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Mutation of Tyrosine Residues Involved in the Alkylation Half Reaction of Epoxide Hydrolase from *Agrobacterium radiobacter* AD1 Results in Improved Enantioselectivity

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Enantiomerically pure epoxides (oxiranes) are uniquely suited building blocks for synthetic purposes.[1] Such epoxides are often prepared by means of remarkably effective synthetic catalysts.[2] There are, however, few enzymatic routes in the repertoire.[3] We have studied the enzyme mediated kinetic resolution of readily available racemic epoxides by selective hydrolysis of one enantiomer to the 1,2-diol, a process for which a synthetic catalyst has recently also been developed.[4] Epoxide hydrolases that perform this conversion have been found in various organisms.[5] An attractive enzyme is the recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1[6] that can be produced in large amounts and which has good potential for the kinetic resolution of styrene oxides.[7] Here we report novel aspects of the catalytic mechanism of the enzyme and a mechanism-based approach that has led to the first site-specific mutant of an epoxide hydrolase that has improved characteristics in kinetic resolutions.

The epoxide hydrolase from *A. radiobacter* AD1[8] belongs to the α/β-hydrolase fold family and contains a catalytic triad in the active site.[9] The catalytic mechanism involves two discrete chemical steps. The first is an S_N2 nucleophilic attack by an Asp107 carboxylate oxygen on the least-hindered carbon atom of the epoxide, resulting in a covalent ester intermediate (Figure 1). In the second step, the ester intermediate is hydrolyzed by a water molecule that is activated by the Asp246-His275 pair.[8]

The chemical opening of an epoxide is facilitated by an acidic functional group that interacts with the ring oxygen. Such an activation likely also takes place in epoxide hydrolases. [10] Earlier speculations were made that the proton donor could be a lysine residue, but evidence in support of this is scant.[8,9c,11] We observed from crystallographic data for epoxide hydrolase from *A. radiobacter* AD1[12] that Tyr152 and Tyr215 are positioned close to the nucleophilic Asp107 in a manner such that their phenolic hydroxyl groups could be proton donor. No backbone amides or other acid groups are present that can serve as oxyanion hole or as proton donor during ring opening. A mechanistical role for Tyr215 was supported by a sequence alignment of known epoxide hydrolase sequences, which revealed that this tyrosine residue is absolutely conserved in the C-terminal part of the cap domain. This is remarkable considering that the overall similarity between various epoxide hydrolase sequences is often less than 20%, and indicates an important role for this residue. The tyrosine residue is conserved within a short stretch of sequence that is different for soluble and microsomal epoxide hydrolases, namely N-W/Y-Y-R and R-F/Y-Y-K, respectively. Sequence alignments were particulary poor in the N-terminal part of the cap domain where Tyr152 is located, and only alignments done by hand indicated that a second tyrosine might be present in the other soluble epoxide hydrolases.

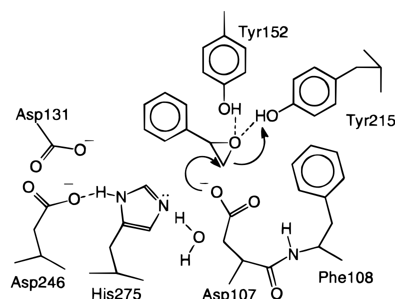

Figure 1. Schematic representation of the first step of the reaction mechanism of epoxide hydrolase.

To investigate the role of Tyr215 and Tyr152, we constructed mutant enzymes in which the tyrosine was replaced by a phenylalanine[13] and the resulting mutant enzymes were expressed and purified to homogeneity.[14] The Tyr215Phe mutant showed a 100- to 1000-fold increase of the K_m for both enantiomers of styrene oxide (SO) and *p*-nitrostyrene oxide (pNSO), a strong decrease of the k_{cat} for the (*S*)-enantiomers, and a small decrease of the k_{cat} for the (*R*)-enantiomers (Table 1).[15] Mutation of Tyr152 to Phe resulted in an enzyme that had an even higher K_m value for (*R*)-SO than the Tyr215Phe mutant whereas the k_{cat} value again remained in the same order of magnitude as the value for wild-type enzyme (Table 1). The similar changes in the steady-state kinetics of the Tyr215Phe and the Tyr152Phe mutant compared to wild-type enzyme indicate that both tyrosines perform similar roles in the kinetic mechanism of epoxide hydrolase. A mutant

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(13) The Tyr215Phe and the Tyr152Phe mutants of epoxide hydrolase were constructed as described before.[8] The primers 5′-caactacttccgtgccaac-3′ and 5′-gagtcgtggttctcgcaattcc-3′ (mutated codons are underlined) were used for constructing the Tyr215Phe mutant and the Tyr152Phe mutant, respectively. Subsequently, the mutated epoxide hydrolase genes were sequenced.

