Biochemical Studies on a Plant Epoxide Hydrolase

Discovery of a Proton Entry and Exit Pathway and the Use of In vitro Evolution to Shift Enantioselectivity

ANN GURELL
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Abstract

The work leading to this thesis has provided additional information and novel knowledge concerning structure-function relationship in the potato epoxide hydrolase.

Epoxide hydrolases are enzymes catalyzing the hydrolysis of epoxides to yield the corresponding vicinal diols. The reaction mechanism proceeds via a nucleophilic attack resulting in a covalent alkylenzyme intermediate, which in turn is attacked by a base-activated water molecule, followed by product release. Epoxides and diols are precursors in the production of chiral compounds and the use of epoxide hydrolases as biocatalysts is growing. The promising biocatalyst SteH1, a plant epoxide hydrolase from potato, has been investigated in this thesis.

In paper I the active site residue Glu\textsuperscript{35}, was established to be important for the formation of the alkylenzyme intermediate, activating the nucleophile for attack by facilitated proton release through a hydrogen bond network. Glu\textsuperscript{35} is also important during the hydrolytic half reaction by optimally orienting the hydrolytic water molecule, aiding in the important dual function of the histidine base. Glu\textsuperscript{35} makes it possible for the histidine to work as both an acid and a base.

In paper II a putative proton wire composed of five water molecules lining a protein tunnel was proposed to facilitate effective proton transfer from the exterior to the active site, aiding in protonation of the alkylenzyme intermediate. The protein tunnel is also proposed to stabilize plant epoxide hydrolases via hydrogen bonds between water molecules and protein.

Enzyme variants with modified enantiospecificity for the substrate (2,3-epoxypropyl)benzene have been constructed by in vitro evolution using the CASTing approach. Residues lining the active site pocket were targeted for mutagenesis. From the second generation libraries a quadruple enzyme variant, W106L/L109Y/V141K/I155V, displayed a radical shift in enantioselectivity. The wild-type enzyme favored the S-enantiomer with a ratio of 2:1, whereas the quadruple variant showed a 15:1 preference for the R-enantiomer.

Keywords: epoxide hydrolase, enantioselectivity, in vitro evolution, proton wire, epoxides, selectivity, CASTing, structure-function

Ann Gurell, Department of Biochemistry and Organic Chemistry, Box 576, Uppsala University, SE-75123 Uppsala, Sweden

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Det finns mitt i skogen en oväntad glänta som bara kan hittas av den som gått vilse

Tomas Tranströmer
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AnEH</td>
<td><em>Aspergillus niger</em> epoxide hydrolase</td>
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<td>ArEH</td>
<td><em>Agrobacterium radiobacter</em> epoxide hydrolase</td>
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<td>CAST</td>
<td>combinatorial active site saturation test</td>
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<td>E</td>
<td>enantiomeric ratio</td>
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<td>ee&lt;sub&gt;p&lt;/sub&gt;</td>
<td>product enantiomeric excess</td>
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<td>EC</td>
<td>Enzyme Commission</td>
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<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<td>EH</td>
<td>epoxide hydrolase</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>HssEH</td>
<td><em>Homo sapiens</em> soluble epoxide hydrolase</td>
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<td>MD</td>
<td>molecular dynamics</td>
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<td>mEH</td>
<td>microsomal epoxide hydrolase</td>
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<td>MmsEH</td>
<td><em>Mus musculus</em> soluble epoxide hydrolase</td>
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<td>MtEH</td>
<td><em>Mycobacterium tuberculosis</em> epoxide hydrolase</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDB</td>
<td>protein data bank</td>
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<td>sEH</td>
<td>soluble epoxide hydrolase</td>
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<td>StEH1</td>
<td><em>Solanum tuberosum</em> epoxide hydrolase 1</td>
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<tr>
<td>TSO</td>
<td><em>trans</em>-stilbene oxide</td>
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<td>wt</td>
<td>wild type</td>
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Introduction to epoxide hydrolases

In the work presented in this thesis epoxide hydrolase 1 (StEH1), an enzyme from potato, *Solanum tuberosum*, has been investigated. In all papers the wild-type enzyme as well as its altered, mutated variants have been studied and compared. From these results conclusions have been drawn. Results are sometimes specific to the potato enzyme but some conclusions regard epoxide hydrolases (EHs) in general. Additionally, new insights into relationships between enzyme structure and enzyme function have been given. The work also aims to open up new possibilities in the biochemistry area as a whole.

Epoxide hydrolases

Enzymes are proteins that catalyze reactions. Catalyzing a reaction is the process of increasing the rate of the reaction. Enzymes are responsible for extraordinary rate enhancements compared to the nonenzymatic uncatalyzed reactions. Decarboxylation of orotic acid is a process which in nature occurs at a time span over almost eighty million years. The enzyme orotidine 5’-phosphate decarboxylase enhances this reaction by a factor of $10^{17}$, decarboxylating forty molecules of orotic acid during a single second (Radzicka and Wolfenden 1995).

A catalyst is not consumed in the reaction nor does it affect the chemical equilibrium. Enzymes enable reactions by stabilizing the transition state so that the activation energy is lower compared to the uncatalyzed reaction. Enzyme can do this *e.g.* by general acid/base catalysis or electrostatic catalysis. Enzymes can also provide a different reaction pathway through covalent catalysis. One way of achieving this is by forming an intermediate that is more reactive than the substrate through nucleophilic or electrophilic attack. The potato epoxide hydrolase, for example, forms a reactive alkylenzyme intermediate to enhance the rate of epoxide hydrolysis.

However, to fully explain the concept of enzyme catalysis solely by transition state stabilization could be misleading. Other forces may underlie, accompany, or replace the energy of transition state stabilization. These concepts could be binding effects, electrostatics, quantum mechanical tunneling, coupled protein motions, low-barrier hydrogen bonds, and near-attack conformations (Radzicka and Wolfenden 1995, Fersht 1999, Borman 2004).
To aid organization of enzymes many classification methods are employed. Enzymes can e.g. be classified by reaction type, structure, properties, subcellular localization, origin of species, and substrate preference.

The most common classification is the one by the Enzyme Commission (EC) which divides enzymes into six major groups by the reaction type they catalyze. These groups are the oxidoreductases (EC 1), transferases (EC 2), hydrolases (EC 3), lysases (EC 4), isomerases (EC 5), and ligases (EC 6).

The epoxide hydrolase from potato belongs to the group of hydrolases. Hydrolases are enzymes catalyzing the hydrolysis of a molecule through the addition of a water molecule. Hydrolases can be further subdivided and classified based on the bonds they act upon. If the chemical bond is an ether bond the enzyme belongs to the subclass EC 3.3. If the molecule is an epoxide and the enzyme is soluble, like the enzyme from potato, it belongs to the group of enzymes with EC number 3.3.2.10.

According to other classification systems the EHs are further divided up, since there is a wide range of epoxide hydrolases there are several groups containing one or several epoxide hydrolase enzymes. EHs can for example be divided into microsomal EHs (mEHs), soluble EHs (sEHs), cholesterol EHs, leukotriene A₄ and hepoxilin A₃ hydrolases, juvenile hormone EH, fosfomycin resistance epoxide hydrolases, limonene-1,2-epoxide hydrolases, and α/β hydrolase fold EHs. The potato epoxide hydrolase belongs to the α/β hydrolase fold EHs (Lu and Miwa 1980, Ollis et al. 1992, van der Werf et al. 1998, Armstrong 1999, Arand et al. 2003a; 2003b, Johansson et al. 2005, Morisseau and Hammock 2005, Fillgrove et al. 2007, Haeggström et al. 2007, Decker et al. 2009, Widersten et al. 2010).

In this thesis, properties of the wild-type epoxide hydrolase as well as enzyme variants originating from it have been studied. In most experiments the reaction when epoxide hydrolase converts epoxide into a vicinal diol is studied. From these investigations results and finally conclusions can be drawn. In paper I and II the study focus lies on properties influencing the catalytic mechanism. In paper III the hydrolysis reaction was followed both to identify active enzyme variants and to investigate a changed activity profile of the enzymes.

Epoxides

In enzymatically catalyzed reactions the molecules interconverted by the enzyme are referred to as the substrate. Epoxide hydrolases catalyze reactions using epoxides as substrates. Epoxides are molecules containing an epoxide unit which is a three-membered heterocyclic ring containing two carbon atoms and an oxygen atom (Figure 1). The oxygen is electonegative rendering the carbon-oxygen bonds polar. The ring structure is highly strained leading to the reactive character of the epoxide. Epoxides belong to
a sub-class of ethers but in comparison to other ethers, which are often used as solvents because of their inertness, epoxides can be reactive even under mild conditions.

Nucleophilic additions to epoxides can be both acid and base catalyzed. Under acidic conditions the nucleophilic attack occurs at the most substituted carbon and under basic conditions at the least substituted one. The substituents on an epoxide ring also influence the overall character of the epoxide, and the point of nucleophilic attack during hydrolysis. In uncatalyzed hydrolysis at neutral pH, the nucleophilic attack normally occurs on the least sterically hindered carbon of the oxirane ring, an effect altered by an electron donating substituent. A phenyl group e.g., directs the attack to the substituted carbon by stabilizing the partial positive charge on the attacked carbon (Parker and Isaacs 1959).

In the studies leading to this thesis two epoxides have been used as substrates (Figure 1). In paper I and paper II the hydrolysis of both enantiomers of trans-stilbene oxide (TSO) was used to investigate the function of StEH1. In paper III the aim was to construct novel epoxide hydrolases with increased catalytic activity for the enantiomers of (2,3-epoxypropyl)benzene. Since most plant epoxide hydrolases are involved in the metabolic pathways of lipid epoxides, the endogenous substrates for the potato enzyme are believed to be epoxide-containing fatty acids. These epoxides differ in size and bulkiness from the substrates used in this study but it has been shown that the potato enzyme exhibits broad substrate specificity (Morisseau et al. 2000, Elfström and Widersten 2005, Newman et al. 2005).

![Figure 1. Hydrolysis of trans-stilbene oxide (1) into the corresponding diol meso-hydrobenzoin (2) and of (2,3-epoxypropyl)benzene (3) into 3-phenylpropane-1,2-diol (4).](image)

**Functions of epoxide hydrolases**

Epoxide hydrolases are present in various organisms ranging from mammals, plants, insects, yeasts, filamenous fungi, to bacteria. They all hydro-

Catabolic EHs are most often found in bacteria, the stress response enzymes in plants, and the regulatory signaling and detoxification enzymes in mammals. In humans epoxide hydrolases convert endogenous as well as exogenous reactive epoxides, often toxic, into vicinal diols, which are less harmful since they are easily excreted and do not possess the same intrinsic reactivity as epoxides. The epoxides themselves are often formed in the oxidation of other compounds. For example, the epoxides formed during the metabolic oxidation of polycyclic aromatic hydrocarbons found in soot and cigarette smoke and known to cause cancer can be made less toxic by the hydrolysis catalyzed by epoxide hydrolases. However, some resulting diols are themselves precursors of highly carcinogenic compounds. Epoxide hydrolases have a dual function both as detoxification enzymes and as activator enzymes producing toxic compounds (Lu and Miwa 1980, Seidegård and Ekström 1997, Benhamou et al. 1998, Szeliga and Dipple 1998, Armstrong 1999).

