Enantioselective biocatalytic conversions of epoxides
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Chapter 8

Summary and concluding remarks
Introduction

This thesis describes the biocatalytic scope and limitations of an epoxide hydrolase and a haloalcohol dehalogenase. These enzymes, obtained from *Agrobacterium radiobacter* AD1, were tested for their suitability to prepare optically pure epoxides and derivatives thereof. These compounds can be used for the synthesis of various biologically active agents such as pharmaceuticals. The gram-negative bacterium *A. radiobacter* AD1 was isolated from polluted freshwater sediment. Its epoxide hydrolase (EchA) and haloalcohol dehalogenase (HheC) are involved in the degradation of epichlorohydrin and dichloropropanols, enabling the organism to grow on these compounds. The 34 kD epoxide hydrolase was purified and the corresponding gene was cloned by means of PCR. The recombinant EchA can be produced at up to 40% of the total protein content in *Escherichia coli*, enabling the isolation of 200 to 300 mg of pure protein from a one-liter culture. This makes the enzyme available in sufficient quantities for applications in (synthetic) organic chemistry. In 1997, it was the first microbial epoxide hydrolase gene to be cloned. Until then, all conversions with microbial epoxide hydrolases were performed with whole cell suspensions or small quantities of (partially) purified enzyme.

Biocatalytic potential of epoxide hydrolase

The epoxide hydrolase is able to hydrolyse a broad range of aliphatic and aromatic epoxides (Chapter 2). Epichlorohydrin, the best substrate for EchA, is converted without enantioselectivity (E<3), enabling the organism to completely degrade it. Other terminal aliphatic epoxides, such as 1,2-epoxyhexane are also hydrolysed with a low enantioselectivity (E<5). Epoxides that have an aromatic group next to the chiral carbon atom of the epoxide, such as styrene oxide, are hydrolysed with moderate to high enantioselectivity. Para-substituted styrene oxides are hydrolysed with highest enantioselectivity, followed by ortho- and meta-substituted styrene oxides, yielding in all cases optically pure remaining (S)-enantiomers.

A styrene oxide derivative bearing a methyl group at the α-position (α-methylstyrene oxide) is hydrolysed with a moderate enantioselectivity. The absolute configuration of the residual (S)-enantiomer of α-methylstyrene oxide was determined by comparison with ambiguous literature values (Chapter 2). A more detailed study showed that this assignment was not correct and that the remaining enantiomer is of the (R)-configuration. The hydrolysis of various substituted α-methylstyrene oxides catalysed by other epoxide hydrolases showed that the enantiopreference is highly unpredictable. An epoxide bearing a substituent on the cis-β-position (cis-β-methylstyrene oxide) is accepted, an epoxide with a substituent on the trans-β-position (trans-β-methylstyrene oxide) not.
Summary and concluding remarks

Most of the aromatic epoxides shown in Figure 1 are converted in an enantioselective way not only by EchA, but also by some other bacterial, fungal or yeast epoxide hydrolases. The substrate range of EchA is most similar to that of the epoxide hydrolase obtained from the organism Aspergillus niger LCP 521. This enzyme hydrolyses the same range of ring-substituted styrene oxides with somewhat higher enantioselectivity. The E-values of kinetic resolutions catalysed by EchA were estimated using competitive Michaelis-Menten kinetics, taking chemical hydrolysis into account (Chapter 2). This method was also used for determining the E-value of kinetic resolutions catalysed by haloalcohol dehalogenases (Chapter 4 and 6) and haloalkane dehalogenases.

The X-ray structure of EchA has been determined at 2.1 Å resolution. The enzyme shows a two-domain structure consisting of a main domain with an α/β hydrolase fold topology and a cap domain. In the structure, the side chain amide group of glutamine 134 is bound at the position where the epoxide is supposed to bind. Since this represents an inactive form of EchA, which is probably due to a crystallographic contact, it is unlikely that it exists during the catalytic cycle. The substrate range and enantioselectivity can be rationalised by modeling studies. Based on structural similarity with other α/β hydrolase fold enzymes, a likely model of the epoxide hydrolase with an empty active site was generated. In this model, the active site residues have undergone (minor) positional changes, which would make an analysis of the structural basis of the substrate range by docking of various epoxides rather unreliable. So far, no structures of EchA with bound substrate or substrate analogues have been obtained.

