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Crystals of DhaA mutants from *Rhodococcus rhodochromus* NCIMB 13064 diffracted to ultrahigh resolution: crystallization and preliminary diffraction analysis

The enzyme DhaA from *Rhodococcus rhodochromus* NCIMB 13064 belongs to the haloalkane dehalogenases, which catalyze the hydrolysis of haloalkanes to the corresponding alcohols. The haloalkane dehalogenase DhaA and its variants can be used to detoxify the industrial pollutant 1,2,3-trichloropropane (TCP). Three mutants named DhaA04, DhaA14 and DhaA15 were constructed in order to study the importance of tunnels connecting the buried active site with the surrounding solvent to the enzymatic activity. All protein mutants were crystallized using the sitting-drop vapour-diffusion method. The crystals of DhaA04 belonged to the orthorhombic space group $P2_12_12_1$, while the crystals of the other two mutants DhaA14 and DhaA15 belonged to the triclinic space group $P1$. Native data sets were collected for the DhaA04, DhaA14 and DhaA15 mutants at beamline X11 of EMBL, DESY, Hamburg to the high resolutions of 1.30, 0.95 and 1.15 Å, respectively.

1. Introduction

Haloalkane dehalogenases (EC 3.8.1.5), which are members of the α/β -hydrolase fold family, catalyze the hydrolytic conversion of a broad spectrum of hydrocarbons to the corresponding alcohols (Janssen, 2004). Dehalogenation is a key step in the aerobic mineralization of many halogenated compounds that are environmental pollutants (Janssen *et al.*, 2005). Haloalkane dehalogenases are considered to be important biocatalysts in bioremediation applications to decontaminate contaminated environments (Stucki & Thuer, 1995). Moreover, they can be used as the active components of biosensors (Campbell *et al.*, 2006), in industrial biocatalysis (Prokop *et al.*, 2004) or in decontamination mixtures for chemical warfare agents (Prokop *et al.*, 2005, 2006).

The haloalkane dehalogenase DhaA was isolated from the Gram-positive bacterium *Rhodococcus rhodochromus* NCIMB 13064 (Kulakova *et al.*, 1997). As well as a wide range of halogenated hydrocarbons, DhaA can slowly convert the serious industrial pollutant 1,2,3-trichloropropane (TCP; Schindler *et al.*, 1999; Bosma *et al.*, 1999). A study of the enhancement of the activity of DhaA towards TCP by directed evolution has been published by Bosma *et al.* (2002). After two rounds of error-prone PCR, they obtained mutants with twofold and eightfold improved activity towards this substrate. The structure–function relationships of the effect of mutations on the enzymatic activity have recently been studied using the crystal structure of DhaA (Newman *et al.*, 1999) and mutations were introduced into the structure by computer modelling (Banas *et al.*, 2006). This study describes the construction, crystallization and structural analysis of one of the mutants, DhaA04 (C176Y), which was previously labelled M1 by Bosma and coworkers.

DhaA is expected to have several pathways (tunnels) that connect the buried active site to the surrounding solvent. The derived mutant enzymes DhaA04 (C176Y) and DhaA14 (I135F) with mutations introduced at residues located in two different tunnels were constructed in order to reveal the importance of these tunnels to the enzymatic activity towards TCP. The mutant enzyme DhaA15 (I135F + C176Y) carries both mutations. The aim of our study was to produce crystals of the haloalkane dehalogenase DhaA mutants



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DhaA04, DhaA14 and DhaA15 of sufficient quality for diffraction experiments. Finally, the results were compared with the known structure of wild-type DhaA (Newman *et al.*, 1999).

