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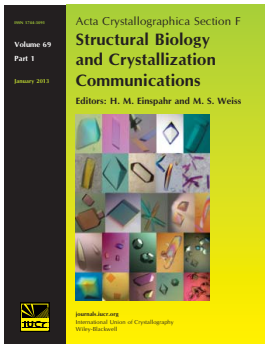

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Crystallographic analysis of new psychrophilic haloalkane dehalogenases: DpcA from *Psychrobacter cryohalolentis* K5 and DmxA from *Marinobacter* sp. ELB17

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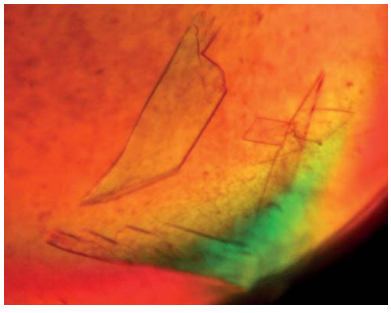

Haloalkane dehalogenases are hydrolytic enzymes with a broad range of potential practical applications such as biodegradation, biosensing, biocatalysis and cellular imaging. Two newly isolated psychrophilic haloalkane dehalogenases exhibiting interesting catalytic properties, DpcA from *Psychrobacter cryohalolentis* K5 and DmxA from *Marinobacter* sp. ELB17, were purified and used for crystallization experiments. After the optimization of crystallization conditions, crystals of diffraction quality were obtained. Diffraction data sets were collected for native enzymes and complexes with selected ligands such as 1-bromohexane and 1,2-dichloroethane to resolutions ranging from 1.05 to 2.49 Å.

1. Introduction

Haloalkane dehalogenases (EC 3.8.1.5; HLDs) are microbial enzymes which catalyse the hydrolytic conversion of halogenated aliphatic compounds to their corresponding alcohols (Janssen *et al.*, 2005). The hydrolytic dehalogenation accomplished by these enzymes is one of the most important steps in the biodegradation of 1-halo-*n*-alkanes and α,ω-dihalo-*n*-alkanes, which are serious halogenated pollutants (Poelarends *et al.*, 2000). HLDs have a broad substrate specificity (Koudelakova *et al.*, 2011) and a high enantioselectivity (Prokop *et al.*, 2010), which makes these enzymes applicable not only in bioremediation (Stucki & Thueer, 1995) but also in biosensing (Campbell *et al.*, 2006; Bidmanova *et al.*, 2010), biocatalysis (Prokop *et al.*, 2010; Westerbeek *et al.*, 2011), cellular imaging and protein analysis (Los & Wood, 2007; Ohana *et al.*, 2009). The application potential of HLDs has recently been reviewed by Koudelakova *et al.* (2013).

Structurally, HLDs belong to the superfamily of α/β-hydrolases (Ollis *et al.*, 1992). The enzyme structure consists of a highly conserved α/β-hydrolase main domain and a smaller helical cap domain (Banáš *et al.*, 2006). The variable cap domain, which is inserted into the sequence of the catalytic main domain near the C-terminus, has been identified in the structure of many α/β-hydrolases and has been found to have an influence on the substrate specificity of HLDs. The active-site cavity, which is located between the main domain and the cap domain (Janssen, 2004), comprises five catalytic residues called the catalytic pentad which are essential for the function of HLDs. The catalytic pentad is formed by the nucleophile (Asp), the catalytic base (His), the catalytic acid (Asp/Glu) and two halide-stabilizing residues (the Trp and Trp/Asn pair) (Chovancová *et al.*, 2007).

Several new crystal structures of HLDs have already been crystallized in the last few years (see, for example, Prudnikova *et al.*, 2009; Bogdanović *et al.*, 2010; Gehret *et al.*, 2012; Stsiapanava *et al.*, 2011; Degtjarik *et al.*, 2013). As more structures of different haloalkane dehalogenases with different specificities become available, a more complete understanding of the structure–function relationship of this interesting class of enzymes is guaranteed.

