization of DL-2-haloacid dehalogenase from *Methylobacterium* sp. CPA1

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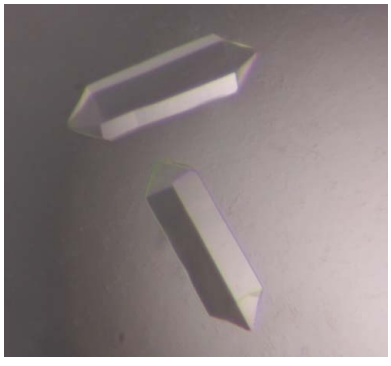

DL-2-Haloacid dehalogenase from *Methylobacterium* sp. CPA1 (DL-DEX Mb) is a unique enzyme that catalyzes the dehalogenation reaction without the formation of an ester intermediate. A recombinant form of DL-DEX Mb has been expressed in *Escherichia coli*, purified and crystallized using the hanging-drop vapour-diffusion method. The crystal belongs to the hexagonal space group $P6_3$, with unit-cell parameters $a = b = 186.2$, $c = 114.4$ Å. The crystals are likely to contain between four and eight monomers in the asymmetric unit, with a V_M value of 4.20–2.10 Å^3 Da^{-1}. A self-rotation function revealed peaks on the $\chi = 180^\circ$ section. X-ray data have been collected to 1.75 Å resolution.

1. Introduction

A variety of halogenated substances have been widely used as pesticides, herbicides and solvents. Although many of these have now been banned from use owing to their toxicity, they are persistent and cause serious environmental pollution. Some of these compounds can be degraded by bacterial enzymes called dehalogenases, which have attracted attention from the viewpoint of environmental technology (Fetzner & Lingens, 1994). These enzymes include haloalkane dehalogenases (EC 3.8.1.5), 2-haloacid dehalogenases (EC 3.8.1.2), haloacetate dehalogenases (EC 3.8.1.3) and 4-chlorobenzoyl-CoA dehalogenases (EC 3.8.1.7). 2-Haloacid dehalogenases are further classified into three types based on their substrate specificities (Soda *et al.*, 1996). L-2-Haloacid dehalogenases (L-DEX) specifically catalyze the dehalogenation of L-2-haloalkanoic acids to produce the corresponding D-2-hydroxyalkanoic acid. D-2-Haloacid dehalogenases (D-DEX) act on D-2-haloalkanoic acids to yield L-2-haloalkanoic acids. DL-2-haloacid dehalogenases (DL-DEX) react with both D- and L-2-haloalkanoic acids, producing L- and D-2-hydroxyalkanoic acids, respectively.

The reaction mechanisms of L-DEX (Liu *et al.*, 1995, 1997; Li *et al.*, 1998), haloalkane dehalogenase (Verschueren *et al.*, 1993; Pries *et al.*, 1994, 1995) and 4-chlorobenzoyl-CoA dehalogenase (Yang *et al.*, 1994; Benning *et al.*, 1996; Crooks *et al.*, 1995) have been analyzed by chemical modification, site-directed mutagenesis, mass spectrometry and X-ray crystallography. These dehalogenases have a common mechanism in which an active-site carboxylate group attacks the substrate C atom bound to the halogen atom to form an ester intermediate and a halide ion. The intermediate is then hydrolyzed. In contrast, DL-DEX was shown to have a reaction mechanism that differed from the ester-intermediate mechanism (Nardi-Dei *et al.*, 1999; Park *et al.*, 2003). Mechanistic analysis of DL-DEX 113 and DL-DEX 312 revealed that the solvent water molecule activated by a catalytic base directly attacks the α-carbon of the substrate to release a halide ion. The reaction catalyzed by DL-DEX is thus unique in that the dehalogenation proceeds without formation of an ester intermediate.

We isolated DL-DEX from *Methylobacterium* sp. CPA1 (DL-DEX Mb), expressed the gene in *Escherichia coli*, purified the product protein and confirmed that the recombinant enzyme has DL-DEX Mb activity for both D- and L-2-chloropropionates. DL-DEX Mb has 301 residues per subunit and a molecular weight of 34 046 Da. Structure determination of DL-DEX Mb should help to clarify the substrate-

recognition mechanism for both D- and L-2-haloalkanoic acids and the role of the active-site residues, as the three-dimensional structure of DL-DEX is not available. Moreover, a comparison of the structure of DL-DEX Mb with those of other dehalogenases such as L-DEX, haloalkane dehalogenase and 4-chlorobenzoyl-CoA dehalogenase will provide new insight into the catalytic mechanism of the dehalogenation of haloalkanoic acids. In this communication, we report the crystallization and preliminary X-ray diffraction studies of DL-DEX Mb.

