Hysteretic Behavior, Regioselectivity and Role of Salt Bridging Residues at the Domain Interface of Potato Epoxide Hydrolase StEH1, Site-Directed Mutagenesis and Kinetic Study

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Abstract

According to an earlier study, StEH1 exhibits slow transitions between different conformational states implying hysteresis. Since, ionic interactions play a great role in protein stability, they are also expected to have significant effects on the structure dynamics of protein. The aim of this work is to remove some ionic interactions from the domain interface of StEH1 and investigate its effects on hysteresis. A number of salt bridging residues have been modified from the surface of StEH1 and mutant variants were constructed in order to test the possibilities of different structural states and variations in catalytic properties. Kinetic properties of wild type StEH1 and mutant variants have been determined by steady-state and pre-steady state measurements. In general, moderate decreases in catalytic efficiencies as compared to the wild type with some exceptions were observed. Some variants displayed better affinity towards specific substrates and that increases catalytic efficiencies better than the wild type. Two different rates were observed in the pre-steady state phase during the reaction with (S,S)-2-MeSO as observed earlier for the wild-type enzyme except with the R236Q mutant, revealing hysteretic behavior. Removal of ionic interactions from the surface of StEH1 affects stabilization of the alkylenzyme intermediates and affects regioselectivity. Ionic interactions at the domain interface of protein provide a significant role in the overall functional behaviors of protein.
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List of abbreviations

EH - Epoxide hydrolase, StEH1 - Solanum tuberosum epoxide hydrolase1, trans-2-MeSO - trans-2-methylstyrene oxide, SO - Styrene oxide
Introduction

Biocatalysis is evolving to a rational, structure-based science though initially it was more focused on empirical approaches like screening or simple extrapolations from known substrates (1). Three-dimensional structures of biocatalysts now increase the possibility of rational design of new reactions and catalysts (1). Structure and mechanism-based approaches may help to better understand the reasons behind the selectivity of an enzyme-catalyzed reaction. Scientists are now more interested in developing biocatalytic processes because of the highly enantioselective and regioselective nature of enzyme-catalyzed reactions. Moreover, the use of enantiopure compounds in pharmaceutical industries is becoming an important issue. Bioactive compounds often exist as chiral compounds since different enantiomers of the same compound generally do not have the same pharmacological effect (2) hence, many pharma companies have been developing single enantiomeric drugs (3). Therefore, chiral synthetic intermediates are increasingly demandable for the production of pure optically active pharmaceuticals (2). Epoxide hydrolases are efficient biocatalytic tools for the hydrolysis of epoxides to give the corresponding vicinal diols (4,5). Since enantiopure epoxides and vicinal diols are valuable intermediates for the preparation of various biologically active compounds (6), proper understanding of structure and selectivity of epoxide hydrolases provides better opportunities to prepare enantiopure compounds.

Epoxides

Epoxides are chemically versatile and important synthons for fine organic synthesis (7). They are also present in many biologically active molecules as a common structural element and undergo facile, stereoselective ring-opening reactions with a wide range of nucleophiles (8).

![Fig 1. Ring-opening reactions of epoxides with nucleophiles (9,10).](image)
Enantiopure epoxides and corresponding vicinal diols are also used for the production of β3-adrenergic receptor agonists, anti-obesity drugs, N-methyl D-aspartate receptor antagonists with neuroprotective properties, nematocides and anticancer agents (7).

Epoxides make up a sub-class of ethers, containing three-membered heterocyclic system and are more reactive than other simple ethers due to the high ring strain. Generally, epoxide ring opening is facilitated by a nucleophilic attack under either acid or base catalyzed conditions to yield the product. In both cases, the nucleophile attacks one of the carbon atom from the opposite side to the oxygen bridge resulting in a inversion of configuration (11).

Epoxide hydrolase

As a prerequisite for biocatalysts, epoxide hydrolases have broad range of substrate specificity (12). Epoxide hydrolases are cofactor-independent enzymes (7), easy-reliable catalysts for organic synthesis. They are ubiquitous in nature and available in bacteria, yeast, fungi, plants, insects and animals (7). These enzymes generally catalyze the hydrolytic cleavage of oxirane derivatives and also have other functions like detoxification of potentially genotoxic epoxides, as regulators of physiological functions facilitated by signaling molecules and are also identified as essential components in microbial catabolic pathways (13).

Epoxide hydrolases belong to the structural family of α/β hydrolase fold enzymes (4,14). The three-dimensional structures of these enzymes indicated that they are composed of a α/β hydrolase fold domain with a lid domain on top and an optional N-terminal domain. The substrate-binding pocket is situated between the α/β hydrolase fold domain and the lid domain (4). There are also two superfamilies within this group of epoxide hydrolases, one consists of cytosolic or soluble epoxide hydrolase and the other one by microsomal epoxide hydrolase which share an N-terminal extension (4).

