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Epoxide hydrolase-catalyzed enantioselective conversion of *trans*-stilbene oxide: Insights into the reaction mechanism from steady-state and pre-steady-state enzyme kinetics

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ABSTRACT

A detailed kinetic study based on steady-state and pre-steady-state measurements is described for the highly enantioselective epoxide hydrolase Kau2. The enzyme, which is a member of the α/β-hydrolase fold family, preferentially reacts with the (*S*,*S*)-enantiomer of *trans*-stilbene oxide (TSO) with an *E* value of ~200. The enzyme follows a classical two-step catalytic mechanism with formation of an alkyl-enzyme intermediate in the first step and hydrolysis of this intermediate in a rate-limiting second step. Tryptophan fluorescence quenching during TSO conversion appears to correlate with alkylation of the enzyme. The steady-state data are consistent with (*S*,*S*) and (*R*,*R*)-TSO being two competing substrates with marked differences in k_{cat} and K_M values. The high enantiopreference of the epoxide hydrolase is best explained by pronounced differences in the second-order alkylation rate constant (k_2/K_S) and the alkyl-enzyme hydrolysis rate k_3 between the (*S*,*S*) and (*R*,*R*)-enantiomers of TSO. Our data suggest that during conversion of (*S*,*S*)-TSO the two active site tyrosines, Tyr^{157} and Tyr^{259}, serve mainly as electrophilic catalysts in the alkylation half-reaction, polarizing the oxirane oxygen of the bound epoxide through hydrogen bond formation, however, without fully donating their hydrogens to the forming alkyl-enzyme intermediate.

1. Introduction

Epoxide hydrolases (EHs) catalyze the opening of the epoxide ring in a substrate by the addition of water, generating a diol as the final reaction product. Specifically, enantioselective or enantioconvergent EHs have found their way into various biotransformations as valuable biocatalysts [1–3]. Kau2 is a metagenome-derived EH and a member of the α/β-hydrolase fold family [4]. It turned out to be an interesting catalyst, often exhibiting a high enantiopreference (represented by a high *E* value) and/or enantioconvergence in conjunction with low product inhibition [4,5]. The enzyme has been used in a number of preparative-scale conversions with substrate loads of up to 80 g l^{-1} [4,5]. As an example, the enzyme reacted with racemic *trans*-stilbene oxide (**1**) in a highly enantioselective manner with an *E* value of ~200, resulting in the formation of *meso*-hydrobenzoin (**2**) and residual (*R*,*R*)-**1** with an *ee* of >99% in isolated yields close to the theoretical maximum of 50% (Fig. 1) [5]. (*R*,*R*)-**1** and **2** are interesting organic intermediates which have found applications as starting materials in various syntheses [6–10]. These valuable properties of the Kau2 EH prompted us to analyze its catalytic mechanism of the enantioselective hydrolysis of **1** in more detail. Moreover, kinetic data on the hydrolysis of **1** have been accumulated for the structurally related potato EH, whose enantioselectivity was shown to be low for this compound [11].

The catalytic mechanism of α/β-hydrolase fold EHs is known to involve the action of a catalytic triad, which consists of a nucleophile (Asp), a general base (His) and an acid (carboxylate function of Asp or Glu) [12]. After substrate binding to the enzyme, the nucleophilic carboxylic acid of Asp is polarized by the His–acid charge-relay system; the activated nucleophile then makes an attack on the epoxide, resulting in the formation of a covalent intermediate with an ester bond between the carboxylic acid of the

Abbreviations: A_{295}, absorbance at 295 nm; *E*, enantiomeric ratio; EH, epoxide hydrolase; TSO, *trans*-stilbene oxide.

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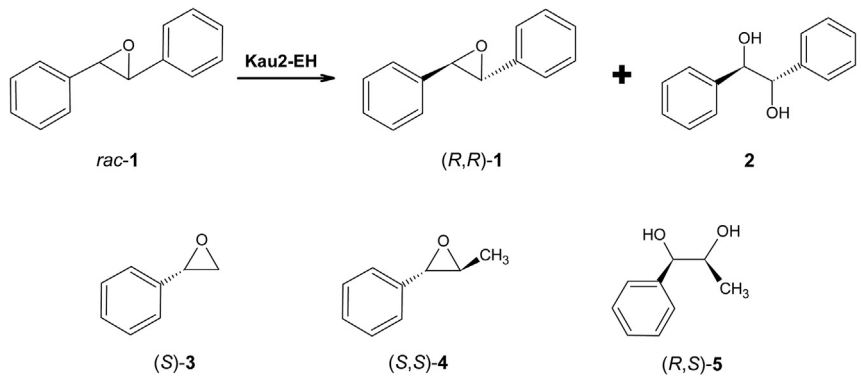

Fig. 1. Kau2 EH-catalyzed hydrolysis of racemic *trans*-stilbene oxide (**1**), and other epoxide and diol compounds used in this work. Kinetic resolution of racemic **1** results in the formation of (*R*,*R*)-**1** and *meso*-hydrobenzoin (**2**). (*S*)-styrene oxide: (*S*)-**3**; (*S*,*S*)-*trans*-1-phenyl-1,2-epoxypropane: (*S*,*S*)-**4**; (*R*,*S*)-1-phenylpropane-1,2-diol: (*R*,*S*)-**5**.