(14) The mutant and wild-type enzyme were overexpressed in *E. coli* BL21(DE3) and purified as described before.[8] The enzymes were stored in TEMAG buffer at 4 °C and remained fully active for at least two month.

(15) The steady-state parameters k_{cat} and K_m for styrene oxide (SO) and *p*-nitrostyrene oxide (pNSO) were obtained from progress curves, using an amount of enzyme sufficient to complete the reaction within 20 min.[8] SO was analyzed by gas chromatography.[8] Substrate depletion curves for pNSO were recorded in TE buffer at 30 °C on a Kontron Uvikon 930 UV/VIS spectrophotometer. The reaction was started by the addition of a stock solution of pNSO in acetonitrile to the cuvette with the enzyme solution to a final concentration of 1% acetonitrile. By using the extinction coefficients for pNSO ($\epsilon_{310} = 4289$ M^{-1} cm^{-1}) and the corresponding diol ($\epsilon_{310} = 3304$ M^{-1} cm^{-1}), the recorded traces were directly fitted with the Michaelis−Menten equation to obtain k_{cat} and K_m values.

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Table 1. Steady State Parameters of Wild-Type and Mutant Epoxide Hydrolase for Both Enantiomers of PNSO and SO Determined at pH 9 and 30 °C

substrate	wild-type enzyme			Tyr215Phe mutant enzyme			Tyr152Phe mutant enzyme		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
(*R*)-pNSO	7.8	0.008	975	>3.4	>0.8	4.2[a]	>1.2	>1.5	0.8[a]
(*S*)-pNSO	>7.7	>0.5	5[a]	>0.02	>1	0.02[a]	>0.005	>1	0.005[a]
(*R*)-SO	3.8	0.0005	8000	2.5	0.6	4.2	1.1	3.5	0.3
(*S*)-SO	10.5	0.021	500	0.7	5	0.14	>0.12	>15	0.008[a]

[a] Due to high K_m values and maximum solubility of pNSO (1 mM) and SO (10 mM) only the k_{cat}/K_m values could be determined, setting lower limits for the k_{cat} and K_m values.

Table 2. Enantioselectivity of Wild-Type and Tyr215Phe Epoxide Hydrolase

racemic substrate	*E* value	
	wild-type	Tyr215Phe
pNSO[a]	100	>200
SO[b]	16	30
mClSO[b]	6.5	12
pClSO[b]	32	130

[a] Determined by measuring k_{cat}/K_m with the pure enantiomers.[15] [b] Determined from a kinetic resolution experiment.[17]

in which both tyrosines were replaced by phenylalanines showed no detectable catalytic activity. These observations suggest that Tyr152 and Tyr215 are involved in hydrogen bonding and/or proton donation to the ring oxygen.

Besides remaining catalytically active, the Tyr215Phe mutant enzyme showed an increased stereoselectivity with styrene oxide and substituted variants thereof (Table 2). The *E* values[16] of the mutant enzyme for SO, *m*-chlorostyrene oxide (mClSO), and pNSO were increased approximately 2-fold compared to the *E* values of wild-type enzyme.[17] A spectacular improvement of the *E* value was found for *p*-chlorostyrene oxide (pClSO), which increased 4-fold from 32 for wild-type enzyme to over 130 for the Tyr215Phe mutant (Figure 2). This corresponded to 96% of the theoretical yield of (*S*)-pClSO, with an enantiomeric excess of 99%. The k_{cat} values with the (*R*)-enantiomers are hardly affected, and the Tyr215Phe mutant enzyme remains an excellent catalyst, considering that a low concentration of the Tyr215Phe mutant is still sufficient to convert (*R*)-pClSO in a reasonable amount of time (Figure 2).

