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Crystallization and preliminary X-ray diffraction analysis of haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* UT26

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Haloalkane hydrolytic dehalogenase LinB from *Sphingomonas paucimobilis* UT26, an enzyme which releases chloride or bromide anion from *n*-halogenated alkanes and has a broad range of substrate specificity, was crystallized using the hanging-drop vapour-diffusion method at 278 K. The best crystals were obtained by microseeding with a precipitant containing 18–20%(*w*/*v*) PEG 6000, 0.2 *M* calcium acetate and 0.1 *M* Tris–HCl pH 8.9. The crystals diffract to at least 1.60 Å using synchrotron X-ray under cryogenic (100 K) conditions. They belong to the orthorhombic space group $P2_12_12$ with unit-cell parameters a = 50.29, b = 71.70, c = 72.73 Å. The asymmetric unit contains one molecule of the enzyme.

1. Introduction

γ-Hexachlorocyclohexane (γ-HCH, also known as γ-BHC or lindane) is a potent halogenated organic insecticide employed for agricultural and public health purposes which has been used worldwide since the 1940s. Because of its toxicity and long persistence in soil, most countries have prohibited the use of γ-HCH. However, there still are many contaminated sites and, because some countries are using γ-HCH for economic reasons, new sites are still continually being contaminated.

Sphingomonas (formerly *Pseudomonas*) *paucimobilis* SS86 was isolated from an upland experimental field to which γ-HCH had been applied once a year for 12 years in succession (Senoo & Wada, 1989; Wada *et al.*, 1989). *S. paucimobilis* UT26, a mutant of SS86, has Nal^r as a genetic marker.

Because γ-HCH is a highly chlorinated compound, dechlorination is a very significant step towards its degradation. It was found that three different types of dechlorination reactions are involved in the degradation of γ-HCH by *S. paucimobilis* UT26 (Imai *et al.*, 1989). The first reaction is dehydrochlorination of γ-HCH to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) *via* γ-pentachlorocyclohexene, the second reaction is hydrolytic dechlorination of 1,4-TCDN to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) *via* 2,4,5-trichloro-2,5-cyclohexane-1-ol and the third reaction is reductive dechlorination of 2,5-dichlorohydroquinone, which is produced from 2,5-DDOL by dehydrogenase LinC (Nagata *et al.*, 1994), to hydroquinone (Miyauchi *et al.*, 1998). Four genes (*linA*, *linB*, *linC* and *linD*) which are involved in the above reactions have been cloned and sequenced (Imai *et al.*, 1991; Nagata *et al.*, 1993, 1994; Miyauchi *et al.*, 1998).

The amino-acid sequence of LinB showed significant similarity to two other known haloalkane hydrolytic dehalogenases, the enzymes from *Xanthobacter autotrophicus* GJ10 (DhlA; Keuning *et al.*, 1985; Janssen *et al.*, 1989) and *Rhodococcus rhodochrous* NCIMB 13064 (Kulakova *et al.*, 1997). DhlA is the only haloalkane dehalogenase tertiary structure so far determined (Franken *et al.*, 1991; Verschueren *et al.*, 1993). Because of the level of similarity between LinB and DhlA (28% sequence identity), it has been suggested (Damborský *et al.*, 1995) that LinB belongs to the same protein superfamily, the α/β-hydrolases (Ollis *et al.*, 1992).

Recently, the dehalogenation activity of LinB with various substrates was assayed by measuring the rates of halide release (Nagata *et al.*, 1997). LinB shows the same level of activity at pH values between 8.0 and 8.6. Not only monochloroalkanes (C_3–C_{10}) but also dichloroalkanes, bromoalkanes and chlorinated aliphatic alcohols were good substrates for LinB. These results indicate that LinB shares properties with DhlA (Keuning *et al.*, 1985), with the exception of substrate specificity: in brief, LinB prefers long-chain haloalkanes as substrates, while DhlA prefers short-chain haloalkanes.

In this paper, we report the crystallization of the *S. paucimobilis* UT26 LinB and preliminary crystallographic characterization of the crystals.

2. Enzyme purification and crystallization

Haloalkane dehalogenase LinB from *S. paucimobilis* UT26 was overexpressed in *E.*

Table 1
Data collection and processing statistics.

Resolution interval (Å)	Number of independent reflections	Multiplicity	Completeness (%)	*R* factor (%)	Percentage with $I > 3\sigma(I)$	$\langle I/\sigma(I)\rangle$
25.0–6.30	588	6.5	88.6	4.1	87.3	44.67
6.30–4.47	1091	7.2	97.7	4.6	97.0	42.39
4.47–3.65	1357	7.3	96.9	4.5	96.0	45.04
3.65–3.16	1613	7.4	97.8	5.2	96.7	39.08
3.16–2.83	1789	7.4	97.1	6.9	92.3	31.89
2.83–2.60	1840	7.6	98.6	8.7	96.2	25.83
2.60–2.39	2335	7.6	98.5	10.6	95.7	22.77
2.39–2.24	2262	6.9	98.7	11.2	95.9	21.13
2.24–2.11	2404	3.4	95.8	6.1	92.3	18.22
2.11–2.00	2526	3.4	94.6	6.6	90.9	16.40
2.00–1.91	2515	3.4	94.0	7.8	88.0	13.99
1.91–1.83	2650	3.4	93.4	9.2	86.4	12.35
1.83–1.75	3084	3.4	92.6	11.2	81.9	10.60
1.75–1.69	2716	3.4	91.6	12.9	78.4	9.54
1.69–1.63	3098	3.4	91.6	14.5	76.0	8.81
1.63–1.60	1707	3.4	89.7	16.2	71.5	8.01
1.60–1.58	1074	2.8	81.4	16.2	59.8	7.49
Total	34649	4.8	94.2	6.5	86.7	18.31