Two novel HLD enzymes, DpcA and DmxA, have recently been isolated from the Gram-negative psychrophilic bacteria *Psychrobacter cryohalolentis* K5 (Drienovska *et al.*, 2012) and *Marinobacter* sp. ELB17 (Chrast *et al.*, in preparation), respectively. Both psychrophilic enzymes exhibited unique temperature profiles with

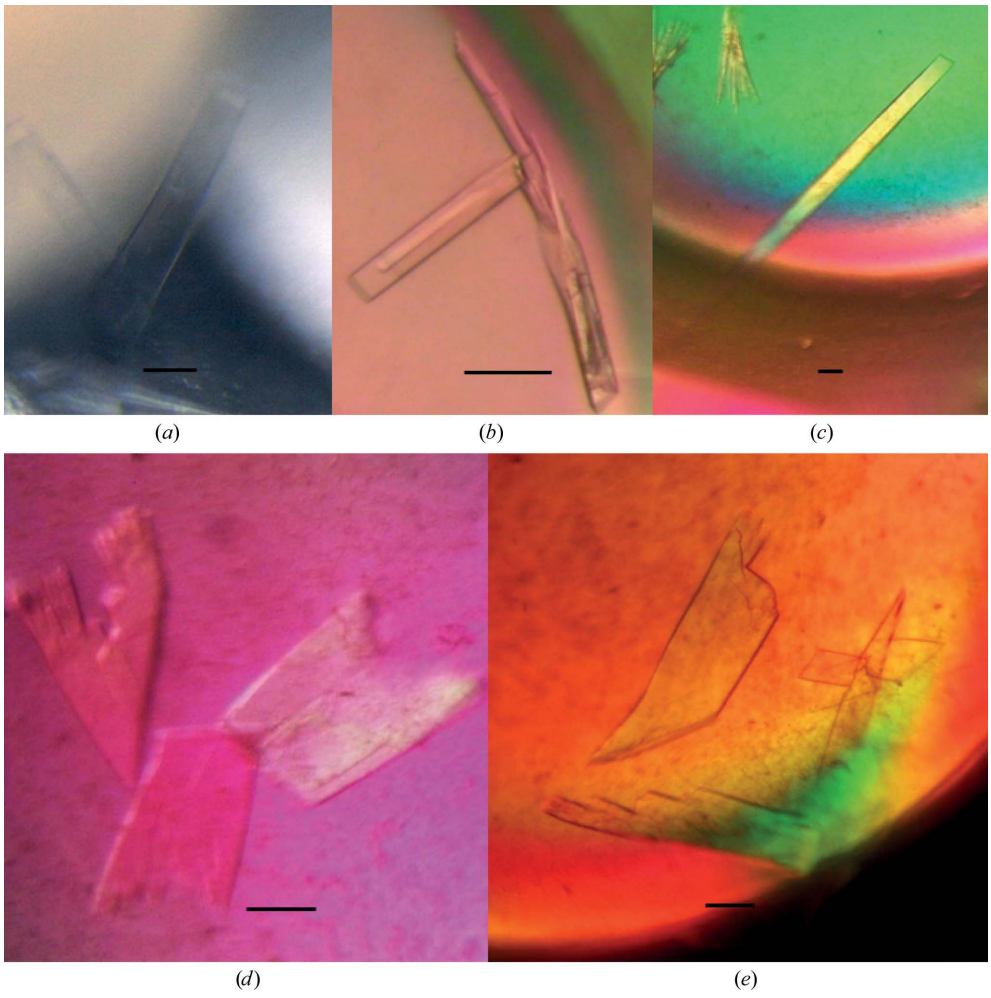

Figure 1
Crystals of the novel haloalkane dehalogenases used for diffraction measurements: DpcA (*a*), DpcA cocrystallized with 1-bromohexane (*b*), DpcA soaked with 1,2-dichloroethane (*c*), DmxA (*d*) and DmxA soaked with 1,2-dichloroethane (*e*). The scale bar represents 100 µm.

exceptionally high activities at low and high temperatures. Moreover, both enzymes exhibited high enantioselectivity towards selected brominated esters, and DmxA also towards β-brominated alkanes. Understanding the structural bases of the extremophilicity of the enzymes allows the construction of HLD variants with improved activity and stability at low and high temperatures and thus broadens their applicability in environmental and biosynthetic applications. Here, we report the crystallization and diffraction data analysis of two extremophilic enzymes, DpcA and DmxA, and their complexes with selected ligands.

