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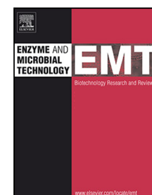

A single point mutation enhances hydroxynitrile synthesis by halohydrin dehalogenase

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ABSTRACT

The cyanide-mediated ring opening of epoxides catalyzed by halohydrin dehalogenases yields β-hydroxynitriles that are of high interest for synthetic chemistry. The best studied halohydrin dehalogenase to date is the enzyme from *Agrobacterium radiobacter*, but this enzyme (HheC) exhibits only low cyanolysis activities. Sequence comparison between a pair of related halohydrin dehalogenases from *Corynebacterium* and *Mycobacterium* suggested that substitution of a threonine that interacts with the active site might be responsible for the higher cyanolytic activity of the former enzyme. Here we report that a variant of HheC in which this substitution (T134A) is adopted displays an up to 11-fold higher activity in cyanide-mediated epoxide ring-opening. The mutation causes removal of the hydrogen bond between residue 134 and the side chain O of the active site serine 132, which donates a hydrogen bond to the substrate oxygen. The mutation also increases dehalogenase rates with various substrates. Structural analysis revealed that the anion-binding site of the mutant enzyme remained unaltered, showing that the enhanced activity is due to altered interactions with the substrate oxygen rather than changes in the nucleophile binding site.

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1. Introduction

β-Hydroxynitriles are valuable building blocks for preparative purposes due to the synthetic versatility of the nitrile group [1–3]. They provide easy access to aminoalcohols which are important precursors in asymmetric synthesis and medicinal chemistry [4–6]. Nitrile groups can in turn be enzymatically converted to amides [7] or carboxylates [8,9]. Some β-hydroxynitriles can be prepared via biocatalytic approaches, such as reduction of β-keto nitriles [10,11] and lipase-catalyzed hydrolysis of β-hydroxynitrile acetates [12–16]. An alternative biocatalytic conversion yielding β-hydroxynitriles is the ring-opening of epoxides with cyanide as a nucleophile, a reaction catalyzed by halohydrin dehalogenases [17–20] (Fig. 1). This reaction has been explored for the synthesis of the side chain of the cholesterol-lowering drug atorvastatin [21,22].

The halohydrin dehalogenases are structurally and mechanistically related to the short-chain dehydrogenase/reductase (SDR) protein family [23–25]. Based on sequence similarities, the enzymes have been grouped into three distinct types, namely type A, B and C [23]. For each of type A and B, two enzymes have been investigated biochemically. The type A enzymes are $HheA_{Cor}$ from *Corynebacterium* sp. strain N-1074 [26] and $HheA_{Art}$ from *Arthrobacter* sp. strain AD2 [23]. Type B enzymes include $HheB_{Cor}$ from strain N-1074 [26] and $HheB_{Myc}$ from *Mycobacterium* sp. strain GP1 [23]. However, the type C enzyme from *Agrobacterium radiobacter* (HheC) is the best studied halohydrin dehalogenase, with several crystal structures [24,27] and biochemical data [28–30] available. The structural and biochemical studies revealed the presence of a substrate binding site in which a conserved tyrosine (Y145 in HheC) abstracts a proton from the substrate alcohol group. This is facilitated by a serine (S132) that is

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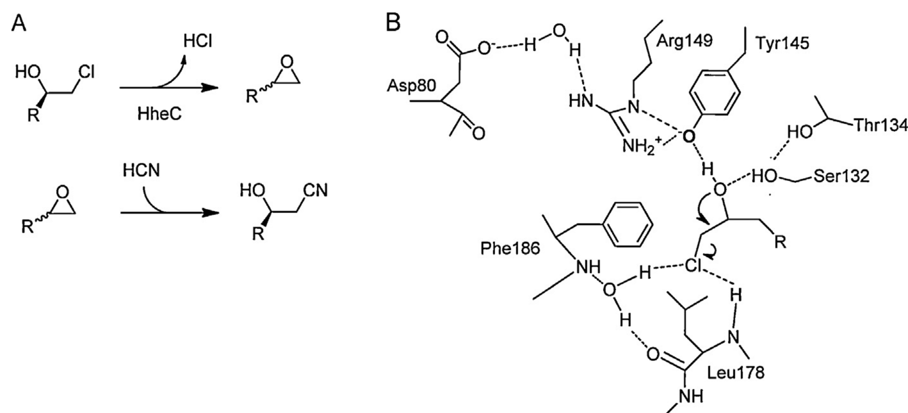

