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Crystallization and crystallographic analysis of the *Rhodococcus rhodochrous* NCIMB 13064 DhaA mutant DhaA31 and its complex with 1,2,3-trichloropropane

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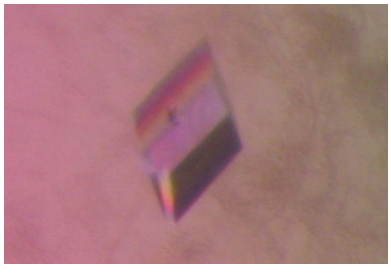
Haloalkane dehalogenases hydrolyze carbon–halogen bonds in a wide range of halogenated aliphatic compounds. The potential use of haloalkane dehalogenases in bioremediation applications has stimulated intensive investigation of these enzymes and their engineering. The mutant DhaA31 was constructed to degrade the anthropogenic compound 1,2,3-trichloropropane (TCP) using a new strategy. This strategy enhances activity towards TCP by decreasing the accessibility of the active site to water molecules, thereby promoting formation of the activated complex. The structure of DhaA31 will help in understanding the structure–function relationships involved in the improved dehalogenation of TCP. The mutant protein DhaA31 was crystallized by the sitting-drop vapour-diffusion technique and crystals of DhaA31 in complex with TCP were obtained using soaking experiments. Both crystals belonged to the triclinic space group *P*1. Diffraction data were collected to high resolution: to 1.31 Å for DhaA31 and to 1.26 Å for DhaA31 complexed with TCP.

1. Introduction

The haloalkane dehalogenase DhaA was isolated from the Gram-positive bacterium *Rhodococcus rhodochrous* NCIMB 13064 (Kulakova *et al.*, 1997). The enzyme catalyzes the hydrolytic dehalogenation of various halogenated aliphatic hydrocarbons. The structures of several DhaA mutant variants have been published (Newman *et al.*, 1999; Klvana *et al.*, 2009; Stsiapanava *et al.*, 2010). The main α/β-hydrolase domain is conserved in the members of the α/β-hydrolase superfamily and serves as a scaffold for the catalytic residues. The second helical cap domain is structurally more variable and is known to influence the substrate specificity of these enzymes (Chovancová *et al.*, 2007). The active-site cavity is located between two protein domains and is connected to the protein surface by two major access tunnels: the main tunnel and the slot tunnel used by the ligands for exchange between the active site and the surrounding solvent. A catalytic pentad of DhaA which performs hydrolysis has been identified. It includes a nucleophile (Asp106), a base (His272), a catalytic acid (Glu130) and two halide-stabilizing residues (Asn41 and Trp107). The dehalogenation reaction proceeds in the active site *via* nucleophilic attack of a nucleophile on a C atom carrying a halogen on the substrates. This leads to cleavage of the carbon–halogen bond, displacement of the halide and the formation of a covalent alkyl-enzyme intermediate. The alkyl-enzyme intermediate is subsequently hydrolyzed by a water molecule activated by a catalytic base (Pavlova *et al.*, 2009).

Some haloalkane dehalogenases are involved in the biodegradation of important environmental pollutants. The enzyme DhaA is of particular interest because it enables a very slow conversion of the toxic artificial compound and suspected human carcinogen 1,2,3-trichloropropane (TCP) to 2,3-dichloropropane-1-ol under laboratory conditions (Schindler *et al.*, 1999; Bosma *et al.*, 1999). To increase the efficiency of this reaction, focused directed evolution was used to construct the mutant DhaA31, which has up to 32-fold higher catalytic activity and 26-fold higher catalytic efficiency than the parent



wild-type enzyme (Pavlova *et al.*, 2009). The mutant DhaA31 has a k_{cat} value of 1.26 s^{-1} and a K_m value of 1.2 m*M*, while the wild type has a k_{cat} value of 0.04 s^{-1} and a K_m value of 1.0 m*M*. The positions for mutagenesis were selected by computer modelling and were randomized experimentally. DhaA31 has large aromatic residues at two of the three randomized positions and at two positions modified by site-directed mutagenesis. These changes enhance activity towards TCP by decreasing the accessibility of the active site to water molecules, thus promoting the formation of an activated complex. Kinetic analyses confirmed that the mutations improved carbon–halogen bond cleavage and shifted the rate-limiting step to the release of products (Pavlova *et al.*, 2009). Comparison of the wild-type DhaA and the mutant DhaA31 structures both in the free form and in complex with TCP could provide valuable structural information on the molecular basis of the enhanced catalysis.