2. Materials and methods

2.1. Gene synthesis, protein expression and purification

Optimized recombinant *dpcA* and *dmxA* genes were prepared by gene synthesis (Mr. Gene, Germany). The synthesized genes were subcloned into the expression vector pET21b (Novagen, USA) using *Nde*I and *Bam*HI restriction endonucleases (TaKaRa, Japan) and then transcribed from the T7 phage promoter under the control of lacUV5. *Escherichia coli* BL21(DE3) ArcticExpress and *E. coli* BL21(DE3) cells carrying recombinant plasmids pET21b::*dpcAHis* and pET21b::*dmxAHis*, respectively, were cultured in an LB medium with ampicillin (100 µg ml^{-1}) at 310 K. When the culture reached an optical density of 0.5–0.6 at 600 nm, protein expression was induced by the addition of IPTG to a final concentration of 0.5 m*M*. The DpcA and DmxA enzymes were expressed overnight at 285 K and for 4 h at 303 K, respectively. After the expression, the cells were harvested by centrifugation at 10 000*g* for 10 min, washed with 20 m*M* potassium phosphate buffer with 10%(*v*/*v*) glycerol, resuspended in 20 ml of the same buffer and frozen at 193 K. The defrosted cultures were disrupted by sonication with a Hielscher UP200S ultrasonic processor (Hielscher Ultrasonics, Germany). Lysates were centrifuged at 21 000*g* for 1 h (Laborzentrifugen, Germany). The crude extracts were purified on a HiTrap Chelating HP 5 ml column charged with Ni^{2+} ions (GE Healthcare, Sweden). Purified proteins containing C-terminal hexahistidine tags were dialysed overnight against 50 m*M* Tris–HCl pH 7.5, sterilized using a 0.22 µm filter and stored at 277 K.

2.2. Crystallization

2.2.1. Crystallization of DpcA and DmxA. The purified DpcA and DmxA enzymes at a concentration of 10 mg ml^{-1} in 50 m*M* Tris–HCl buffer pH 7.5 were used for crystallization experiments. Initial crystallization trials were carried out using the sitting-drop vapour-diffusion technique (Ducruix & Giegé, 1999) in CombiClover crystallization plates (Emerald BioSystems, Bainbridge Island, USA)

Table 1
Data-collection statistics for the crystals of DpcA, DpcA cocrystallized with 1-bromohexane and DpcA soaked with 1,2-dichloroethane.

Values in parentheses are for the outermost resolution shell.

Crystal	DpcA	DpcA cocrystallized with 1-bromohexane	DpcA soaked with 1,2-dichloroethane
Beamline	BESSY MX 14.2	BESSY MX 14.2	BESSY MX 14.2
Wavelength (Å)	0.978	0.978	0.918
Detector	Rayonics MX-225 CCD	Rayonics MX-225 CCD	Rayonics MX-225 CCD
Crystal-to-detector distance (mm)	90	120	120
Rotation range per image (°)	1	1	1
Total rotation range (°)	300	231	360
Exposure time per image (s)	2	4	1
Resolution range (Å)	50–1.05 (1.09–1.05)	50–1.35 (1.40–1.35)	50–1.25 (1.74–1.65)
Space group	$P2_1$	$P2_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 41.3, b = 79.4, c = 43.5, $\alpha = \gamma$ = 90.0, β = 95.0	a = 42.5, b = 78.9, c = 43.5, $\alpha = \gamma$ = 90.0, β = 93.7	a = 41.3, b = 79.3, c = 43.5, $\alpha = \gamma$ = 90.0, β = 95.2
Mosaicity (°)	0.42	0.45	0.12
Matthews coefficient (Å^3 Da^{-1})	2.04	2.04	2.03
No. of molecules in asymmetric unit	1	1	1
Solvent content (%)	39.8	39.7	39.6
Total No. of measured intensities†	687041	258865	563629
No. of unique reflections	120315 (7363)	60638 (5474)	76107 (12016)
Multiplicity	5.7	4.3	7.41
Average $I/\sigma(I)$	39.7 (3.4)	25.64 (2.06)	8.56 (2.1)
Completeness (%)	92.2 (56.2)	98.7 (89.7)	98.7 (96.6)
$R_{\rm merge}$‡ (%)	5.0 (30.7)	5.3 (37.7)	
$R_{\rm meas}$§ (%)			24.8 (128.6)