Fig. 1. Halohydrin dehalogenase catalyzed-reactions. (A) Dehalogenation and cyanide-mediated epoxide-ring opening. (B) Catalytic mechanism of the dehalogenase reaction, with formation of an epoxide.

hydrogen-bonded to the hydroxyl and an arginine (R149) involved in transfer of the proton to the solvent, via a conserved aspartate (D80). The negative charge resulting from proton abstraction results in an intramolecular nucleophilic attack of the oxygen on the halogen-substituted carbon. Displacement of the halogen, with formation of a halide and epoxide, is facilitated by the halide-binding site. The latter can accommodate other small anions, allowing the reverse reaction (Fig. 1B).

The role of halohydrin dehalogenases in their natural bacterial host is the conversion of vicinal halohydrins to epoxides during the microbial degradation of haloalcohols [23]. The unusual carbon–carbon bond formation occurring during cyanide-mediated epoxide ring opening is an important example of the catalytic versatility in the reverse reaction, in which various anionic nucleophiles are accepted [20,31–36]. HheC catalyzes epoxide ring formation with chemically diverse halohydrins with rates up to 47 μmol min^{-1} mg^{-1} [29]. In the case of ring-opening reactions, $HheA_{Art}$, $HheB_{Myc}$ and HheC display initial activities of 0.1 to 2.2 μmol min^{-1} mg^{-1} for a range of epoxides [19]. Thus, cyanolysis rates are lower than dehalogenation activities. For example, the rate of cyanolysis of *rac-p*-nitrostyrene oxide catalyzed by HheC was more than 17-fold lower than corresponding epoxide ring-opening reaction with chloride [32]. The highest rate in cyanide-mediated epoxide ring opening with HheC was 5.4 μmol min^{-1} mg^{-1} with epoxybutane (**1**) as the substrate [34]. In contrast, with $HheB_{Cor}$ rates of epoxybutane **1** and epichlorohydrin cyanolysis were 90.9 and 27.8 μmol min^{-1} mg^{-1}, respectively [17,18].

In the present study we use sequence and activity comparisons to generate a HheC mutant with higher cyanolysis activity and study the effect of mutations on the catalytic properties of HheC.

2. Materials and methods

2.1. Sequence-structure analysis and modelling

Multiple sequence alignments of of $HheA_{Art}$ (GenBank: AAK92100), $HheA_{Cor}$ (BAA14361), $HheB_{Cor}$ (BAA14362), $HheB_{Myc}$ (AAK73175) and HheC (AAK92099) were generated using Expresso v9.02 [37]. The SWISS-MODEL server was used in alignment mode [38] to generate structural models for $HheB_{Cor}$, $HheB_{Myc}$ and T134A + C153S HheC based on Expresso alignments (Fig. 2) using HheC (PDB 1ZMT) [27] as template.

2.2. Mutagenesis and enzyme preparation

All mutations were introduced in the less oxygen susceptible C153S mutant of HheC [39] using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. For T134A + C153S HheC, plasmid pBAD_C153S_HheC [40] was used as a template together with primer pair FwT134A (5′-TTATTACCTCTGCA**GCG**CCCTTCGGGCCTTGGAAG, mutated codon in bold) and RvT134A (5′-CAAGGCCCGAAGGG**CGC**TGCAGAGGTAATAAAGAT) resulting in plasmid pBAD_T134A + C153S_HheC. T134A + C153S + W249F HheC was constructed similarly using plasmid pBAD_T134A + C153S_HheC as template and primers used previously [28]. Mutations were confirmed by sequencing (GATC Biotech, Konstanz, Germany). Enzymes were recombinantly expressed in *Escherichia coli* MC1061 and purified as described before [41].

