Purification, crystallization and preliminary crystallographic analysis of DehIVa, a dehalogenase from Burkholderia cepacia MBA4

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Purification, crystallization and preliminary crystallographic analysis of DehIVA, a dehalogenase from *Burkholderia cepacia* MBA4

DehIVA is one of two dehalogenases produced by the soil- and water-borne bacterium *Burkholderia cepacia* MBA4. It acts to break down short-chain halogenated aliphatic acids through a nucleophilic attack and subsequent hydrolysis of an enzyme–substrate intermediate to remove the halide ions from l-enantiomers substituted at the C2 position (e.g. 1,2-monochloropropanionic acid). Dehalogenases are an important group of enzymes that are responsible for breaking down a diverse range of halogenated environmental pollutants. The *dhlIVA* gene coding for DehIVA was expressed in *Escherichia coli* and the protein was purified and crystallized using the hanging-drop method. Crystals grown in PEG 4000 and ammonium sulfate diffracted to 3.1 Å. The crystals had a primitive hexagonal unit cell, with unit-cell parameters *a* = *b* = 104.2, *c* = 135.8 Å, *α* = *β* = 90, *γ* = 120°. Determining this structure will provide valuable insights into the characterization of the catalytic mechanisms of this group of enzymes.

1. Introduction

Halogenated organics are an industrially important group of chemicals that in recent decades have been the focus of considerable international attention regarding their detrimental effects on the environment. Their harmful qualities stem from the recalcitrant, carcinogenic and toxic properties of many of these compounds and there is an obvious need to determine strategies for their removal from contaminated systems (Schwarze *et al.*, 1997). Numerous microorganisms have been isolated that degrade halogenated organics through the action of enzymes called dehalogenases. These enzymes form a diverse group with a broad range of substrate specificities and catalytic mechanisms (Slater *et al.*, 1995). Within this group are the 1,2-haloacid dehalogenases (1,2-HADs), which catalyze the dehalogenation of the l-enantiomers of C2-substituted short-chain organic acids. The reaction proceeds with the inversion of configuration at the chiral centre to produce a d,2-hydroxyacid. This stereospecificity, common throughout dehalogenases, also makes them useful in stereoselective industrial applications (Kurihara *et al.*, 1995) in addition to their usefulness in bioremediation.

*Burkholderia cepacia* MBA4 was isolated by Tsang *et al.* (1988) by growing cultures containing monobromoacetate (MBA) as the sole carbon source and was shown to produce two dehalogenases, though only the dehalogenase DehIVA was expressed under batch conditions. DehIVA is a member of the 1,2-HAD family of enzymes and exists as a homodimer with a molecular weight of ~45 kDa. Each monomer is 231 amino acids in length (Tsang & Pang, 2000).

Structural information is available for other dehalogenases, including a number of haloalkane dehalogenases (Streltsov *et al.*, 2003; Oakley *et al.*, 2004; Verschueren *et al.*, 1993) and two haloacid dehalogenases, DhiB and L-DEX YL (Ridder *et al.*, 1997; Hisano *et al.*, 1996). DehIVA has a high amino-acid sequence identity with the latter two enzymes (40 and 39%, respectively; Slater *et al.*, 1997). The structures and reaction mechanisms of these enzymes have been determined to be very different from those of the haloalkane dehalogenases (Ridder *et al.*, 1997, 1999; Janssen *et al.*, 2001; Liu *et al.*, 1995). It should be noted that there is still some uncertainty surrounding the roles of specific catalytically important residues; in particular, histidines 20 and 56 and arginines 42 and 156 (Liu *et al.*, 1994; Pang & Tsang, 2001; Ridder *et al.*, 1999; Li *et al.*, 1998). 1-HADs...
are an important group of enzymes; further structural characterization of related proteins would benefit our understanding of the groups’ reaction mechanisms.

Although there have been a number of rigorous biochemical studies reported regarding this enzyme (Murdia et al., 1992; Tsang et al., 1988; Tsang & Pang, 2000; Tsang & Sze, 2002), this is the first report of the crystallization and preliminary crystallographic analysis of the enzyme DehVα.

2. Materials and methods

2.1. Overexpression and purification of DehVα

The construct pHKU201 (Tsang & Pang, 2000) bearing the structural gene for DehVα (hdlVα) was cloned into Escherichia coli BL21 (DE3). Tsang & Pang (2000) have developed protocols for the overexpression and purification of DehVα. After confirming the sequence of the hdlVα gene, our own protocols were developed from those of Tsang and Pang. In summary, three 21 cultures were grown in Luria Broth media at 310 K until the OD₆₀₀ reached 2.7 and were then induced by the addition of IPTG (to 1 mM). The culture-growth temperature was not modified. After a 4 h induction, cells were spun down at 7500 rev min⁻¹ for 8 min. Cell pellets were harvested and resuspended in 275 ml 50 mM Tris buffer pH 7.4. PMSF was added to a final concentration of 1 mM and the cell suspension then lysed in a French Pressure cell. Cell lysate (270 ml) was then spun down at 15 000 rev min⁻¹ for 15 min to remove the insoluble fraction.