2. Materials and methods

2.1. Construction of mutants, expression, purification and holoprotein reconstitution

The mutant recombinant gene *dhaA04* with a C176Y mutation was obtained by inverse PCR using primers 5'-TACGTCGTCCTCCGCTTAC-3' and 5'-TTTCGGGAGCGCACCTC-3'. The methodology has been described previously (Chaloupkova *et al.*, 2003). Plasmid pUC18::*dhaAHis* was used as a template. The gene *dhaA14* was constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) according to the manufacturer's instructions. The same specific complementary primers 5'-GGAATTCATCCGGCCTTCCCGACGTGG-3' and 5'-CCACGTCGGGAAAGCCGGATGAATTCC-3' were used to introduce the I135F mutation into both genes. Different DNA templates were used in mutagenesis: plasmid pAQN::*dhaA04His* in the construction of *dhaA15* and pUC18::*dhaAHis* in the construction of *dhaA14*. The recombinant gene *dhaA14* was recloned into expression vector pAQN identically to *dhaA04*. The mutant recombinant genes *dhaA04* and *dhaA14* were recloned into pAQN vector downstream from the *tac* promoter (*P tac*), which is under the control of *lacIq*, using *Bam*HI and *Hind*III restriction endonucleases and T4 DNA ligase (New England BioLabs, Ipswich, USA; Nagata *et al.*, 1999).

Recombinant plasmids were used to transform chemically competent *Escherichia coli* BL21 cells. The cells were grown at 310 K to an optical density of about 0.6 ($\lambda = 600$ nm) in 2.1 l Luria-Bertani (LB) medium (Sigma-Aldrich, St Louis, USA) with ampicillin (0.1 mg ml^{-1}). Protein expression was induced at 303 K by the addition of isopropyl β -D-1-thiogalactopyranoside to a final concentration of 0.5 mM . After 4 h cultivation, the cultures were harvested by 12 min centrifugation at 3700g. The cells were disrupted by sonication with a Hielscher UP200S ultrasonic processor (Hielscher Ultrasonics, Teltow, Germany) and the lysate was centrifuged at 21 000g for 1 h.

The crude extract was applied onto a HiTrap Chelating column with affinity resin (Amersham Biosciences, Freiburg, Germany) charged with Ni^{2+} and equilibrated using a purification buffer consisting of 20 mM potassium phosphate pH 7.5, 0.5 M sodium chloride and 10 mM imidazole. Purification buffer containing 50 mM imidazole was used to elute unbound and weakly bound fractions. Histidine-tagged proteins were eluted with purification buffer containing 300 mM imidazole. Proteins were purified to homogeneity

using a Superdex 75 100/300 GL gel-filtration column (Amersham Biosciences, Freiburg, Germany) equilibrated with gel-filtration buffer (50 mM Tris-HCl pH 7.5 and 150 mM NaCl).

Eluted proteins were dialyzed against 50 mM Tris-HCl buffer pH 7.5. Protein concentrations were determined by the Bradford method (Sigma-Aldrich, St Louis, USA). SDS-PAGE was run using 15% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R-250 dye (Fluka, Buchs, Switzerland) and the molecular weights of the proteins were determined based on Protein Molecular Weight Markers (MBI Fermentas, Burlington, Canada).

2.2. Crystallization

The purified DhaA04 protein was used for crystallization experiments at a concentration of $15\text{--}18 \text{ mg ml}^{-1}$ in 50 mM Tris-HCl buffer pH 7.5. Crystallization trials were performed at room temperature using Emerald BioStructures CombiClover crystallization plates (EBS plates; Emerald BioSystems, Bainbridge Island, USA) with the sitting-drop vapour-diffusion technique (Ducruix & Giegé, 1999). $1 \mu\text{l}$ DhaA04 protein solution was mixed with an equal volume of reservoir solution (80 mM Bicine pH 9.0, 8% PEG 8000 and 80 mM MgCl_2). Colourless single crystals with dimensions of about $0.20 \times 0.15 \times 0.06 \text{ mm}$ were obtained within a week (Fig. 1a).