2. Experimental

2.1. Expression and purification

The DL-DEX Mb gene (DDBJ accession No. AB301951) from *Methylobacterium* sp. CPA1, which assimilates 2-chloropropionate as a sole carbon source, was amplified by PCR using the primers 5′-CATGCCATGGCACACCGTTCGGTTCT-3′ and 5′-CGCGGATCCCTAATTGGTGTACTTATTCGCCG-3′. The amplified DNA fragment was ligated into the expression vector pHCE19 (Bio-Leaders, Daejeon, South Korea) at the *Nco*I/*Bam*HI sites. The recombinant plasmid, pHCE19/DL-DEX Mb, was used to transform *E. coli* strain JM109. LB medium (0.9 l) containing 100 µg ml^{-1} ampicillin was inoculated with a 5 ml overnight culture and the cells were grown at 310 K for 16 h. Cells were harvested by centrifugation and stored at 193 K until use.

The cells harvested from 5.4 l culture were suspended in 50 m*M* potassium phosphate pH 7.5 and then disrupted by sonication. The cell debris was removed by centrifugation. The crude extract was treated with 1%(*w*/*v*) streptomycin sulfate and 2 *M* potassium phosphate pH 7.5 was added to the resulting supernatant to a final concentration of 1 *M*. The solution was applied onto a Butyl-Toyopearl 650M column (Tosoh, Tokyo, Japan) equilibrated with 1 *M* potassium phosphate pH 7.5. The flowthrough fraction was collected and dialyzed against 50 m*M* potassium phosphate pH 7.5. The fraction was applied onto a Butyl-Toyopearl 650M column equilibrated with 50 m*M* potassium phosphate pH 7.5 containing 30% saturated ammonium sulfate. DL-DEX Mb was eluted with a linear gradient of 30–0% saturated ammonium sulfate in 50 m*M* potassium phosphate pH 7.5. The active fraction was dialyzed against 10 m*M* potassium phosphate pH 7.5 and loaded onto a DEAE-Toyopearl column (Tosoh) equilibrated with 10 m*M* potassium phosphate pH 7.5. DL-DEX Mb was eluted with a linear gradient of 10–100 m*M* potassium phosphate pH 7.5. The active fractions were collected as purified enzyme, concentrated to 15.0 mg ml^{-1} by ultrafiltration after dialysis against 10 m*M* HEPES–NaOH pH 7.5 and stored at 193 K.

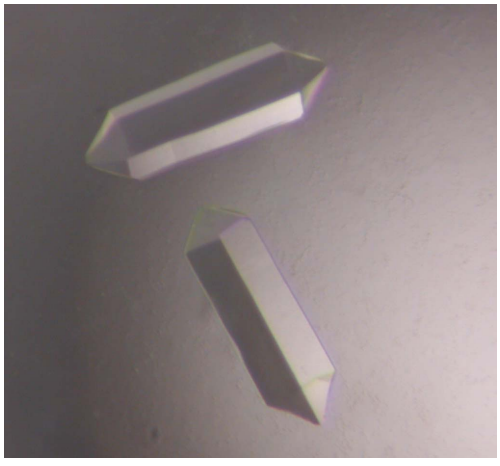

Figure 1
Crystals of DL-DEX from *Methylobacterium* sp. CPA1.

2.2. Enzyme assay

DL-DEX Mb was routinely assayed by measuring the chloride ions released from L-2-chloropropionate. The standard assay mixture (100 µl) comprised 25 m*M* L-2-chloropropionic acid, 25 m*M* sodium hydroxide, 100 m*M* Tris–sulfate buffer pH 9.0 and the enzyme. After incubation at 303 K for 1–5 min, the reaction was terminated by the addition of 11.1 µl 1.5 *M* sulfuric acid. The chloride ions released were determined spectrophotometrically using mercuric thiocyanate and ferric ammonium sulfate (Iwasaki *et al.*, 1956). The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The specific activity of the purified DL-DEX Mb was 30 µmol min^{-1} mg^{-1}.

