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Crystallization and preliminary X-ray analysis of an enantioselective halohydrin dehalogenase from *Agrobacterium radiobacter* AD1

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Halohydrin dehalogenases are key enzymes in the bacterial degradation of vicinal halopropanols and structurally related nematocides. Crystals of the enantioselective halohydrin dehalogenase HheC from *Agrobacterium radiobacter* AD1 have been obtained at room temperature from hanging-drop vapour-diffusion experiments against 50–70% saturated ammonium sulfate solution at pH 6.5–7.3. The crystals belong to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 104.5$, $c = 121.4$ Å, and contain two monomers in the asymmetric unit. The crystals diffract to 3.0 Å resolution with X-rays from a Cu $K\alpha$ rotating-anode generator.

1. Introduction

Haloorganic compounds are widespread in nature. While most of these compounds originate from both biotic and abiotic natural sources, many short-chain halogenated compounds are produced industrially on a large scale. The intentional and accidental introduction of several of these latter compounds into the environment has caused important pollution problems because of their persistency and toxicity. Fortunately, bacteria have been found that are able to degrade some of these halogenated hydrocarbons and even use them as a growth substrate. Often, these bacteria use dehalogenases to break the bond between a carbon and a halogen. It is no surprise that some of these enzymes have been extensively studied in view of their detoxifying properties (Fetzner & Lingens, 1994; Fetzner, 1998).

Enzymes that convert vicinal halohydrins form a special dehalogenase subclass. These halohydrin dehalogenases are also referred to in the literature as haloalcohol dehalogenases, halohydrin hydrogen-halide lyases or halohydrin epoxidases. They catalyze the reversible conversion of a vicinal haloalcohol such as 2,3-dichloro-1-propanol to its corresponding epoxide by the intramolecular nucleophilic substitution of the halogen by the vicinal hydroxyl group, thereby releasing a proton and a halide ion (Fig. 1) (Castro & Bartnicki, 1968; Bartnicki & Castro, 1969; van den Wijngaard *et al.*, 1991; Poelarends *et al.*, 1999; van Hylckama Vlieg *et al.*, 2001). Substrates include not only chlorinated and brominated C_2 and C_3 alcohols such as 2-chloroethanol, 1,3-dichloro-2-propanol and 2,3-dichloro-1-propanol and their brominated analogues, but also aromatic halohydrins such as 1-chloro-2-phenylethanol. The observation that the dehalogenation of chiral halohydrins can proceed with high enantioselectivity has attracted broad interest in these enzymes (Nakamura *et al.*, 1992, 1994; Assis *et al.*, 1998; Lutje Spelberg *et al.*, 1999, 2001; van Hylckama Vlieg *et al.*, 2001).

The amino-acid sequences of six halohydrin dehalogenases have been obtained so far (Yu *et al.*, 1994; Poelarends *et al.*, 1999; van Hylckama Vlieg *et al.*, 2001). Based on their sequences, the six enzymes have been classified into three groups of two enzymes each, which are referred to as groups A, B and C (van Hylckama Vlieg *et al.*, 2001). Within the groups, the members share more than 80% sequence identity, whereas the pairwise sequence identity between the groups is only 25–35%. There are striking differences in enantioselectivity for the conversion of substrates by enzymes of different groups. For example, the enantioselectivities of $HheB_{GP1}$ from group B and HheC from group C are the opposite of each other for aromatic halohydrins such as 2-chloro-1-phenylalcohol (van Hylckama Vlieg *et al.*, 2001).

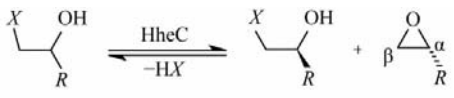

Figure 1
Schematic representation of the reversible reaction catalysed by the enantioselective halohydrin dehalogenase from *A. radiobacter* AD1. The enzyme preferentially converts one enantiomer of a racemic mixture of a vicinal halohydrin. The backward reaction, the epoxide ring opening by a nucleophile, is not only enantioselective but also highly β-regioselective.

Sequence comparisons with other protein sequences showed that halohydrin dehalogenases are homologous to members of the short-chain dehydrogenase/reductase (SDR) family (Jörnvall *et al.*, 1995; van Hylckama Vlieg *et al.*, 2001). SDR proteins catalyze the reversible conversion of a hydroxyl group into a ketone or aldehyde group using $NADP^+$ or NAD^+ as a cofactor. Halohydrin dehalogenases lack the specific signature of a nucleotide-binding sequence motif, which is in agreement with the observation that they do not need a cofactor. On the other hand, the catalytic serine and tyrosine residues of the SDR proteins are strictly conserved in halohydrin dehalogenases, although the catalytic lysine is conservatively replaced by an arginine (van Hylckama Vlieg *et al.*, 2001). van Hylckama Vlieg and coworkers have proposed a catalytic mechanism for the halohydrin dehalogenases based on the well studied catalytic mechanism of the SDR proteins (Jörnvall *et al.*, 1995). The hydroxyl group of a halohydrin is thought to be hydrogen bonded to the catalytic serine and tyrosine residues. The arginine residue is proposed to increase the pK_a of the tyrosine residue, thereby enabling the tyrosine to abstract a proton from the vicinal hydroxyl group of the halohydrin. The activated hydroxyl group can then perform a nucleophilic attack on the β-carbon atom, resulting in the cleavage of the carbon–halogen bond and the concurrent ring closure of the epoxide. To favour the breaking of the carbon-halogen bond, the halogen atom/halide ion is possibly stabilized by a halide-binding site, as has been observed in the structures of several other types of dehalogenases (Verschueren *et al.*, 1993; Ridder *et al.*, 1999).