Fig 2. Ring-opening mechanism of epoxides.

\[
\text{Nu}^- : \text{OH}^-, \text{OMe}^-, \text{NH}_2^- \text{etc}.
\]
\[
\text{X}^- : \text{Cl}^-, \text{Br}^- \text{etc}.
\]
Potato epoxide hydrolase, StEH1

StEH1, a soluble monomeric epoxide hydrolase from potato, is functionally similar to other soluble epoxide hydrolases and also have broad substrate specificity (5,15). The main catalytic groups involved in StEH1 are Asp105 as nucleophile, His300 as general base, Asp265 as charge relay residue and Tyr154 and Tyr235 (5). Both tyrosine residues are situated in the lid domain and are involved to assist ring opening by hydrogen bonding to the substrate's oxirane oxygen. Another important catalytic residue, Glu35, was identified from the crystal structure of StEH1 (16). Two important roles of Glu35 in catalysis have been reported, activation of Asp105 and by properly orienting the water molecule which then attacks the alkylenzyme intermediate to facilitate the hydrolysis half reaction (17).
The catalytic mechanism of StEH1 consists of two main steps. In the first step, two tyrosine residues (Tyr154, Tyr235) from the lid domain form hydrogen bonds with the epoxide ring to facilitate a nucleophilic attack \((18,19)\), where the tyrosine phenols stabilize the excess electronic charge on the leaving group oxygen originating from the incoming carboxylate (Asp105) nucleophile \((20)\). Nucleophilic attack by Asp105 on one of the epoxide ring carbons form the alkylenzyme intermediate. In the following step, the protonated alkylenzyme is hydrolyzed with the help of nucleophilic attack by a water molecule activated by His300 and Glu35 residues \((20)\).

![Catalytic mechanism of potato epoxide hydrolase, StEH1](image)

**Fig 5.** Catalytic mechanism of potato epoxide hydrolase, StEH1 \((20)\).

Enzyme mediated epoxide ring opening by the nucleophilic attack end up with inversion of configuration. The nucleophile can attack at either carbon center of the epoxide ring which results in hydrolysis thus is not uniformly regioselective. Epoxide hydrolase from different species exists with different regioselectivity \((8)\). Different enantiomers of styrene oxide (SO) derivatives have
been tested with StEH1 to evaluate substrate specificity and stereospecificity (5,20-25). StEH1 shows enantioselective preference towards (S) or (SS)-enantiomers of styrene oxide (SO) and its derivatives 2-methylstyrene oxide (2-MeSO) and trans-stilbene oxide (TSO) (21). The regiospecificity of StEH1 catalyzed reaction is due to the initial nucleophilic attack by the enzyme on the stereo center of the substrates (22). In the same observation, influence on enantiospecificity due to alkylation step in SO hydrolysis by Agrobacterium radiobacter AD1 enzyme was also reported (26). Whereas, rat microsomal epoxide hydrolase displays stereospecificity with a certain substrate due to the hydrolysis half reaction (27). In addition, kinetic mechanisms and reaction conditions can vary with different enantiomers of trans-stilbene oxide for the enzymatic hydrolysis (17).

![Styrene Oxide and its derivatives](image)

**Fig. 6. Styrene Oxide and its derivatives.**

**Hysteretic behavior**

Slow responses of enzymes, in terms of kinetic characteristics, with a rapid change of substrate or ligand concentration related to conformational change are termed as hysteretic enzymes (27) invoking a slow conformational change as the rate limiting step in the enzymatic reactions (28). Hysteretic behavior is also observed in the potato epoxide hydrolase, StEH1, show as a slow conformational change between two conformal states of substrate-free enzyme (21). This behavior is observed during the pre-steady state phase of the enzyme-catalyzed reaction with (S,S)-2-methylstyrene oxide and detected as two observed rates for the formation of the alkylenzyme intermediate. One of the rate displays negative dependence of substrate concentration which implies the presence of slow transitions between the conformers of free enzyme (21). A kinetic mechanism (Scheme 1) of StEH1 catalyzed hydrolysis has been proposed (21) to explain the observed behavior related to hysteresis, and that might also help to explain the regioselectivity of StEH1 catalyzed reactions.
Weak interactions between the charged amino acid side chains are common on protein surfaces and are termed salt bridges \((29)\). Though their free energy contribution to protein stability is a controversial issue, they are expected to play a role in protein stabilization \((30)\). Earlier studies reported StEH1 exhibits slow conformational changes between two conformers of free enzyme. There is no evidence of the existence of different conformational states from the available crystal structures. Removal of salt bridges can affect the stabilization of those different structural states and finally the catalytic properties. There are some salt bridges identified at the interface of StEH1 wild type enzyme from its available 3D structure, located between the core and lid domains of the enzyme. The aim is to remove some salt bridges from the domain interface of StEH1 and investigate the effects on hysteresis and catalytic properties. In this work, a number of salt bridges have been removed by site-directed mutagenesis and investigated in terms of kinetic properties, thermostability and regioselectivity and compared with the wild type enzyme.
Materials and Methods