The biological role of the epoxide hydrolase in the potato plant is not fully established but the expression of the enzyme has been shown to be regulated by both developmental as well as environmental signals. The EH is mostly expressed in the leaves of the potato plant suggesting a role in formation of the protective cutin layer. The potato EH is also suggested to play a role defending against exogenous epoxides. It appears that epoxide-containing fatty acids are the preferred endogenous substrate for the potato EH, like for most plant EHs (Stapleton et al. 1994, Morisseau et al. 2000, Newman et al. 2005).

In the last decades the use of epoxide hydrolases as biocatalysts to produce chiral compounds has grown and their potential has been explored. Both wild-type epoxide hydrolases and engineered variants have been investigated and utilized. The search for novel EHs with new or improved substrate specificities, enantioselectivities and regioselectivities is an on-going process. Many epoxide hydrolases are highly enantio- and regioselective and could be used to produce chiral synthons for e.g. the production of pharmaceuticals, agrochemicals, and fine chemicals. The high enantio- and regioselectivity shown by the enzymes allow both the epoxide and the diol to be prepared in high enantiomeric purity enabling kinetic resolution as well as enantioconvergent resolution. EHs with complementary enantio- and regioselectivity are needed for enantioconvergent hydrolysis of racemic epox-
ides and will theoretically produce vicinal diols at high yield. To date epoxide hydrolases from microbial sources are most used but the potato epoxide hydrolase is a promising biocatalytic candidate. In paper III new enzyme variants with changed enantioselectivity compared to wild-type StEH1 with the substrate (2,3-epoxypropyl)benzene were constructed (Archelas and Furstoss 1998; 2001, Orru and Faber 1999, Steinreiber and Faber 2001, de Vries and Janssen 2003, Monterde et al. 2004, Smit 2004, Lee and Shuler 2007, Lee 2008, Choi 2009, Widersten et al. 2010).

α/β Hydrolase fold epoxide hydrolases

The α/β hydrolase fold enzymes are a group of enzymes with diverse catalytic functions. Many lipases, esterases, peptidases, proteases, peroxidases, and dehalogenases are members of the family. The sequence similarity among these enzymes is low, often below 20%, and the functions they carry out and the substrates they convert are diverse. The α/β hydrolase fold enzymes form a superfamily of proteins based on two characteristics: a shared structural fold and catalytic triad. The first similarity, the canonical α/β hydrolase fold consists of a central eight-stranded β-sheet, surrounded by α-helices (Figure 2). All epoxide hydrolases belonging to the superfamily have a lid domain, covering the core, which consists mostly of α-helices. In α/β hydrolase fold enzymes the core domain has been preserved from a common ancestor to provide a stable scaffold and to conserve catalytic residues’ positions. The substrate binding site has diverged during evolution giving rise to the many catalytic functions seen within the superfamily.

The second similarity between the members of the superfamily is the catalytic triad, which consists of a nucleophile, a histidine and an acid. The histidine residue is conserved in all α/β hydrolase fold enzymes but the length and shape of the loop it is connected to can vary. The nucleophile can be a serine, cysteine, or an aspartic acid and it is positioned in a sharp turn to provide space for the substrate and the hydrolytic water molecule. Epoxide hydrolases from this superfamily always have an aspartate functioning as a nucleophile. The position of the nucleophile also contributes to the formation of the oxyanion hole. The oxyanion hole is most often formed by two backbone amide nitrogens, one coming from the residue next to the nucleophile (Ollis et al. 1992, Nardini and Dijkstra 1999, Holmquist 2000, Barth et al. 2004, Bugg 2004, van Loo et al. 2006).

Epoxide hydrolases belonging to the α/β hydrolase fold superfamily can be divided into two major groups; the microsomal epoxide hydrolases and the soluble ones. There are microsomal epoxide hydrolases from mammalian, fungal, insect, and bacterial origin whereas the soluble epoxide hydrolase family includes enzymes from mammals, plants, and bacteria. Soluble EHz are generally more substrate selective than mEHs and are not able to
convert as bulky substrates. The microsomal enzymes have an additional N-terminal extension serving as membrane anchor, attaching them to the endoplasmatic reticulum (Lu and Miwa 1980, Stapelton et al. 1994, Seidegård and Ekström 1997, Zou et al. 2000, Barth et al. 2004).

Structures of six epoxide hydrolases belonging to the α/β hydrolase fold enzyme superfamily are known: two epoxide hydrolases from bacteria, the EH from *Agrobacterium radiobacter* (ArEH), and the EH from *Mycobacterium tuberculosis* (MtEH), one EH from fungi, *Aspergillus niger* (AnEH), and two from mammals, the sEH from *Mus musculus* (MmsEH) and the sEH from human (HssEH). The potato epoxide hydrolase is the only plant EH with known structure (Figure 2) (Nardini et al. 1999, Biswal et al. 2008, Zou et al. 2000, Argiriadi et al. 2000, Gomez et al. 2004, Mowbray et al. 2006).

*Figure 2.* Overall structure of the α/β hydrolase-fold potato epoxide hydrolase (StEH1) (PDB entry: 2cjp, Mowbray et al. 2006) (A) Core domain in white and lid domain in black. (B) The surface of the enzyme shows a globular monomeric enzyme. (C) A close-up of the active site with the conserved active site residues shown in stick representation: the two tyrosines (Tyr<sup>154</sup>, Tyr<sup>235</sup>), and the catalytic triad: the nucleophile (Asp<sup>105</sup>), the histidine base (His<sup>300</sup>), and the charge relay residue (Asp<sup>265</sup>). All images were made using PyMol (DeLano 2002).
All epoxide hydrolases show a high degree of similarity in the core α/β-hydrolase fold domain. The α-helical lid domains differ most in structure between the EHs. The lid domain together with the core domain builds the active site pocket. The lid is suggested to have the most influence on the substrate specificities of the EHs. Most reported epoxide hydrolases are homodimers, but plant EHs are monomers. The lid domains of plant EHs have an additional loop preventing dimerization. The lid of AnEH, in contrast, contains an additional loop stabilizing a dimeric structure. The HssEH has been shown to be active both in its monomeric form and as a dimer. In addition, the mammalian enzymes also have an N-terminal domain with phosphatase activity (Dietze et al. 1990, Cronin et al. 2003, Newman et al. 2003, Barth et al. 2004, Mowbray et al. 2006).

Catalytic mechanism of α/β hydrolase fold epoxide hydrolases

When epoxide hydrolases convert epoxides into vicinal diols the reaction proceeds via a two-step catalytic mechanism (Figure 3). The first step is the formation of a covalent alkylenzyme intermediate and the second step is the actual hydrolysis reaction. The catalytic triad is located in the core and two tyrosines involved in positioning the epoxide during catalysis are located in the lid domain. The catalytic triad consists of an aspartate acting as nucleophile, a histidine functioning as a general base, and aspartic or glutamic acid acting as a charge relay residue. Their roles have been confirmed by e.g. site-directed mutagenesis, where an enzyme variant with the nucleophilic aspartate exchanged for a serine resulted in a totally inactive enzyme. Enzyme variants with a mutation in the general base histidine were still able to form the alkylenzyme intermediate but lacked the ability to hydrolyze it (Dubois et al. 1978, Lacourciere and Armstrong 1993, Bell and Kasper 1993, Borhan et al. 1995, Pinot et al. 1995, Tzeng et al. 1998, Armstrong 1999, Armstrong and Cassidy 2000, Arand et al. 2003a, Morisseau and Hammock 2005).

Preceding the nucleophilic attack is the formation of the Michaelis complex. The epoxide has to enter the active site in close proximity to the catalytic residues for the enzyme-substrate complex to form. Aiding in the positioning of the epoxide are two tyrosine residues from the lid domain. The tyrosines are located 5-8 Å away from the nucleophile, giving room for the oxirane to position itself in the active site. By donated hydrogen bonds the oxirane orients in a position optimal for nucleophilic attack. The nucleophile attacks one of the carbons creating a covalent intermediate.
Figure 3. Two-step catalytic mechanism of α/β-hydrolase fold epoxide hydrolases. In I the epoxide enters the active site and the Michaelis complex is formed. Two tyrosines position the epoxide for nucleophilic attack leading to formation of the alkylenzyme intermediate in step 1. The histidine base, in concert with the aspartic charge relay residue, activates the water molecule in II making it ready for the hydrolysis step 2. The catalytic cycle ends in III with the diol formation and product release.

The negative charge on the alkylenzyme intermediate is stabilized by the two tyrosine residues and the whole intermediate is also stabilized by the oxyanion hole. The covalent alkylenzyme intermediate is then attacked by a water molecule. The conserved histidine residue working in concert with the charge relay residue base-activates the water. The resulting vicinal diol exits the active site and a new catalytic cycle can start (Tzeng et al. 1996, Rink et al. 2000, Yamada et al. 2000, Elfström and Widersten 2006).

The reaction mechanism described is a simplified, minimal version and at the beginning of this thesis work the exact mechanism was still unclear. In paper I and II additional residues involved in the mechanism are explored and an extended new mechanism is proposed.
Kinetic mechanism of α/β hydrolase fold epoxide hydrolases

The kinetic mechanism of epoxide hydrolases can in its simplest form be described as shown in scheme 1 and has been deduced using both steady state kinetics and pre-steady state kinetics.

\[
\begin{align*}
E & \underset{k_{-2}}{\rightleftharpoons} ES & k_2 & \rightarrow E\text{-alkyl} & k_3 & \rightarrow E + P
\end{align*}
\]

(Scheme 1)

The equilibrium dissociation constant, \(K_S\), describes the formation and breakdown of the Michaelis complex. The formation of the alkylenzyme intermediate is described by \(k_2\), and its breakdown to the enzyme-substrate complex is described by \(k_{-2}\). \(k_3\) is the rate of alkylenzyme complex hydrolysis leading to product release. This last step is virtually irreversible. Formation of the enzyme-substrate complex is a rapid equilibrium and for almost all substrates and EHs the first alkylation step is faster than the hydrolytic half-reaction. The turnover number, \(k_{\text{cat}}\) almost equals \(k_3\), thus indicating that the hydrolytic half-reaction is the rate-limiting step. Reactions with the substrates used in the papers included in this thesis, trans-stilbene oxide and (2,3-epoxypropyl)benzene, can adequately be explained by the mechanism in scheme 1 (Tzeng et al. 1996, Laughlin et al. 1998, Armstrong and Cassidy 2000, Rink et al. 2000, Elfström and Widersten 2005, Lindberg et al. 2008, Widersten et al. 2010).