The DhaA14 and DhaA15 proteins were crystallized using protein concentrations of $7\text{--}10 \text{ mg ml}^{-1}$ in 50 mM Tris-HCl buffer pH 7.5 by the same crystallization technique at the lower temperature of 277 K. Colourless plate-shaped crystals with similar dimensions of $0.16 \times 0.06 \times 0.02 \text{ mm}$ for DhaA14 and $0.17 \times 0.07 \times 0.02 \text{ mm}$ for DhaA15 grew within 5 d in drops containing $1 \mu\text{l}$ protein solution plus $1 \mu\text{l}$ reservoir solution composed of 25% PEG 4000, 8% 2-propanol and 100 mM sodium acetate (Figs. 1b and 1c). Drops were equilibrated over $600 \mu\text{l}$ reservoir solution in Emerald BioStructures CombiClover crystallization plates.

2.3. Data collection and processing

Diffraction data for the DhaA04 and DhaA14 mutants were collected at beamline X11 of the DORIS storage ring at the EMBL Hamburg Outstation with a monochromatic fixed wavelength of 0.81 \AA using a MAR CCD 165 mm detector. Crystals were mounted in nylon loops (Hampton Research, Aliso Viejo, USA) directly from the drop and flash-frozen in a cold nitrogen stream at 100 K without additional cryoprotection. Reflections for DhaA04 and DhaA14 crystals were observed to resolutions of 1.30 \AA (Fig. 2a) and 0.95 \AA (Fig. 2b), respectively. DhaA04 data sets were collected in two steps. 152 images were recorded in a low-resolution shell ($30\text{--}2.30 \text{ \AA}$) with an oscillation angle of 1° and a crystal-to-detector distance of 200 mm . A high-resolution set of 318 images ($30\text{--}1.20 \text{ \AA}$) was collected with a 0.5° oscillation angle and a crystal-to-detector

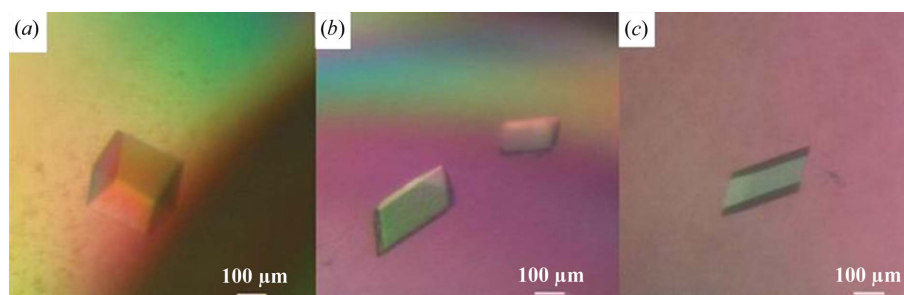


Figure 1 Crystals of three mutants of DhaA protein derived from *R. rhodochrous* NCIMB 13064: (a) DhaA04, (b) DhaA14 and (c) DhaA15.

Table 1

Data-collection statistics for the DhaA04, DhaA14 and DhaA15 crystals.

Values in parentheses are for the highest resolution shell.

Data set	DhaA04	DhaA14	DhaA15
Beamline	X11	X11	X11
Detector	MAR CCD 165 mm	MAR CCD 165 mm	MAR555 flat panel
Resolution range (Å)	30–1.30 (1.32–1.30)	30–0.95 (0.97–0.95)	40–1.15 (1.16–1.15)
Unit-cell parameters (Å, °)	$a = 42.68, b = 76.30, c = 93.10,$ $\alpha = \beta = \gamma = 90$	$a = 42.74, b = 44.40, c = 46.62,$ $\alpha = 115.5, \beta = 97.9, \gamma = 109.5$	$a = 42.46, b = 44.33, c = 46.48,$ $\alpha = 115.5, \beta = 98.4, \gamma = 109.6$
Space group	$P2_12_12_1$	$P1$	$P1$
Redundancy	7.6 (6.3)	5.8 (4.0)	3.9 (3.7)
Measured reflections	575026	941405	356502
Unique reflections	75623	163495	91358
Completeness (%)	100 (99.9)	94.2 (90.6)	94.3 (89.5)
R_{merge}^\dagger (%)	7.5 (45.0)	5.9 (41.8)	4.2 (25.8)
$I/\sigma(I)$	32.0 (4.9)	26.4 (4.4)	11.27 (2.6)
Mosaicity (°)	0.44	0.20	0.21

$$^\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$
, where $I_i(hkl)$ is the intensity of the i th measurement of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of the reflection.