2. Materials and methods

2.1. Construction of the mutant, protein expression and purification

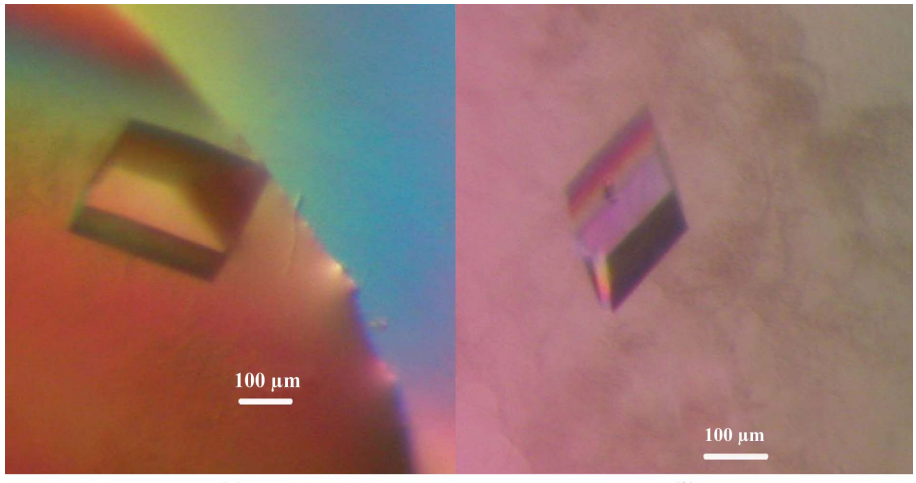
The recombinant gene *dhaA31* carrying I135F, C176Y, V245F, L246I and Y273F mutations was obtained by focused directed evolution. The methodology has been described previously by Pavlova *et al.* (2009). The *dhaA31* gene was carried by a pAQN vector downstream of the *tac* promotor (P *tac*), which was under the control of *lacI*^{q}. *Escherichia coli* BL21 cells with the plasmid were cultured in 4 l Luria–Bertani medium at 310 K. The induction of enzyme expression at 303 K was initiated by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 m*M*. The cells were harvested and disrupted by sonication using a Soniprep 150 (Sanyo Gallenkamp PLC, Loughborough, England). The supernatant was used after centrifugation at 21 000*g* for 1 h. The enzyme was purified by immobilized metal-affinity chromatography as described previously (Pavlova *et al.*, 2009). The enzyme was bound to the resin in equilibration buffer (20 m*M* potassium phosphate buffer pH 7.5, 0.5 *M* sodium chloride and 10 m*M* imidazole). Unbound and non-specifically bound proteins were washed out with buffer containing 37.5 m*M* imidazole. The target enzyme was eluted using buffer containing 300 m*M* imidazole. The active fractions were pooled and dialyzed overnight against 100 m*M* Tris–HCl buffer pH 7.5 without additives. The DhaA31 enzyme was stored in the same buffer. The entire purification process and storage took place at 277 K. The active fractions were pooled and dialyzed overnight against 100 m*M* Tris–HCl buffer pH 7.5 without additives. Protein concentrations were determined by the Bradford method (Bradford, 1976). SDS–PAGE was run on 15% polyacrylamide gels to check the purity of the protein.

2.2. Crystallization

Freshly isolated and purified DhaA31 enzyme in 100 m*M* Tris–HCl buffer pH 7.5 was used in crystallization experiments. Initial crystallization experiments were carried out by the sitting-drop vapour-diffusion method (Ducruix & Giegé, 1999) using Combi-Clover crystallization plates (EBS plates; Emerald BioSystems, Bainbridge Island, USA) at room temperature and subsequently at the lower temperature of 277 K. Protein microcrystals were obtained using a couple of precipitants from the commercial crystallization kit Crystal Screen (Hampton Research, Aliso Viejo, California, USA) and PEG-based conditions from the JBScreen Classic kit (Jena Bioscience GmbH, Jena, Germany). Three-dimensional crystals of the mutant protein DhaA31 were obtained at 277 K during a four-month optimization procedure. The droplets, which consisted of a 2–4 µl total volume with precipitant:protein ratios of 1:1 (drop volume 2 µl), 1:2 (3 µl) and 1:3 (4 µl), were equilibrated over 800 µl reservoir solution. DhaA31 monocrystals were grown in 2 µl droplets consisting of equal volumes of protein solution at a concentration of 7 mg ml^{-1} in 50 m*M* Tris–HCl buffer pH 7.5 and reservoir solution consisting of 29%(*w*/*v*) PEG 4000 and 100 m*M* MES sodium salt pH 6.4.