2.3. Enzyme assays and kinetic measurements

Epoxide cyanolysis activities were determined based on cyanide depletion rates in biocatalytic reactions with 20 mM epoxide [1,2-epoxybutane (**1**), 2-methyl-1,2-epoxybutane (**2**), 3,3-dimethyl-1,2-epoxybutane (**3**), or 1,2-epoxyhexane (**4**)] in 50 mM phosphate buffer, pH 8.0 at 30 °C. The concentration of cyanide was monitored over reaction time via the specific absorption at 267 nm of the complex ion $[Ni(CN)_4]^{2-}$, which formed after mixing of each 150 μL of sample and 2 mM $NiCl_2$ in 1 M NH_3 [42]. Reactions were initiated by addition of a suitable amount of purified enzyme in a total volume of 2 mL containing varying concentrations of cyanide. The absorbance of the complex ion $[Ni(CN)_4]^{2-}$ was read at 267 nm in UV-Star 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) and changes in cyanide concentration were obtained with the help of a standard curve (linear up to 2 mM cyanide).

Kinetic resolution reactions with racemic epoxides were carried out in a total volume of 20 mL at 30 °C in 50 mM potassium phosphate buffer, pH 8.0, containing 5 mM racemic epoxides **1**, **2**, **3** and racemic styrene oxide (**5**) with 15 mM cyanide. Conversions were initiated by addition of 0.1 to 1 mg enzyme, dependent on the activity of the protein under study. Samples were taken periodically and extracted with equal volumes of diethyl ether containing mesitylene as internal standard. Organic extracts were dried over anhydrous sodium sulphate and were analyzed by chiral GC as described elsewhere [19]. Both conversion and enantiomeric excess values were calculated from relative peak areas, providing numbers for the enantiomeric ratio E according to Chen et al. [43].

Kinetic constants for the dehalogenation of 1,3-dichloro-2-propanol (**6**), 1,3-dibromo-2-propanol (**7**), 1-chloro-2,3-propanediol (**8**), 1-bromo-2,3-propanediol (**9**), 1-chloro-2-propanol (**10**), 1-chloro-2-methyl-2-propanol (**11**), and 2-chloro-1-phenylethanol (**12**) were obtained from reactions in 50 mM Tris·SO_4 buffer, pH 7.5, at 30 °C. Reactions were carried out in a volume of 2 mL and started by addition of purified enzyme. Halide release rates were measured using a colorimetric assay described by Bergmann and Sanik [44]. Briefly, 150 μL sample was mixed with 150 μL assay solution which was prepared freshly before use by mixing equal volumes of solution I (0.25 M $NH_4Fe(SO_4)_2$ in 9 M HNO_3) and solution II (saturated solution of $Hg(SCN)_2$ in absolute ethanol). Halide release led to an increase of absorption at 460 nm. The linear range of this assay is up to 1 mM for bromide. For chloride concentrations up to 1 mM, the standard curve was fitted by a 3rd degree polynomial.

Kinetic parameters were obtained by fitting the Michaelis–Menten equation, or in case of substrate inhibition by fitting with the following equation:

$$V = \frac{k_{cat} \times S}{K_M + S \times \left(1 + \left(S/K_i\right)\right)} \quad (1)$$

2.4. Determination of ligand binding constants by tryptophan fluorescence

Changes in protein fluorescence properties upon titration with chloride, bromide or cyanide were followed on a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA). Enzyme solutions were diluted to 50 μg mL^{-1} in 50 mM Tris·SO_4 buffer, pH 7.5, at 30 °C. Emission spectra from 300 to 400 nm were recorded after excitation at 290 nm. The change in fluorescence intensity during titration with ligand stock solution was determined at the respective maximum emission wavelengths (λ_{max} values: HheC, 343 nm; C153S HheC, 344 nm; T134A + C153S HheC, 347 nm). Values were corrected for dilution (total volume change below 10%).

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