distance of 85 mm. For DhaA14, measurements extended to a Bragg spacing of 0.95 Å and image frames were collected in three steps. 360 low-resolution image frames (30–2.20 Å) were recorded using an oscillation range of 1° and a crystal-to-detector distance of 210 mm. 1° oscillations and a 100 mm crystal-to-detector distance were used to collect 720 images in the medium-resolution shell (30–1.25 Å). Finally, 720 frames were collected for a high-resolution shell (30–0.95 Å) using 0.5° oscillations with a crystal-to-detector distance of 50 mm. Data for the DhaA04 and DhaA14 mutant proteins were integrated and scaled using the *HKL* package (Otwinowski & Minor, 1997).

A single data set for the DhaA15 protein was collected to a resolution of 1.15 Å (Fig. 2c) at beamline X11 at the EMBL Hamburg Outstation using a MAR555 flat-panel detector (MAR Research) with 0.5° oscillation angle and a crystal-to-detector distance of 171 mm. Intensity data were indexed and integrated using *XDS* (Kabsch, 1993). X-ray data-collection statistics for all three mutants are presented in Table 1.

2.4. Preliminary structure solution

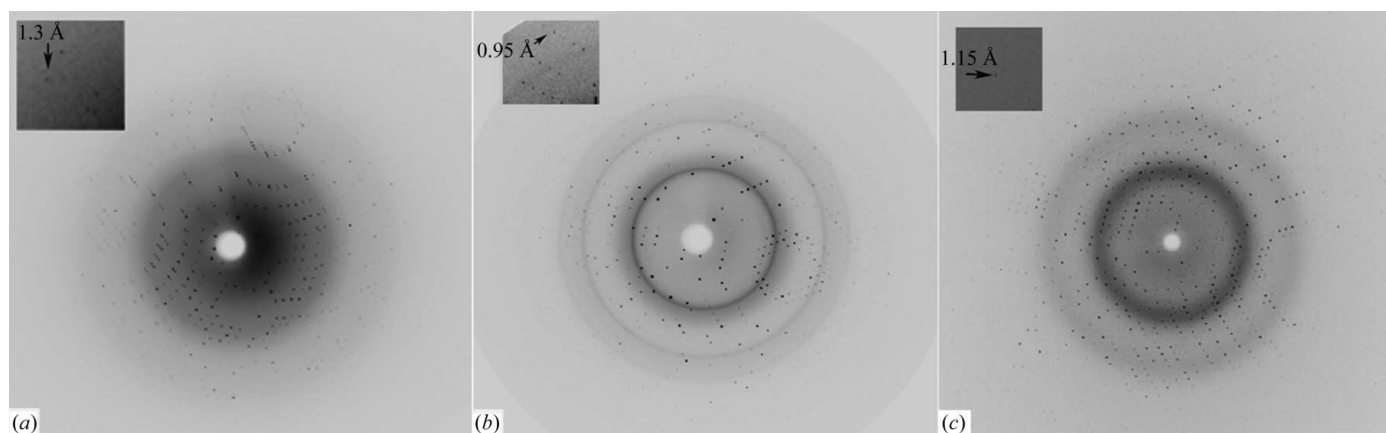
The known structure of the haloalkane dehalogenase from *Rhodococcus* sp. (PDB code 1bn6; Newman *et al.*, 1999), renumbered according to the gene sequence (Kulakova *et al.*, 1997), was used as a template for molecular replacement. Phase calculation and initial

rounds of refinement were performed using *MOLREP* (Vagin & Teplyakov, 1997), *CNS* (Adams *et al.*, 1997) and *REFMAC5* (Murshudov *et al.*, 1997) programs as implemented in the *Auto-Rickshaw* software pipeline (Panjikar *et al.*, 2005).