However, there are situations where the simple mechanism cannot explain the observed kinetic behaviors. Additional steps involving isomerizations between conformers of the alkylenzyme intermediate have for example been proposed for the reaction with (1R)-styrene oxide. Also the fact that an EH can attack either one of the two electrophilic carbons of an epoxide, which forms two different alkylenzyme intermediates is not addressed in scheme 1. An extended kinetic model therefore has to be used which describes separate pathways for formation of two different diols from one epoxide. This alternative model includes isomerization between two unliganded enzyme forms as well as between two Michaelis complexes (Rink et al. 1998, Lindberg et al. 2008; 2010).
Methodological basis of the methods used in this thesis

Enzyme kinetics

Studies of the rates of enzyme catalysis, enzyme kinetics, provide information on enzyme specificities and mechanisms. Investigations of enzymes during steady state conditions following Michaelis-Menten kinetics can give values of the kinetic parameters $k_{\text{cat}}$, $K_M$, and $k_{\text{cat}}/K_M$.

The turnover number, $k_{\text{cat}}$ is a first order rate constant and a measure of the number of substrate molecules each enzyme active site converts to product per unit time. $k_{\text{cat}}$ refers to the properties of all enzyme-substrate, enzyme-intermediate, and enzyme-product complexes, i.e. $k_{\text{cat}}$ cannot be greater than any first-order rate constant on the forward reaction pathway. In the case of the two-step reaction of StEH1 described in scheme 1, $k_{\text{cat}}$ will be the composite of all rate constants in the forward and reverse reaction pathway, $k_2$, $k_{-2}$, and $k_3$.

The Michaelis constant $K_M$ is experimentally determined as the substrate concentration at half the maximum velocity. $K_M$ is an apparent dissociation constant of all enzyme-bound species and is in its simplest form a direct measure of the dissociation constant, $K_S$.

The specificity constant, $k_{\text{cat}}/K_M$ is an apparent second-order rate constant and its value cannot be greater than any of the second-order rate constants on the forward reaction pathway. It sets a lower limit on the rate constant for association of enzyme and substrate. Some enzymes exhibit impressive efficiencies, almost as soon as the enzyme encounters the substrate it converts it into product. These are called diffusion-controlled enzymes and enzymes with specificity constants of $10^8$-$10^9$ M$^{-1}$s$^{-1}$ are enzymes working close to diffusion-controlled conditions.

Studying enzyme kinetics in the steady state phase is a good way to get information of overall rate constants, but to elucidate the rates of all individual steps in catalysis, transient kinetic methods have to be used. In the pre-steady state phase the concentration of reaction intermediates has not reached equilibrium. It is during this time period the individual rate constants can be observed.

Since turnover numbers of enzymes are high, the time span to measure in pre-steady state can be as short as $10^{-7}$ s. To detect these events rapid mixing
of the enzyme with substrate and a direct monitoring of events occurring during this short time span is necessary. A stopped flow apparatus was used to detect the pre-steady state phase for the potato EH in the experiments described in this thesis. In the stopped flow the mixing is rapid and the reaction can be detected by a spectroscopic signal on a millisecond scale.

In the TSO hydrolysis reaction catalyzed by StEH1 the intrinsic tryptophan fluorescence is detected over time. Quenching of the fluorescence by a tryptophan in the active site of StEH1 occurs when the alkylenzyme intermediate is formed and this signal is recovered during the subsequent steps of the reaction. Detection and analysis of the burst kinetics as well as the recovery of the fluorescence signal give values of the individual rate constants for the reaction catalyzed by StEH1 (Kyte 1995, Fersht 1999, Johnson 2003, Elfström and Widersten 2005, Frey and Hegeman 2007).

pH dependence in enzymatic reactions

If an enzyme-catalyzed reaction is dependent on amino acids either functioning as acids or bases, the activity of the enzyme will change as the pH is altered. Many amino acid residues in an enzyme can be protonated or deprotonated. The pKₐs of amino acids integrated in proteins are seldom the same as the pKₐs they display in small peptides, due to the effect of the microenvironment surrounding the residue. Sometimes the pKₐ can be severely perturbed, e.g. the pKₐ of glutamate and aspartate in an enzyme usually vary between 2 to 5.5. Histidines usually display pKₐs of 5-8 and the pKₐs of tyrosines and cysteines range from 8-12.

When detecting the effect of changes in pH of a catalyzed reaction it is not the acid/base behaviors of all residues that are seen. Only the ionizing behaviors of those residues directly important for the catalysis at the active site, or residues responsible for keeping the enzyme in an active form, are detected.

Analyzing the pH-dependency of the rate of an enzyme-catalyzed reaction gives information about the catalytic mechanism and help pinpoint residues of interest and their ionization states during the catalytic cycle. When assuming Michaelis-Menten kinetics and following the pH dependency of kₐ the process of concern is the reaction, starting from the enzyme-substrate complex to product release. The assigned pKₐ is then a weighted mean of all substrate bound enzyme complexes. The pH dependence of kₐ/Kₐ follows the ionization of free enzyme and substrate. If the rate-determining step is not changed with pH, the pH-dependence of kₐ/Kₐ will be the same for all non-ionizing substrates (Kyte 1995, Fersht 1999, Frey and Hegeman 2007).
Solvent kinetic isotope effects

Isotopic substitutions can be used to provide information about the reaction pathway. An isotopic substitution of the atom directly participating in the rate-limiting step of a reaction gives rise to a primary isotope effect. Isotopically substituting an atom not directly participating in the reaction gives rise to secondary isotope effects. Isotope effects can also be detected in solvent. Solvent kinetic isotope effects can be detected when measuring the rate of a reaction performed in the isotopic form of a solvent. Comparing the rate with the rate in solvent with the naturally occurring isotope can give insight into proton transfer reactions accompanying the bond making/breaking steps between the heavier atoms. The solvent isotopic effects on enzymatic reactions are detected by substituting H₂O for D₂O and can give information about the mechanism of a hydrolysis reaction.

In the reaction with the potato epoxide hydrolase the hydrolysis reaction is most often the rate-limiting step and thus a proton transfer from water or from the deuterium oxide would be possible to probe. A shift in pH optimum towards more basic pH is a possible effect of exchanging H₂O for D₂O. It is also expected that the ratio of $k_{H}/k_{D}$ will be approximately 2, as reported for general base-assisted ester hydrolysis (Jencks and Carriuolo 1961, Bender et al. 1964, Kyte 1995, Fersht 1999).

Construction of new enzymes

In the quest of finding or constructing enzymes with novel functions or improved specificities scientists have successfully used in vivo, in vitro, and in silico approaches. The search for epoxide hydrolases for biocatalytic processes includes the exploration and mining of yet undiscovered proteins e.g. by screening samples for epoxide hydrolase function, or by starting with a computer-aided genome analysis. The most common approach is to modify and improve desired properties like enantioselectivity in presently known EHs (de Vries and Janssen 2003, van Loo et al. 2006, Lee and Schuler 2007, Choi 2009, Damborsky and Brezovsky 2009).

Directed evolution and rational design are methods used to redesign enzymes. In directed evolution a library of enzyme variants is created by random mutagenesis and followed by screening and/or selecting variants possessing the improved desired properties. Mimicking evolution in the test tube, in vitro evolution, is an iterative process with several rounds of mutagenesis and screening. After each round the protein variant showing most desired improvement is used as template for the next round of mutagenesis. DNA shuffling and error-prone polymerase chain reaction are two of the most used methods to introduce mutations. No prior structure information of the enzyme is needed to conduct directed evolution. When using the method
of rational design, sequence and structure information is necessary and specific amino acids are targeted for site-specific mutagenesis. Both approaches have resulted in numerous enzyme variants with impressive results regarding improved specificities (Leung et al. 1989, Cadwell and Joyce 1991, Stemmer 1994, Moore and Arnold 1996, Turner 2009).

There are of course limitations to both approaches. Even though knowledge of structure-function relationships in enzymes is good there are still extreme challenges to overcome before a rational design can be used to alter all enzymes in the desired direction. In directed evolution, library creation and screening procedures can pose problems. A selection-based variant identification system is desired but hard to develop. Constructing a screen testing the variants for improvements in the desired properties is somewhat easier but still challenging. For a selection or a screen to be truly successful it has to test the enzyme variants for several desired properties simultaneously, because the outcome of a selection round depends on the constraints decided in the screen itself.

Depending on the properties to change, for example solvent resistance, substrate specificity, thermostability, and enantiospecificity, different screening approaches have to be taken. The number of variants to screen or select can be too high to be manageable in an ordinary laboratory setup. One approach to overcome some of these limitations is the combination of rational design and directed evolution, creating small focused libraries of enzyme variants by introducing mutations only at defined functional sites (You and Arnold 1994, Lutz and Patrick 2004, Chica et al. 2005, Kazlauskas and Lutz 2009, Otten et al. 2010).

The iterative CASTing method (combinatorial active site saturation test) developed by Reetz and co-workers have proven to be successful when changes in substrate scope and enantioselectivity are desired. For example, the group has improved the selectivity factor for the reaction with phenyl glycidyl ether catalyzed by an AnEH from 5 to 115. From the structure, which has to be solved, sets of one to three amino acid residues lining the active site pocket are categorized and divided into “hot spots”. These hot spots are then randomly mutared creating small focused libraries. Enzyme variants identified as hits in the first round are used as templates for further rounds of CASTing, revisiting the hot spots once more, and hopefully generating additive as well as cooperative effects in the constructed enzyme variants (Reetz et al. 2005; 2006a; 2006b; 2007; 2009a; 2009b).

Focused libraries reduce the protein sequence space and the screening efforts tremendously but still the process of screening is a bottle-neck in laboratory-based enzyme evolution. By constricting the number of possible amino acid substitutions in each position, the time for screening is decreased. Table 1 shows the effects of reducing the number of possible amino acids for substitution from twenty to twelve. Screening a focused library in which, for example, two amino acids are targeted for mutagenesis, would when using
the NNK codon degeneracy during construction, demand that at least 3,000 variants were screened to get 95% coverage of the library. In comparison, only 430 variants have to be screened when using the NDT codon. However, it should be taken into consideration that the possibility of incorporating all amino acids will not be present in the new variants, which could result in the loss of potentially improved variants (Reetz et al. 2008). In paper III the CASTing approach combined with the use of a restricted amino acid alphabet was used to construct new enzyme variants of StEH1 with the aim of finding improvement in enantioselectivity for either enantiomer of (2,3-epoxypropyl)benzene while still maintaining a high activity.

Table 1. Number of enzyme variants to screen for 95% coverage as a function of codon degeneracy and number of amino acids positions targeted for mutagenesis

<table>
<thead>
<tr>
<th>Degenerate codon</th>
<th>No. of codons</th>
<th>No. of amino acids</th>
<th>No. of stops</th>
<th>Amino acids encoded</th>
<th>1 position (95% coverage)</th>
<th>2 positions (95% coverage)</th>
<th>3 positions (95% coverage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNK</td>
<td>32</td>
<td>20</td>
<td>1</td>
<td>All</td>
<td>94</td>
<td>3066</td>
<td>98163</td>
</tr>
<tr>
<td>NDT</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>FLIVY HNDCR SG</td>
<td>34</td>
<td>430</td>
<td>5175</td>
</tr>
</tbody>
</table>

Adapted from Reetz et al. 2008.
Aims and scope of the thesis

The investigations presented here concern the potato epoxide hydrolase, StEH1. However, the conclusions drawn from the work presented are more general and not just specific to this enzyme. In paper I a new catalytic mechanism is proposed. In paper II a proton-water channel found in the potato enzyme, believed to increase the thermostability of the enzyme, is suggested to be present in other plant EHs as well. In paper III new StEH1 enzyme variants were constructed. Variants with shifted enantio preference were constructed opening the door to the possibility of StEH1 variants being used as biocatalysts. All studies have also contributed to new insight into structure-function relationships in enzymes in general.