The overexpression and purification of DehVα was monitored as the dominant 25 kDa band on SDS–PAGE as described by Tsang & Pang (2000). The 25 kDa band was clearly evident after induction with IPTG (Fig. 1a).

The estimated percentage purity of DehVα throughout its purification is summarized in Table 1. As the pl of DehVα is ~5.1 (determined experimentally), the lysate supernatant was applied to a Q-Sepharose (Pharmacia/Pfizer, New York, USA) anion-exchange chromatography column pre-equilibrated with 50 mM Tris pH 7.4. DehVα was eluted with a linear salt gradient from 0 to 1 M NaCl at a flow rate of ~1.0 ml min⁻¹ for 5 h in the same Tris buffer. Samples containing DehVα as indicated by SDS–PAGE were combined. This was then treated with two rounds of ammonium sulfate precipitation, first at 50% (the precipitate was discarded) and then at 90% ammonium sulfate. Precipitate was resuspended in 50 mM Tris pH 7.4 and run on a 400 ml Sephacryl (Pharmacia) column. The DehVα peak was diluted down in a 1:5 ratio with double-distilled H₂O to a buffer concentration of 10 mM Tris pH 7.4 and then run through a weak anion-exchange column (DEAE Sephacel, Pharmacia) and eluted with a linear salt gradient to 10 mM Tris pH 7.4, 0.5 M NaCl (~0.5 ml min⁻¹ over ~10 h). This sample was then desalted, the buffer changed to 30 mM Tris pH 7.4 and spun down in an ultracentrifuge (90 000 rev min⁻¹ for 30 min) to remove an unwanted contaminant. As a final step, the sample (~10 ml) was applied onto a hydroxyapatite (Biorad, Hercules, USA) column (five runs of 2 ml) and eluted off with a linear gradient to 70% 30 mM Tris pH 7.4, 90 mM Na₂HPO₄ at 1 ml min⁻¹ for 40 min. The purified eluants were concentrated (6 ml Vivaspin concentrator tube with 3500 Da molecular-weight cutoff; Vivascience, Hanover, Germany) and pooled (5 ml at ~1.14 mg ml⁻¹; Fig. 1b). All chromatography except the hydroxyapatite runs was conducted at 277 K (hydroxyapatite at ~290 K). In preparation for crystallization trials, the hydroxyapatite- purified DehVα was desalted and concentrated (6 ml Vivaspin with 3500 Da molecular-weight cutoff) to ~7.5 mg ml⁻¹ in a final volume of 800 µl in Tris buffer pH 7.4.

2.2. Crystallization

Crystallization screens were set up using JBL Screens 2 and 3 from Jena Bioscience (Jena Biosciences, Jena, Germany). The hanging-

| Table 1 Purification of DehVα from overexpression in E. coli strain BL21. |
|-----------------------------|----------------|
| Step                        | Purity (%)     |
| Lysate                      | 5              |
| Q-Sepharose                 | 15             |
| Ni₃SO₄₃                   | 20             |
| Sephacryl                  | 40             |
| DEAE                        | 70             |
| Hydroxyapatite              | 98             |

| † Estimated by SDS–PAGE analysis. |

*Figure 1* SDS–PAGE monitoring of the overexpression and purification of DehVα. (a) Pre- and post-induction bacterial suspensions (lanes 1 and 2, respectively). DehVα is clearly induced with the addition of IPTG (~25 kDa band). (b) Purified DehVα: 15 and 7.5 µl sample loads of protein solution. The lane marked BMW is a broad molecular-weight ladder (kDa).

*Figure 2* Microphotographs of three morphologically different crystal forms of DehVα. Crystals form 1, 2 and 6 are shown in (a), (b) and (c), respectively. The largest crystal dimensions for each were ~0.3, 0.2 and 0.2 mm, respectively.
The crystals typically grew to $\sim 0.2 \times 0.2 \times 0.2$ mm over 10–24 h. The crystals did not grow any larger beyond this time. Preliminary data were collected in-house using a Rigaku RUH2R X-ray source with a rotating copper anode equipped with Osmic confocal optics, a MAR Research MAR345 detector and an Oxford Cryosystems 600 Series Cryostream. Crystals were transferred to a 10 µl drop composed of mother liquor modified to include the cryoprotectant glycerol at 20% (v/v) for approximately 5 s prior to flash-freezing to 100 K using the Cryostream. An X-ray diffraction image collected in-house is shown in Fig. 3.

Data processing was conducted using the HKL suite of programs (Otwinski & Minor, 1997) and a summary of the data-processing statistics are shown in Table 2. Crystals were found to belong to the primitive hexagonal space group $P\bar{3}1$ with unit-cell parameters $a = b = 104.2$, $c = 135.8$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ and a maximum diffraction resolution of $\sim 3.1$ Å. Based upon the Matthews coefficient of 4.3 Å$^3$ Da$^{-1}$, we anticipate that the asymmetric unit will contain between one and four monomers of DhlVa.

A full structural determination using molecular replacement (with the DhlB structure, PDB code 1qqs) is currently under way.

Crystals were taken to beamline 14-ID-B, BioCARS Facility, Advanced Photon Source (APS) in Argonne for analysis with synchrotron radiation; however, owing to the deterioration of the crystals during transport or handling no usable X-ray diffraction data could be measured.

### References