2.3. Data collection and processing

Diffraction data for DhaA31 and for DhaA31 complexed with TCP were collected on beamline X12 at EMBL Hamburg Outstation (Germany) at a fixed wavelength of 1.033 Å using a MAR CCD 225 mm detector. Prior to data collection, several crystals of DhaA31 were soaked with TCP (Fig. 1*b*). In this soaking experiment, 50 µl TCP was added to 800 µl reservoir solution and left for 6 h at room temperature. Crystals were mounted in nylon loops (Hampton Research, Aliso Viejo, USA; Teng, 1990) directly from the test drop and were flash-frozen in a stream of nitrogen gas at 100 K without additional cryoprotection. The diffraction data for DhaA31 were collected in two steps. 120 low-resolution images were recorded with an oscillation angle of 3° and a crystal-to-detector distance of



(*a*) (*b*)

Figure 1
Crystals of the mutant haloalkane dehalogenase DhaA from *R. rhodochrous* NCIMB 13064: (*a*) DhaA31 and (*b*) DhaA31 complexed with TCP.

Table 1
Data-collection statistics for crystals of DhaA31 and DhaA31 complexed with TCP.

Values in parentheses are for the highest resolution shell.

	DhaA31	DhaA31–TCP
Beamline	DESY X12	DESY X12
Detector	MAR CCD 225 mm	MAR CCD 225 mm
Wavelength (Å)	1.033	1.033
Resolution range (Å)	50–1.31 (1.35–1.31)	50–1.26 (1.27–1.26)
Space group	*P*1	*P*1
Unit-cell parameters (Å, °)	a = 42.55, b = 44.37, c = 46.41, α = 115.3, β = 98.5, γ = 109.5	a = 42.49, b = 44.39, c = 46.53, α = 115.3, β = 97.7, γ = 109.5
Measured reflections	580117	537263
Unique reflections	297181	162936
Multiplicity	4.3	2.4
Completeness (%)	94.6 (90.3)	92.4 (82.7)
$R_{\rm merge}$† (%)	4.4 (8.1)	3.3 (6.1)
$\langle I/\sigma(I)\rangle$	35.6 (18.3)	41.1(17.6)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity of the reflection.

300 mm. A high-resolution set of 360 images (50–1.31 Å) was collected with an oscillation angle of 1° and a crystal-to-detector distance of 100 mm. Similarly, diffraction data for DhaA31 in complex with TCP were collected in two steps. A set of 400 high-resolution images (50–1.26 Å) was recorded with a 0.5° oscillation angle and a crystal-to-detector distance of 100 mm and this was followed by the collection of 130 low-resolution image frames with a 1.5° oscillation angle and a crystal-to-detector distance of 300 mm. Data for both protein crystals were integrated and scaled using the *HKL*-2000 program package (Otwinowski & Minor, 1997).