coli, purified to homogeneity and concentrated to 2.0 mg ml^{-1} in 20 m*M* potassium phosphate pH 6.8, 1 m*M* 2-mercaptoethanol buffer, as previously reported (Nagata *et al.*, 1997). The purification buffer was exchanged by centrifugation for 10 m*M* Tris–HCl pH 7.5 and the enzyme was concentrated to 14.4 mg ml^{-1} for crystallization experiments. All crystallization experiments were performed at 278 K by the hanging-drop variant of the vapour-diffusion method in VDX plates (Hampton Research). Drops contained 4 µl of the respective reservoir solution and 4 µl of the enzyme solution.

The initial crystallization trials were carried out using reservoirs each consisting of 1 ml of Hampton Research Crystal Screen (Jancarik & Kim, 1991) and Crystal Screen II (Cudney *et al.*, 1994). We observed formation of crystalline material in Jancarik & Kim's conditions 36 [8%(*w*/*v*) polyethylene glycol 8000 (PEG 8000), 0.1 *M* Tris–HCl pH 8.5] and 46 [18%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* calcium acetate]. pH, PEG and calcium acetate concentrations were optimized and the best crystals grew at 17–19%(*w*/*v*) PEG 6000, 0.2 *M* calcium acetate and 0.1 *M* Tris–HCl pH 8.8–9.0. The LinB crystals took two to three weeks to appear and grew as very thin (thickness <0.01 mm) two-dimensional crystals from heavily precipitated protein. These crystals were used for microseeding and macroseeding (Stura & Wilson, 1992). The best result we obtained from the following microseeding experiment. Droplets containing 10 µl of enzyme and 10 µl reservoir solution [18%(*w*/*v*) PEG 6000, 0.2 *M* calcium acetate and 0.1 *M* Tris–HCl pH 8.9] were equilibrated for 24 h, after which they were centrifuged at 10000*g* to remove precipitate. A seed stock was produced by washing five previously prepared small crystals in 100 µl precipitant solution. Subsequently, the crystals were crushed and the stock was homogenized. Crystallization was initiated by mixing droplets composed from 4.2 µl supernatant and 1.4 µl seed stock. Large plates of LinB grew to an average size of about 0.8 × 0.4 × 0.02–0.04 mm after two to three weeks.

3. Data collection and analysis

A single crystal of LinB with dimensions 0.6 × 0.35 × 0.03 mm was mounted in a nylon cryoloop (Hampton Research), immersed in cryoprotectant [20%(*w*/*v*) PEG 6000, 10%(*w*/*v*) sucrose, 10%(*v*/*v*) PEG 400] for a few seconds and then rapidly exposed to a cold nitrogen stream (Oxford Cryosystems Cryostream). Diffraction data were collected at the crystallographic beamline BL711 at the MAX-II synchrotron in Lund (Sweden) at 100.0 K using a MAR 345 image-plate detector (X-ray Research). The wavelength used was λ = 0.9420 Å. Data were recorded in two sweeps at different exposure times (10 and 60 s) in order to accurately record both the strongest low-resolution and the weakest high-resolution diffraction intensities. Oscillation angles used for data collection were 0.5° for the first (slow) run and 1.5° for the second (fast) run. All data were processed and merged using the *XDS* system (Kabsch, 1993).

As shown in Table 1, the crystal of LinB from *S. paucimobilis* UT26 diffracted to at least 1.60 Å which was surprisingly well for its size and shape. The crystal belongs to the orthorhombic space group $P2_12_12$ with unit-cell parameters a = 50.29, b = 71.70, c = 72.73 Å. The asymmetric unit containing one molecule of the enzyme gives a V_m of 2.1 Å^3 Da^{-1}, which is within the acceptable range (Matthews, 1968). The solvent content is then approximately 42%.

Initial attempts to solve the phase problem by molecular replacement using DhlA coordinates were unsuccessful. Therefore we initiated a search of heavy-atom derivatives for structure determination. We obtained two useful heavy-atom derivatives by soaking crystals for 3 d at 278 K in 1 m*M* potassium tetrachloroplatinate(II), $K_2[PtCl_4]$, and in 2 m*M* potassium dicyanoaurate, $K[Au(CN)_2]$. We collected data to 2.5 Å and 99% completeness from both derivatives using the same equipment and conditions as for the native data, and we are currently applying the MIR method in order to solve the crystal structure.

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