† The criterion for an observed reflection was $I/\sigma(I) > 0$. ‡ $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is an individual intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity of reflection hkl with summation over all data. § $R_{\rm meas}$ is the redundancy-independent merging R factor, also known as $R_{\rm r.i.m.}$ (Diederichs & Karplus, 1997; Weiss & Hilgenfeld, 1997; Weiss *et al.*, 1998). $R_{\rm meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean of the $N(hkl)$ individual measurements $I_i(hkl)$ of the intensity of reflection hkl.

Table 2
Data-collection statistics for the crystals of DmxA and DmxA soaked with 1,2-dichloroethane.

Values in parentheses are for the outermost resolution shell.

Crystal	DmxA	DmxA soaked with 1,2-dichloroethane
Beamline	BESSY MX 14.2	BESSY MX 14.2
Wavelength (Å)	0.918	0.918
Detector	Rayonics MX-225 CCD	Rayonics MX-225 CCD
Crystal-to-detector distance (mm)	180	180
Rotation range per image (°)	1	1
Total rotation range (°)	360	200
Exposure time per image (s)	1	1
Resolution range (Å)	50–2.49 (2.64–2.49)	50–2.10 (2.23–2.10)
Space group	$C222_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 45.8, b = 100.1, c = 153.6, $\alpha = \beta = \gamma$ = 90.0	a = 45.6, b = 152.7, c = 46.4, $\alpha = \gamma$ = 90.0, β = 116.3
Mosaicity (°)	0.48	0.27
Matthews coefficient (Å^3 Da^{-1})	2.52	2.07
No. of molecules in asymmetric unit	1	1
Solvent content (%)	51.2	40.7
Total No. of measured intensities†	173086 (25427)	135806 (21100)
No. of unique reflections	12411 (1846)	32442 (5006)
Multiplicity	13.95	4.19
Average $I/\sigma(I)$	5.64 (3.03)	11.42 (1.97)
Completeness (%)	96.9 (90.9)	97.9 (94.2)
$R_{\rm meas}$‡ (%)	30.02 (58.5)	10.5 (78.8)

† The criterion for an observed reflection was $I/\sigma(I) > 0$. ‡ $R_{\rm meas}$ is the redundancy-independent merging R factor, also known as $R_{\rm r.i.m.}$ (Diederichs & Karplus, 1997; Weiss & Hilgenfeld, 1997; Weiss *et al.*, 1998). $R_{\rm meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean of the $N(hkl)$ individual measurements $I_i(hkl)$ of the intensity of reflection hkl.

at 293 K. For screening the initial crystallization conditions we used Crystal Screen and Crystal Screen 2 (Hampton Research, Aliso Viejo, California, USA), several screens from JBScreen Classic (Jena Bioscience, Jena, Germany), Counterdiffusion Screening Kits (GCB–CSK, 24 conditions and PEG screening; Triana Science and Technology, Granada, Spain) and MD-2 (Molecular Dimensions Ltd, Suffolk, England). Drops contained different ratios of protein and precipitant solution (1:1, 1:2, 1:3 and 2:1 with drop size 3 µl). Experiments were set up at 277, 285, 293 and 298 K.