What is the cause of this behavior? During the conversion of SO by the wild-type epoxide hydrolase, the hydrolysis of the covalent intermediate to product is the rate-limiting step and its rate is close to the overall k_{cat}.[18] The low K_m values of the wild-type enzyme are due to very high alkylation rates which are over 100-fold faster than the hydrolysis rates. This results in a high accumulation of the alkyl-enzyme during turnover as was also observed for rat microsomal epoxide hydrolase.[19] The Tyr215Phe and the Tyr152Phe mutant enzymes showed a large increase of the K_m for (*R*)-SO whereas the k_{cat} values were only slightly reduced. This suggests that for (*R*)-SO only the alkylation half reaction, which comprises the Michaelis complex and the reversible formation of the alkyl-enzyme, was affected by the mutation. The X-ray structure of epoxide hydrolase indicates that only the hydroxyl groups of Tyr152 and Tyr215 are in a position to form hydrogen bonds with the oxirane oxygen. Consequently, the loss

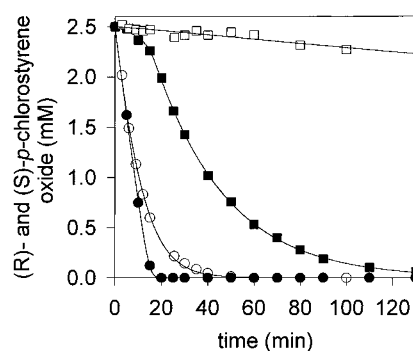

Figure 2. Kinetic resolution of 5 mM *p*-chlorostyrene oxide with 1 μM of wild-type epoxide hydrolase (●, ■) and with 3 μM of Tyr215Phe mutant enzyme (○, □). The round symbols represent the (R)-enantiomer and the square symbols represent the (S)-enantiomer.

of one hydrogen bond in the Tyr152Phe and the Tyr215Phe mutant would affect the binding of the substrate in the Michaelis complex and destabilize the transition state of the alkyl-enzyme formation leading to lower alkylation rates. How the dealkylation rate, the back reaction, is influenced by the mutations depends on the stability of the covalent intermediate. If a reduction of the alkylation rate is also the main effect of the mutation for (*S*)-SO conversion, the rate of formation of the alkyl enzyme must have become rate-limiting in agreement with a reduced k_{cat}. Thus, the increase of the K_m with SO is accompanied by a relatively small reduction in the k_{cat} for the (*R*)-enantiomer, which caused a smaller decrease of k_{cat}/K_m for the (*R*)-enantiomer than for the (*S*)-enantiomer and hence an increase in enantioselectivity. The same changes may explain the observed increase in enantioselectivity with the other substrates. Indeed, the k_{cat} for the hydrolysis of the (*R*)-enantiomer of pClSO is close to that of wild-type enzyme, but the k_{cat} for the (*S*)-enantiomer has decreased virtually to zero in the mutant (Figure 2).

The above considerations suggest a general principle for the observed increase in enantioselectivity for the Tyr215Phe mutant. Owing to the strongly reduced stabilization of the transition state and/or the Michaelis complex, the alkylation rate of the enzyme has become rate-limiting for (*S*)-enantiomers, which are already the poorer substrates for wild-type enzyme. The resulting reduction of the k_{cat}/K_m with (*S*)-enantiomers for the mutant enzyme enhances the enantioselectivity. The hypothesis that Tyr215 and Tyr152 act as proton donor in the reaction mechanism of epoxide hydrolase, which is indicated by the X-ray structure, is supported by the steady-state kinetics of the mutant enzymes.

In conclusion, we have observed that mutation of a tyrosine, which interacts with the epoxide during ring opening, yields an enzyme with reduced turnover number for the nonpreferred enantiomer and with increased enantioselectivity. Since Tyr215 is conserved among epoxide hydrolase sequences, an activating role of this residue is suggested for other epoxide hydrolases as well. Replacing this tyrosine residue, an operation in principle deleterious to the catalytic machinery, might also result in mutants with improved enantioselectivity for these enzymes.

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(16) The *E* value is defined as the ratio $(k_{cat,R}/K_{m,R})/(k_{cat,S}/K_{m,S})$, with R and S representing the enantiomers. For a discussion on *E* values and enantiomeric ratios, see: Straathof, A. J. J.; Jongejan, J. A. *Enzyme Microb. Technol.* **1997**, *21*, 559.

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