A range of optically pure para-substituted styrene oxides was used to investigate the kinetic properties of EchA (Chapter 3). The influence of a substituent on the aromatic ring on
the rate of hydrolysis indicated that conversion of the two enantiomers occurs with different kinetic mechanisms. Previous investigations showed that the hydrolysis of \((R)\)-styrene oxide occurs with four distinguishable kinetic steps, whereas only three steps are observed for the conversion of the \((S)\)-enantiomer. This additional unimolecular step, which takes place between the formation and hydrolysis of the ester intermediate, can be a (re)protonation step, a conformational change or an intramolecular acyl transfer. The possibility that tyrosine reprotonation takes place during this unknown step would be in agreement with the observed fluorescence change, although protonation steps are generally too fast to observe. On the other hand, (non-enzyme catalysed) acyl transfer steps are normally much slower than the observed rates for the additional step, although acyl transfers could be faster in the active site of the enzyme. It is difficult to obtain evidence for such an intermediate acyl transfer since its occurrence cannot be established by labeling studies and no change in identity or configuration of the product occurs. A possible approach to demonstrate the existence of such an intermediate would be to perform a single-turnover experiment with a His275Arg mutant. In this mutant enzyme, the last hydrolysis step does not take place, probably allowing trapping of the ester intermediate, of which the structure could be studied.

Although the enantioselectivity of EchA towards some substrates is reasonably high, some aspects can hamper a large-scale application. One problem is the peculiar kinetic behaviour that is most prominent during the conversion of styrene oxide (Chapter 2). The remaining \((S)\)-styrene oxide is converted at an almost 3-fold higher rate than the first-reacting \((R)\)-enantiomer. In a large-scale application, careful monitoring of the conversion is necessary to determine the optimal moment to terminate the reaction.

A second problem is the low solubility of (substituted) styrene oxides, which is generally between 3 mM and 10 mM in Tris buffer (pH 9.0, 30 °C). When a liquid epoxide was added to a concentration higher than the solubility, the formed emulsion quickly inactivated the enzyme (data not shown). The addition of 30% DMSO increases the solubility of the epoxides 3- to 4-fold. EchA is stable and active in the presence of up to 30% of this miscible cosolvent (research report Marlon Gons 1998). The low solubility of epoxides was also circumvented by using a two-phase system with octane as a second phase. A kinetic resolution of styrene oxide that was carried out at a concentration of 39 g/L in octane yielded \((S)\)-styrene oxide (> 95% e.e.) in a yield of 30%. The somewhat lower yield compared to what is obtained in an one-phase system is caused by mass transfer limitations and enzyme inactivation.

The solubility of 2-pyridyl oxirane is very high, making the use of above-described organic (co)solvents not necessary. Kinetic resolutions of this substrate using EchA were performed at concentrations up to 400 mM. At this very high substrate concentration, the rate of hydrolysis was reduced due to enzyme inactivation and product inhibition. However, in a larger-scale experiment (2 g) at the lower substrate concentration of 127 mM, the remaining \((S)\)-2-pyridyl oxirane was isolated as an optically pure product in 35% yield.
Summary and concluding remarks