Microcrystals of both proteins were obtained using the commercial crystallization screening kit Crystal Screen (Hampton Research), composed of 0.2 *M* ammonium acetate, 30%(*w*/*v*) PEG 4000 and 0.1 *M* sodium acetate trihydrate pH 4.6 for DpcA or 0.1 *M* sodium citrate tribasic dihydrate pH 5.6 for DmxA. Precipitating solutions with various pH and PEG 4000 concentrations as well as different temperature conditions were tested to optimize protein crystal size by using the sitting-drop and hanging-drop vapour-diffusion techniques in CombiClover crystallization plates (Emerald BioSystems) and EasyXtal 15-Well Tools (Qiagen, Hilden, Germany), respectively. The crystallization conditions were further optimized using the Additive Screen kit (Hampton Research). Each reservoir well contained 300 µl precipitant solution in the case of the sitting-drop technique and 600 µl of precipitant solution in the case of hanging-drop vapour-diffusion experiments. Crystallization drops were prepared by mixing 2 µl protein solution and 1 µl precipitant solution plus 0.3–0.6 µl 0.1 *M* L-proline. For the crystallization of DmxA, 0.1 *M* L-proline or 0.1 *M* sarcosine were added to the crystallization drops. The experiments were carried out at 292 K.

2.2.2. Crystallization of DpcA and DmxA with selected ligands. Similar optimized conditions with different ratios of protein, precipitant solution, substrate and additive were used for the cocrystallization of DpcA with 10%(*v*/*v*) 1-bromohexane in a solution of 0.1 *M* glycine buffer pH 8.6. Crystals of DpcA cocrystallized with 1-bromohexane (Fig. 1*b*) were obtained by the sitting-drop vapour-diffusion method from a solution prepared by mixing protein, precipitant solution and 1-bromohexane in a 1:1:1.3 µl ratio and adding 0.33 µl 0.1 *M* L-proline as an additive. The DpcA (Fig. 1*c*) and DmxA (Fig. 1*e*) complexes with 1,2-dichloroethane were obtained by soaking experiments after the addition of 50 µl 1,2-dichloroethane to the reservoir well containing 800 µl of the precipitating solution with the crystals and incubation for 7 d.