In paper I the aim was to elucidate the function of the active site residue Glu$^{35}$. After studying the previously known structure of StEH1 it was suggested that this glutamate residue is interacting with the hydrolytic water molecule and is the first residue in a network of hydrogen bonds connecting the active site with the exterior and solvent. One specific aim of the study was to answer questions about the functions of the interaction with the water molecule. Does the glutamate residue aid in the correct positioning of the water molecule, and is this interaction essential for the reaction to occur? What is the function of the network of hydrogen bonds connecting the active site with the exterior? Is the network preserved in other EHs? Additional questions arose during the investigation that needed response. What is the explanation for the observed pH-dependencies that were experimentally determined for the reaction with the enantiomers of trans-stilbene oxide with wild-type enzyme and with the E35Q (Glu→Gln residue replacement) mutant variant? Are there solvent isotope effects arising from the hydrolysis reaction and if so, how are these to be interpreted? Is the simplified catalytic mechanism insufficient to explain the results seen in this study? Can a new catalytic mechanism be deduced and proposed? Kinetic studies (steady state and pre-steady state) of wild-type enzyme and a constructed E35Q mutant variant as well as computer simulations probing the acid-base characteristics of His$^{300}$ were used to answer these questions.

In paper II a putative proton wire in the wild-type enzyme composed of five water molecules lined up in a tunnel was investigated. Water molecules from the exterior to the catalytic Tyr$^{154}$, could be transporting protons in to
the active site. In the enzyme variants constructed, the residues coordinating the waters in the tunnel were exchanged for residues without hydrogen bonding properties. The enzyme variants were studied kinetically for the reaction with both enantiomers of trans-stilbene oxide. For one variant the structure was solved. The results from the study would hopefully answer the following questions. What is the function of the tunnel lined with coordinated water molecules? Is there a tunnel with the same function in other EHs? Will the mutations lead to effects seen on kinetic parameters? Does the wire provide any additional functions?

In paper III the aim was to construct enzyme variants with modified specificity for the substrate (2,3-epoxypropyl)benzene. Since StEH1 is a promising biocatalytic candidate it was interesting to see if it would be possible to alter the substrate specificity of the enzyme in a chosen direction. Residues lining the active site pocket were chosen and divided into four hot spots. These spots were exposed to mutagenesis. The constructed variants were screened for activity with both substrate enantiomers. A mutant gene identified as a hit arising from the first generation libraries was then used as template for a second round of saturation mutagenesis. The produced enzyme variants would answer the following questions. Were the residues chosen for mutagenesis involved in determining specificity? If so, what is the rationale behind the observed shift in specificity? Did the iterative methodology of library construction result in a rapid in vitro evolution? Did cooperative or additive effects arise in enzyme variants from the second generation libraries? Can the potato enzyme be altered in a desirable way and can it be sculpted into a useful biocatalyst?
Active site residue Glu$^{35}$ is important in formation and breakdown of the alkylenzyme intermediate (Paper I)

Disruption of the hydrogen bond pattern in the active site of StEH1

The active site residue Glu$^{35}$ had from structure analysis of StEH1 been identified as a residue that could be involved in catalysis. The glutamate is the first residue in a chain of hydrogen bonds connecting the active site with bulk solvent. A mutant variant, E35Q, with the glutamate residue exchanged for a glutamine, was constructed and analyzed (Figure 4).

![Diagram of wild-type StEH1 and E35Q mutant variant showing hydrogen bonds](image)

*Figure 4. Possible effects on the hydrogen bonds in the active site of StEH1 as a function of a glutamate → glutamine mutation at position 35.*

The mutation was proposed to interfere with the hydrogen bonding pattern in the active site and disrupt the chain of hydrogen bonds from the active site to the exterior. The hydrogen bond between the carboxylate oxygen of Glu$^{35}$ and the side chain amide nitrogen of Gln$^{304}$ positions the glutamate residue optimally for accepting a proton from the hydrolytic water molecule. This hydrogen bond pattern is disrupted when introducing a glutamine, changing the whole hydrogen bond acceptor/donor milieu around the water. In the mutant variant the amide NH$_2$ group is thought to be oriented towards the water molecule, thereby shifting the orientation of the water. These proposed effects of the mutation were supported by computer simulations. The position of Gln$^{35}$ always converged in the orientation shown in Figure 4 in performed molecular dynamics (MD) simulation experiments. In addition the
results from the simulations also suggested that the water molecule seems to be pushed out of the active site in the substrate-free E35Q mutant variant.

Conservation of a putative hydrogen bond network connecting the active site with solvent

Glu\textsuperscript{35} is interacting with the putative hydrolytic water molecule and is the first residue in a chain of hydrogen bonds connecting the active site with the exterior. Glu\textsuperscript{35} is within hydrogen bond distance to the hydroxyl group of Ser\textsuperscript{39}, which in turn is hydrogen bonded to the hydroxyl of Tyr\textsuperscript{219}. The tyrosine is connected to the guanidinium group of Arg\textsuperscript{41}, which in turn is connected to Glu\textsuperscript{215}. Glu\textsuperscript{215} is positioned at the surface of StEH1 making it possible to release protons to the solvent. A strong conservation of the glutamate at positions corresponding to position 35 in StEH1 are found in other soluble epoxide hydrolases (Figure 5). Chemical functionalities as well as structure arrangement of residues proposed to be integral parts of the hydrogen bond network are also well conserved.

Figure 5. Sequence alignment of soluble epoxide hydrolases from plants and mammals. Residues directly participating in the putative hydrogen bond network are indicated with white letters. StEH1 (Swiss-Prot entry Q41415_SOLTU), soybean (Q39856_SOYB), rough lemon (Q76E11_9ROSI), rape (Q8L5G6_BRANA), rice (Q9S7P1_ORYSA), pineapple (Q8H289_ANACO), E. lagascae (Q84ZZ3_EUPLA), mouse-ear cress (Q42566_ARATH), tobacco (Q9ZP87_TOBAC), pig (HYES_PIG), human (HYES_HUMAN), rat (HYES_RAT), and mouse (HYES_MOUSE).

TSO hydrolysis is effected when glutamate is mutated into glutamine at position 35 in StEH1

The kinetic effects of the glutamate to glutamine exchange at position 35 in StEH1, seen in the hydrolysis of both enantiomers of TSO, were analyzed both in the steady state as well as in the pre-steady state phases (Table 2). In
the reaction with S,S-TSO the specificity constant was almost unaffected by the mutation. However, the turnover number was decreased. For the reaction with R,R-TSO the catalytic turnover number decreased forty-fold. The hydrolysis rate was decreased as was the rate of formation of alkylenzyme.

The alkylation rates for wild-type StEH1 with S,S-TSO and for the E35Q mutant variant with both enantiomers of TSO are similar and more than ten-fold lower than that for wild-type StEH1 with R,R-TSO. The explanation for the high $k_2$ rate in the wild type with R,R-TSO could be that when the wild type binds R,R-TSO the salt bridge between His$^{300}$ and Asp$^{105}$ is disrupted. The salt bridge is not disrupted to the same extent in the other cases investigated. It could be that the greater the disruption of the salt bridge, the more accelerated the proton liberation from Glu$^{35}$ through the hydrogen bond network becomes.

Table 2. Kinetic parameters of TSO hydrolysis catalyzed by wild-type StEH1 and E35Q mutant variant at pH 6.8

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_S$ (μM)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$k_3$ (s$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (μM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$μM$^{-1}$)</th>
<th>$k_2/K_S$ (s$^{-1}$μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S,S-TSO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>11±6</td>
<td>18±2</td>
<td>14±2</td>
<td>3.2±0.06</td>
<td>4.7±0.5</td>
<td>0.80±0.07</td>
<td>1.8±1.4</td>
</tr>
<tr>
<td>E35Q</td>
<td>≤5</td>
<td>≤38 b</td>
<td>≤5 b</td>
<td>1.2±0.06</td>
<td>1.1±0.1</td>
<td>1.1±0.1</td>
<td>-</td>
</tr>
<tr>
<td>R,R-TSO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>36±20</td>
<td>260±60</td>
<td>16±20</td>
<td>24±3</td>
<td>23±2</td>
<td>2.4±0.2</td>
<td>7.7±3</td>
</tr>
<tr>
<td>E35Q</td>
<td>27±20</td>
<td>16±3</td>
<td>6.7±2</td>
<td>0.95±0.04</td>
<td>0.52±0.02</td>
<td>0.48±0.05</td>
<td>0.86±0.6</td>
</tr>
</tbody>
</table>

$^a$ Data adapted from Elfström and Widersten 2005.

$^b$ Poorly determined due to low signal-to-noise ratios at low substrate concentrations.
An unexpected pH dependence for the hydrolysis of $R,R$-TSO is displayed by the E35Q mutant variant

**Figure 6.** Effects of pH on the catalytic turnover number, $k_{\text{cat}}$ (A and B) and on the hydrolysis of the alkylenzyme intermediate, $k_3$ (C and D) catalyzed by wild-type StEH1 (unfilled symbols) and E35Q mutant variant (filled symbols) with $S,S$-TSO (A and C) and $R,R$-TSO (B and D). Results from reactions performed in 100% H$_2$O (circles) and in 84% D$_2$O (triangles) are shown in D.

The plots of pH as a function of the logarithm of $k_{\text{cat}}$ and $k_3$ are expected to resemble each other since the main rate-limiting step is the hydrolysis reaction. The E35Q mutant variant displayed similar behavior as previously reported by the wild type (Figure 6) (Elfström and Widersten 2005). The plots for wild-type StEH1 with both enantiomers of TSO and for E35Q mutant with $S,S$-TSO display two distinct titrations, the titration of a base at the acidic limb of the plot and that of an acid at the basic limb. The pH dependence displayed by E35Q in hydrolysis of $R,R$-TSO results in similar p$K_a$ values but the profile is inverted, the rates reaching the minima where others display their maxima (Table 3). The p$K_a$ of the acidic limb resembles that of
an acid titration and the basic limb that of a base. Simulations of the displayed pH dependence of $k_{\text{cat}}$ performed to explain the unusual pH profile, suggest that the rate of formation of the tetrahedral intermediate during the catalytic cycle is substantially decreased together with a drastic shift in acidity of all enzyme-substrate/intermediates. For the $S,S$-TSO reaction catalyzed by E35Q the rate decrease in tetrahedral intermediate formation was not as drastic.