3. Results and discussion

Initial screening experiments for all three mutants of the haloalkane dehalogenase DhaA were performed using Emerald BioStructures CombiClover crystallization plates (EBS plates; Emerald BioSystems, Bainbridge Island, USA) using the Hampton Research Crystal Screen kit (Hampton Research, Aliso Viejo, USA), the MDL crystal screen kit (Molecular Dimensions Ltd, Suffolk, England) and the JBScreen Mixed Kit (Jena Bioscience GmbH, Jena, Germany). All proteins were crystallized at room temperature using the vapour-diffusion method. Conditions D2 and CS-103 (25% PEG 4000, 8% 2-propanol and 100 mM sodium acetate) of the JBScreen kit were found to be the most suitable for crystallization of the DhaA14 and DhaA15 mutants. The quality and stability of the crystals were further improved by carrying out the experiments at 277 K.

Following unsuccessful trials with DhaA04 using commercial screening kits, in-house solutions with various PEGs as precipitant agents in the pH range 5–10 were tested in crystallization experiments. The first crystals of DhaA04 were obtained at room


Figure 2

Diffraction images of the DhaA4 (a), DhaA14 (b) and DhaA15 (c) mutant proteins. The crystals of DhaA04, DhaA14 and DhaA15 diffracted to resolutions of 1.30, 0.95 and 1.15 Å, respectively.

temperature in a solution containing 100 mM Tris–HCl pH 8.75, 10% PEG 8000 and 100 mM MgCl₂. These crystals dissolved within 3 d. Changing the buffer and decreasing the PEG and MgCl₂ concentrations further improved the crystallization conditions. Stable protein crystals suitable for diffraction experiments were obtained at room temperature within 5 d in drops composed of 80 mM Bicine pH 9.0, 8% PEG 8000 and 80 mM MgCl₂.

Single crystals of the DhaA04, DhaA14 and DhaA15 mutant proteins were used for synchrotron diffraction measurement. Native data sets were collected from DhaA04, DhaA14 and DhaA15 crystals to 1.30, 0.95 and 1.15 Å resolution, respectively. The crystals of DhaA04 belonged to the orthorhombic space group *P*2₁2₁2₁, with unit-cell parameters *a* = 42.68, *b* = 76.30, *c* = 93.10 Å. Scaling and merging of data from low- and high-resolution shells resulted in an overall *R*_{merge} of 7.5%. The Matthews coefficient (*V*_M; Matthews, 1968) is 2.30 Å³ Da⁻¹ and the solvent content of 46% corresponds to the presence of one molecule in the asymmetric unit.

Diffraction data for the DhaA14 mutant were collected in three steps and were processed in the triclinic space group *P*1, with unit-cell parameters *a* = 42.74, *b* = 44.40, *c* = 46.62 Å, *α* = 115.5, *β* = 97.9, *γ* = 109.5°. The presence of one molecule in the asymmetric unit yields a Matthews coefficient (*V*_M) of 2.14 Å³ Da⁻¹, with 43% solvent content.

The crystals of the DhaA15 mutant belong to the triclinic space group *P*1, with unit-cell parameters *a* = 42.46, *b* = 44.33, *c* = 46.48 Å, *α* = 115.5, *β* = 98.4, *γ* = 109.6°. The presence of one molecule in the asymmetric unit yields a Matthews coefficient (*V*_M) of 2.11 Å³ Da⁻¹, with 42% solvent content.

The structures of the three DhaA mutant proteins from *R. rhodochrous* NCIMB 13064 are currently in the process of being further refined and interpreted.

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