Residue selection

Three dimensional structure of wild type potato epoxide hydrolase StEH1 (PDB entry 2CJP) was analyzed by Pymol viewer (DeLano scientific LLC) to identify residues involved in salt bridges between the lid and core domain at the interface of protein. Three residues, arginine (R) at position 236 adjacent to the catalytic tyrosine (235), Glutamate (E) at position 215 and a lysine (K) at 179 position were picked for mutation as they have ionic interactions with a glutamate (E) at position 165, arginine (R) at 41 and aspartate (D) at 202 position respectively. Moreover, some other hydrogen bonding interactions with backbone residues are also observed.

Structure alignment

Structure based sequence alignments were performed by using ClustalW2, a multiple sequence alignment programme, from EMBL-EBI nucleotide sequence database (www.ebi.ac.uk/Tools/clustalw2/index.html). The sequences of other soluble plant epoxide hydrolases were collected from the ExPASy proteomics server using UniProt knowledgebase database (www.uniprot.org/uniprot) with the Swiss-Prot entries Q41415_SOLTU (StEH1), A2Q320_MEDTR (barrel medic), Q8LPE6_CICAR (chickpea), Q39856_SOYBN (soybean), Q8L5G6_BRANA (rape), Q8H289_ANACO (pineapple) and Q9S7P1_ORYSA (rice) (25).

Site-directed mutagenesis

Site-directed mutagenesis was performed in order to construct mutants by the polymerase chain reaction using mutagenic primers (supplied by Thermo Scientific) and plasmid pGTacStEH1-5H as a template encoding wild-type StEH1 (5). The two mutants R236K and R236Q were constructed so that, the codon CGT was replaced by KAG (K=A/C) in the cDNA at position 236 to obtain both mutants and subcloned into pGTacStEH1-5H between MunI and SacI restriction sites. The mutant K179Q was constructed by using codon CAA instead of AAA at the 179th position in the cDNA and subcloned in to same plasmid stated above, between the same restriction sites. The fourth mutant, E215Q, was constructed in the same way, by changing codon GAG to CAG and subcloned in to the same plasmid between XhoI and SacI restriction sites. All plasmids with mutated cDNA insert were sequenced in order to fully investigate the expected mutations, with no
other alterations. The restriction enzymes, buffers and other enzymes were purchased from Promega and Fermentas Corporations.

**Protein expression and purification**

All mutated cDNAs were subcloned into the pGTacStEH1-5H expression plasmid and transformed into E. coli XL1-Blue (Stratagene) bacteria by electroporation using a Bio-Rad Gene Pulser. Expression and purification of all four mutant proteins were performed according to a previously published protocol (5) involving Ni(II)-IMAC and size exclusion chromatography (Chromatographic resins from GE Healthcare).

**Steady state kinetics**

Steady state kinetic parameters were determined spectrophotometrically from the initial rates of enzyme catalyzed hydrolysis of both enantiomers of SO (styrene oxide) and 2-MeSO (phenylpropylene oxide) in the presence of wild-type StEH1 or mutants. Initial rates were measured in 0.1 M sodium phosphate (pH 7.5) buffer at 30°C. Substrate dilutions were made by dissolving substrate in acetonitrile so that the final concentration of acetonitrile in the reaction mixture was 1% (v/v) and the substrate concentrations varied in a range of 0.02 to 0.50 mM. The enzyme concentrations used were 0.02 µM in the reaction with S-SO and SS-MeSO and 0.20 µM – 0.35 µM with R-SO and RR-MeSO. The enzyme-catalyzed hydrolysis of epoxides to diol were followed by a decrease in absorbance at 225 nm (Δε = 2.75 mM$^{-1}$ cm$^{-1}$ and Δε = 4.3 mM$^{-1}$ cm$^{-1}$ for SO and 2-MeSO, respectively) and recorded reaction time was 15 to 60 s. The Michaelis-Menten equation was fitted to the experimental data using MMFIT and RFFIT in the SIMFIT program (http://www.simfit.man.ac.uk).

**Pre-steady state kinetics**

The enzyme-catalyzed hydrolysis of the two enantiomers of 2-MeSO was followed during the pre-steady state phase in order to determine microscopic rate constants. Formation of the alkyl enzyme intermediate can be detected by a concomitant decrease in the intrinsic tryptophan fluorescence of the enzyme (5). Measurements were performed in a sequential stopped-flow spectrophotometer (Applied Photophysics SX.20MV) with an excitation wave length of 290 nm, detecting fluorescent light collected through a 320nm cut-off filter. The reaction was performed at 30 °C in 0.1 M sodium phosphate buffer (pH 7.5). Substrate concentrations were in a range between
25-1200 μM and 85-1200 μM for \((S,S)-2\text{-MeSO}\) and \((R,R)-2\text{-MeSO}\), respectively. Enzyme concentrations were 2 or 10 μM with the \((S,S)-2\text{-MeSO}\) or \((R,R)-2\text{-MeSO}\), respectively. The apparent rate constants \((k_{\text{obs}})\) were determined by fitting a single (equation 1) or a double (equation 2) exponential function with floating endpoints to averaged (8-12 traces) progression curves, depending on the complexity of the enzyme catalysed reaction (21).