2.3. Dynamic light-scattering assessment

DLS analyses of DL-DEX Mb were performed using a Dynapro instrument (DynaPro-801, Protein Solutions) by injecting 20 µl 2–4 mg ml^{-1} protein solution containing 20 m*M* HEPES–NaOH pH 7.0. The DL-DEX Mb sample possesses a monomodal distribution and the molecular weight was estimated to be 140 kDa.

2.4. Crystallization

DL-DEX Mb was crystallized by the hanging-drop vapour-diffusion method at 277 K. The initial screening for crystallization conditions was performed using the sparse-matrix screen Crystal Screen I from Hampton Research (Jancarik & Kim, 1991). Minute crystals of DL-DEX Mb appeared from solution Nos. 19 [100 m*M* Tris–HCl pH 8.5, 0.2 *M* ammonium acetate, 30%(*v*/*v*) 2-propanol] and 33 (4.0 *M* sodium formate). Crystallization condition No. 19 was optimized by changing the concentration of the precipitants and additives, but

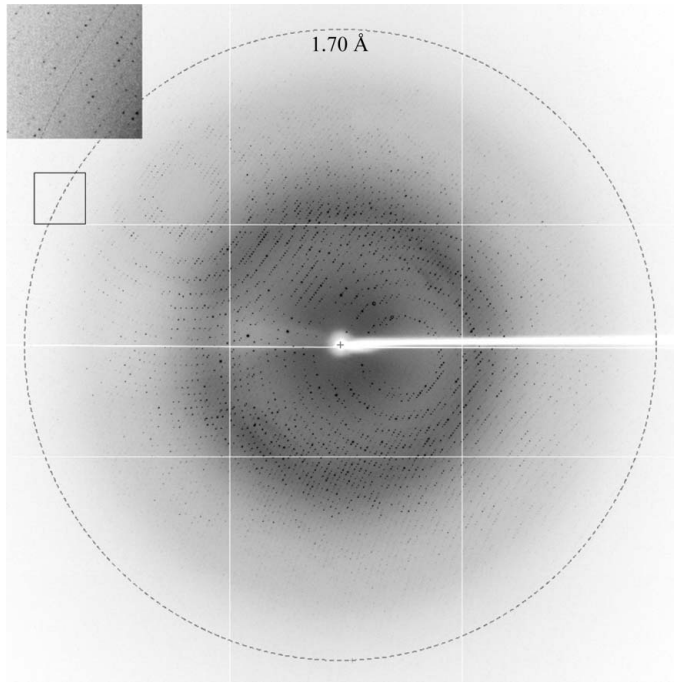

Figure 2
X-ray diffraction pattern from a crystal of DL-DEX Mb. A higher magnification view is shown at the top left of the image.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P6_3$
Unit-cell parameters (Å)	$a = b = 186.2$, $c = 114.4$
Temperature (K)	100
Wavelength (Å)	1.0
Resolution (Å)	50.0–1.75 (1.81–1.75)
Total No. of reflections	4424952
No. of unique reflections	225607
Completeness (%)	99.7 (100.0)
$R_{\rm merge}$† (%)	7.3 (31.9)
Mean $I/\sigma(I)$	27.1 (4.6)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_{hkl,i} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i I_{hkl,i}$, where I is the observed intensity and $\langle I\rangle$ is the average intensity for multiple measurements.

crystals diffracting to high resolution suitable for X-ray studies could not be obtained. Crystals suitable for X-ray analysis were obtained from condition No. 33 (Fig. 1). The optimized condition involves the equilibration of a mixture containing 2 µl protein solution (15.0 mg ml^{-1} protein, 10 m*M* HEPES–NaOH pH 7.5) and 2 µl reservoir solution [3.0 *M* sodium formate, 5%(*v*/*v*) glycerol] against the reservoir solution.

2.5. Data collection

For preliminary characterization, intensity data for unit-cell parameter and space-group determination were collected at 100 K on a Rigaku R-AXIS IV^{++} image-plate detector equipped with Osmic MaxFlux confocal optics using a wavelength of 1.54 Å (Cu $K\alpha$) from a Rigaku rotating-anode generator operated at 40 kV and 100 mA. Prior to flash-freezing, the crystal was soaked for a few seconds in reservoir solution containing 30%(*v*/*v*) glycerol. The crystal was then mounted in a 0.5 mm cryoloop (Hampton Research) and flash-frozen in a cold nitrogen stream at 100 K. Data collection for the DL-DEX Mb crystal was performed at 100 K with a wavelength of 1.00 Å using the synchrotron-radiation source at Photon Factory BL5 and an ADSC Quantum 315 detector system (Tsukuba Japan) (Fig. 2). The data were processed using *HKL*-2000 (Otwinowski & Minor, 1997).