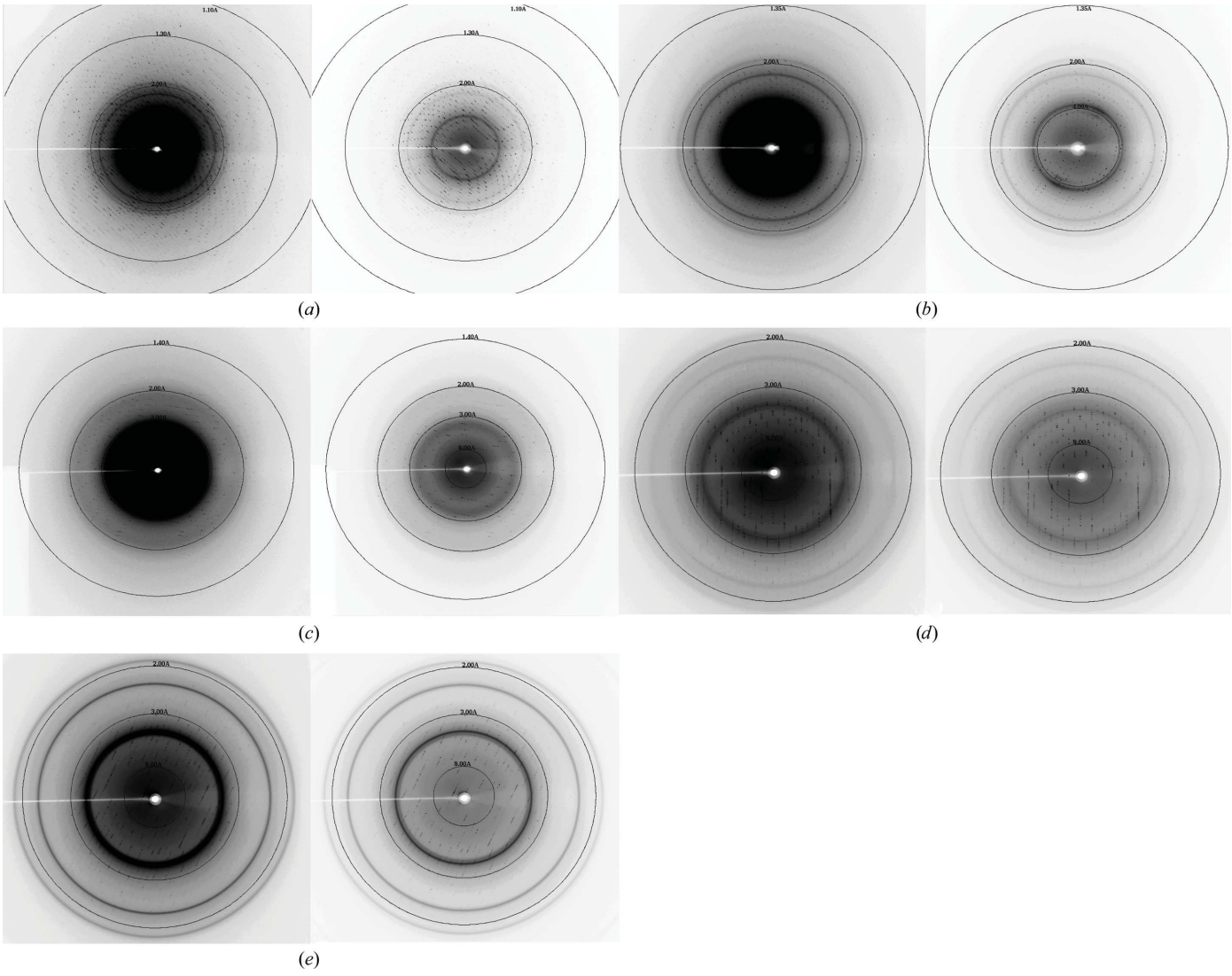

Figure 2
Diffraction images of DpcA (*a*), DpcA cocrystallized with 1-bromohexane (*b*), DpcA soaked with 1,2-dichloroethane (*c*), DmxA (*d*) and DmxA soaked with 1,2-dichloroethane (*e*).

2.3. Data collection and processing

Diffraction data were collected on beamline 14.2 at the BESSY II electron-storage ring (Mueller *et al.*, 2012) operated by the Joint Berlin MX-Laboratory (Berlin-Aldershof, Germany) and equipped with a Rayonics MX-225 CCD detector at wavelengths of 0.978 Å for DpcA and DpcA in complex with 1-bromohexane, and of 0.918 Å for DmxA and for DmxA and DpcA in complex with 1,2-dichloroethane. All diffraction experiments were carried out in a liquid-nitrogen stream at 100 K using a Cryojet XTL system (Oxford Instruments).

Single crystals of DpcA and its complex with 1-bromohexane were mounted in a nylon loop (Hampton Research) and SPINE loop (MiTeGen; from Jena Bioscience) and directly flash-cooled in a nitrogen stream without additional cryoprotection. Crystals of DmxA, DmxA with 1,2-dichloroethane and DpcA with 1,2-dichloroethane were cryoprotected by soaking in a drop containing 50%(*w*/*v*) PEG 3350 for 5 s, mounted in a nylon loop (Hampton Research) and flash-cooled in a liquid-nitrogen stream.

The crystal-to-detector distance was 90 mm for DpcA, 120 mm for the DpcA complexes with both ligands and 180 mm for DmxA and its complex with 1,2-dichloroethane. An oscillation range of 1.0° for all crystals and exposure times from 1 to 4 s per image were used. Complete data-collection and processing statistics are summarized in Tables 1 and 2.

The diffraction data for DpcA and its complex with 1-bromohexane were indexed, integrated and scaled by *HKL*-3000 (Minor *et al.*, 2006), while *XDS* (Kabsch, 2010) and the graphical user interface *XDSAPP* (Krug *et al.*, 2012) were used for the data sets of DmxA, DmxA in complex with 1,2-dichloroethane and DpcA in complex with 1,2-dichloroethane. Matthews coefficients were calculated with *MATTHEWS_COEF* (Matthews, 1968) using the the *CCP*4 software package (Winn *et al.*, 2011).

3. Results and discussion

3.1. Crystallization and characterization of DpcA and its complexes with ligands

Needle-shaped crystals of DpcA with dimensions of approximately 600 × 100 × 50 µm (Fig. 1*a*) were grown in a drop consisting of 2 µl protein solution and 1 µl crystallization solution containing 0.2 *M*

ammonium acetate, 0.1 *M* sodium acetate trihydrate pH 5.88, 30%(*w*/*v*) PEG 4000 and 0.01 *M* L-proline.