\[
F(t) = A \exp(-k_{\text{obs}}t) + C \quad (1)
\]

\[
F(t) = A_1 \exp(-k_{\text{obs}1}t) + A_2 \exp(-k_{\text{obs}2}t) + C \quad (2)
\]

\(F\) is the fluorescence signal, \(A\) the initial fluorescence value at \(t=0\), \(k_{\text{obs}}\) is the apparent rate constants and \(C\), the floating end point. F-tests were performed to validate the higher order equation.

When the apparent rates displayed a hyperbolic substrate concentration dependence, indicative of a mechanism as depicted in Scheme 2, Equation 3 was fitted to the data in order to determine rate constants for alkylenzyme formation, \(k_2\), and decomposition, \(k_2\) and \(k_3\), and the ES dissociation constant, \(K_S\).

\[
E + S \xrightleftharpoons[k_{-2}]{k_2} E\cdot ES \xrightarrow{k_3} E\text{-alkyl} \xrightarrow{k_0} E + \text{diol} \quad \text{(Scheme 2)}
\]

In the cases of a negative dependence on substrate concentration, a situation described by Scheme 1, involving rate limiting hysteretic steps, Equation 4 was fitted to the data. This fit does not allow for determination of individual rate constants but provides the sums of the rates of interconversion of the \(E\leftrightarrow E' (k_0+k_{0})\), which is the ordinate, and \(E'S\leftrightarrow E'S (k_5+k_{3})\) determined by the end point rate at \([S] \rightarrow \infty\) (21).

\[
k_{\text{obs}} = \frac{k_2[S]}{K_S + [S]} + (k_{-2} + k_3) \quad (3)
\]

\[
k_{\text{obs}} = \frac{k_0 + (k_5/K_S)[S]}{1 + ([S]/K_S)} + \frac{k_{-0} + (k_5'/K_S')[S]}{1 + ([S]/K_{S}')} \quad (4)
\]

**Thermostability of enzyme activity**

The enzyme activity was measured after incubation for different time periods at three different temperatures: 30 °C, 45 °C and 55 °C in order to investigate thermostability of all mutants and wild-type StEH1. The activity was measured spectrophotometrically at 225 nm, by observing the decrease in absorbance due to hydrolysis of epoxides to diol, in 0.1 M sodium
phosphate (pH 7.5) at 30 °C as stated before. (S,S)-2-MeSO at 0.30 mM concentration was used as a substrate for all StEH1 variants and the enzyme concentration was 0.02 µM in the reaction mixture. Apparent rate constants of inactivation ($k_{\text{inactive}}$) were determined from fitting an equation for single exponential decay to the experimental data (equation 5). The half-lives were calculated from $t_{1/2} = \ln 0.5 / -k_{\text{inactive}}$.

$$activity\ (t) = A \exp (-k_{\text{inactive}}\ t)$$

(5) 

**Regioselectivity**

To investigate the regioselectivity of all enzyme variants, initially reactions were performed with both enantiomers (1R,2R/1S,2S) of 2-MeSO in a final volume of 500 µl at 30 °C with continuous agitation for 24 h, where acetonitrile (3%), substrate (3.66 µM), enzyme (2 µM) and 0.05 M sodium phosphate buffer (pH 7.5) were used in the reaction mixture. After reaction completion, the reaction mixture was evaporated and the diol products were dissolved with the same solvent used as mobile phase in HPLC separation system. Samples were loaded into a HPLC straight phase system, with a mobile phase mixture of hexane / isopropanol (93:7) and separated over a chiral column (CHIRALPAK, AS-H, 0.46 cm Ø × 25cm). The column was coupled with a Shimadzu Prominence LC-20AD pump and peaks were detected at 220 nm using a Prominence SPD-M20A diode array detector. Enantiomeric excess, ee (%), was measured from the peak areas of the diols.
Results

Residue selection, site-directed mutagenesis, protein expression and purification

Salt bridging residues were identified from the 3D structure of StEH1 (PDB entry 2CJP) and analyzed by Pymol viewer (DeLano scientific LLC). Three residues (Arg236, Lys179, Glu215) located on the surface of the StEH1 were selected for mutation. The mutant variants were constructed by site-directed mutagenesis using mutagenic primers in the polymerase chain reactions. The selected residues were replaced by a glutamine (Gln) or lysine (Lys). Aiming at keeping the polar character on the surface and hence avoid unexpected problems during purification associated to solubility, such as precipitation.

