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Exploration of the biocatalytic potential of a halohydrin dehalogenase using chromogenic substrates

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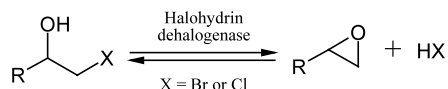
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Abstract—Halohydrin dehalogenases are bacterial enzymes that catalyse the reversible formation of epoxides from *vicinal* halohydrins. A spectrophotometric assay for halohydrin dehalogenases based on the absorption difference between the halohydrin *para*-nitro-2-bromo-1-phenylethanol and the epoxide *para*-nitrostyrene oxide was developed. The enantioselectivity of ring-closure reactions catalysed by three different halohydrin dehalogenases could be estimated from the shape of progress curves. Evaluation of ring-opening reactions catalysed by halohydrin dehalogenase from *Agrobacterium radiobacter* AD1 established that, in addition to Cl⁻ and Br⁻, nucleophiles such as N₃⁻, CN⁻ and NO₂⁻ are also accepted for the ring opening of *para*-nitrostyrene oxide. The ring-opening reactions with these nucleophiles resulted in highly enantioselective kinetic resolutions, which expands the scope of synthetically valuable conversions catalysed by a halohydrin dehalogenase. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Chiral non-racemic epoxides are important building blocks in the synthesis of various pharmaceutical products. Halohydrins can be considered as direct precursors of epoxides, since base-catalysed ring closure of an enantiomerically pure halohydrin generally yields an enantiomerically pure epoxide. Halohydrin dehalogenases catalyse the ring closure of a *vicinal* halohydrin and the reverse reaction, the ring opening of an epoxide by a halide (Scheme 1).



Scheme 1.

This class of enzymes can be used to prepare various optically active epoxides and halohydrins.^{1–3} Several genes encoding halohydrin dehalogenases have been cloned and overexpressed making these enzymes

available in abundant quantities.^{4,5} Sequence similarities and substrate specificities suggest that halohydrin dehalogenases obtained from *Agrobacterium radiobacter* AD1, *Arthrobacter* sp. AD2 and *Mycobacterium* sp. GP1 belong to three different groups.⁴ A general mechanism for enzymatic dehalogenation of halohydrins was proposed based on significant sequence similarity to short chain dehydrogenases/reductases and confirmed by site-directed mutagenesis of the conserved catalytic residues. The mechanism is fundamentally different from that of hydrolytic dehalogenases since it does not include a covalent enzyme–substrate intermediate.⁶

An enzyme-catalysed (enantioselective) ring-closure or ring-opening reaction can be monitored by extracting the substrate or product from the aqueous phase using an organic solvent and analysing the yield and enantiomeric purity by (chiral) GC or HPLC. This method is tedious and laborious and cannot be applied easily for an examination of numerous reactions or reaction conditions. Colorimetric assays for measuring dehalogenase activity are based on the detection of chloride ions or the use of pH indicators.^{7,8} The method based on measuring chloride concentrations requires continuous sampling from the reaction mixture. Use of a pH

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indicator such as phenol red in a weakly buffered medium allows continuous monitoring of the dechlorination reaction, but the sensitivity is low⁹ and furthermore, a weak buffer does not allow the addition of compounds that influence the pH of the medium such as strong nucleophiles.

Thus, a spectrophotometric assay would be very valuable. However, chromogenic substrates for measuring halohydrin dehalogenase activity have not yet been described in the literature. The goal of the work described herein is the development of such an assay for continuous monitoring of reactions catalysed by halohydrin dehalogenases. The substrates should be suitable for studying ring-closure and ring-opening reactions. Evaluation of the shape of the progress curve of a reaction with a racemic substrate can give information about the enantioselectivity of the conversion since a sequential conversion of the two enantiomers will result in a biphasic curve. The assay must also be applicable for studying the reverse reaction, the ring opening of an epoxide by a nucleophile. A halohydrin dehalogenase from *Corynebacterium* sp. N-1074 catalyses the ring opening of epoxides by cyanide, resulting in the formation of β -hydroxynitriles.¹⁰ More recently, we have shown that azide is accepted as a nucleophile by the halohydrin dehalogenase from *A. radiobacter* AD1.¹¹ Apart from these examples, ring-opening reactions with alternative nucleophiles catalysed by a halohydrin dehalogenase have not been described.

Herein, we describe a convenient on-line spectrophotometric assay for studying halohydrin dehalogenase-catalysed ring-closure and ring-opening reactions. The method is based on the difference in the absorbance spectrum of the epoxide and the corresponding ring opened product.