Table 3. Experimentally determined $pK_a$ values for the TSO hydrolysis catalyzed by wild-type StEH1 and E35Q mutant variant in H$_2$O and D$_2$O

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_3$</th>
<th>$k_{\text{cat}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S,S$-TSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild type (H$_2$O)</td>
<td>n.d.</td>
<td>7.4±0.2*</td>
</tr>
<tr>
<td>E35Q (H$_2$O)</td>
<td>6.0±0.4</td>
<td>7.4±0.1</td>
</tr>
<tr>
<td></td>
<td>5.2±0.5</td>
<td>7.4±0.2</td>
</tr>
<tr>
<td></td>
<td>5.3±0.2</td>
<td>8.1±0.1</td>
</tr>
<tr>
<td>$R,R$-TSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild type (H$_2$O)</td>
<td>6.8±0.2</td>
<td>7.8±0.2</td>
</tr>
<tr>
<td>wild type (D$_2$O)</td>
<td>7.2±0.5</td>
<td>7.9±0.4</td>
</tr>
<tr>
<td>E35Q (H$_2$O)</td>
<td>7.0±0.1</td>
<td>7.9±0.3</td>
</tr>
<tr>
<td>E35Q (D$_2$O)</td>
<td>7.3±0.2</td>
<td>8.4±0.4</td>
</tr>
</tbody>
</table>

* Data adapted from Elfström and Widersten 2005.

The deuterium solvent kinetic isotope effects on the hydrolysis with $R,R$-TSO for wild-type StEH1 and E35Q mutant variant were examined (Figure 6). The pH/pD optimum is shifted 0.5 pH units towards the basic region, a result shown by another epoxide hydrolase and by chymotrypsin. The wild type shows a clear solvent isotope effect, indicating that proton transfer from the water molecule is indeed rate-limiting within the pH range tested. In E35Q proton transfer from the water is rate-limiting between pH 7.5 and 9.0. At high pH/pD values the solvent isotope effect is not as distinct (Jencks and Caniolo 1961, Bender et al. 1964, Armstrong et al. 1980).

The $pK_a$ of His$^{300}$ shifts during the catalytic cycle

The pH dependencies seen in the wild-type StEH1 catalyzed reactions with TSO resembles the ones reported for other epoxide hydrolases and the acidic $pK_a$ has been assigned to the histidine base corresponding to His$^{300}$ in StEH1 (Armstrong et al. 1980, Blée and Schuber 1992, Bellevik et al. 2002, Elfström and Widersten 2005). Consensus of the assignment of the basic $pK_a$ to a residue has not priorly been reached. MD simulations and continuum electrostatic $pK_a$ calculations together with the experimentally determined data suggest the basic $pK_a$ to be assigned to His$^{300}$ as well. The calculated
values of $\text{His}^{300} \text{pK}_a$ show the same trend as the experimentally determined ones (Table 4). In the substrate-free wild-type enzyme the $\text{pK}_a$ value suggests that the histidine residue is protonated at neutral pH, forming a hydrogen bond to Asp$^{105}$. In the E35Q mutant the calculated $\text{pK}_a$ is two units lower. The formation of the Michaelis complex does not influence the $\text{pK}_a$ of $\text{His}^{300}$ but when the alkylenzyme intermediate is formed the $\text{pK}_a$ drops to be restored when the tetrahedral intermediate is formed. $\text{His}^{300}$ in its deprotonated form, hydrogen bonds to the putative hydrolytic water molecule and base-activates it for nucleophilic attack. The acid-base behavior of $\text{His}^{300}$ is analogous to the role of the histidine in trypsin which also displays the same shift in $\text{pK}_a$ during the catalytic cycle (Kosiakoff and Spencer 1981).

Table 4. Calculated $\text{pK}_a$ values for $\text{His}^{300}$

<table>
<thead>
<tr>
<th>enzyme</th>
<th>$S, S$-TSO</th>
<th>$R, R$-TSO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>free enzyme</td>
<td>Michaelis complex</td>
</tr>
<tr>
<td>wild type</td>
<td>10±2</td>
<td>n.d.</td>
</tr>
<tr>
<td>E35Q</td>
<td>8.2±1</td>
<td>n.d.</td>
</tr>
</tbody>
</table>


A novel proposed catalytic mechanism

The experimentally determined results combined with the computer simulations made it possible to propose a new catalytic mechanism explaining the catalytic cycle in more detail (Figure 7). The catalytic mechanism in Figure 3 is here expanded including explanations to for example, the protonation and deprotonation of $\text{His}^{300}$ and the role of Glu$^{35}$ as first in a chain of hydrogen bonds connecting the active site with the bulk solvent.
Figure 7. Proposed mechanism for the hydrolysis of TSO catalyzed by StEH1. (I) At neutral pH, at the start of the catalytic cycle, His$^{300}$ is in its fully protonated form, making ion-ion interactions with the charge relay residue, Asp$^{265}$ (not drawn), and the nucleophile, Asp$^{105}$. (II) Substrate enters the active site and by the aid of Tyr$^{154}$ and Tyr$^{235}$ the enzyme orients the epoxide oxygen in the right direction, forming the Michaelis complex. The substrate interacts with the protein via nonpolar interactions and through a hydrogen bond between the oxirane oxygen and the hydroxyl of Tyr$^{154}$. His$^{300}$ is concurrently deprotonated by a water-mediated base abstraction by Glu$^{35}$. (III) The hydrogen exits the active site through a network of hydrogen bonds donors/acceptors, Ser$^{39}$, Tyr$^{219}$, Arg$^{41}$, and Glu$^{215}$ to the solvent. (IV) Asp$^{105}$ is then released from its ion-ion interaction with His$^{300}$ and attacks the epoxide ring forming a negatively charged alkylenzyme intermediate, which is stabilized by the hydroxyls of the tyrosine pair. (V and VI) The alkylenzyme intermediate is then attacked by a His-activated water molecule forming a tetrahedral intermediate which is stabilized by the backbone amides of Trp$^{106}$ and Phe$^{33}$ through electrophilic catalysis. (V) Simultaneously as the nucleophilic attack by the activated water, a proton enters the active site via a channel lined with water molecules ending at Tyr$^{154}$, which protonates the charged alkylenzyme intermediate. (VI and VII) His$^{300}$ mediates breakdown of the tetrahedral intermediate by donating a proton, but as the product is released, abstracts a proton back from Asp$^{105}$. A water molecule enters the active site which is restored to the initial conformation and the next catalytic cycle can start.

Conclusions

Glu$^{35}$ is proposed to be a catalytic residue in the active site of the potato epoxide hydrolase. The hydrogen bond network, in which Glu$^{35}$ is a part, connecting the active site with the exterior is structurally and functionally preserved in other sEHs. During the hydrolytic half-reaction Glu$^{35}$ aids in positioning the water molecule through a hydrogen bond in an optimal orientation. The imidazolium of His$^{300}$ is then able to work as both an acid and a base. When Glu$^{35}$ is mutated into a glutamine this interaction is lost and the reaction is hampered. Glu$^{35}$ also influences the alkylenzyme formation by activating Asp$^{105}$ for nucleophilic attack. This is facilitated by the release of protons through the hydrogen bond network.
A proton wire in potato epoxide hydrolase adds thermostability (Paper II)


A protein channel lined with water molecules

During the catalytic cycle of StEH1 a charged alkylenzyme intermediate is formed (Figure 7, V). The intermediate is protonated by one of the catalytically important tyrosines, Tyr^{154}, located in the active site pocket. The tyrosine is in turn proposed to be protonated by a water molecule. The proton is transported to the active site through a tunnel in the protein lined with water molecules. The water molecules are coordinated by the backbone carbonyls of Pro^{186} and His^{269}, the hydroxyl of Tyr^{149}, the backbone carbonyl of Leu^{266}, the imidazole moiety of His^{153}, and at the active site the hydroxyl of Tyr^{154}. To assess the importance of the proton wire three mutant variants, Y149F, H153F, and double mutant Y149F/H153F were constructed (Figure 8).

Figure 8. A schematic overview of the putative proton wire in wild-type StEH1 (A). The anticipated effects of the residue replacements in the mutant variants, Y149F (B), H153F (C), and Y149F/H153F (D).
The hydroxyl of Tyr\textsuperscript{149} is an integral part of the chain, hydrogen bonding to water molecules on both sides. In the Y149F mutant variant the hydroxyl group is removed. The anticipated effect of the mutation on the hydrogen bond pattern is seen in Figure 8B. In the structure of StEH1, His\textsuperscript{153} is seen coordinating a water molecule. Replacing the histidine residue with phenylalanine removes a protein-water hydrogen bond interaction (Figure 8C). In the double mutant Y149F/H153F three protein-water hydrogen bond interactions are lost that are present in the wild type (Figure 8D).

Conservation of a putative proton wire in plant epoxide hydrolases

The tyrosyl residue corresponding to Tyr\textsuperscript{149} in StEH1 is strongly conserved in plant epoxide hydrolases. A structure-based sequence alignment of ten plant EHs indicates that the residue is more conserved than is required by structural settings (Figure 9). However, a histidine at the position corresponding to position 153 is not essential, a residue shared among the ten EHs only with the rice EH. In most other EHs the histidine is replaced by a tyrosine. A tyrosine still participates in protein-water hydrogen bond interactions, the conserved feature in that position is the hydrogen bonding capacity. The residues interacting with the proton wire through backbone carbonyls, Pro\textsuperscript{186}, His\textsuperscript{269}, and Leu\textsuperscript{266} are reasonably conserved. Their conservation is based on the preservation of the correct structure. The putative proton wire seems to be conserved in plants but a similar tunnel cannot be identified in the structure of the other epoxide hydrolases from the \(\alpha/\beta\) hydrolase fold enzyme superfamily (ArEH, MtEH, AnEH, MmsEH, HssEH).

Figure 9. Sequence alignment of soluble plant epoxide hydrolases. Residues directly participating in the putative proton wire are indicated with white letters. StEH1 (Swiss-Prot entry Q41415_SOLTU), soybean (Q39856_SOYB), rough lemon (Q76E11_9ROSI), rape (Q8L5G6_BRANA), rice (Q9S7P1_ORYSA), pineapple (Q8H289_ANACO), \textit{E. lagasca} (Q84ZZ3_EUPLA), barrel medic (A2Q320_MEDTR), chickpea (Q8LPE6_CICAR), and mouse-ear cress (Q42566_ARATH).
A water molecule replaces the hydroxyl group when tyrosine is mutated into a phenylalanine at position 149 in StEH1

The structure of the substrate-free form of the Y149F mutant variant was solved and refined to a resolution of 2 Å. Only very subtle changes were found when comparing the Y149F mutant variant with the wild type. The space occupied by a hydroxyl group in the wild-type structure is filled by a water molecule in Y149F. The other water molecules lining the proton wire remained in the same positions and the hydrogen bond pattern in Figure 8B is an adequate representation of the structure of the proton wire in the Y149F mutant variant.