Fig. 8. Mutated amino acid residues (Arg 236, Lys179, Glu215) on the surface of StEH1. Catalytic residues (Asp105, Tyr154, Tyr235, His300 and Glu35) are also shown together with other residues taking part in ionic interactions with the mutated residues (PBD entry 2CJP).

R236K/Q

Arginine236 is located just after the catalytic tyrosine235 in the core domain α-helix, engaged in ionic interactions with the glutamate165 from the lid domain. The arginine side chain contains a guanidinium group involved also in interactions with asparagine233 and with the backbone of phenylalanine158 and valine160. The interactions are expected to be important to
maintain the native structure of the protein and also for positioning the catalytically important tyrosine235 in the active side pocket. Arginine236 was replaced by lysine and glutamine to construct two different mutants. Lysine contains a long side chain terminated by an ε-ammonium group whereas glutamine contains an amide functional group.

Fig. 9. Weak interactions of Arg236 and Glu215 (From 3D structure, 2CJP).

E215Q

Glutamate215 is located in one of the end side helices of the lid domain and makes an ionic interaction with arginine41 from the core domain, which is also hydrogen bonded to the side chains of Tyr219 and Gln304. Glutamate215 make other interactions with Ser212 from lid domain. The salt bridge between Glu215 and Arg41 seems to have an effective role in maintaining the fine structure of the protein since it connects the two domains at the interface of the protein. Glu215 was replaced by Gln, obtaining a replacement of the carboxylate group by an amide group.

K179Q

Lysine179 is located in a helix on top of the lid domain and engaged in a salt bridge with Asp202. Since this interaction is situated in the same domain on the top surface of the protein, it may not have any significant effect on the overall stability of protein structure. Lys179 was replaced by a Gln to investigate how it effects on the overall kinetic and catalytic properties.

The StEH1 mutant variants were expressed in E. coli XL1-Blue and purified in the same way according to an established procedure. Approximately 8-10 mg of purified protein per liter of cultured medium was obtained which is slightly lower than the wild type. The yield from purification of the R23Q mutant was relatively lower than the other mutants with difficulties during purification. Purified proteins were stored at 4 ºC until further analysis.

Steady-state kinetics

Steady state kinetic parameters were obtained from the enzyme catalyzed hydrolysis of both enantiomers of styrene oxide (SO) and its derivative trans-2-methyl styrene oxide by StEH1 and
mutant variants at 30 °C and pH 7.5 in 0.1 M sodium phosphate buffer. The parameter values are listed in the Table 1 and 2 and discussed below.

In general, it was evident from the steady state-parameter values that (S)- or (S,S)-enantiomers are more favored than their corresponding (R)- or (R,R)-enantiomers of SO and trans-2-MeSO for the wild type StEH1 and all mutant variants, as reported previously (21). Moreover, all the mutant variants showed higher catalytic efficiencies towards the trans-2-MeSO than the SO in the same manner displayed by wild type StEH1.

**K179Q** : StEH1 K179Q mutant showed an almost two-fold increase in $k_{cat}/K_M$ values towards both enantiomers of SO as compared to the wild type enzyme. Although the turn-over rates were slightly lower than the wild type, in both cases the total efficiency was increased by low $K_M$ values. On the other hand, this mutant displayed almost the same efficiency to (1R,2R)-2-MeSO as compared to wild type, with a 50% decrease in efficiency observed for the (S,S) enantiomer.

**E215Q** : The catalytic efficiency of E215Q was almost three fold higher than the wild type with relatively much lower $K_M$ value to the (R)-enantiomer of SO, indicating that (R)-SO is a favorable substrate for this mutant.
R236K: The catalytic efficiency of StEH1 R236K mutant towards the enantiomers of SO was almost the same as wild type StEH1. The catalytic turnover rate of R236K catalyzed hydrolysis of (1R)-SO displayed a half fold decrease as compared to wild type, but the lower $K_M$ value maintained the overall efficiency as wild type. R236K displayed half fold decrease in catalytic efficiency towards (S,S)-2-MeSO as compared to the wild type. Overall, the mutation effected catalytic efficiencies a lot for the trans-2-MeSO, one of the favored substrates for wild type StEH1.

R236Q: The catalytic efficiencies of the R236Q mutant decreased drastically with all the tested substrates except for (R)-SO as compared with wild type. The catalytic turnover was much lower than with the wild type but exhibited comparable $k_{cat}/K_M$ value with (R)-SO which is due to a low $K_M$ value.

Pre-steady state kinetics

Formation of alkylenzyme intermediates, in the catalytic pathways of StEH1 and mutant variants-catalyzed trans-2-methyl styrene oxide hydrolysis, was determined by following the concomitant decreases in intrinsic tryptophan fluorescence of the enzyme (5). The double exponential function was applied during the reaction with (S,S)-2-MeSO except for when catalyzed by the StEH1 R236Q mutant whereas single exponential function was applied with (R,R)-2-MeSO. Fitting with double exponential function gave two apparent rate constants ($k_{obs1}$ and $k_{obs2}$) with individual amplitudes. The faster rate displayed hyperbolic dependence with substrate concentration and the slower rate displayed negative dependence of substrate concentration.