2. Results

2.1. Development and validation of the assay

Racemic *para*-nitro-2-bromo-1-phenylethanol **1** was considered to be a suitable chromogenic substrate based on previous work that demonstrated that halohydrin dehalogenase from *A. radiobacter* AD1 catalysed the enantioselective ring closure of aromatic halohydrins such as 2-chloro-1-phenylethanol (enantioselectivity factor, $E=73$).³ A spectrophotometric continuous assay for measuring epoxide hydrolase activity has been described.¹² This assay is based on the difference in extinction coefficients of *para*-nitrostyrene oxide (ϵ_{310} 4289 M⁻¹ cm⁻¹) and *para*-nitrophenylethanediol (ϵ_{310} 3304 M⁻¹ cm⁻¹), the product of epoxide hydrolysis. The substrate is broadly applicable since it can be used to investigate various aspects of epoxide hydrolase-catalysed conversions such as kinetic properties,¹³ enzyme stability in various media,¹⁴ and enantioselectivity.¹⁵

Racemic *para*-nitro-2-bromo-1-phenylethanol **1** was synthesised by reduction of ω -bromo-*para*-nitroaceto-

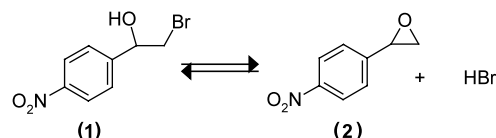
phenone. Racemic *para*-nitrostyrene oxide **2** was prepared by ring closure of the halohydrin **1**. The extinction coefficient of the halohydrin **1** was found to be 3050 M⁻¹ cm⁻¹ at 310 nm. The rate of ring-closure without the presence of enzyme was 2.3×10^{-5} s⁻¹ at 22°C (Tris buffer pH 7.5). Under similar conditions, no degradation of *para*-nitrostyrene oxide **2** was observed.

Halohydrin dehalogenases from *A. radiobacter* AD1, *Arthrobacter* sp. AD2 and *Mycobacterium* sp. GP1 catalysed the ring closure of racemic *para*-nitro-2-bromo-1-phenylethanol **1**, resulting in the formation of *para*-nitrostyrene oxide **2** (Scheme 2). The difference in extinction coefficient of the halohydrin and epoxide enabled the on-line monitoring of the reaction by following the increase in absorbance at 310 nm (Fig. 1).

2.2. Enantioselectivity of the ring-closure reaction

The progress curve of the ring-closure reaction of racemic *para*-nitro-2-bromo-1-phenylethanol (at 250 μ M concentration) catalysed by the halohydrin dehalogenase from *A. radiobacter* AD1 showed a biphasic shape (Fig. 2). An explanation for this can be a different rate of conversion of the two enantiomers. The biphasic shape was not observed in reactions catalysed by the halohydrin dehalogenases obtained from *Arthrobacter* sp. AD2 and *Mycobacterium* sp. GP1.

To determine the enantioselectivity of the ring-closure reactions, kinetic resolution experiments were performed with racemic halohydrin **1** at an initial substrate concentration of 3 mM. The conversion was monitored by taking samples from the reaction mixture at differ-



Scheme 2.

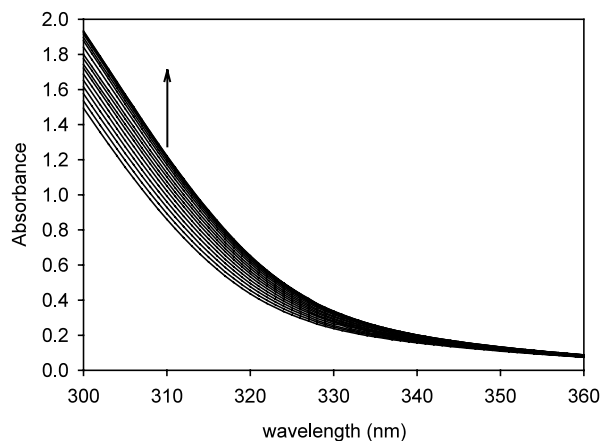


Figure 1. Absorption spectrum changes during the conversion of (*R*)-*para*-nitro-2-bromo-1-phenylethanol **1** to (*R*)-*para*-nitrostyrene oxide **2** catalysed by the halohydrin dehalogenase from *A. radiobacter* AD1. Concentration of (*R*)-**1**, 285 μ M.