The kinetic effects of the reaction with TSO reveals an increase in catalytic turnover for the Y149F mutant variant

The kinetic effects of the mutations introduced in the putative proton wire in StEH1, seen in the hydrolysis of both enantiomers of \textit{trans}-stilbene oxide, were analyzed in the steady state phase. The hydrolysis of both enantiomers of TSO was also followed in the pre-steady state phase for the Y149F mutant variant. With \textit{R,R}-TSO it was impossible to reach enzyme saturation within the solubility range of the substrate, why pre-steady state parameters could not be determined with high accuracies (Table 5). The Y149F mutant variant displayed an increased catalytic turnover with both enantiomers of TSO. The Michaelis constants were at the same time increased, resulting in catalytic efficiencies comparable to that for the wild type or even decreased. The increase in $k_{\text{cat}}$ is an effect of an increased hydrolysis rate. With the H153F mutant variant the largest effect was seen in the nine-fold increase in the Michaelis constant for the reaction with \textit{R,R}-TSO. The $k_{\text{cat}}/K_M$ for the reaction with \textit{R,R}-TSO with the Y149F/H153F mutant variant was ten-fold decreased compared to the wild-type value. For the reaction with \textit{S,S}-TSO the catalytic efficiency was twice as high. The wild-type StEH1 show higher catalytic efficiency with \textit{R,R}-TSO and the enantioselectivities displayed by the wild type and the Y149F/H153F mutant variant are more than thirty-fold different.
Table 5. Kinetic parameters for TSO hydrolysis catalyzed by wild-type StEH1, and the Y149F, H153F, and Y149F/H153F mutant variants, at pH 6.8

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kinetic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>S,S-TSO</td>
<td></td>
</tr>
<tr>
<td><strong>enzyme</strong></td>
<td>$K_S$ (μM)</td>
</tr>
<tr>
<td>wild type</td>
<td>11±6$^a$</td>
</tr>
<tr>
<td>Y149F</td>
<td>15±8</td>
</tr>
<tr>
<td>H153F</td>
<td>-</td>
</tr>
<tr>
<td>Y149F/H153F</td>
<td>-</td>
</tr>
</tbody>
</table>

| R,R-TSO   |                    |
| **enzyme** | $K_S$ (μM) | $k_2$ (s$^{-1}$) | $k_2$ (s$^{-1}$) | $k_3$ (s$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ (s$^{-1}$μM$^{-1}$) | $k_2/K_S$ (s$^{-1}$μM$^{-1}$) |
| wild type | 36±20     | 260±60   | 16±20    | 24±3     | 23±2$^a$  | 10±1     | 2.4±0.2$^a$  | 2.7±0.3    |
| Y149F    | 500±900   | 300±400  | 30       | 60       | 44±2     | 36±3     | 1.2±0.06    | 0.80±0.6   |
| H153F    | -        | -        | -        | -        | 15±2     | 94±20    | 0.16±0.01   | -         |
| Y149F/H153F | -        | -        | -        | -        | n.d.     | n.d.     | 0.20±0.01   | -         |

$^a$Data adapted from Elfström and Widersten 2005.
n.d. Not determined due to inability to reach saturation within the solubility range of the substrate.

Proton transfer between electronegative atoms, in the StEH1 case from the exterior to the active-site Tyr$^{154}$, is expected to be a much faster ($>10^{10}$ s$^{-1}$) process than the catalytic turnover of the enzyme (<300 s$^{-1}$). A facilitated proton transfer would thus have no influence on the catalytic turnover. The increased $k_{cat}$ is then more likely to be an effect of a decreased energy barrier for the formation of the transition state intermediate in the hydrolysis half-reaction, which is the rate-limiting step of the reaction. It is proposed that the observed effect is due to the facilitated protonation of the alkylenzyme intermediate. If the intermediate is not protonated a doubly negatively charged high-energy specie would be formed. The electrostatic repulsion in this intermediate would create a much higher energy barrier for the hydrolysis reaction than is seen now that the protonation occurs.

The effects of thermal inactivation are additive

Thermal inactivation after incubation of enzyme variants at 55 °C over time was determined (Figure 10A). The activity was measured for the reaction with R,R-TSO. The wild-type activity was decreased to half after 2 hours and 15 minutes. Both the Y149F mutant variant and H153F mutant variant displayed half-lives of one hour at 55 °C. The double mutant Y149F/H153F displayed a severely affected activity with a half-life of only twenty minutes at 55 °C. The effects on the activity at elevated temperatures seem to be additive, the sum of the single mutants gives a good approximation of the thermal sensitivity of the double mutant. The thermal inactivation of the wild
type and the Y149F mutant variant was also examined after five minute long incubations at temperatures up to 70 °C (Figure 10B). The Y149F mutant was not as thermally stable as the wild type. At 60 °C the wild-type enzyme retained around 65 % of its initial activity but the Y149F mutant had lost 70% of its activity. The thermal inactivation was not reversible as shown in Figure 10C where the relative activity of wild-type StEH1 and Y149F mutant variant incubated at 50 °C for three hours and then at room temperature for an additional three hours is plotted.

Figure 10. Thermal inactivation of enzyme activity. (A) The effects of incubation at 55 °C over time on the activity using 50 μM R,R-TSO with wild-type StEH1 (square), Y149F mutant variant (circle), H153F mutant variant (diamond), and double mutant variant Y149F/H153F (triangle). (B) Remaining enzyme activity in measurements using 50 μM R,R-TSO, after five minutes of incubations at temperatures ranging between 50 °C and 70 °C with wild-type StEH1 (square), and Y149F mutant variant (circle). (C) The effects of incubation at 50 °C over three hours on the activity with 50 μM R,R-TSO for wild-type StEH1 (square), and Y149F mutant variant (circle), and the remaining activity using 50 μM R,R-TSO at incubation temperature 22 °C over three hours. Activities prior to incubations at elevated temperatures were normalized to 100%.

The negative effects of the elevated temperatures on the activity of the enzyme variants could be an effect of removed hydrogen bonds. Hydrogen bonds between water molecules and protein residues contribute to stabilization of protein structure. The removal of one to three hydrogen bonds in the mutant variants compared to the wild type severely affected the thermal re-
sistance. Even though it is not a direct analysis of structural unfolding the thermal inactivation studies indicate that the removal of hydrogen bonds affected the structure of the mutant variants. However, the loss in protein stability could increase protein flexibility. An increased flexibility could decrease the energy barrier for the formation of the tetrahedral intermediate during the rate-limiting hydrolysis half-reaction. Other studies have shown the plant EHs to be more stable than the mammalian sEHs. The difference in stability between the EHs could be an effect of their in vivo roles and functions (Yamagata et al. 1998, Morisseau et al. 2000).

Conclusions
The putative proton wire in the potato epoxide hydrolase is proposed to facilitate effective proton transfer from the exterior to the active site Tyr^{154}, aiding in protonation of the alkylenzyme intermediate during the catalytic cycle. The protein tunnel lined with water molecules is also proposed to be present in other plant EHs and there via hydrogen bonds between water molecules and protein contribute to stability of the enzyme.
Proton entry and exit pathway in StEH1 (Paper I & II)

The results from paper I regarding the plausible exit route for protons through a hydrogen bond network, combined with the result regarding the putative proton wire in paper II led to the proposal of a combined entry and exit pathway for protons in StEH1 (Figure 11).

*Figure 11.* Possible lateral translocation of protons in and out of the active site of StEH1 (PDB entry: 2cjp, Mowbray *et al.* 2006). (A) Arrow indicates the possible proton entry and exit pathway in StEH1. (B) Surface image of StEH1 with position of the residues involved in proton entry and proton release indicated. (C) In blue, residues involved in the entry pathway of protons (Tyr$_{149}$, Pro$_{186}$, His$_{269}$, Leu$_{266}$, and His$_{153}$), in black, the active site residues (Tyr$_{154}$, Tyr$_{535}$, Asp$_{105}$, His$_{300}$, and Asp$_{265}$), and in purple, residues together with a water molecule involved in the exit route of protons (Glu$_{35}$, Ser$_{39}$, Tyr$_{219}$, Arg$_{41}$, and Glu$_{215}$). All images were made using PyMol (DeLano 2002).

It is conceivable that the potato epoxide hydrolase has evolved to efficiently provide protons into the buried active site to be used for the protonation of the alkylenzyme intermediate. At the same time His$_{300}$ has to be deprotonated to be able to act as an effective base and thus a proton has to be shuffled out of the active site during the initial stages of the catalytic cycle. This release is proposed to proceed through the hydrogen bond network composed of side chain functionalities of residues Glu$_{35}$, Ser$_{39}$, Tyr$_{219}$, Arg$_{41}$, and Glu$_{215}$. The exit pathway of protons is a conserved structural element in mammals and plants and residues corresponding to Glu$_{35}$ are present in 95% of unique, non-potato, sequences annotated as established or putative epoxide hydrolases. The combined entry and exit pathway of protons are proposed to be a conserved structural feature of epoxide hydrolases from plants.
Construction of epoxide hydrolases with altered enantiopreference (Paper III)

The use of enzymes as catalysts in asymmetric catalysis is increasing. Enzymes catalyze reactions in aqueous solvents and at moderate temperatures which contributes to an environmentally friendly and safe production line. The specificity epoxide hydrolases show for some substrates is a valuable property in the synthesis of chiral diols. The demand on ultra-pure epoxides is then low since a racemic reactant mixture would still generate an optically pure product.

More than 80% of all active compounds in the pipeline for use in the pharmaceutical industry are chiral. Mono-substituted epoxides with an unsaturated hydrocarbon side chain are important chiral intermediates in the synthesis of more complex chiral compounds and are thus of interest for the industry. The epoxide (2,3-epoxypropyl)benzene and corresponding vicinal diol could be useful anti-inflammatory agents (Bysouth and Musgrave Wild 1972, Breuer et al. 2004, Turner 2009). The wild-type potato epoxide hydrolase catalyze the reaction with (2,3-epoxypropyl)benzene but the enantioselectivity is low. In this study, construction of enzymes with enhanced enantioselectivity towards (2,3-epoxypropyl)benzene was attempted using an evolutionary approach in a laboratory setting.

Restricting mutagenesis to defined hot spots reduces library sizes

In the attempt to produce new enzyme variants with altered enantioselectivity compared to the wild-type enzyme with the substrate (2,3-epoxypropyl)benzene, residues lining the active site pocket in StEH1 were identified and divided into four hot spots (Figure 12). Targeting residues close to the active site for mutagenesis has been proven more effective than targeting distant ones when enantioselectivity is the desired property to alter (Morley and Kazlauskas 2005, Reetz et al. 2005). Saturation mutagenesis was restricted to these residues thereby reducing protein sequence space and screening efforts. Amino acids identified as directly participating in catalysis were not included in the hot spots since catalytically active enzyme variants were desired.
Limited codon degeneracy was applied when constructing the enzyme libraries; however residues with aromatic, aliphatic, uncharged polar, positively charged, and negatively charged properties were present in the reduced assortment of amino acids. Mutations in library A are directed at the phenylalanine at position 33, a residue positioned deep in the active site pocket. The phenyl ring of Phe$^{33}$ interacts with, and could be involved in the positioning of Tyr$^{235}$. The residues targeted for mutagenesis in library B, Trp$^{106}$ and Leu$^{109}$, are positioned at the entry pathway for the substrate where Trp$^{106}$ also is interacting with Tyr$^{235}$. In addition, the backbone amides of Phe$^{33}$ and Trp$^{106}$ form the oxyanion hole which stabilizes the tetrahedral intermediate during the catalytic cycle. Residues targeted for mutagenesis in library C, Val$^{141}$, Leu$^{145}$, and Ile$^{155}$, are positioned in the entry pathway for the epoxide. Residues targeted for mutagenesis in library D, Ile$^{180}$ and Phe$^{189}$, are located deep in the active site pocket.
Screening enzyme libraries in the quest of finding improved enzyme variants

The cDNA libraries were constructed by using mutagenic primers in the polymerase chain reaction, PCR. In the first generation libraries the plasmid coding for wild-type SteH1 was used as a template. In the second generation libraries the plasmids encoding the selected first generation library hit variants were used as templates. The cycle of library construction and screening using the enzyme variants with the desired improvements in activity from the first generation libraries as template was repeated creating seven second generation libraries originated from three first generation library hit variants. Iteratively revisiting the same hot spots when creating the second generation libraries generates additive, and hopefully cooperative effects on the activity in the mutant variants. An outline of the sites visited during library construction is shown in Figure 13.