Mutant epoxide hydrolase with enhanced biocatalytic capabilities

The X-ray structure of EchA shows that the tyrosine residues Y215 and Y152 present in the cap domain play an important role in catalysis. Replacement of tyrosine 215 with a phenylalanine (Y215F) results in an enzyme with a higher enantioselectivity towards substituted styrene oxides (Chapter 3). The increased enantioselectivity is mainly due to a relatively strong decrease of the $k_{\text{cat}}$ for (S)-enantiomers. This low activity towards the remaining enantiomers improves the synthetic applicability of the mutant enzyme. As mentioned above, to obtain optically pure epoxides (e.e. >99%) by kinetic resolution, the point at which the reaction is terminated is essential. If the reaction is terminated too early, a non-enantiopure epoxide will be obtained in a high yield. If the reaction is terminated too late, the enantiopure remaining epoxide will be obtained in a low yield. The yield of the remaining optically pure epoxide is predominantly dependent on the activity towards the remaining enantiomer, which has decreased drastically with the Y215F epoxide hydrolase. Increasing the enantioselectivity by mutating a tyrosine residue can also be applicable to other epoxide hydrolases since sequence alignments show that the tyrosine residues are conserved.

No other examples have yet been described in which the enantioselectivity of an epoxide hydrolase was increased using site-directed mutagenesis. It has been claimed, however, that the enantioselectivity of epoxide hydrolases was improved using protein-imprinting techniques. Imprinting with optically pure epoxides resulted either in an increased enantioselectivity or a reversed enantiospecificity.

Another advantage of the Y215F epoxide hydrolase is the enhanced stability at elevated temperatures and pH-values. Together with the increased enantioselectivity and the strongly reduced activity towards the remaining enantiomer makes the Y215F epoxide hydrolase an attractive biocatalyst for the preparation of optically pure epoxides.

Biocatalytic potential of haloalcohol dehalogenase: the ring-closure reaction

Haloalcohol dehalogenases have been used for preparing optically active haloalcohols and epoxides by kinetic resolution of vicinal haloalcohols. The reverse reaction, the enantioselective ring opening of an epoxide by a nucleophile, will yield the remaining epoxide and the formed β-substituted alcohol, optically active. The haloalcohol dehalogenase from A. radiobacter (HheC) was initially investigated because of its role in the degradation of halogenated compounds. The observation that 2,3-dichloro-1-propanol is only 50% degraded by the organism triggered us to investigate the enantioselectivity of this haloalcohol dehalogenase. Kinetic resolution experiments performed with whole cell suspensions
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showed that HheC is moderately to highly enantioselective towards a variety of aliphatic haloalcohols.

The expression level of the enzyme in *A. radiobacter* is low, making the use of the whole organism not attractive for synthetic applications. The genes encoding HheC and the corresponding enzymes from *Arthrobacter sp.* AD2 and *Mycobacterium sp.* GP1 were cloned and the proteins were expressed in *E. coli* at a level of up to 15 to 25% of the total protein content.19 The purified recombinant HheC catalyses the conversion of a range of aliphatic and aromatic haloalcohols containing a primary or secondary hydroxyl group (Chapter 4). Enantioselective conversions of aromatic haloalcohols such as 2-chloro-1-phenylethanol (E = 73) catalysed by a haloalcohol dehalogenase were not described in the literature before. HheC shows the highest enantioselectivity towards 2,3-dichloro-1-propanol and 2,3-dibromo-1-propanol (E>100), which was also described earlier for other haloalcohol dehalogenases.16

The highly enantioselective ring closure of 2,3-dichloro-1-propanol yielded, surprisingly, racemic epichlorohydrin. This is due to the ring opening of epichlorohydrin with chloride, yielding 1,3-dichloro-2-propanol. Ring closure of this prochiral compound results in racemic epichlorohydrin. The HheC-catalysed racemisation of epichlorohydrin based on these reactions was applied in Chapter 7 to obtain a dynamic kinetic resolution of epihalohydrins.