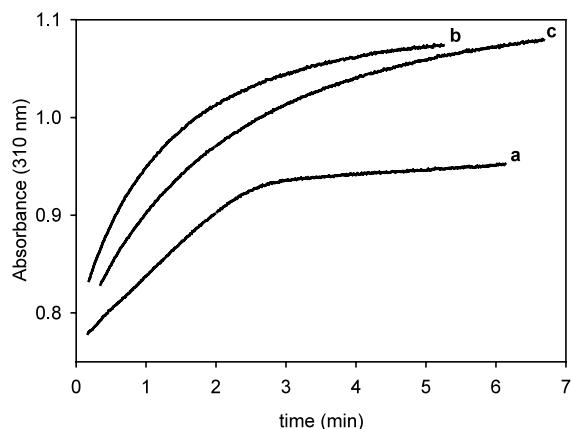


Figure 2. Progress curves of the conversion of racemic *para*-nitro-2-bromo-1-phenylethanol **1**, catalysed by the halohydrin dehalogenase from *A. radiobacter* AD1 (a), *Arthrobacter* sp. AD2 (b) and *Mycobacterium* sp. GP1 (c). Concentration **1**, 255 μM .

ent times and determining the enantiomeric excess of both the halohydrin **1** and the formed epoxide **2** by chiral HPLC. The enantioselectivity factor (*E*-value) was estimated from the enantiomeric excess values of the substrate and product.¹⁶ An *E*-value of 92 (± 4) was obtained for the kinetic resolution catalysed by the halohydrin dehalogenase from *A. radiobacter* AD1 (Fig. 3).

The observed *E*-value is lower than the intrinsic *E*-value, since the non-enzyme-catalysed ring-closure reaction ($k_c = 2.3 \times 10^{-5} \text{ s}^{-1}$) affected the enantiomeric excess of the formed product. Correction of the enantiomeric excess of the product epoxide for the chemical side reaction yielded an *E*-value of 124 (± 10). The (*R*)-enantiomer was converted preferentially, followed by a much slower conversion of the (*S*)-enantiomer, causing the biphasic shape of the progress curve observed when the increase in absorbance was monitored at 310 nm. The halohydrin dehalogenases from *Arthrobacter* sp.

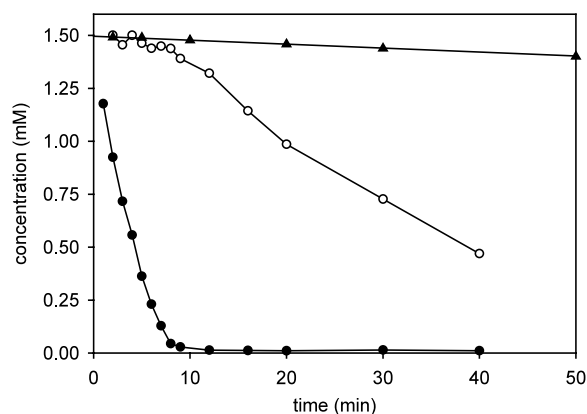


Figure 3. Kinetic resolution of racemic *para*-nitro-2-bromo-1-phenylethanol **1**, catalysed by the halohydrin dehalogenase from *A. radiobacter* AD1. Concentration **1**, 3 mM. Symbols: \circ , (*S*)-enantiomer; \bullet , (*R*)-enantiomer; \blacktriangle , non-enzyme-catalysed reaction.

AD2 and *Mycobacterium* sp. GP1 converted the halohydrin **1** with a low enantioselectivity ($E < 3$). The enantioselectivity of these two enzymes is opposite to that of *A. radiobacter* AD1 as the (*S*)-enantiomer was converted at a slightly higher rate than the (*R*)-enantiomer.

The exact *E*-value of the conversion could not be determined from the spectrophotometric progress curve, but the bend in the curve gives an approximate indication of the enantioselectivity of the conversion (Fig. 2). The monophasic progress curves for the conversion of racemic **1** with the enzymes obtained from *Arthrobacter* sp. AD2 and *Mycobacterium* sp. GP1 indicated a low enantioselectivity, which was confirmed by monitoring the enantiomeric excess values of the substrate and product. The structurally similar 2-chloro-1-phenylethanol was converted by both enzymes with comparably low enantioselectivity ($E < 10$).⁴ Because our main interest is to use halohydrin dehalogenases to synthesise enantiomerically pure building blocks, further experiments focused on the enzyme from *A. radiobacter* AD1.