The presence of two observed rates during the reaction with (1S,2S)-2-MeSO was reported earlier (21) and explained by a model outlined in the scheme 1. The model describes two additional reaction steps: (1) between two conformational states of free enzyme, $E\leftrightarrow E'$ and (2) between the corresponding Michaelis complexes, $ES\leftrightarrow ES'$ (21). In the case of two observed rates, the faster rate $k_{obs1}$ indicates the mechanism on pathway $E\leftrightarrow ES\leftrightarrow ES'\rightarrow E$-alkyl$_1\rightarrow E$+diol$_1$ that displays hyperbolic dependence of substrate concentration. The lower rate displayed negative dependence of substrate concentration, which indicates a different pathway for the formation of alkylenzyme intermediate and reported in the scheme 1 as an off-pathway mechanism outlined as $E\rightarrow E'\leftrightarrow ES'\leftrightarrow ES\rightarrow E$-alkyl$_1\rightarrow E$+diol$_1$ (21). The negative substrate dependence of apparent rate ($k_{obs2}$) also indicates that at lower substrate concentrations, conformational changes between two free enzyme states tends to display faster rates ($k_0+k_o$) whereas with the increase in substrate concentration it turns towards the conformational change between two different ES complex and displays slower rates ($k_1+k_3$) due to the substrate binding that stabilizes one conformer (21). Again, during the reaction with (R,R)-2-MeSO a single apparent rate was observed and also explained by the same model outlined in the
scheme 1 (21). Here, the observed rate displayed negative dependence of substrate concentration that describes by the mechanism as $E \rightarrow E' \leftrightarrow ES' \leftrightarrow ES \rightarrow E$ alkyl$_1 \rightarrow E +$ diol$_1 / E' \rightarrow ES' \rightarrow E$ alkyl$_2 \rightarrow E +$ diol$_2$ (21) which also would explain the formation of two types of diol products.

The observations from the pre-steady state measurements for all mutant variants were in agreement with the proposed kinetic mechanism reported earlier except for the R236Q mutant. R236Q mutant gave single apparent rate constant ($k_{obs}$) with (S,S)-2-MeSO, which displayed hyperbolic dependence of substrate concentration. The absence of two observed rates could be an indication that the interconversion rates were undetectable and not affected by the substrate concentrations or that the structures of such different conformational states were affected by mutation. The lowest alkylenzyme formation rate ($k_3$) displayed by this mutant also an indication for the high $K_M$ value observed during the reaction with (S,S)-2-MeSO as compared with the wild type and other mutants. K179Q mutant displayed a higher alkylenzyme formation rate in comparison with wild type and other mutants, but finally catalytic efficiency decreased by 50 %, which was also reflected in a lower hydrolysis rate. The sum of interconversion rates between two different ES complexes displayed by wild-type and mutant variants during the reaction with trans-2-MeSO may affect the overall catalytic efficiencies, by the stabilization of different ES complexes or intermediates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_s$ (mM)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$k_2/K_s$ (s$^{-1}$ mM$^{-1}$)</th>
<th>$k_3$ (s$^{-1}$)</th>
<th>$(k_2 + k_3)$ (s$^{-1}$)</th>
<th>$(k_3 + k_2)$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.46±0.1</td>
<td>350±20</td>
<td>760±200</td>
<td>180±20</td>
<td>120±6</td>
<td>33±0.6</td>
</tr>
<tr>
<td>K179Q</td>
<td>0.75±0.2</td>
<td>650±50</td>
<td>870±200</td>
<td>230±20</td>
<td>69±7</td>
<td>25±10</td>
</tr>
<tr>
<td>E215Q</td>
<td>0.84±0.4</td>
<td>370±70</td>
<td>440±200</td>
<td>290±20</td>
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<td>29±20</td>
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<tr>
<td>R236K</td>
<td>0.16±0.1</td>
<td>160±50</td>
<td>1000±700</td>
<td>200±70</td>
<td>83±30</td>
<td>40±50</td>
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<tr>
<td>R236Q</td>
<td>0.68±0.3</td>
<td>157±24</td>
<td>231±61</td>
<td>109.5±10</td>
<td>76.5±2</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3. Pre-steady state kinetic parameters in 0.1 M sodium phosphate, pH 7.5, 30 °C

(55)
Thermostability

The long-term temperature effect on enzyme activity was measured spectrophotometrically after incubation at three different temperatures (30 °C, 45 °C and 55°C) for different time periods. The apparent rate constant for the heat inactivation of enzyme activity was measured after fitting with the equation for single exponential decay to the experimental data. Half-life of enzyme activity was measured.