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![Chemical Structure](image)

**Figure 2** *Kinetic resolution of 2-chloro-1-phenylethanol catalysed by HheC, combined with in situ epoxide removal catalysed by EchA, resulting in optically pure (S)-2-chloro-1-phenylethanol.*

Due to the reversibility of the reaction, the HheC-catalysed kinetic resolution of 2-chloro-1-phenylethanol yielded the remaining (S)-haloalcohol in only 92% e.e. The reversible ring closure could be drawn to completion by adding an excess of EchA resulting in a tandem enzyme reaction.20 By removal of the inhibiting epoxide, a total conversion of (R)-2-chloro-1-phenylethanol occurred, allowing the remaining (S)-2-chloro-1-phenylethanol to be obtained optically pure (Figure 2). The HheC-catalysed conversions of the various haloalcohols, combined with the product removal by EchA, were performed at a substrate concentration of 5 mM (Chapter 4). What are the prospects for scaling up these tandem reactions to concentrations of 100 mM or higher? The presence of substrate or product emulsions will cause inactivation of the epoxide hydrolase and possibly also of the haloalcohol dehalogenase. Alternative ways to achieve a complete conversion of the
haloalcohol can be the addition of a second organic phase to remove epoxide. Such a two-phase system can also make separation of substrate and product during work-up easier, since the residual haloalcohol remains in the aqueous phase and formed epoxide is transferred to the organic phase. Another alternative is the use of bromoalcohols instead of chloroalcohols since the equilibrium of the reversible ring-closure reaction is more towards the epoxide. The conversion of para-nitro-2-bromo-1-phenylethanol yields almost optically pure (S)-haloalcohol (98% e.e.) (Chapter 5).

Chromogenic substrates for haloalcohol dehalogenases

The progress of the kinetic resolution of various haloalcohols catalysed by HheC is commonly followed by measuring conversion and enantiomeric excess using chiral HPLC or GC (Chapter 4). This method is tedious and laborious since it requires multiple sampling and analysis. A convenient spectrophotometric assay for haloalcohol dehalogenases based on the absorption difference between the para-nitro-2-bromo-1-phenylethanol and the epoxide para-nitrostyrene oxide was developed (Chapter 5). HheC converts para-nitro-2-bromo-1-phenylethanol with high enantioselectivity, resulting in a biphasic progress curve. The enzymes obtained from Arthrobacter sp. AD2 and Mycobacterium sp. GP1 showed a low enantioselectivity towards this substrate, resulting in a monophasic progress curve. This illustrates that this assay can be used to quickly determine enantioselectivity of new haloalcohol dehalogenases or mutant enzymes obtained from directed evolution experiments.

The enantiomers of both para-nitro-2-bromo-1-phenylethanol and para-nitrostyrene oxide were obtained optically pure by preparative chiral HPLC column. The resulting enantiopure chromogenic substrates were useful for determining steady state kinetic parameters, for studying the range of accepted nucleophiles in ring-opening reactions (Chapter 5), for studying mutants with increased stability, and for a detailed investigation of the kinetic mechanism (Tang et al., manuscript in preparation).

A drawback of the chromogenic assay is that only the conversions of para-nitro-2-bromo-1-phenylethanol and para-nitrostyrene oxide can be monitored. The use of para-nitrostyrene oxide as a reporter substrate for following the EchA-catalysed conversion of other non-chromogenic substrates has been described previously for EchA. For example, addition of a haloalcohol to a reaction mixture containing HheC and para-nitro-2-bromo-1-phenylethanol will give information about the enzymatic conversion of the non-chromogenic haloalcohol. If the enzyme converts this haloalcohol, an effect on the progress curve of para-nitro-2-bromo-1-phenylethanol will be observed. By fitting the progress curves with Michaelis-Menten kinetics for competing substrates, steady state parameters for the non-chromogenic substrate can be obtained.
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Screening for accepted nucleophiles

The range of nucleophiles accepted by HheC was investigated with optically pure enantiomers of para-nitrostyrene oxide (Chapter 5). The occurrence of an enzyme-catalysed reaction can be measured by incubating enzyme with epoxide and various nucleophilic compounds and monitoring absorbance at 310 nm. Incubation of (R)-para-nitrostyrene oxide with the enzyme and the nucleophiles Br\(^-\), Cl\(^-\), N\(_3\)\(^-\), NO\(_2\)\(^-\) and CN\(^-\) resulted in a decrease in absorbance. A reaction rate with I\(^-\) could not be determined reliably. When 250 µM para-nitrostyrene oxide was incubated with 25 mM of I\(^-\), an increase in absorbance was observed, possibly due to chemical ring opening at the α-carbon atom. Subsequent addition of enzyme caused an immediate, but relatively small decrease in absorbance, indicating that HheC accepted I\(^-\) as a nucleophile. However, the position of the equilibrium, which is at the side of the epoxide, makes the use of this reaction to prepare iodoalcohols unfeasible.