The kinetic basis of the high enantioselectivity can be established by studying the steady-state kinetic parameters of the separate enantiomers of *para*-nitro-2-bromo-1-phenylethanol **1**. The *E*-value is defined by Eq. (1), where k_{cat} and K_m represent the Michaelis–Menten parameters for both enantiomers. The enantiomers of the halohydrin **1** and epoxide **2** were obtained in enantiomerically pure form (e.e. >99%) by separating them using a HPLC equipped with a chiral column (Chiralpak AS, Daicel). Enantiomerically pure (*R*)- and (*S*)-*para*-nitro-2-bromo-1-phenylethanol **1** (250 μM) were converted by the halohydrin dehalogenase from *A. radiobacter* AD1 with an initial activity of 35 and 2.6 $\mu\text{mol min}^{-1} \text{ mg}^{-1}$, respectively. The K_m for the (*R*)-enantiomer was too low to be determined by initial rate measurements ($K_m < 50 \mu\text{M}$) allowing only the calculation of a k_{cat} value of 16.3 s^{-1} .

For the (*S*)-enantiomer, on the other hand, no saturation kinetics were observed ($K_m > 250 \mu\text{M}$), allowing only the determination of a k_{cat}/K_m value of 4740 $\text{s}^{-1} \text{ M}^{-1}$. The above described kinetic parameters and the *E*-value of 124 allowed calculation of a K_m value of 28 μM for the (*R*)-enantiomer using Eq. (1).

2.3. Evaluation of nucleophiles for the ring-opening reaction

The enzyme-catalysed ring opening of *para*-nitrostyrene oxide was investigated by allowing different nucleophilic compounds to react with *para*-nitrostyrene oxide. In Fig. 4, the progress curves are shown of the reaction between (*R*)-*para*-nitrostyrene oxide **2** and Br^- at various concentrations. An equilibrium constant of 480 mM was calculated from the ring opening of (*R*)-**2** at a Br^- concentration of 100 mM.

The halide ions I^- , Cl^- , Br^- and F^- , the ions OCN^- , SCN^- , N_3^- , CN^- , CH_3COO^- and NO_3^- , and the non-

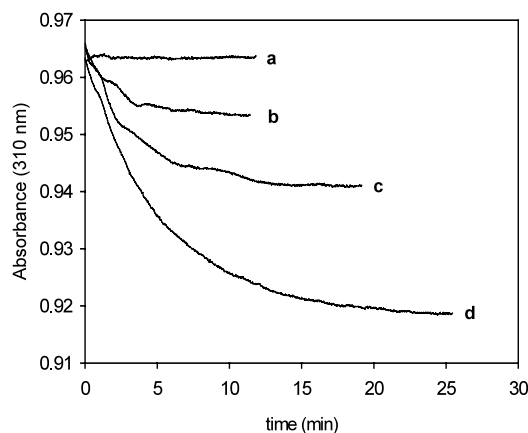


Figure 4. Progress curves of the ring opening of (*R*)-*para*-nitrostyrene oxide **2** by Br⁻, catalysed by halohydrin dehalogenase from *A. radiobacter* AD1. Incubations contained: 0.25 mM Br⁻ (a), 25 mM Br⁻ (b), 50 mM Br⁻ (c), 100 mM Br⁻ (d); 225 μM (*R*)-**2**.

ionic nucleophiles isopropylamine and ethanol were also tested. The conversion rates are expressed in $\Delta A \text{ min}^{-1} \text{ mg}^{-1}$ since, apart from the reaction with Br⁻, nucleophilic ring opening leads to products with unknown extinction coefficients. The enantioselectivity of the ring opening with a certain nucleophile was determined by allowing the (*R*)- and (*S*)-enantiomers of the epoxide to react separately. The effect of the non-enzyme-catalysed ring opening was minimised by measuring the initial rate within the first 2 min. During this period, no substantial decrease or increase of absorbance was observed in a control reaction in which the enzyme was omitted. An exception is I⁻, which gave an apparent chemical reaction that caused an increase in absorbance.

