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Differences in crystallization of two LinB variants from *Sphingobium japonicum* UT26

Haloalkane dehalogenases are microbial enzymes that convert a broad range of halogenated aliphatic compounds to their corresponding alcohols by the hydrolytic mechanism. These enzymes play an important role in the biodegradation of various environmental pollutants. Haloalkane dehalogenase LinB isolated from a soil bacterium *Sphingobium japonicum* UT26 has a relatively broad substrate specificity and can be applied in bioremediation and biosensing of environmental pollutants. The LinB variants presented here, LinB32 and LinB70, were constructed with the goal of studying the effect of mutations on enzyme functionality. In the case of LinB32 (L117W), the introduced mutation leads to blocking of the main tunnel connecting the deeply buried active site with the surrounding solvent. The other variant, LinB70 (L44I, H107Q), has the second halide-binding site in a position analogous to that in the related haloalkane dehalogenase DbeA from *Bradyrhizobium elkanii* USDA94. Both LinB variants were successfully crystallized and full data sets were collected for native enzymes as well as their complexes with the substrates 1,2-dibromoethane (LinB32) and 1-bromobutane (LinB70) to resolutions ranging from 1.6 to 2.8 Å. The two mutants crystallize differently from each other, which suggests that the mutations, although deep inside the molecule, can still affect the protein crystallizability.

1. Introduction

Halogenated organic compounds represent one of the largest group of environmental pollutants. Microbial enzymes, haloalkane dehalogenases (EC 3.8.1.5), are able to cleave the carbon–halogen bond in a broad range of such halogenated aliphatic pollutants, leading to the formation of a corresponding alcohol, a halide ion and a proton (Janssen *et al.*, 2005). Besides the potential use of these enzymes in bioremediation (Stucki & Thueer, 1995), they can also be applied in biosensing (Campbell *et al.*, 2006; Bidmanova *et al.*, 2010), biosynthesis (Prokop *et al.*, 2010; Westerbeek *et al.*, 2011), cellular imaging and protein analysis (Los & Wood, 2007; Ohana *et al.*, 2009).

Haloalkane dehalogenase LinB was isolated from the Gram-negative soil bacterium *Sphingobium japonicum* UT26 (Nagata *et al.*, 1997). It consists of two domains: an α/β -hydrolase core domain and a helical cap domain, which lies on the top of the core domain. Active-site residues are located in a hydrophobic cavity at the interface between the two domains and are connected to the protein surface by several tunnels (Marek *et al.*, 2000). The size, shape, physico-chemical properties and dynamics of the tunnels are believed to affect the substrate specificity and enzymatic activity of haloalkane dehalogenases (Koudelakova *et al.*, 2011). This work forms part of continuing structural studies of haloalkane dehalogenase LinB.

The studied LinB variants, LinB32 and LinB70, were constructed in order to explore the effects of the introduced mutations on the catalytic properties of LinB. The variant LinB32 (L177W) with a bulky tryptophan residue, blocking the enzyme main tunnel, showed changes in the substrate specificity and limitations in the halide ion release (Chaloupková *et al.*, 2003; Biedermannova *et al.*, 2012). The variant LinB70 (L44I, H107Q) has the second halide-binding site in a position analogous to that of the related haloalkane dehalogenase DbeA from *Bradyrhizobium elkanii* USDA94 (Chaloupkova *et al.*, unpublished results). The variant LinB70 exhibited differences in the

catalytic activity. The crystallization and initial X-ray diffraction analysis of two LinB variants, LinB32 and LinB70, and their complexes with the halogenated substrates 1,2-dibromoethane (LinB32) and 1-bromobutane (LinB70) are reported.

2. Materials and methods

2.1. Protein preparation

The variant LinB32 (L177W) was constructed by inverse PCR with the following oligonucleotides (5'-GCG CAG GAT GTG TCC GGG GAG-3' and 5'-AAC TTG TTC GAC AAA AAC-3') as described previously (Chaloupková *et al.*, 2003). The gene of LinB70 (L44I, H107Q) was synthesized artificially (Entelechon, Regensburg, Germany) according to the wild-type sequence. Both LinB variants had a C-terminal hexahistidyl tail enabling purification by metal-affinity chromatography. To overproduce LinB32 and LinB70 in *Escherichia coli*, the corresponding genes were subcloned into the expression vector pET21b under the control of the T7lac promoter (Novagen, Madison, USA) and gene expression was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG). *E. coli* BL21 (DE3) cells containing the plasmids were cultured in 1 l of Luria broth medium at 310 K. When the culture reached an optical density 0.6 at 600 nm, the induction of enzyme expression (at 293 K) was initiated by the addition of IPTG to a final concentration of 0.5 mM. The cells were harvested and disrupted by sonication using a Soniprep 150 (Sanyo Gallenkamp PLC, Loughborough, UK). The supernatant was used after centrifugation at 277 K and 100 000g for 1 h. The crude extract was further purified on a HiTrap Chelating HP 5 ml column charged with Ni²⁺ ions (GE Healthcare, Uppsala, Sweden) as described previously (Chaloupkova *et al.*, 2013). The purity (~99%) and the protein yield (16 and 20 mg of LinB32 and LinB70, respectively) obtained from 1 l of cell culture after single-step purification were sufficient for the following crystallization experiments. The purified proteins were pooled and dialysed against 50 mM Tris-HCl buffer (pH 7.5) overnight. Both LinB variants containing a C-terminal hexahistidyl tail were stored in the same buffer at 277 K until use.