To obtain crystals of DpcA with 1-bromohexane (Fig. 1*b*), the haloalkane substrate was added directly into the drop to cocrystallize with the protein. The drop was prepared by mixing 1 µl protein solution, 1 µl precipitant solution, 1.3 µl 1-bromohexane, 0.33 µl 0.1 *M* L-proline.

The DpcA complex with the second ligand was obtained by the addition of 1,2-dichloroethane into the optimized precipitating solution with the enzyme crystals (Fig. 1*c*) and the addition of 0.6 µl 0.1 *M* L-proline. Soaking experiments with 1,2-dichloroethane were performed for 7 d.

3.2. Crystallization and characterization of DmxA and its complexes with ligands

Plate-shaped crystals of DmxA (Fig. 1*d*) with dimensions of approximately 320 × 180 × 10 µm were grown from an optimized precipitating solution consisting of 0.2 *M* ammonium acetate, 0.1 *M* sodium citrate tribasic dihydrate pH 5.7, 30%(*w*/*v*) PEG 4000, 0.02 *M* L-proline. Several selected crystals of DmxA were soaked with 1,2-dichloroethane for 7 d (Fig. 1*e*).

3.3. Preliminary diffraction data analysis

Crystals of the DpcA enzyme diffracted to 1.05 Å resolution (Fig. 2*a*) and belonged to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 41.3, b = 79.4, c = 43.5 Å, $\alpha = \gamma = 90.0$, $\beta = 95.0^\circ$. Crystals of the DpcA complexes with 1-bromohexane and 1,2-dichloroethane diffracted to 1.35 Å (Fig. 2*b*) and 1.65 Å (Fig. 2*c*) resolution, respectively. Both crystals belonged to the primitive monoclinic space group $P2_1$, with unit-cell parameters similar to those of the free enzyme.

DmxA crystals diffracted to a resolution of 2.49 Å (Fig. 2*d*), while crystals of DmxA soaked with 1,2-dichloroethane diffracted to a resolution of 2.10 Å (Fig. 2*e*). The crystals of native DmxA belonged to the centred orthorhombic $C222_1$ space group, with unit-cell parameters a = 45.8, b = 100.1, c = 153.6 Å, $\alpha = \beta = \gamma = 90.0^\circ$ and DmxA crystals soaked with 1,2-dichloroethane belonged to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 45.6, b = 152.7, c = 46.4 Å, $\alpha = \gamma = 90.0$, $\beta = 116.3^\circ$.

According to the calculated Matthews coefficient V_M (see Tables 1 and Table 2), crystals of DpcA and its complexes with ligands contained one molecule in the asymmetric unit, while in the case of DmxA soaked with 1,2-dichloroethane two molecules of enzyme were present in the asymmetric unit. The crystal parameters and data-collection statistics for DpcA and DmxA native crystals and their complexes with ligands are summarized in Tables 1 and 2.

The diffraction data will be used to determine the structures of DpcA, DmxA and their complexes. The structures of both enzymes will be solved by molecular replacement with *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP*4 software suite (Winn *et al.*, 2011). The coordinates of haloalkane dehalogenases from *Xanthobacter autotrophicus* (PDB entry 1b6g; 40% sequence identity for 121 residues and 53% sequence similarity with DpcA; Ridder *et al.*, 1999) and *Plesiocystis pacifica* (PDB entry 2xt0; 43% sequence identity for 129 residues and 58% sequence similarity with DpcA; Hesseler *et al.*, 2011) will be used as search models for the DpcA structure. For DmxA, the coordinates of haloalkane dehalogenases from *Rhodococcus* sp. (PDB entry 1bn6; 48% sequence identity for 137 residues and 64% sequence similarity with DmxA; Newman *et al.*, 1999) and *R. rhodochrous* (PDB entry 3fbw; 48% sequence identity for 138 residues and 64% sequence similarity with DmxA; Stsiapanava *et al.*, 2010) will be used as search models.

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