![Diagram of library construction](image)

**Figure 13.** Outline of combinations of hot spots in the constructed cDNA libraries. Site A: Phe^{33}, B: Trp^{106} and Leu^{109} C: Val^{141}, Leu^{145}, and Ile^{155} D: Ile^{180} and Phe^{189}. Numbers in parentheses indicates number of screened variants.

A 96-wells plate format was utilized, both for protein expression and screening. The screen was adapted from the protocol described by Cedrone et al. (2005), where the epoxide forms a blue-colored conjugate and the diol does not. A well containing a highly active enzyme variant where all epoxide is converted into diol is hence colorless. In the first generation libraries the cleared lysates from 5,700 enzyme variants were screened for activity with the racemic mixture of (2,3-epoxypropyl)benzene. During the next round of library screening the lysates from 4,400 enzyme variants from seven libraries were screened for improved enantioselectivity with the pure enantiomers of (2,3-epoxypropyl)benzene.
First generation enzyme variants with enhanced preference for S-(2,3-epoxypropyl)benzene

From the first generation libraries approximately four hundred functional variants were identified as hits. An enzyme variant was identified as a hit if it showed comparable or higher activity than the wild type with the racemic epoxide. From these hits the cDNA encoding seventy enzyme variants were sequenced and the resulting amino acid substitutions in the targeted positions are shown in Table 6. All libraries contained wild-type plasmids to some degree, why oversampling sometimes was made to ensure a high coverage of the library.

Table 6. Sequence analysis of enzyme hits selected from screening for activity with (2,3-epoxypropyl)benzene.

<table>
<thead>
<tr>
<th>Mutated residue (library)</th>
<th>Identity and frequency of residue replacements in sequenced variants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>1st generation</td>
<td></td>
</tr>
<tr>
<td>Phe33 (A)</td>
<td>0.5</td>
</tr>
<tr>
<td>Trp106 (B)</td>
<td>1.0</td>
</tr>
<tr>
<td>Leu109 (B)</td>
<td>0.8</td>
</tr>
<tr>
<td>Val141 (C)</td>
<td></td>
</tr>
<tr>
<td>Leu145 (C)</td>
<td>0.5</td>
</tr>
<tr>
<td>Ile155 (C)</td>
<td></td>
</tr>
<tr>
<td>Ile180 (D)</td>
<td>0.1</td>
</tr>
<tr>
<td>Phe189 (D)</td>
<td>0.2</td>
</tr>
<tr>
<td>2nd generation</td>
<td></td>
</tr>
<tr>
<td>Phe33 (A)</td>
<td>0.1</td>
</tr>
<tr>
<td>Trp106 (B)</td>
<td>0.6</td>
</tr>
<tr>
<td>Leu109 (B)</td>
<td></td>
</tr>
<tr>
<td>Val141 (C)</td>
<td>0.1</td>
</tr>
<tr>
<td>Leu145 (C)</td>
<td></td>
</tr>
<tr>
<td>Ile155 (C)</td>
<td></td>
</tr>
<tr>
<td>Ile180 (D)</td>
<td></td>
</tr>
<tr>
<td>Phe189 (D)</td>
<td></td>
</tr>
</tbody>
</table>

In library A an aromatic residue seems to be preferred at position 33 and in library B the tryptophan residue was, if mutated, always exchanged for a phenylalanine. The aromatic interactions between the residues Phe33, Trp106, and Tyr335 are, in most active enzyme variants, a conserved feature, and seem to be important for a retained activity. At position 109 the hydrophobic property was conserved in the incorporated residues. At position 141 the polar residue lysine was the residue most often incorporated in the novel active variants, indicating a favorable influence on catalysis of a positively
charged residue at that position. In half of the cases a polar residue was also incorporated replacing Leu\textsuperscript{145}. Ile\textsuperscript{155} was in all cases exchanged for a less bulky valine. Residues in library D were in most cases exchanged for residues with hydrophobic character. In position 189 the residues were also of less bulk.

Out of the seventy sequenced enzyme variants from the first generation libraries eighteen were expressed in \textit{Escherichia coli} and purified. The stereochemistry of the produced diols from hydrolysis of \textit{rac}-(2,3-epoxypropyl)benzene catalyzed by the enzyme variants were detected using chiral high performance liquid chromatography (chiral HPLC). The regiospecificity in the catalyzed reaction has not been established but the nucleophilic attack is assumed to occur at the least hindered carbon. The enzymes are not promiscuous in their point of attack since only one diol enantiomer is formed in the hydrolysis of a pure enantiomer epoxide. Assuming attack at the least hindered carbon, the \textit{S}-epoxide is converted to a \textit{S}-diol, and the \textit{R}-diol is a product of the hydrolysis of the \textit{R}-epoxide.

![Figure 14](image-url)

\textit{Figure 14.} Product outcomes after reactions with \textit{rac}-(2,3-epoxypropyl)benzene catalyzed by wild type, and StEH1 mutant variants. Bars in black indicate relative concentration of formed \textit{S}-3-phenylpropane-1,2-diol, bars in white indicate relative concentrations of formed \textit{R}-3-phenylpropane-1,2-diol. Error bars indicates standard deviations.

The enantioselectivity displayed by most tested enzyme variants was similar to the wild-type StEH1 (Figure 14). In the hydrolysis of a racemic mixture of (2,3-epoxypropyl)benzene the wild-type enzyme forms 67\% \textit{S}-3-phenylpropane-1,2-diol and only 33\% \textit{R}-3-phenylpropane-1,2-diol. Aiming at increasing the preference for both enantiomers, two enzyme variants, F189V and V141K/I155V, which displayed an increased preference for the \textit{R}-epoxide were chosen as starting points for the next generation of enzyme libraries. An enzyme variant with increased preference for the \textit{S}-epoxide compared to the wild-type was also chosen as template for the next generation libraries. This enzyme variant, W106F/L109I, showed a product ratio of
the S-diol vs. R-diol of 4:1, resulting in a product enantiomeric excess, $ee_p$, of 60% for the S-diol.

A second generation enzyme variant with shifted enantiopreference

The second generation libraries were screened with both enantiomers of (2,3-epoxypropyl)benzene individually. Out of 4,400 screened enzyme variants from the second generation libraries around 350 were identified as hits. The hit frequencies in the libraries differed for the two enantiomers. There were more enzyme variants identified with improved or retained activity compared with wild type for the S-epoxide than for the R-epoxide. The cDNA from eighty of these variants active with either epoxide were sequenced. The results are shown in Table 6.

The quality of the second generation libraries was much poorer than that of the first generation libraries and almost three quarters of the sequenced plasmids were coding for wild-type StEH1. A total of twenty-two new enzyme variants were identified. The distribution of the incorporated residues at position 33 is much higher in the second generation libraries than in the first generation libraries, either retaining the phenylalanine or introducing a polar residue. A leucine is found in some enzyme variants at position 106. This removes the aromatic interaction with Tyr$^{235}$, conserved in all sequenced first generation enzyme variants. A tyrosine or an asparagine was introduced in position 109. The enzyme variants with the polar residue removed in position 106 are the same ones with a tyrosine residue introduced at position 109. It could be that this aromatic residue is introduced as compensation to the aromatic loss in position 106 or just to provide space close to the nucleophile, Asp$^{105}$. The introduction of an asparagine at position 109 introduces a polar residue into a totally non-polar micro-environment. Polarity is also introduced at positions 141 and 145 through incorporated residues. In position 155 the hydrophobic character is still conserved, as in the first generation libraries.

The enantioselectivity of eight selected, expressed and purified enzyme variants were detected using chiral HPLC. Some displayed poor activity or purification yields why the result for only three variants, V141K/I155V, V141Q/I155L, and W106L/L109Y/V141K/I155V, are shown in Figure 14. The enzyme variant V141K/I155V from the second generation library CA (Figure 13) is on protein level identical to its parent but on DNA level a distinct clone. The enzyme displayed low enantiopreference for the hydrolysis of (2,3-epoxypropyl)benzene. V141Q/I155L originated from library DC showed only a modest enantiopreference for S-(2,3-epoxypropyl)benzene. However, the enzyme variant, W106L/L109Y/V141K/I155V, found in the screen of library CB displayed a drastic shift in enantiopreference compared...
to wild-type StEH1. The enzyme variant produces 84% R-3-phenylpropane-1,2-diol and only 16% S-3-phenylpropane-1,2-diol during the hydrolysis with the racemic mixture of epoxide, resulting in a $ee_p$ of 69% in favor of the R-diol.

In Table 7 the steady state parameters for the hydrolysis reactions with both enantiomers of (2,3-epoxypropyl)benzene catalyzed by wild-type StEH1, V141K/I155V, and W106L/L109Y/V141K/I155V enzyme variants are shown. The results were determined by detecting the formed diols using reverse-phase HPLC.

**Table 7.** Steady state kinetic parameters for the hydrolysis of (2,3-epoxypropyl)benzene catalyzed by wild-type StEH1, and the V141K/I155V, and W106L/L109Y/V141K/I155V mutant variants.

<table>
<thead>
<tr>
<th>substrates</th>
<th>enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$mM$^{-1}$)</th>
<th>$E$ (R/S)</th>
<th>$E$ (S/R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R$-(2,3-epoxypropyl)benzene</td>
<td>wild type</td>
<td>13±0.9</td>
<td>0.78±0.1</td>
<td>17±2</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V141K/I155V</td>
<td>9.1±0.2</td>
<td>1.0±0.04</td>
<td>9.3±0.2</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W106L/L109Y/V141K/I155V</td>
<td>7±3</td>
<td>2±1</td>
<td>12±0.6</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>$S$-(2,3-epoxypropyl)benzene</td>
<td>wild type</td>
<td>43±3</td>
<td>1.0±0.1</td>
<td>42±3</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V141K/I155V</td>
<td>13±4</td>
<td>1.6±0.4</td>
<td>8.3±0.9</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W106L/L109Y/V141K/I155V</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.77±0.08</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

The $E$-value for $R$-(2,3-epoxypropyl)benzene goes from 0.4 → 1.1 → 15 when moving from wild-type enzyme, via the double mutant to the quadruple mutant. The basis for the effect is not a significant increase in the catalytic efficiency in hydrolysis with the $R$-epoxide, since it is slightly decreased compared to the wild-type catalytic efficiency. Instead it is the catalytic efficiency with the $S$-epoxide that is severely hampered. A 55-fold decrease in activity compared to the wild type for $S$-(2,3-epoxypropyl)benzene for the W106L/L109Y/V141K/I155V mutant variant was detected.