Wild type StEH1 and all other mutant variants were quite stable at 30 °C, with an estimated half life of more than 500 min. The enzyme activity at 45 °C remained the same for the wild type, K179Q and E215Q mutant whereas it drastically decreased with R236K and R236Q. At 55 °C, wild type enzyme remains stable, with an estimated half life of 53 min, but the other mutants lost their activity just after few minutes, which was an indication of how removing these salt bridges affects thermostability.

Regioselectivity

Regioselectivity of wild type StEH1 and mutants variants was determined from the enzyme-catalyzed hydrolysis reaction of trans-2-MeSO enantiomers. The reaction was performed at 30 °C in 0.05 M sodium phosphate buffer for 24 hrs and diol products were analyzed by chiral column chromatography using a HPLC separation system. Percent enantiomeric excess (% ee) and the difference in the transition state free energies were determined from the peak areas of diol products.

The major product obtained from the enzyme catalyzed reaction with (S,S)-2-MeSO was mostly (R,S)-diol which is due to the nucleophilic attack at C-1 carbon of the oxirane ring of substrates. On the other hand, a mixture of the diol products were obtained during the reaction with (R,R)-2-MeSO giving a higher percentage of (S,R)-diol as major product by favoring the nucleophilic attack at C-1 carbon. The results of the hydrolysis of both enantiomers of trans-2-
MeSO were almost the same as observed in the previous studies (21).

<table>
<thead>
<tr>
<th>enzyme</th>
<th>ee (%)</th>
<th>$\Delta \Delta G^\ddagger$ (kJ/mol)</th>
<th>major:minor product</th>
<th>ee (%)</th>
<th>$\Delta \Delta G^\ddagger$ (kJ/mol)</th>
<th>major:minor product</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>98.2±0.006</td>
<td>-11.8</td>
<td>(1R,2S):(1S,2R)</td>
<td>15±4</td>
<td>-0.7</td>
<td>(1S,2R):(1R,2S)</td>
</tr>
<tr>
<td>K179Q</td>
<td>99.8±0.05</td>
<td>-17.4</td>
<td>(1R,2S):(1S,2R)</td>
<td>20±2</td>
<td>-1.0</td>
<td>(1S,2R):(1R,2S)</td>
</tr>
<tr>
<td>E215Q</td>
<td>99.5±0.2</td>
<td>-14.6</td>
<td>(1R,2S):(1S,2R)</td>
<td>1.8±2</td>
<td>-0.09</td>
<td>(1S,2R):(1R,2S)</td>
</tr>
<tr>
<td>R236K</td>
<td>99.7±0.007</td>
<td>-16.4</td>
<td>(1R,2S):(1S,2R)</td>
<td>42±3</td>
<td>-2.3</td>
<td>(1S,2R):(1R,2S)</td>
</tr>
<tr>
<td>R236Q</td>
<td>99.7±0.02</td>
<td>-16.4</td>
<td>(1R,2S):(1S,2R)</td>
<td>44±6</td>
<td>-2.4</td>
<td>(1S,2R):(1R,2S)</td>
</tr>
</tbody>
</table>

All mutant variants displayed prominent regioselectivity as observed with the wild-type during the reaction with (S,S)-2-MeSO. R236K and R236Q mutant displayed three fold increase in regioselectivity towards the major (S,R)-dial product in the reaction with (R,R)-2-MeSO, in comparison with wild type while E215Q mutant demonstrated lower regioselectivity with an ee of 1.8%.
Discussion

The present work was aimed to understand more about the structure dynamics of StEH1 and finding out the relationships between the structure-based mechanism and enantioselectivity. Enzyme-substrate interaction is one of the regulating factors for enzyme specificity (32). Moreover, knowledge of the structure dynamics will lead to a better understanding of enzyme-substrate interactions and other possible factors controlling stereoselectivity and catalysis. A kinetic model (Scheme 1) has already been suggested for the StEH1 during the reaction with trans-2-MeSO enantiomers that indicated different conformational states of free enzyme or ES complexes (21). Under native state, proteins have ability to adopt different conformations that fulfill functional requirements (33). The results from the present study also reveals that StEH1 probably exhibits different conformational states implied from its hysteretic behavior.