The enzyme-catalysed nucleophilic ring opening of *para*-nitrostyrene oxide resulted in products with lower extinction coefficients (310 nm), which allowed the monitoring of the conversion by following the decrease in absorption. The initial rate of enzymatic ring opening of epoxide (*R*)-**2** with Br⁻ and Cl⁻ was more than 100-fold higher than that of (*S*)-**2** (Table 1). A decrease in absorption was also observed when the (*R*)-enantiomer was subjected to the anions N₃⁻, CN⁻ and NO₂⁻, whereas no absorbance change was observed with the (*S*)-enantiomer. No decrease in absorbance was observed with either enantiomer was observed with F⁻, OCN⁻, SCN⁻, NO₃⁻, CH₃COO⁻, ethanol and isopropylamine. To validate the absence of a reaction between the epoxide and these latter compounds, the concentration of remaining epoxide was monitored in time using (chiral) HPLC. In all cases where no change in absorbance ($\Delta A < 0.01 \text{ min}^{-1} \text{ mg}^{-1}$) occurred, no decrease in epoxide concentration due to an enzymatic conversion was observed.

In order to investigate if nucleophiles that do not react with the epoxide can bind in the active site, the inhibiting effect of these compounds on the initial rate of the conversion of 250 μM halohydrin (*R*)-**2** was determined (Table 2). Nucleophiles that reacted with the epoxide were in general good inhibitors of the ring-closure reaction. Inhibition by halide ions decreased in the order of I⁻ > Br⁻ > Cl⁻ > F⁻, which is also the order of decreasing nucleophilicity. The non-ionic nucleophiles ethanol and isopropylamine did not inhibit the ring-closure reaction. OCN⁻ was a good inhibitor and therefore binds in the active site, although no enzyme-catalysed reaction with the epoxide took place. On the other hand, CN⁻, which reacted selectively with one enantiomer of the epoxide, did not inhibit the ring-closure reaction when added at a concentration of 10 mM.

Table 1. Initial rates of ring opening of (*R*)- and (*S*)-*para*-nitrostyrene oxide **2** by various nucleophiles, catalysed by the halohydrin dehalogenase from *A. radiobacter* AD1

Nucleophile	Concentration of nucleophile (mM)	(<i>R</i>)- 2 ($\Delta A \text{ min}^{-1} \text{ mg}^{-1}$)	(<i>S</i>)- 2 ($\Delta A \text{ min}^{-1} \text{ mg}^{-1}$)
Br ⁻	25	2.33	0.02
Cl ⁻	25	2.83	0.01
N ₃ ⁻	2.5	0.17	<0.01
NO ₂ ⁻	2.5	0.25	<0.01
CN ⁻	2.5	0.45	0.01

Table 2. Inhibition of the ring closure of (*R*)-*para*-nitro-2-bromo-1-phenylethanol **1** by various nucleophiles, catalysed by the halohydrin dehalogenase from *A. radiobacter* AD1

Nucleophile	Inhibition constant I ₅₀ (mM) ^a	Nucleophile	Inhibition constant I ₅₀ (mM) ^a
I ⁻	2.3	OCN ⁻	4.5
Br ⁻	4.3	SCN ⁻	30
Cl ⁻	13	NO ₃ ⁻	44
F ⁻	29	CH ₃ COO ⁻	33
N ₃ ⁻	2.7	CH ₃ CH ₂ OH	> 50
NO ₂ ⁻	20	(CH ₃) ₂ CHNH ₂	> 50
CN ⁻	> 50		

^a I₅₀ represents the nucleophile concentration at which the initial rate of ring closure of 250 μM (*R*)-**1** is 50% of the initial rate in the absence of the nucleophile.

Table 3. Enantioselective ring opening of racemic *para*-nitrostyrene oxide **2**^a with various nucleophiles, catalysed by halohydrin dehalogenase from *A. radiobacter* AD1

Nucleophile	Nucleophile conc. (mM)	Init. activity ^b ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	<i>E</i> -value	Conversion (%)	e.e. epoxide ^c (%) ^d
Cl ⁻	100	1.57	>40	26	36 (<i>S</i>)
CN ⁻	10	0.09	45	51	92 (<i>S</i>)
NO ₂ ⁻	10	0.55	105	52	97 (<i>S</i>)
N ₃ ⁻	1.3	0.18	>200	51	>99 (<i>S</i>)

^a Concentration racemic **2**, 3 mM, except with N₃⁻, 2 mM.

^b Initial activity towards (*R*)-*para*-nitrostyrene oxide **2** in a kinetic resolution experiment.

^c Highest observed e.e.

^d Value between parentheses is the absolute configuration of the unreacted enantiomer.

On the basis of the screening, we conclude that Br⁻, Cl⁻, N₃⁻, NO₂⁻ and CN⁻ are accepted and F⁻, OCN⁻, SCN⁻, NO₃⁻, CH₃COO⁻, ethanol and isopropylamine are not accepted as nucleophiles in the ring opening of epoxide **2** catalysed by the halohydrin dehalogenase from *A. radiobacter* AD1. The substantial differences in initial activities towards both enantiomers indicate that kinetic resolutions will likely occur with high enantioselectivity.