2.2. Crystallization

All crystallization trials were performed in Emerald BioStructures CombiClover 24-well crystallization plates (Emerald BioSystems, Bainbridge Island, USA) with enzymes not older than 4 d at the concentration of 2–10 mg ml⁻¹ in 50 mM Tris-HCl buffer pH 7.5 using the sitting-drop vapour-diffusion method. 1 or 2 μ l of protein

solution was mixed with the reservoir solution in the ratios 1:3, 1:1 and 3:1 and equilibrated against 800 μ l of the reservoir solution. Various commercial screens were employed for screening of LinB32 and LinB70 initial crystallization conditions including Crystal Screen and Crystal Screen 2 (Hampton Research, Aliso Viejo, USA), PACT, The JCSG+ Suite, The PEGs Suite (Qiagen Ltd, Crawley, UK), Low Ionic Strength Kit (Sigma-Aldrich, St Louis, USA). The obtained crystallization conditions were optimized and a macroseeding procedure (Bergfors, 1999) was used to improve the size and crystal quality. For the macroseeding experiments, LinB70 needle clusters were crushed into smaller pieces by a sharp needle (Hampton Research) and transferred with a nylon loop (Hampton Research) into a receiving drop, containing 2 μ l of protein solution at a concentration of 2.5 mg ml⁻¹ and the reservoir solution in the ratio 1:1. After 7 d of incubation at 277 K the round of macroseeding was repeated. The crystals reached their final size after two to three cycles of such a procedure.

2.3. Diffraction data collection and soaking

2.3.1. LinB32 variant, crystal form A. Diffraction data collection for LinB32 crystal form A was performed on beamline 14.2 operated by the Joint Berlin MX-Laboratory at the BESSY II electron storage ring (Berlin-Adlershof, Germany) (Mueller *et al.*, 2012) equipped with the Rayonics CCD 225 mm detector. The crystal of LinB32 was mounted in a polymer microloop (MiTeGen, Ithaca, USA) directly from the crystallization drop and flash-cooled in a 100 K liquid-nitrogen stream without additional cryoprotection. 120 images were recorded at a wavelength of 0.918 Å, with an oscillation angle of 1° per image and a crystal-to-detector distance of 240 mm. The data set was indexed, integrated and scaled using the graphical user interface XDSAPP (Krug *et al.*, 2012) for running XDS (Kabsch, 2010).

2.3.2. LinB32 variant, crystal form B. The diffraction data for LinB32 crystal form B were collected on the EMBL Hamburg beamline X13 (DESY, Hamburg, Germany) using the MAR CCD 165 mm detector. Crystals were mounted in a nylon loop (Hampton Research) and flash-cooled in a 100 K liquid-nitrogen stream without cryoprotection. 249 images were recorded at a wavelength of 0.812 Å, with an oscillation angle of 0.45° per image and a crystal-to-detector distance of 140 mm. The data were processed using the HKL-3000 package (Minor *et al.*, 2006).

2.3.3. LinB32 variant, crystal form B in complex with 1,2-dibromoethane. The complex of LinB32 with 1,2-dibromoethane was obtained by the addition of 50 μ l of volatile haloalkane (1,2-dibromoethane) to the reservoir solution [6%(v/v) final concentration]. After 10 h of incubation at 298 K and as a result of

Figure 1

Three forms of LinB32 crystals: (a) hexagonal-shaped crystals grown in the presence of KNO₃ (crystal form A), (b) dendritically shaped crystals appeared after initial screening, (c) tetragonal prisms obtained in the presence of MgCl₂ (crystal form B).

Table 1

Data-collection and processing statistics for the crystals of LinB32 and LinB70.

Values in parentheses correspond to the highest-resolution shell.