![Nucleophiles accepted by HheC](image)

The non-halide nucleophiles accepted by HheC are ambident anions with a (close to) linear shape. This suggests that the binding site of the nucleophile has the shape of a tunnel and that binding is stabilised by groups that donate a hydrogen bond. A correlation between the occurrence of a reaction and binding of a nucleophile was established by determining the inhibition of the nucleophiles on the enzyme-catalysed ring-closure of para-nitro-2-bromo-1-phenylethanol. Generally, ions that are accepted by the enzyme are moderate to good inhibitors of the ring-closure reaction. The nucleophile OCN\(^-\) is an exception to this rule since it is a very good inhibitor of the ring-closure reaction, but it is not accepted by the enzyme in the ring-opening reaction. An explanation can be that the binding of this nucleophile is in such a way that the nucleophilic oxygen cannot interact with the epoxide.

A limitation to the assay is the sensitivity since the lowest observable initial rate of ring opening reactions is approximately 0.01 ΔA.min\(^{-1}\).mg\(^{-1}\). Depending on the nucleophile, this detection limit corresponds to an activity range of 0.005 to 0.02 µmol.min\(^{-1}\).mg\(^{-1}\). Currently, the binding of various nucleophiles is investigated using site-directed mutagenesis and preliminary information obtained from the X-ray structure.

Enantioselective nucleophilic ring opening of aromatic epoxides

The HheC-catalysed ring-opening reactions of para-nitrostyrene oxide by N\(_3\)\(^-\), NO\(_2\)\(^-\) and CN\(^-\), were scaled up from 200 µM to 2 mM. The products of these reactions are
precursors of biologically active compounds. Further investigations focused on ring opening by \( \text{N}_3^- \) since the use of \( \text{CN}^- \) was reported previously\(^{23}\) and ring opening by \( \text{NO}_2^- \) yielded multiple unidentified compounds (Chapter 6).

The ring opening of substituted styrene oxide by \( \text{N}_3^- \) is highly regio- and enantioselective. The regioselectivity of the enzyme-catalysed ring opening is opposite to that of the non-catalysed reaction. The enantioselectivity of the ring opening of para-nitrostyrene oxide was very high (\( E > 200 \)). A limitation is the parallel chemical conversion leading to the other regio-isomer. In a large-scale reaction (7.8 g/L) this limitation was minimized by adding para-nitrostyrene oxide as a second phase combined with a slow addition of \( \text{N}_3^- \). The isolated product consisted of (S)-para-nitrostyrene oxide in 46% yield and 98% e.e. and the (R)-azidoalcohol in 47% yield and 97% e.e.

The ring opening of an epoxide by \( \text{N}_3^- \) catalysed by an immobilized enzyme preparation from \textit{Rhodococcus} sp. was described previously.\(^{24}\) Since in the enzyme preparation an epoxide hydrolase was present, the suggestion was made that this enzyme catalysed the ring opening by \( \text{N}_3^- \). However, the two-step mechanism of epoxide hydrolases makes the occurrence of such a reaction not likely. A more plausible explanation would be the presence of a haloalcohol dehalogenase in this organism.