2.4. Kinetic resolution of *para*-nitrostyrene oxide by ring opening with selected nucleophiles

Kinetic resolutions of 3 mM racemic epoxide **2** catalysed by halohydrin dehalogenase from *A. radiobacter* AD1 were performed in the presence of the nucleophiles Cl⁻, N₃⁻, NO₂⁻ or CN⁻ (Table 3). The Br⁻ nucleophile was not included since the high equilibrium constant makes the preparation of halohydrin **1** starting from epoxide **2** and Br⁻ impractical (since very high concentrations of NaBr e.g. >10 M) are needed to achieve complete conversion (>99%) of 3 mM epoxide **2**.

All kinetic resolutions occurred with high enantioselectivity towards the (*R*)-enantiomer leaving the (*S*)-enantiomer of the epoxide behind. With 100 mM Cl⁻ the e.e. of the epoxide reached a maximum of 36% (Fig. 5). The low conversion with this nucleophile, due to an unfavorable equilibrium position, did not allow an exact determination of the *E*-value (*E* >40). The ring opening by NO₂⁻ occurred with high enantioselectivity (*E* = 105) resulting in an almost enantiomerically pure unreacted (*S*)-*para*-nitrostyrene oxide. To our knowledge, the enzyme-catalysed nucleophilic ring opening of an epoxide by NO₂⁻ has not been described before. Ring opening by CN⁻ occurred with a low conversion rate (0.09 $\mu\text{mol min}^{-1} \text{mg}^{-1}$) and moderate enantioselectivity (*E* = 45), resulting in (*S*)-*para*-nitrostyrene oxide with an e.e. of 92% after 24 h incubation. Since CN⁻ is a very poor leaving group, it is highly unlikely that ring closure of the formed β -hydroxynitrile occurs, making the reaction irreversible. The low enantiomeric purity of the remaining (*S*)-enantiomer of the epoxide is more likely to be due to the low reaction rate or inactivation.

A complete conversion of the (*R*)-enantiomer of the epoxide could be achieved with a small excess of N₃⁻. Racemic epoxide **2** was converted with high enantioselectivity

towards the (*R*)-enantiomer (*E* >200) and the remaining (*S*)-*para*-nitrostyrene oxide **2** was obtained with an e.e. of more than 99%.

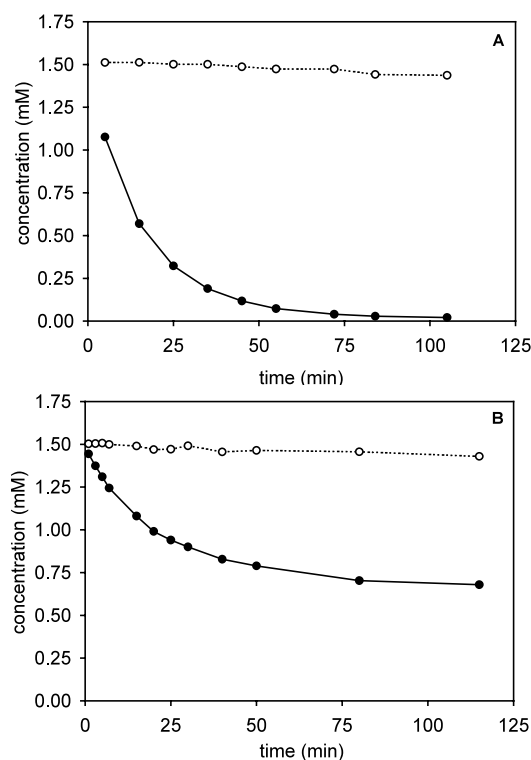


Figure 5. Enantioselective ring opening of racemic *para*-nitrostyrene oxide by NO₂⁻ (A) and Cl⁻ (B), catalysed by halohydrin dehalogenase from *A. radiobacter* AD1. Incubations contained: (A), 10 mM NO₂⁻, 3 mM **2** and 5.3 μM enzyme; (B), 100 mM Cl⁻, 3 mM **2** and 0.74 μM enzyme. Symbols: \circ , (*S*)-enantiomer; \bullet , (*R*)-enantiomer.