**Conclusions**

Enzyme variants with modified specificity for both enantiomers of the substrate (2,3-epoxypropyl)benzene have been constructed. The potato epoxide hydrolase have the ability to be altered in a desired way making it a promising candidate for use as a biocatalyst. The residues targeted for mutagenesis were indeed involved in determining the enantioselectivity displayed by the enzyme variants. Especially residues in position 106 and in position 109 could be important for deciding the enantiopreference for the substrate (2,3-
epoxypropyl)benzene, possibly due to the importance of an aromatic residue in the vicinity of the nucleophile, Asp$^{105}$. The process of in vitro evolution did in only two generations result in enzyme variants with shifted enantio-preference. The wild type favored the $S$-enantiomer in a ratio of 2:1 and the quadruple mutant variant W106L/L109Y/V141K/I155V, displaying the most radical shift, favored the $R$-enantiomer in a ratio of 15:1. Additive, and possibly also cooperative effects on the enantiospecificity were detected in the second generation enzyme variants.
Concluding remarks and future perspectives

The work summarized in this thesis has provided additional information and novel knowledge concerning structure-function relationship in the potato epoxide hydrolase.

The studies described in papers I and II resulted in the proposal of a combined entry and exit pathway of protons in potato epoxide hydrolase. The entry pathway is suggested to be structurally conserved in the epoxide hydrolases from plants and being responsible for efficient protonation during the alkylenzyme intermediate formation as well as providing stability to the enzyme structure. The exit pathway of protons is proposed to be conserved in soluble epoxide hydrolases from both mammals and plants. The residue Glu\textsuperscript{35} has been shown to be a catalytically important residue both in the formation of the alkylenzyme intermediate and during the hydrolytic half-reaction. A catalytic mechanism integrating the new results with previously established knowledge was also proposed. The mechanism provides explanations to formerly unsolved questions regarding protonation of the alkylenzyme intermediates and proton entry and release from the active site pocket.

Even though the novel catalytic mechanism described in paper I explains the behavior of the potato epoxide hydrolase with the substrate $\text{trans}$-stilbene oxide it would be of interest to further study how and why the mechanism differs between substrates. This information could give answer to more general structure-function questions. It would also be of interest to investigate proton liberation through the conserved hydrogen bond network in other epoxide hydrolases. Is the disruption of the proton exit pathway affecting the individual rates of the reaction similarly to the observed behavior in StEH1? How do protons enter and exit the active site in other epoxide hydrolases? Are there similar networks where protons are shuffled in and out of the active site in other structurally related enzymes?

A highly resolved structure with the alkylenzyme intermediate trapped in the catalytic cycle before hydrolysis would provide information of great interest, e.g. in studies of the mechanism. The structure would, for example aid in interpreting experimental data, and in more detail reveal the interactions between enzyme and substrate.

Further studies on the proton wire would also be interesting to conduct. What if the detection of the supposed unfolding of the protein variants could be followed directly? Is the detected stability at high temperatures an evolved feature in plant EHs due to their biological function? If the struc-
tures of other plant epoxide hydrolases were solved the prediction of the presence of a protein tunnel lined with water molecules in those EHs could be proven valid or not.

In paper III *in vitro* evolution was applied with the aim to shift the enantioselectivity of the potato epoxide hydrolase for the substrate (2,3-epoxypropyl)benzene. It is interesting to see if the wild-type enzyme could be redesigned to possess improvements in desired properties, since StEH1 is a potential biocatalyst in the production of chiral diols. Indeed the applied iterative approach of CASTing resulted in several enzyme variants with shifted enantiopreference compared to the wild-type enzyme. Variants with improved enantioselectivity for the S-epoxide were identified in the first round of library screening. The best variant identified, W106F/L109I, produced 80% of the resulting S-3-phenylpropane-1,2-diol and only 20% of the R-diol when hydrolyzing the racemic epoxide mixture. From the second generation libraries an enzyme variant, W106L/L109Y/V141K/I155V, with four residues replaced compared to wild type displayed a drastic shift in enantiopreference. The mutant variant produce more than 80% R-3-phenylpropane-1,2-diol and less than 20% S-diol when hydrolyzing rac-(2,3-epoxypropyl)benzene. The drastic effect on enantiopreference resulted in a 38-fold difference in enantioselectivity between wild-type StEH1 and the quadruple mutant, W106L/L109Y/V141K/I155V.

It would be of great interest to see if further rounds of CASTing will result in novel enzyme variants with further improved enantioselectivities. Reetz and coworkers (2009b) have enhanced the enantioselectivity for the S-enantiomer of phenyl glycidyl ether, starting with an E-value of 3 displayed by the wild-type AnEH reaching an E-value of 193 as displayed by the new enzyme variant. The enzyme variant was evolved using five rounds of CASTing and accumulated nine residue replacements. Will the same magnitude of improvement be possible with the potato epoxide hydrolase and the chosen hot spots for mutagenesis? Was the approach of targeting only residues lining the active site pocket for mutagenesis the right decision? Is the used screen optimal for detection of enantioselective enzyme variants for this system? Could a different screening approach be applied?

The investigations presented in this thesis have had different research foci. Some studies are aiming at direct applications of the results, such as the constructed enzyme variants in paper III, whereas other studies have been conducted to provide knowledge not to be immediately utilized, such as the discovery of the proton entry and exit pathway. Fundamental research on epoxide hydrolases increases the understanding of underlying principles and in the long term adds to the knowledge base for further applications using epoxide hydrolases.

The work summarized in the thesis has to a large extent been curiosity-driven and the results open up further questions and possibilities. Could the proton shuffling in the potato epoxide hydrolase in the future be used in an
industrial application? Did the structure-function relationships provide information that in the future can be utilized when constructing enantioselective epoxide hydrolases for a wide range of epoxides? Will the determining factor of selectivity be assessed and assigned to all residues in the active site pocket? If the desired end product is not a diol the epoxide hydrolase could still be used. Mixing of engineered, highly specific, enzyme variants of the potato epoxide hydrolase with other enzymes, for example enzyme variants of diol dehydrogenases, would make it possible to produce pure enantiomer product molecules in a one-pot reaction.
Sammanfattning

Denna avhandling beskriver arbetet kring ting som är för små för att se och som sker på tidskalor för korta för att vanligtvis uppfatta. Undersökningarna och experimenten har gällt ett enzym och de processer enzymet påverkar och utför. Studierna har haft varierat forskningsfokus, undersökningarna i delarbete I och delarbete II kan till största del definieras som grundforskning. Mycket av dagens grundforskning ämnar åt att söka ny kunskap genom att undersöka var gränserna går för nyligen introducerade metoder och teorier. Vetenskapsrådet är en statlig myndighet med mål att göra Sverige till en världssedande forskningsnation och försöker implementera det målet genom att finansiera just grundforskning. All grundforskning leder inte till kunskap som direkt kan appliceras i en industriell process eller användas för att snabbt lösa ett i dagsläget reellt problem, men besvarar ofta frågeställningar, stödjer eller omkullkastar hypoteser, samt bidrar med vetande som tillsammans med annan tillgänglig kunskap kan appliceras till samhällets nytta. Studierna sammanställda i delarbete III hade som syfte att skapa resultat med direkt applicerbarhet, även om det grundläggande forskningsintresset inte gick förlorat.

Enzymer katalyserar reaktioner, d.v.s. de påskyndar kemiska processer utan att själva förbrukas. Att förstå hur katalysen sker på molekylnivå är av intresse för forskning bedriven både i grundforskningssyfte och industriellt syfte. Frågeställningar som ställts under detta avhandlingsarbete är bl. a. följande. Vilka delar av enzymet interagerar med molekylen som skall omvandlas? Vilka interaktioner kan förväntas bidra mest till katalysen? Går det att förändra dessa delar och få en eftersökt effekt?

För att kunna använda enzymer i produktionen av organiska molekyler, t. ex. i framställandet av läkemedel, krävs det ofta att den naturliga funktionen hos enzymet ändras eller förbättras. Kunskap om vilka delar av ett enzym som måste förändras, muteras, för att erhålla en eftersökt effekt är då nödvändig. Ett syfte med studierna i denna avhandling var att bidra till den kunskapen. I delarbete I och II så undersöktes vilka delar av det enzym som studerats i denna avhandling som ökar hastigheten för dess katalys, samt vilka delar som kan bidra till att enbart vissa molekyler binder till enzymet och omvandlas och inte andra. I delarbete III användes genteknik och den nyvunna kunskapen för att mutera fram nya förbättrade enzymvarianter.
Figur 15. (A) Epoxider bildar dioler genom addition av vatten. (B) Enantiomerer är molekyler som är varandras spegelbilder. (C) En protonpump identifierad i potatisenzyme epoxidhydrolas är viktig för effektiv katalys.


Asymmerisk syntes, handlar om att framställa den ena enantiomeren i överskott och det senaste decenniet har alternativet att använda enzymer för att framställa rena enantiomerer börjat spira. Enzymer är, då de själva är
kirala, ofta selektiva för vilken enantiomer de binder och omvandlar till produkt. Biokatalysatorer kan skapa en miljövänligare och kostnadseffektivare framställningsprocess, än den traditionella organiska syntesprocessen, då t. ex. antalet produktionssteg ofta kan minskas när enzymer används. Enzymer katalyserar ofta reaktioner i vanligt vatten och vid rumstemperatur, det behövs inga miljöfarliga organiska lösningsmedel eller förhöjda temperaturer vilket är ytterligare fördelaktiga effekter till följd av användandet av biokatalysatorer.


Figur 16. (A) Processen att skapa och identifiera förbättrade enzymvarianter genom *in vitro* evolution. (B) Fördelning av bildade diolenantiomerer efter katalys av ursprungsenzym samt två förbättrade enzymvarianter.

I delarbete III konstruerades enzymvarianter som skulle kunna katalysera reaktionen med antingen den ena eller den andra enantiomeren av en för läkemedelsframställning intressant epoxid. Ett stort antal enzymvarianter undersökt och de varianter med förbättrade egenskaper användes som mall för nästa generation av varianter (Figur 16A). I en process som efterliknar evolutionen skapades det i laboratoriet flera enzymvarianter av ursprungsenzymet från potatis som hade förbättrade egenskaper. De omvandlade antingen den ena av enantiomeren i högre grad än ursprungsenzymet (Figur 16B, enzymvariant 1) eller den andra enantiomeren av epoxiden. Genom att använda *in vitro* evolution framställdes t. ex. ett enzym som helt skiftat enantiopreferens (Figur 16B, enzymvariant 2).
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