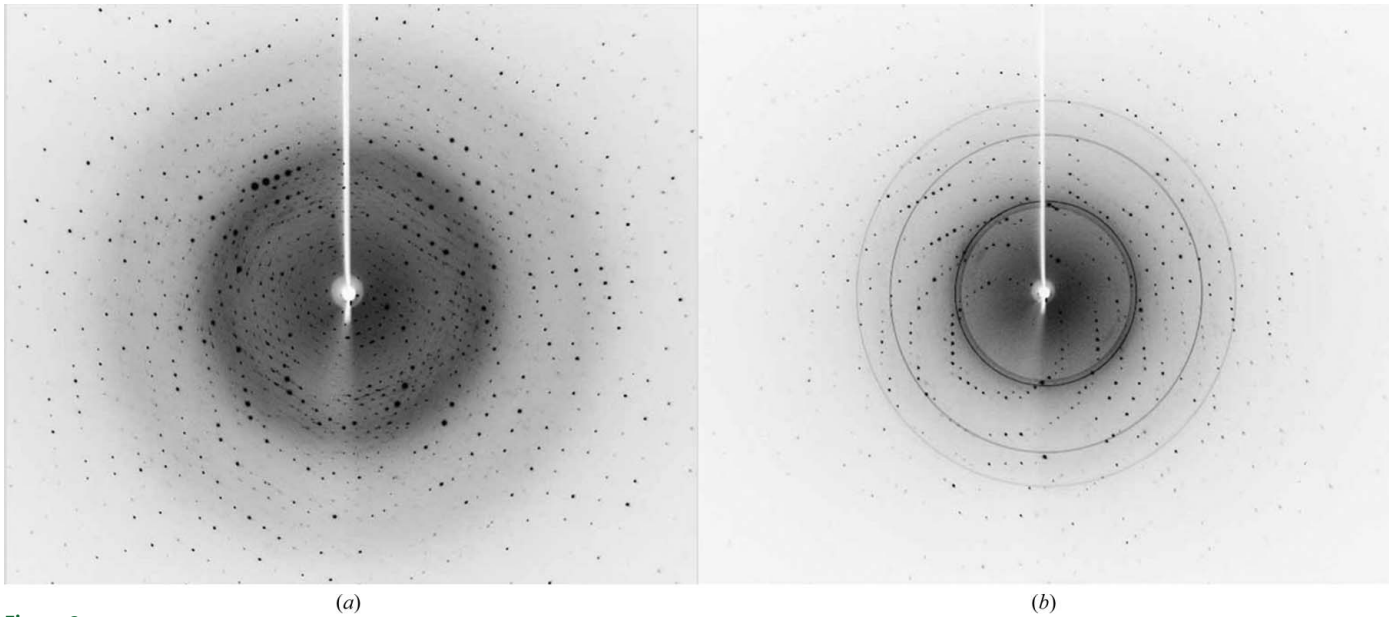
2.4. Preliminary structure solution

The structures of DhaA31 and of its complex with TCP were solved by the molecular-replacement method in *MOLREP* (Vagin & Teplyakov, 2010) using the structure of haloalkane dehalogenase DhaA from *R. rhodochrous* (PDB code 3fbw; Stsiapanava *et al.*, 2010) as the search model. Rigid-body refinement was performed using the *REFMAC*5 program from the *CCP*4 program suite (Murshudov *et al.*, 1997). R and $R_{\rm free}$ were 0.234 and 0.257, respectively, for DhaA31 and 0.251 and 0.279, respectively, for DhaA31 complexed with TCP.

3. Results and discussion

The initial crystallization conditions for mutant haloalkane dehalogenase DhaA31 were identified using solution C3 of Crystal Screen (Hampton Research, Aliso Viejo, USA), which consisted of 0.1 *M* Na HEPES pH 7.5, 2%(*v*/*v*) PEG 400, 2.0 *M* ammonium sulfate, and solution D4 of the JBScreen Classic kit (Jena Bioscience GmbH, Jena, Germany), composed of 30%(*w*/*v*) PEG 4000, 100 m*M* sodium acetate pH 4.6, 200 m*M* ammonium sulfate. Using these conditions, DhaA31 protein microcrystals were grown in one week. During the optimization procedure, the initial crystallization conditions were slightly modified and resulted in the use of a final precipitant solution consisting of 29%(*w*/*v*) PEG 4000 and 100 m*M* MES sodium salt pH 6.4. Three-dimensional crystals of DhaA31 were grown at 277 K and reached final dimensions of 0.28 × 0.19 × 0.13 mm (Fig. 1*a*) in three weeks.

Three-week-old single crystals of DhaA31 were used for soaking experiments followed by diffraction data collection at a synchrotron-radiation source. Single crystals of DhaA31 and of DhaA31 complexed with TCP were used for X-ray diffraction measurements. Data sets for DhaA31 and DhaA31 complexed with TCP were collected to maximum resolutions of 1.31 Å (Fig. 2*a*) and 1.26 Å (Fig. 2*b*), respectively. The crystals belonged to the triclinic space group *P*1, with unit-cell parameters a = 42.55, b = 44.37, c = 46.41 Å, α = 115.3, β = 98.5, γ = 109.5° for DhaA31 and a = 42.49, b = 44.39, c = 46.53 Å, α = 115.3, β = 97.7, γ = 109.5° for DhaA31 complexed with TCP. The asymmetric units of the crystals of DhaA31 and DhaA31 complexed with TCP contained one monomer, with solvent contents of 40.0 and 40.6%, respectively. The calculated Matthews coefficient ($V_{\rm M}$; Matthews, 1968) for one monomer of DhaA31 in the asymmetric unit was 2.05 Å^3 Da^{-1} and that for DhaA31 in complex with TCP was



(*a*) (*b*)

Figure 2
Diffraction images of crystals of the mutant haloalkane dehalogenase DhaA from *R. rhodochrous* NCIMB 13064: (*a*) DhaA31, (*b*) DhaA31 complexed with TCP. The crystals diffracted to resolutions of 1.31 and 1.26 Å for DhaA31 and DhaA31 complexed with TCP, respectively.

2.07 Å^3 Da^{-1}. Data-collection statistics for DhaA31 and its complex with TCP are presented in Table 1.

The structures are currently being refined and their interpretation will follow.

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