3. Conclusion and discussion

This paper describes the use of chromogenic substrates to investigate diverse halohydrin dehalogenase-catalysed reactions. The ring closure of *para*-nitro-2-bromo-1-phenylethanol **1** and the ring opening of *para*-nitrostyrene oxide **2** by Br⁻ can be monitored on-line since these reactions result in an increase and decrease in absorbance, respectively. The bend in the progress curve gives an indication of the enantioselectivity

tivity of the conversion of halohydrin. An obvious limitation of the assay is that its predictive value with regard to the activity and enantioselectivity of other substrates will be limited to compounds that are structurally related to the chromogenic substrates. On the other hand, this method seems to be suitable since three distinctly different types of halohydrin dehalogenases converted the halohydrin **1**.

A range of nucleophiles was tested for their ability to react with epoxide **2** in the presence of halohydrin dehalogenase from *A. radiobacter* AD1. Ring opening of racemic epoxide **2** with Cl^- , N_3^- , NO_2^- and CN^- resulted in enantioselective kinetic resolutions, in which the (*R*)-enantiomer was in all cases the preferred enantiomer. A complex product mixture was obtained during the ring opening reactions with NO_2^- and CN^- , as judged by chiral HPLC.

The enzyme-catalysed ring opening of an epoxide by NO_2^- is the most remarkable since, to our knowledge, enzymes that accept NO_2^- as a nucleophile have not been described in the literature before. The ambient nucleophilic nature of NO_2^- (possible attack by either the oxygen or nitrogen atom) and the regioselectivity of ring opening of the epoxide (α - or β -attack) make the formation of several products possible. If a nitro ester (R-O-N=O) is formed, various side products, such as a diol, can be expected since this class of compounds is unstable in aqueous media.¹⁷ A further study into the regioselectivity and enantioselectivity of these ring-opening reactions, which will require the synthesis of all possible products, is now in progress.

The enantioselective ring opening of epoxides by N_3^- catalysed by halohydrin dehalogenase from *A. radiobacter* AD1 was studied in more detail recently.¹¹ Various (substituted) styrene oxides were converted to the corresponding azido alcohols with high enantioselectivity and regioselectivity. The regioselectivity towards the β -carbon is opposite to the selectivity of the non-enzyme-catalysed reaction. The highly enantioselective ring opening of the epoxide by N_3^- gives access to optically active azido alcohols, which are direct precursors to highly valuable and biologically active amino alcohols.^{18,19}

Enantioselective enzymatic conversions of epoxides can occur in different ways. Hydrolysis of epoxides is catalysed by epoxide hydrolases from various sources.²⁰ Enantioselective ring opening by amines has been carried out in the presence of liver microsomes and lipases.^{21,22} The ring opening of an aliphatic epoxide by N_3^- was catalysed by an immobilised enzyme preparation from a *Rhodococcus* sp.²³ A halohydrin dehalogenase from *Corynebacterium* sp. N-1074 catalysed the ring opening of epichlorohydrin by cyanide, yielding (*R*)- γ -chloro- β -hydroxybutyronitrile in low enantiomeric purity.²⁴ The decrease in enantiomeric purity of the product, caused by a competing non-enzyme-catalysed conversion, could be overcome by in-situ generation of the epoxide from 1,3-dichloro-2-propanol catalysed by the same enzyme. The combined ring

closure and ring opening reaction gave (*R*)- γ -chloro- β -hydroxybutyronitrile in 95% e.e. and 65% yield.²⁴

This paper described the use of chromogenic substrates for halohydrin dehalogenases which can be used to study various aspects of halohydrin-catalysed conversions, such as steady state kinetics, enantioselectivity of the ring-opening and ring-closure reaction and nucleophile selectivity. The assay can be adopted to a 96-wells format which provides an easy method for screening large number of mutant colonies, such as those produced in directed evolution experiments.

4. Experimental

4.1. General

The enantiomeric excess (e.e.) and yields of various compounds were determined by chiral HPLC (Chiralpak AS, Daicel). Spectrophotometric assays were conducted in a Perkin Elmer Lambda BIO 40 spectrophotometer. NMR spectra were recorded in CDCl_3 . Halohydrin dehalogenases from *A. radiobacter* AD1, *Arthrobacter* sp. AD2 and *Mycobacterium* sp. GP1 were overexpressed in *E. coli* and purified as described before.⁴

