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Biochemical and structural characterisation of a haloalkane dehalogenase from a marine *Rhodobacteraceae*

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ABSTRACT

A putative haloalkane dehalogenase has been identified in a marine *Rhodobacteraceae* and subsequently cloned and over-expressed in *Escherichia coli*. The enzyme has highest activity towards the substrates 1,6-dichlorohexane, 1-bromooctane, 1,3-dibromopropane and 1-bromohexane. The crystal structures of the enzyme in the native and product bound forms reveal a large hydrophobic active site cavity. A deeper substrate binding pocket defines the enzyme preference towards substrates with longer carbon chains. Arg136 at the bottom of the substrate pocket is positioned to bind the distal halogen group of extended di-halogenated substrates.

1. Introduction

Haloalkane dehalogenases (HLDs) (EC 3.8.1.5) catalyse the conversion of halogenated alkanes into their corresponding alcohol products and hydrogen halides. They have potential applications in biocatalysis, biosensors and cell imaging, as well as in the bioremediation of recalcitrant and carcinogenic halogenated by-products from organic synthetic reactions and halogenated pesticides and insecticides [1,2].

The HLDs from the bacteria *Xanthobacter autotrophicus* (DhlA) [3,4], *Rhodococcus rhodochrous* (DhaA) [5], *Sphingomonas paucimobilis* (LinB) [6,7], *Bradyrhizobium japonicum* USDA110 (DbjA) [8,9], *Mycobacterium tuberculosis* (DmbC) [10] and *Plesiocystis pacifica* SIR-1 (DppA) [11] have been biochemically and structurally characterised and are two domain proteins. The core domain belongs to the α/β hydrolase protein fold family with the active site located on the interface of the core and the cap domain, the latter determining substrate specificity. More recently, the first eukaryotic HLD from a purple sea urchin has been biochemically characterised [12].

The HLD enzymatic reaction proceeds via a S_N2 type nucleophilic substitution mechanism [1]. The first step is the nucleophilic attack by the carboxyl group of the conserved aspartate on the carbon atom attached to the halogen of the substrate. This produces an ester intermediate complex where the carboxyl oxygen of the catalytic aspartic acid replaces the halogen. The second step of the reaction is the nucleophilic attack on the carboxyl group of the ester intermediate by a water molecule to release the alcohol product. For the reaction to proceed, the catalytic aspartate oxygen which is not involved in the ester intermediate adduct has to be positioned in the oxyanion hole formed by the main chain nitrogens. The catalytic water is activated by a histidine/carboxylic acid dyad.

Crystal structures of different HLD enzymes have allowed the identification of the catalytically important residues, which have been confirmed by site-directed mutagenesis [4,6,13]. A catalytic pentad has been identified consisting of a catalytic triad of Asp, His and Asp/Glu and a Trp-Trp or Trp-Asn pair, which stabilises the halide leaving group [1]. The Trp-Trp or Trp-Asn pair of residues are essential for activity and are responsible for binding

Abbreviations: HLD, haloalkane dehalogenase; L-HAD, L-Haloacid dehalogenase; DhlA, *Xanthobacter autotrophicus* haloalkane dehalogenase; DhaA, *Rhodococcus rhodochrous* haloalkane dehalogenase; DmbC, *Mycobacterium tuberculosis* haloalkane dehalogenase; LinB, *Sphingomonas paucimobilis* haloalkane dehalogenase; DbjA, *Bradyrhizobium japonicum* USDA110 haloalkane dehalogenase; DppA, *Plesiocystis pacifica* SIR-1 haloalkane dehalogenase; DspA, *Stronglocentrotus purouratus* haloalkane dehalogenase; HanR, *Rhodobacteraceae* family haloalkane dehalogenase; 1HO, 1-hexanol; BAM, benzamidine; PMSF, phenylmethysulfonyl fluoride

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the substrate in the correct orientation by stabilizing both the transition state and the halide ion released during the carbon–carbon bond break. The division of HLDs into three phylogenetic subfamilies was defined by Chovancova et al. in 2007 [14], depending on the amino acid content of the catalytic pentad. Classification of these HLDs into four groups based on substrate specificity revealed there was no clear correlation between substrate specificity and phylogenetic subfamilies [15]. More recently, the characterisation of a HLD from *Agrobacterium tumefaciens* that has a unique halide-stabilizing tyrosine residue (Tyr109) in place of the conserved Trp residue [16] has required an extension of the existing phylogenetic families.

Over the last decade the marine environment has been recognised as a potential source of novel enzymes [17]. The oceans are known to contain abundant organohalogens, which are thought to be produced mainly by marine algae. Some species of polychaete tube worms also produce a wide range of structurally diverse halogenated compounds [18]. These compounds are often cytotoxic and are thought to be part of the organism's defence mechanisms. Microorganisms living in symbiosis with algae and tube worms may have evolved detoxification enzymes such as dehalogenases.

In the search of novel dehalogenase enzymes, a marine *Rhodobacteraceae* species was isolated from the surface of a tube worm. The wild-type bacterium tested positive for L-haloacid dehalogenase (L-HAD) activity. In order to obtain sufficient quantities of this enzyme for biochemical characterisation, the *Rhodobacteraceae* genome was partially sequenced so that the gene could be cloned and the protein over-expressed [19]. In addition to the presence of a novel L-HAD which has been biochemically and structurally characterised [19], a putative HLD was also identified called HanR.