Enantiomers of different styrene oxide derivatives have been tested with StEH1 in previous studies for substrate specificity and enantioselectivity (5, 16, 17, 20, 21). Styrene oxide derivatives contain a benzylic carbon atom on their oxirane ring and a carbocation formed on that atom stabilized by the adjacent aromatic moiety (34). Although nucleophilic attack on the benzylic carbon is sterically unfavorable, but electronically more feasible and hence, it allows the nucleophile to attack on either of the oxirane carbon atoms. A review reported that StEH1 attacked predominantly at the less hindered terminal carbon of the (R)-enantiomer, whereas preferential attack observed at more hindered benzylic carbon of the (S)-enantiomer (12). In several cases, 2,2-disubstituted epoxides displayed absolute regioselectivity during the EH catalyzed hydrolysis, that is by attacking exclusively at the less hindered unsubstituted oxirane carbon, whereas ring opening observed by attack at both positions of carbon atoms at different ratios with the 2,3-disubstituted oxiranes (34). In addition, StEH1-catalyzed hydrolysis of trans-2-MeSO exhibited absolute regioselectivity (by exclusively attacked at the benzylic carbon) with the (S,S)-enantiomer while mixed regioselectivity showed by the (R,R)-enantiomer along with preferential attack at benzylic carbon atom (21). The present study with trans-2-MeSO, catalyzed by wild-type StEH1 and mutant variants, supported the same trends. Moreover, spatial alignment of the catalytically important residues at the active site of enzyme could also play an important role in regioselectivity by affecting catalytic rates and reactivities of oxirane carbons (21), which were partially proven by the regioselectivity results (Table 5) of StEH1 mutant variants. Mutation of salt bridging residues may have altered the active site structure that allowed repositioning of catalytically important residues in
such a way that they preferentially directed nucleophilic attack towards one specific carbon atom. Interconversion rates between the different conformational states of free enzyme and ES complex could also be affected by mutation (as indicated by the observation in the case with R236Q mutant) through stabilizing different transition state intermediates and finally an influential factor for both regioselectivity and catalysis.

Fig. 10. Sequence alignment of some soluble plant epoxide hydrolases. Residues, replaced by mutation in StEH1, are shown and compared with other isoenzymes (highlighted in gray).

Ionic interactions play an important role in protein thermostability (35). Removal of those interactions may alter stability to a large extent. Mutant variants constructed by destroying some salt bridges not only affects thermostability, but also regulates substrate binding, catalytic efficiency and regioselectivity.

K179Q: Lys179 is fairly conserved (Fig. 10) and mutation with Gln may remove all the existing salt bridges since Gln side chain is shorter than the Lys. The actual picture of structure after mutation is not clear but the kinetic study ended up with some interesting results (Tables 1-3). The catalytic efficiency increased almost 2-fold to that of wild type by showing better affinity for the SO enantiomers. Highest alkylenzyme formation rate was observed with this variant with ($S,S$)-2-`MeSO that may be linked to affording a 99.8% ee of diol product.

E215Q: Glu215 may have some special role maintaining the native structure of StEH1 by adding one more carbon atom in the side chain since aspartate is strictly conserved in other isoenzymes. Exchange of Glu with Gln displayed highest catalytic efficiency (as compared to the wild type and other variants) in hydrolysis of (1$R$)-SO by lowering $K_M$ value.

R236K and R236Q: Thses two variants demonstrating lower efficiencies as compared to the wild type and affected thermostability in a larger degree due to the absence of highly conserved arginine residue. The catalytic efficiency of R236K is much better than that of R236Q but almost same consequences observed in the regioselectivity. The structural change, after disturbing ionic interactions in both cases, stabilizes one of the transition state intermediates that provides major diol product more efficiently than the wild type (Table 5). The variation in microscopic rates observed in
the pre-steady state phase finally affects $k_{\text{cat}}$ and regioselectivity.

**Conclusion**

Although there is no clear evidence from available 3-D structures, kinetic observations proved that StEH1 exhibits flexible different structural orientations. The spatial alignment of the catalytically important residues at the active site of enzyme is affected by the mutation. It may alter the reactivities of oxirane carbons of epoxides and directs the nucleophilic attack to specific carbon atom. The interconversion rate between the different alkylenzyme intermediates is one of the regulating factors for regioselectivity of StEH1. Mutant variants constructed by destroying some salt bridges displayed distinct substrate specificities as compared to wild type. The salt bridging residues provide a significant role in the overall functional behaviors of protein. Mutation of some other salt bridging residues may open possibilities to explore different functionalities, substrate specificity and enantiocovertent enzyme catalyzed hydrolysis.

**Acknowledgements**

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References


27. Frieden, C. (1970) Kinetic aspects of regulation of metabolic processes, the hysteretic
enzyme concept, *J. Biol. Chem.* 245, 5788-5799.


Appendix

*Sequences of mutagenic primers:*

Mutagenic Primer- StEH1 R236KQ
TAA CTT GAG CTC CTG TCC ATG GTG CTG TGA GTT CCC AGT TTA TGG GTA AAG CCT
KGTAAT AGT TAA CTG CAC CAG T, K= T/G

Mutagenic Primer- StEH1 K179Q
TTG CTC CAA TTG GTG CTA AGT CTG TTC TTA AGC AAA TAT TGA CAT ACC GCG ATC

Mutagenic Primer-StEH1 E215Q-1
CTT GTT GGC ATA GTA ATC CAA CTG TTC CTC AGA AAG CCA CGA TG

Mutagenic Primer-StEH1 E215Q-2
GTT GGA TTA CTA TGC CAA CAA G