nucleophile and a carbon atom of the opened oxirane. This ester is formed in the so-called alkylation half-reaction and is termed the alkyl-enzyme intermediate; its formation appears to be associated with the observed tryptophan fluorescence quenching when rapidly mixing EH and substrate [13,14]. As a next step in the kinetic mechanism, the alkyl-enzyme intermediate is hydrolyzed, which is usually rate-limiting. In this so-called hydrolytic half-reaction the His–acid charge-relay system activates a water molecule which attacks the carbonyl of the alkyl-enzyme ester. After hydrolysis the diol product is released with the enzyme being restored to its original form (Fig. 2). There is substantial evidence that the catalytic triad is assisted by two conserved active site tyrosines. The orientation of their hydroxyl groups in crystal structures of EHs indicates an involvement of these residues in the polarization of the epoxide oxygen prior to the hydrolysis of the C–O bond [12]. Specifically, it was proposed that one of the two conserved tyrosines would establish a hydrogen bond with the epoxide oxygen of the bound substrate and thereby enhance its polarization (Fig. 2); in a consecutive step a proton transfer from the tyrosine hydroxyl to the generated alkoxide (alkyl-enzyme intermediate) would occur [12]. Recently, it was suggested that one more conserved histidine residue, which is linked to the general base histidine through the hydrolytic water molecule in the active site of the potato EH, plays a significant role in EH catalysis [15].

Several issues concerning the EH catalytic mechanism are still controversial, including the role of the two active site tyrosines. Questions to be asked in this respect are: Are tyrosinate ions being transiently formed during catalysis? Do these tyrosines serve as a general acid catalyst or as an electrophilic catalyst where no complete proton transfer to the transiently formed alkoxide intermediate occurs? In this paper we analyzed the kinetics of the conversion of **1** catalyzed by the Kau2 EH using steady-state and pre-steady-state measurements with the objective to untangle the kinetic basis of the very high enantiopreference of this enzyme and the catalytic role of its active site tyrosines during the enzymatic hydrolysis.

2. Materials and methods

2.1. Chemicals

N-cyclohexyl-*N*′-decylurea (CDU) and *N*-cyclohexyl-*N*′-(4-iodophenyl)urea (CIU) were synthesized as previously described [16,17]. The racemic compound **1**, the *meso*-diol **2**, and (*S*)-styrene oxide ((*S*)-**3**) (Fig. 1) were purchased from Sigma–Aldrich. Enantiopure (*S*,*S*)-**1** and (*R*,*R*)-**1** were obtained by preparative chiral HPLC from *rac*-**1** using a Chiralpak IC column (Daicel Corp.). (*S*,*S*)-*trans*-1-phenyl-1,2-epoxypropane ((*S*,*S*)-**4**) (Fig. 1) was synthesized as previously described [18]. (*R*,*S*)-1-phenylpropane-1,2-diol ((*R*,*S*)-**5**) (Fig. 1) was obtained by preparative kinetic resolution of *rac*-**4**

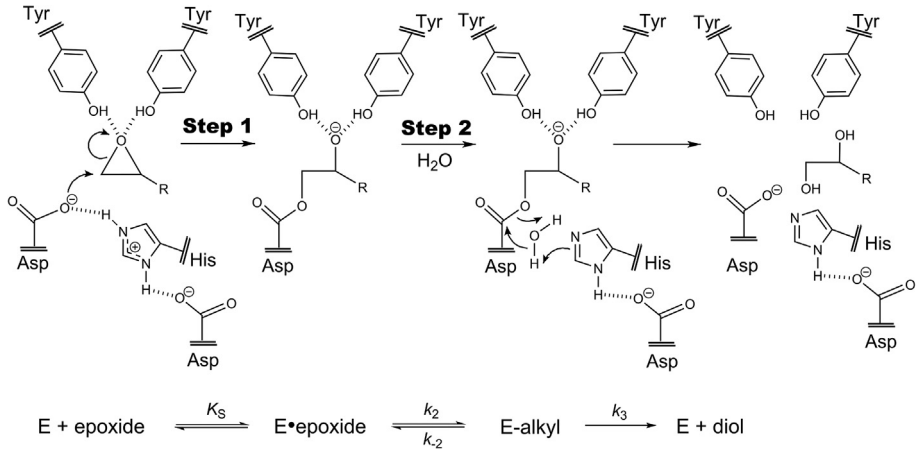

Fig. 2. Catalytic mechanism of α/β-hydrolase fold EHs. Usually, catalytic reaction mechanisms of EHs with α/β-hydrolase folds are represented by three steps: (1) formation of an enzyme–substrate complex with an equilibrium dissociation constant K_S; (2) nucleophilic attack of the active-site Asp results in the reversible formation (k_2, k_{-2}) of a covalent alkyl-enzyme intermediate (E-alkyl); (3) the enzyme intermediate is irreversibly hydrolyzed (k_3) by a base-activated water molecule, which leads to the release of the diol product.