4.2. Synthesis of substrates

4.2.1. Racemic *para*-nitro-2-bromo-1-phenylethanol, **1.** To a cooled solution of ω -bromo-*para*-nitroacetophenone (5.0 g, 20 mmol) in methanol (50 mL), was added sodium borohydride (1.0 g, 26 mmol) and stirred for 3 h. Water (50 mL) was added and the mixture was extracted with diethyl ether. After separation, the organic phase was washed with brine, dried with MgSO_4 and removed by a rotary evaporator yielding an orange solid (4.1 g) that consisted of a 7:3 mixture of **1** and *para*-nitrostyrene oxide **2**. Pure **1** was obtained by flash chromatography on silica 60 H using petroleum ether/diethyl ether (ratio 7:3). ^1H NMR δ : 2.69 (d, 1H, OH, $J=3.7$ Hz); 3.52 (m, 2H); 5.00 (m, 1H); 7.54 (d, 2H_{ar}, $J=8.8$ Hz); 8.20 (d, 2H_{ar}, $J=8.8$ Hz). ^{13}C NMR δ : 36.8 (C-1); 70.1 (C-2); 121.3; 124.4; 144.8; 145.3 (C_{ar}).

4.2.2. Racemic *para*-nitrostyrene oxide, **2.** Racemic *para*-nitrostyrene oxide **2** was prepared by treating a 7:3 mixture of **1** and **2** (1.0 g) dissolved in diethyl ether with aqueous KOH solution (15 mL, 1 M). The mixture was heated under reflux for 15 min, cooled, diluted with sulfuric acid (20 mL, 1 M) and extracted with diethyl ether. After separating, the organic layer was dried with MgSO_4 and removed using a rotary evaporator to yield pure **2** (0.76 g), which was used without further purification. ^1H NMR δ : 2.72 (dd, 1H, $J=2.6$ Hz and 5.5 Hz); 3.17 (dd, 1H, $J=4.0$ Hz and 5.5 Hz); 3.91 (dd, 1H, $J=2.6$ Hz and 4.0 Hz); 7.40 (d, 2H_{ar}, $J=8.8$ Hz); 8.16 (d, 2H_{ar}, $J=8.8$ Hz). ^{13}C NMR δ : 48.9 (C-1); 49.2 (C-2); 121.3; 123.7; 142.7; 145.3 (C_{ar}).

4.2.3. Enantiomerically pure *para*-nitro-2-bromo-1-phenylethanol, **1 and *para*-nitrostyrene oxide, **2**.** Enantiomerically pure **1** and **2** were obtained by preparative HPLC using an analytical Chiralpak AS column with hexane/isopropanol (95:5) as eluent. Retention times of individual enantiomers (e.e. >99%): (*R*)-**1**, 45.4 min; (*S*)-**1**, 52.8 min; (*R*)-**2**, 17.2 min; (*S*)-**2**, 25.3 min.

4.3. Enzyme assays with chromogenic substrates

In a typical experiment, a stock solution of **1** or **2** in DMSO (2 μ L) was added to a cell containing buffer (1.00 mL) at 22°C to give a final concentration of approximately 250 μ M. In ring-opening and inhibition experiments the pH of the medium was 7.2 (300 mM Tris-SO₄) and in ring-closure reactions 7.5 (100 mM Tris-SO₄). In ring-opening and inhibition experiments, the nucleophile was added as its sodium salt (except for ethanol and isopropylamine) from a concentrated aqueous stock solution. The conversion was started by addition of the enzyme and the change in absorbance at 310 nm was monitored. The extinction coefficients of **1**, 3050 M⁻¹ cm⁻¹ and **2**, 4289 M⁻¹ cm⁻¹ at 310 nm were used to calculate concentrations of **1** and **2**.

4.4. Kinetic resolution experiments

Substrate **1** or **2** was added to a screw-capped bottle containing Tris buffer (20 mL) at 22°C to give a final concentration of 3 mM. In ring-opening experiments the pH of the medium was 7.2 (300 mM Tris-SO₄) and in ring-closure reactions 7.5 (100 mM Tris-SO₄). In ring-opening experiments, the nucleophile was added to a concentration indicated in Table 3. The reaction was started by addition of the enzyme. The reaction was monitored by periodically taking samples from the reaction mixture and extracting them with diethyl ether containing an internal standard such as mesitylene or acetophenone. The e.e. and conversion of **1** and **2** were determined by chiral HPLC analysis as described above. In ring-closure experiments the *E*-value of a kinetic resolution was calculated from the enantiomeric excess of the halohydrin **1** and the epoxide **2** and in ring-opening experiments the *E*-value was calculated from the conversion and the enantiomeric excess of epoxide **2**.¹⁶

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