Protein	LinB32 (crystal form A)	LinB32 (crystal form B)	LinB32 (crystal form B) –1,2-dibromoethane	LinB70	LinB70 –1-bromobutane
Beamline	HZB BL 14.2, BESSY, Berlin	X13, DESY, Hamburg	HZB BL 14.3, BESSY, Berlin	HZB BL 14.2, BESSY, Berlin	HZB BL 14.2, BESSY, Berlin
Detector	Rayonics MX-225	MAR CCD 165 mm	MAR CCD 165 mm	Rayonics MX-225	Rayonics MX-225
Wavelength (Å)	0.91841	0.8123	0.8912	0.91841	0.91841
Crystal–detector distance (mm)	240	140	110	280	150
No. of images	120	249	200	90	135
Oscillation per frame (°)	1	0.45	1	1	1
Resolution range (Å)	50–2.10 (2.23–2.10)	40.5–1.57 (1.63–1.57)	50.0–1.65 (1.74–1.65)	50.0–2.8 (2.9–2.8)	50–1.90 (2.02–1.90)
Space group	$P3_12$ or $P3_22$	$P2_12_12_1$	$P2_12_12_1$	$P6_2$ or $P6_4$	$P6_2$ or $P6_4$
Unit-cell parameters (Å, °)	$a = 155.5$, $b = 155.5$, $c = 122.6$, $\alpha = \beta = 90$, $\gamma = 120.0$	$a = 46.73$, $b = 84.49$, $c = 81.06$, $\alpha = \beta = \gamma = 90.0$	$a = 45.89$, $b = 68.11$, $c = 80.46$, $\alpha = \beta = \gamma = 90.0$	$a = b = 81.57$, $c = 121$, $\alpha = \beta = 90$, $\gamma = 120.0$	$a = b = 81.93$, $c = 121.9$, $\alpha = \beta = 90.0$, $\gamma = 120.0$
Measured reflections	743440	251850	250960	85310	316202
Unique reflections	99134	36500	30859	11225	36369
$R_{\text{meas}}^{\dagger}$ (%)	23.7 (95.0)		15.4 (71.9)		25.7 (126.7)
$R_{\text{merge}}^{\ddagger}$ (%)		8.2 (32.6)		21.0 (48.0)	
Completeness (%)	99.7 (99.6)	98.3 (85.2)	99.5 (98.5)	98.1 (84.0)	99.8 (99.3)
Multiplicity	7.5	6.9	8.1	7.6	8.69
$\langle I/\sigma(I) \rangle$	8.16 (2.31)	21.9 (4.5)	14.7 (3.7)	10.6 (1.9)	8.58 (2.18)

$\dagger R_{\text{meas}}$ is the redundancy-independent merging R factor, also known as R_{int} (Diederichs & Karplus, 1997; Weiss & Hilgenfeld, 1997; Weiss *et al.*, 1998). $R_{\text{meas}} = \sum_{hkl} [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean of the $N(hkl)$ individual measurements $I_i(hkl)$ of the intensity of reflection hkl . $\ddagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is an individual intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of reflection hkl with summation over all data.

evaporation the 1,2-dibromoethane penetrated into the protein crystals. These crystals were mounted in a nylon loop (Hampton Research) and flash-cooled in a 100 K liquid-nitrogen stream without cryoprotection. 200 images were collected on BL14.3 operated by the Joint Berlin MX-Laboratory at the BESSY II electron storage ring (Berlin-Adlershof, Germany) equipped with the MAR CCD 165 mm detector at a wavelength of 0.890 Å, with an oscillation angle of 1° per image and a crystal-to-detector distance of 110 mm. The data set was indexed, integrated and scaled using the graphical user interface *XDSAPP* for running *XDS*.

2.3.4. LinB70 variant and LinB70 in complex with 1-bromobutane.

To obtain the complex of LinB70 with 1-bromobutane, 0.5 µl of haloalkane was added to the drop with crystals to the final concentration of 12% (v/v) and incubated for 20 min. Prior to data collection, the crystals were cryoprotected by soaking them in 50% (w/v) PEG 3350 for 5 s. Diffraction data collection was performed on BL14.2 operated by the Joint Berlin MX-Laboratory at the BESSY II electron storage ring (Berlin-Adlershof, Germany) equipped with the Rayonics CCD 225 mm detector. 90 images from the LinB70 crystal and 135 images from the crystals of LinB70 in complex with 1-bromobutane were recorded at a wavelength of 0.918 Å, with an oscillation angle of 1° per image and a crystal-to-detector distance of 280 and 150 mm, respectively. The data sets of LinB70 and LinB70 in complex with 1-bromobutane were indexed, integrated and scaled using *HKL-3000* (Minor *et al.*, 2006) and the graphical user interface *XDSAPP* for running *XDS*, respectively.

The Matthews coefficient (Matthews, 1968) for all data sets was calculated using the program *MATTHEWS_COEF*, which is a part of the *CCP4* package (Winn *et al.*, 2011).

3. Results and discussion

The wild-type haloalkane dehalogenase LinB from *S. japonicum* UT26 was crystallized previously by Smatanová *et al.* (1999). The

same crystallization conditions were applied for LinB32 and LinB70 variants, but did not yield any promising results. The crystallization experiment resulted in heavy amorphous precipitate.