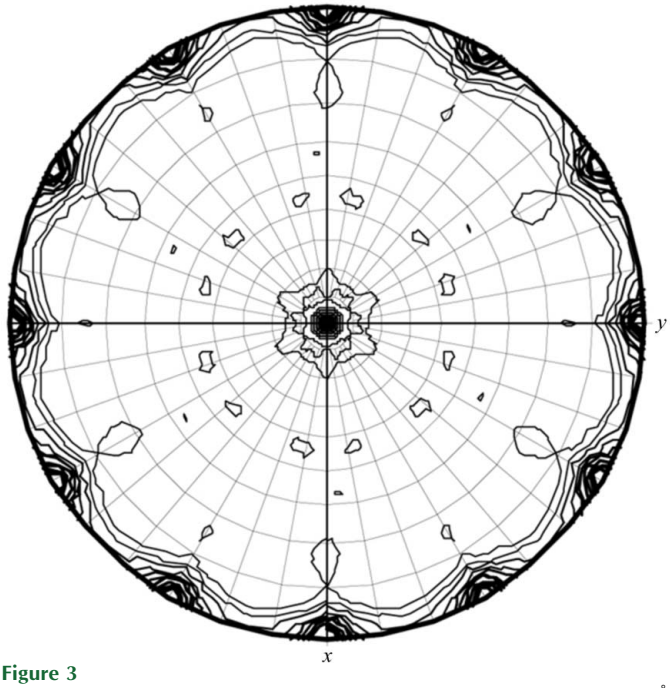

Figure 3
The $\chi = 120^\circ$ section of the self-rotation function using data from 34 to 4 Å resolution and a sphere of 30 Å, showing the noncrystallographic twofold axis at $\theta = 90.0$, $\varphi = -0.3^\circ$ with a peak height of 7.3σ. Contour lines are drawn at 0.5σ from a lowest level of +0.5σ. The rotation function was calculated in *MOLREP* (Collaborative Computational Project, Number 4, 1994; Vagin & Teplyakov, 1997).

3. Results and discussion

The DL-DEX Mb crystal appeared within a week of incubation and grew to maximum dimensions of 0.3 × 0.05 × 0.05 mm (Fig. 1). The crystal belongs to the hexagonal space group $P6_3$, with unit-cell parameters $a = b = 186.2$, $c = 114.4$ Å and a unit-cell volume of 3.43×10^6 Å^3. Assuming the presence of four or eight monomers in the asymmetric unit, the Matthews coefficient ($V_{\rm M}$) values were calculated to be 4.20 or 2.10 Å^3 Da^{-1}, respectively, indicating estimated solvent contents of 70 and 41% in the unit cell (Matthews, 1968). These values are just within the range typical for protein crystals. Dynamic light-scattering measurements showed that the purified protein was monodisperse, with an estimated molecular weight of 140 kDa. These results suggest that DL-DEX Mb exists as a homotetramer or a homohexamer with a subunit molecular weight of 34 046 Da, as the molecular weight of the homooctamer is too large compared with the molecular weight estimated by dynamic light-scattering measurements. Self-rotation functions were calculated at $\chi = 60$, 90, 120 and 180° to detect twofold, threefold, fourfold and sixfold noncrystallographic symmetry. No peaks higher than 0.5σ were observed on the projection map at $\chi = 60$, 90 and 120° except for $\theta = 0^\circ$. The projection map at $\chi = 180^\circ$ showed a noncrystallographic twofold axis at $\theta = 90.0$, $\varphi = -0.3^\circ$ (Fig. 3), suggesting that the enzyme might be a homohexamer with D_3 symmetry. It is possible that the noncrystallographic threefold axis of the homohexamer is almost parallel to the crystallographic threefold axis. An X-ray diffraction data set for the DL-DEX Mb crystal has been collected with 225 607 unique reflections (Fig. 2), giving a data-set completeness of 99.7% in the resolution range 50.0–1.75 Å with an $R_{\rm merge}$ of 7.3% (Table 1). These data indicated that the crystals were of good quality for X-ray structural analysis. The crystal showed no significant decay upon exposure. We are now preparing selenomethionyl-substituted DL-DEX Mb in order to solve the phase problem by the multi-wavelength anomalous dispersion (MAD) method using synchrotron radiation since there were no suitable models for molecular replacement in the Protein Data Bank.

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