**Table 1**  
\textit{Proposed chemo-enzymatic cascade reactions combining an enantioselective reaction catalysed by a haloalcohol dehalogenase with a chemical conversion of the formed product.}

<table>
<thead>
<tr>
<th>Nucleophile (Nu)</th>
<th>Chemical conversion</th>
<th>( X )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{N}_3^- ) or ( \text{NO}_2^- ) (N-attack)</td>
<td>reduction</td>
<td>-NH2</td>
</tr>
<tr>
<td>( \text{NO}_2^- ) (O-attack)</td>
<td>hydrolysis</td>
<td>-OH</td>
</tr>
<tr>
<td>( \text{CN}^- )</td>
<td>hydrolysis</td>
<td>-COOH</td>
</tr>
<tr>
<td>( \text{I}^- )</td>
<td>nucleophilic attack</td>
<td>various nucleophiles</td>
</tr>
</tbody>
</table>

In future research, the nucleophilic ring opening of epoxides catalysed by haloalcohol dehalogenases should be combined with chemical conversions of the formed products in a one-pot process. Advantages of such chemo-enzymatic processes are the direct access to the desired compounds and the avoidance of the isolation of hazardous ring opening products, such as azides. The azidoalcohols and \( \beta \)-hydroxynitriles can be reduced or oxidized to the
corresponding aminoalcohols and hydroxyacids, respectively (Table 1, entries a and c). If the ring opening by NO$_2^-$ occurs by attack of the nitrogen atom, the formed nitro-alcohols can also be converted to amino-alcohols (Table 1, entry a). If the ring opening occurs by attack of oxygen, the product can be hydrolysed to yield the diol (Table 1, entry b). Although the unfavorable position of the equilibrium between an epoxide and I$^-$ requires a large excess of this ion, the small amount of formed product will be receptive for a nucleophilic attack. This gives access to a wide range of ring opening reactions with various nucleophiles (Table 1, entry d).

The above described chemo-enzymatic cascade reactions can potentially contribute to the introduction of “green chemistry”: the use of chemical processes that reduce production of environmentally hazardous compounds. It is a real challenge for organic chemists to adapt synthetic processes to work together with biocatalysts in aqueous environments.

**Dynamic kinetic resolution by ring opening of epihalohydrins with N$_3^-$**

A drawback of a kinetic resolution is that yields of the remaining substrate and formed product are maximally 50%. This limitation can be overcome by an *in-situ* racemisation of the remaining enantiomer during the reaction. This results in a dynamic kinetic resolution in which theoretically the product can be obtained in 100% yield and 100% e.e. The HheC-catalysed reversible ring closure of an epihalohydrin causing racemisation, combined with ring opening of epoxide by an N$_3^-$, catalysed by the same enzyme, resulted in such an enzymatic dynamic kinetic resolution. With epichlorohydrin as a substrate, the rate of ring opening by N$_3^-$ is higher than the rate of racemisation, resulting in a mixed kinetic resolution and dynamic kinetic resolution. With epibromohydrin, the rate of racemisation was higher than the rate of ring opening, resulting in a dynamic kinetic resolution. By optimising the pH of the buffer and the concentration of N$_3^-$ and Br$, the product (S)-1-azido-3-bromo-2-propanol could be obtained in 84% yield and 94% e.e. A sequential ring closure of this haloalcohol, catalysed by the same enzyme, causes a simultaneously occurring kinetic resolution by which (S)-1-azido-3-bromo-2-propanol can be obtained optically pure (>99% e.e.) in 77% yield (Chapter 7). This latter compound and the ring-closed product glycidyl azide can be used as pharmaceutical building blocks.

A similar dynamic kinetic resolution was described using racemic epichlorohydrin and TMSN$_3$. In this reaction, a (salen)Cr(III)N$_3$ complex catalyses racemisation and enantioselective ring opening. As with the biocatalytic reaction described here, the relatively low rate of racemisation limited an optimal resolution. This problem was overcome by a slow addition (16 h) of the nucleophile leading to the desired ring-opening product (S)-3-azido-1-chloro-2-trimethylsiloxypropane in 97% e.e. and 76% yield. This product was used for the synthesis of an aryl oxazolidinone antibacterial reagent. We have also tested whether the slow addition of N$_3^-$ would improve the dynamic kinetic resolution of epichlorohydrin by HheC.
(data not shown). However, this led to accumulation of glycidyl azide and 1,3-diazido-2-propanol as main products.

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