3.1. LinB32 variant

Various commercial screens were employed for screening of the crystallization conditions of LinB32. Two different crystal forms of LinB32 were obtained at 295 K and the protein concentration of 10 mg ml^{−1}. The crystal form A (Fig. 1a) grew during the initial screening and no further optimization of the crystallization conditions was necessary. The hexagonally shaped crystals with dimensions of about 0.1 × 0.1 × 0.02 mm appeared in condition No. 66 of The PEGs Suite consisting of 0.2 M KNO₃, 25% (w/v) PEG 3350. These crystals were used for data collection without additional cryoprotection and a complete data set was collected at 2.1 Å resolution. The crystals belonged to a primitive hexagonal space group $P3_12$ with systematic absences indicating the presence of 3₁ or 3₂ screw axes with unit-cell parameters of $a = b = 155.5$, $c = 122.6$ Å. Consistent with a Matthews coefficient of $V_M = 3.2$ Å³ Da^{−1}, these crystals contained one molecule per asymmetric unit which corresponds to a solvent content of 61.8%.

Dendritic needles branching in one or more directions were grown in condition No. 95 of The JCSG+ Suite consisting of 0.1 M bis-tris, pH 5.5, 0.2 M MgCl₂, 25% (w/v) PEG 3350 (Fig. 1b). Substitution of bis-tris buffer by MES buffer and variation of the pH resulted in the growth of long tetragonal crystals, which appeared on the second day from the solution consisting of 0.1 M MES, pH 5.6–5.8, 0.2 M MgCl₂, 25% (w/v) PEG 3350 and reached final dimensions of 0.7 × 0.03 × 0.03 mm within the next 2 d (Fig. 1c). These crystals were used for X-ray diffraction data collection and a complete data set was collected to a resolution of 1.6 Å. Crystals belonged to the primitive orthorhombic space group $P2_12_12_1$ with unit-cell parameters of $a = 46.7$, $b = 68.5$, $c = 81.1$ Å. Crystals soaked with 1,2-dibromoethane

Figure 2

Crystallization of LinB70. (a) Microcrystals appeared after decreasing the ionic strength of the precipitant and the protein concentration. (b) One-dimensional needle clusters grew after decreasing the temperature. (c) Three-dimensional crystals grown from macroseeds.

belonged to the same crystal form and diffracted to 1.65 Å resolution. The calculated value of the Matthews coefficient (V_M) is 2.0 Å³ Da⁻¹ with a solvent content of 36.8% considering one molecule in the asymmetric unit. For data-collection statistics see Table 1.

3.2. LinB 70 variant

In order to find preliminary crystallization conditions, initial screening was performed at 295 K and the protein concentration of 10 mg ml⁻¹, yielding heavy amorphous precipitations. Reduction of the protein concentration to 5 mg ml⁻¹ together with reduction of ionic strength of the precipitant by employing a Low Ionic Strength Kit (Sigma) led to the formation of microcrystals in conditions No. 22 [0.05 M MES–Na buffer pH 6.0, 12% (w/v) PEG 3350], No. 23 [0.05 M MES–Na pH 6.0, 20% (w/v) PEG 3350] and No. 30 [0.05 M imidazole–HCl pH 7.0, 12% (w/v) PEG 3350] after 5 d of incubation (Fig. 2a). Further optimization by varying the buffer pH and PEG concentration together with lowering of the incubation temperature to 277 K yielded tiny needle clusters (Fig. 2b), which were grown within 2 weeks in conditions consisting of 50 mM MES pH 5.8, 20% (w/v) PEG 3350. In order to improve the crystal quality, a series of macroseeding cycles was carried out. Large needles with dimensions of approximately 0.6 × 0.02 × 0.02 mm (Fig. 2c) suitable for X-ray diffraction and soaking experiments were grown in about 20 d after two to three cycles of macroseeding using previously optimized crystallization conditions. Crystals of LinB70 and LinB70 soaked with 1-bromobutane diffracted to 2.2 and 1.9 Å resolution, respectively, and both belonged to the primitive hexagonal *P*6 space group with systematic absences indicating the presence of 6₂ or 6₄ screw axes with unit-cell parameters of $a = b = 81.9$, $c = 121.6$ Å. Analysis of the Matthews coefficient suggested one LinB70 molecule per asymmetric unit ($V_M = 3.5$ Å³ Da⁻¹ with 65% solvent content). All data-collection and processing statistics are summarized in Table 1.

Further work on the determination of LinB32 and LinB70 structures by molecular replacement, using the coordinates of LinB wild-type (PDB code 1cv2, Marek *et al.*, 2000) is currently in progress. The structure determination of LinB32 and LinB70 variants will help us to obtain detailed information about structural changes affecting the catalytic properties of enzymes.

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