This paper describes the biochemical and structural characterisation of a HLD enzyme from a marine source which has been isolated from the surface of a tube worm.

2. Material and methods

2.1. Gene identification, cloning and overexpression

A *Rhodobacteraceae* family bacterium (Rhb) isolated from a Polychaeta worm was collected from Tralee beach, Argyll, UK. The Rhb genomic DNA was extracted and the genome was sequenced using an Illumina GA2 sequencer. As there was low genome identity to previously sequenced genomes, *De novo* assembly was used to arrange the 72 bp paired-end reads into 1082 contigs using Velvet, version 0.7.63 [20]. A preliminary genome of 3.77 Mbp was assembled into 795 contigs. A putative HLD gene called *HanR* was identified using the NCBI BLAST tool [21] on a Galaxy bioinformatics pipeline [22]. The *HanR* gene was cloned from genomic DNA into the pET-28a plasmid with a N-terminal His-tag. The HanR protein was over-expressed in BL21-CodonPlus (DE3) *Rosetta2 Escherichia coli*.

The cell pellet was re-suspended in buffer A (0.1 M Tris–HCl, 0.1 M NaCl, 0.5 mM EDTA, 1 mM BAM, 1 mM PMSF, pH 8.2) containing 0.01 M imidazole, lysed by sonication and centrifuged to remove cell debris. A nickel affinity chromatography column (GE Healthcare) was equilibrated with buffer A, the cell extract loaded and the unbound protein washed off with buffer A containing 0.01 M imidazole. The bound protein was eluted in buffer A with 0.5 M imidazole.

The fractions corresponding to the protein were concentrated using a 10 kDa membrane (Vivaspin 20, Vivascience) at 3000×*g*, at 4 °C until the final volume reached 1 ml. The concentrated protein sample was further purified on a 120 ml Superdex 75 GF chromatography column which was eluted over 1 column volume of buffer A.

2.2. Enzyme activity

The HLD activity was measured using a modified colorimetric assay, based on the method of Holloway et al. [23]. The assay solution had a final concentration of 1 mM HEPES, 1 mM EDTA, 20 mM sodium sulfate, 10 mM substrate, 20 μg/ml phenol red, pH 8.2. To initiate the assay, 180 μl of dehalogenase assay solution was mixed with 20 μl of purified protein (2 mg/ml). The reaction was followed by measuring a decrease in absorbance at 540 nm over 1 h. To produce a standard curve, the dehalogenase assay solution was mixed with HCl to a final concentration between 0 and 2 mM in a total volume of 200 μl.

2.3. Crystallization

The purified HanR enzyme was concentrated until a final concentration of 10 mg/ml was reached. The concentrated protein was subjected to microbatch crystallization screens at 18 °C using an Oryx Robot (Douglas Instruments). The best crystals were grown from a mixture of equal volumes of protein and precipitant solution containing 0.15 M $MgCl_2$, 0.1 M Tris–HCl and 15% PEG 4000, pH 8.0. The native crystals were frozen using a cryoprotectant containing 0.1 M Tris–HCl pH 8.0, 0.1 M NaCl, 25% PEG 4000 and 30% PEG 400. The substrate soak was conducted by soaking the HanR crystal for 1 min in 0.1 M Tris–HCl, 20% PEG 3350, 30% PEG 400, 25 mM 1-bromohexane, pH 4.0.

2.4. Data collection and structure determination

Diffraction data were collected at the Diamond Light Source synchrotron, UK. Data were processed and scaled with the programs XDS [24] and Aimless [25] using the Xia2 [26] pipeline. Further data analysis and refinement were performed using the CCP4 suite of programs [27].

The HanR native structure was solved by molecular replacement (MR) with the program MOLREP [28] using the LinB model (PDB: 1CV2; [6]). The MR solution was submitted to an automated refinement procedure using ARP/wARP version 7.0.1 [29]. The resulting model was manually rebuilt in COOT [30] and refined with REFMAC5 [31].

The structure of the product bound 1-hexanol (1HO) complex was solved by MR using the refined structure of the native HanR. The dictionary definition for the ligand was built using JLIGAND [32]. The quality of the structures was checked using the program PROCHECK [33]. The program PyMOL [34] was used to produce Figs. 2a, 3 and 4.

3. Results and discussion

3.1. Biochemical characterisation

The specific activity of the HanR was measured with a variety of substrates (Fig. 1). The HanR is active towards both chloroalkanes and bromoalkanes with preference towards longer chain linear substrates.

Activity of HanR towards brominated compounds appears to be higher than towards their chlorinated equivalents, which is not surprising, as the energy of C–Br bond is lower than that of C–Cl. This is also in line with the abundance of brominated organic compounds in the marine environment. The DppA HLD from the marine bacteria *Plesiocystis pacifica* [11] is not active towards any chlorinated compounds. Substrate variations between the HLD enzymes are thought to arise from differences in the size, shape and hydrophobicity of the cap domain [14]. The differences in substrate specificity are difficult to infer from protein sequence only.