The reverse reaction, the ring opening of epoxides by a nucleophile catalyzed by halohydrin dehalogenases, has also been studied. This resulted in the observation that not only chloride and bromide ions but also azide and cyanide ions can serve as a nucleophile in the reaction catalyzed by these enzymes (Nakamura *et al.*, 1991; Lutje Spelberg *et al.*, 2001). For some substrates, the nucleophilic ring opening of epoxides also proceeds with high enantioselectivity (Nakamura *et al.*, 1991; Lutje Spelberg *et al.*, 2001). Furthermore, the nucleophilic epoxide-ring opening catalyzed by halohydrin dehalogenases may proceed with a different regioselectivity compared with the chemical ring opening. It has been shown that the azidolysis of (substituted) styrene oxides by the halohydrin dehalogenase from *A. radiobacter* AD1 (HheC) is not only highly enantioselective but also highly β-regioselective (Lutje Spelberg *et al.*, 2001). These properties give this enzyme a great potential for use as a biocatalyst in the regio- and enantioselective synthesis of chiral epoxides, haloalcohols, azidoalcohols and cyanoalcohols.

We have started a crystallographic investigation of halohydrin dehalogenases to gain insight into the structural details of the catalytic machinery of these enzymes. Such knowledge may assist in the rational improvement of the catalytic properties and substrate/product preferences of these enzymes.

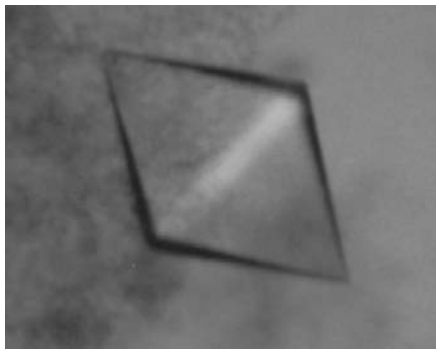

Figure 2
A crystal of halohydrin dehalogenase HheC from *A. radiobacter* AD1.

2. Crystallization, data collection and processing

A. radiobacter halohydrin dehalogenase C (HheC) was prepared as described previously (van Hylckama Vlieg *et al.*, 2001). Crystals were obtained from hanging-drop experiments at room temperature using drops consisting of 2 µl of protein and 2 µl of reservoir solution suspended over 1 ml of reservoir solution. A reservoir solution containing 50–70% saturated ammonium sulfate solution (saturated at room temperature) in 100 m*M* bis-Tris [bis-(2-hydroxyethyl)imino-tris(hydroxymethyl)-methane] buffer pH 6.5–7.1 resulted in the formation of multiple bipyramidal crystals of dimensions up to $0.4 \times 0.3 \times 0.3$ mm within two weeks (Fig. 2). The hanging drops initially show some protein precipitation, but this does not prevent crystal formation. Addition of 5 m*M* DTT to the reservoir solution prevents this precipitation, which suggests that free cysteine residues in the protein are its likely cause.

A native data set to 3.0 Å was collected in-house at room temperature on a MacScience image-plate system using Cu $K\alpha$ radiation from a rotating-anode generator. The data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The data set contained 148 242 measurements, which were reduced to 13 874 unique reflections with an average $R_{\rm merge}$ of 13.0% on intensities. The completeness of the data is 99.5%. The crystals belong to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 104.5$, c = 121.4 Å. Data statistics are given in Table 1. This results in a unit-cell volume of 1.33×10^6 Å^3. With two monomers of approximately 27.5 kDa in the asymmetric unit, the volume per unit mass, $V_{\rm M}$, is 3.01 Å^3 Da^{-1} (Matthews, 1968). These values are within the range (1.7–3.5 Å^3 Da^{-1}) usually found for proteins. The calculated solvent content is 59%, assuming a specific volume of 0.74 cm^3 g^{-1} for the protein molecule.

Table 1
Crystal data and X-ray diffraction data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Cu $K\alpha$
Wavelength (Å)	1.5418
Temperature (K)	293
Space group	$P4_32_12$ or $P4_12_12$
Unit-cell parameters (Å)	$a = b = 104.51$, $c = 121.44$
Resolution (Å)	3.11 (3.17–3.11)
Completeness (%)	99.5 (100)
$R_{\rm merge}$ (%)	13.0 (38.0)
$I/\sigma(I)$	12.1 (4.4)
Total observations	148242
Unique reflections	13874

Preliminary phases for HheC crystals have been obtained by a multiple anomalous dispersion (MAD) experiment around the Br edge on HheC crystals obtained in the presence of NaBr. A full description of the procedure for obtaining useful phase information will be published elsewhere.

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