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Laboratory evolution of an epoxide hydrolase – Towards an enantioconvergent biocatalyst

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ABSTRACT

We performed a laboratory evolution study with the epoxide hydrolase from *Aspergillus niger* M200. This enzyme exhibits no enantioconvergence with the substrates styrene oxide or *para*-chlorostyrene oxide, i.e. racemic vicinal diols are produced from the racemic substrates. After saturation mutagenesis, screening by chiral gas chromatography revealed enzyme variants with improved enantioconvergence as manifested by an increased enantiomeric excess of the diol product. Nine amino acid exchanges accumulated in the active site and the substrate access tunnel over the course of 5 productive rounds of iterative saturation mutagenesis, resulting in an enantioconvergent epoxide hydrolase variant. The final mutant enzyme transformed racemic styrene oxide and *para*-chlorostyrene oxide to (*R*)-diol enantiomers with enantiomeric excesses of 70%. Sequential bi-enzymatic reactions using the wild-type EH and/or its evolved variants enabled preparation of the chiral building blocks (*R*)-phenyl-1,2-ethanediol and (*R*)-*para*-chlorophenyl-1,2-ethanediol from inexpensive racemic epoxides with enantiomeric excesses of 91% and 88%, respectively.

1. Introduction

Two types of epoxide hydrolases (EHs) are very useful biocatalysts for organic chemists: (1) Enantioselective EHs generate enantiopure epoxides, which are valuable chiral building blocks for the synthesis of various biologically active compounds (Archelas and Furstoss, 1997; Finney, 1998; Kasai et al., 1998; Yudin, 2006). (2) Enantioconvergent EHs allow the formation of enantiopure vicinal diols, which are also useful chiral synthons and can additionally be often chemically transformed to the corresponding epoxides with no reduction in enantiopurity (Kolb and Sharpless, 1992; Kolb et al., 1994; Hattori et al., 1995). Whereas enantioselective EHs are applied in hydrolytic kinetic resolutions with an intrinsic yield limitation of 50%, enantioconvergent EHs theoretically yield 100% enantiopure diol in a type of reaction which is generally denoted as deracemization (Strauss et al., 1999; Lee and Shuler, 2007). In contrast to enantioselective EHs, enantioconvergent EHs are less frequent, the main reason being the requirement for an exquisite interplay of two reaction parameters, the regioselectivity coefficients α_S and α_R, which determine the enantiomeric purity of the diol product and thus the degree of enantioconvergence (Moussou et al., 1998). Their values are a consequence of the structural characteristics of the binding site of the enzyme and the structure of the epoxide substrate. From a practical point of view, there is an additional important parameter for enantioconvergent EHs: the *E*-value, which is a measure of the enantioselectivity of a given enzyme–substrate pair. Only enantioconvergent enzymes with a low or moderate *E*-value can be considered useful, since high *E*-values would only prolong the reaction time to reach 100% conversion.

Amino acid substitutions in the substrate access tunnels of α/β-hydrolase fold enzymes are of considerable importance in enzyme engineering studies (Pavlova et al., 2009). The replacement of residues at these sites can lead to significant beneficial effects, such as improved activities and increased enantioselectivities (Chaloupková et al., 2003; Kotik et al., 2007). Combining the randomization of amino acids by saturation mutagenesis with selection procedures is a starting point for Darwinian laboratory evolution, in which the selected clone of the first round of mutagenesis is used as the template for the second round of mutagenesis at a different randomization site (Reetz, 2004; Bershtein and Tawfik, 2008). This process of *in vitro* evolution usually consists of several rounds of mutagenesis and selection, and is continued until a biocatalyst with the desired characteristics has been discovered. This evolutionary strategy

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Table 1
Primers used for saturation mutagenesis.[a]

Name	Site	Sequence (5′ → 3′)
fwdmut-A	A	CCAAGGAC**NNKNNK**CCCGTGCC[b]
revmut-A		GGAAGAACGAGAATCCGAATGGTTTGTG[b]
fwdmut-B	B	CTACCGTGAG**NNKNNK**CCCACCGCC[b]
revmut-B		GAATAAATAGCCCGTGGAAAACTCTCAG[b]
fwdmut-C	C	TATGCGCA**NNKNNK**GCTCCCCC[b]
revmut-C		GGTTCAAATGTACCGCTTTGCAGG[b]
fwdmut-D	D	GACGCCTGCAAAGCGGTACATTTGAAC**NNK**TGC**NNK**TGGGAGGCTCCCCCTGAA
revmut-D		TTCAGGGGGAGCCTCCCA**MNN**GCA**MNN**GTTCAAATGTACCGCTTTGCAGGCGTC
fwdmut-E	E	CAACGGAGCGACG**NNKNNK**CAGAACGAATTA[b]
revmut-E		GGCACAGAGGCGGTGGGGGTAG[b]
fwdmut-F	F	CCGTGAGTGG**NNKNNK**ACCGCCTCTGTG[b]
revmut-F		TAGGAATAAATAGCCCGTGGAAAACTCTCAG[b]
fwdmut-G	G	GGTGATATTGGT**NNKNNK**GTAGGGCGGGTTC[b]
revmut-G		TCCCTGGATAACATAGCCACTGCC[b]
fwdmut-H	H	AATGGAGAAA**NNKNNK**ACCAATGGCCTAG[b]
revmut-H		CGCGCGACTCCCTCTTTCTC[b]
fwdmut-I	I	GGCTATGTTATCCAGGGAGGTGAT**NNK**GGT**NNK**TTTGTAGGGCGGGTTCTGGGCGTA
revmut-I		TACGCCCAGAACCCGCCCTACAAA**MNN**ACC**MNN**ATCACCTCCCTGGATAACATAGCC

[a] Positions that introduced mutations are in bold; M: A/C, N: A/C/G/T, K: G/T.
[b] Primers were 5′-phosphorylated.

using saturation mutagenesis for library creation is a powerful tool for generating enzyme variants with improved characteristics, as long as the sites for mutagenesis are properly selected (Georgescu et al., 2003; Reetz, 2004, 2011; Reetz et al., 2006).

In the past, mutagenesis techniques such as saturation mutagenesis, error-prone PCR, or DNA shuffling were used to improve the enantioselectivities of EHs (Reetz et al., 2004; van Loo et al., 2004; Rui et al., 2005). More recent work dealing with EH engineering towards enhanced enantioselectivity includes the directed evolution of the EHs from *Aspergillus niger* LCP 521 (Reetz et al., 2006) and *Solanum tuberosum* (Gurell and Widersten, 2010), and the limonene EH from *Rhodococcus erythropolis* DCL 14 (Zheng and Reetz, 2010).

For the first time, a laboratory evolution study of an EH towards enantioconvergence is reported. As the model enzyme we selected the EH from *A. niger* M200, which is active on styrene oxide (*rac*-**1**) and its derivatives, preferentially hydrolyzing the (*R*)-epoxide (Kotik et al., 2007). The enzyme exhibits no enantioconvergence, the product of the enzymatic reaction being a racemic 1,2-diol (Scheme 1) (Kotik et al., 2005; Kotik and Kyslík, 2006). There is no X-ray crystal structure available; however, taking into account the high degree of sequence identity (90% on protein level) with the EH from *A. niger* LCP 521 (Arand et al., 1999), whose structure has been solved (Zou et al., 2000), this related structural data provides a reasonable initial basis for selecting appropriate sites for saturation mutagenesis.

The motivation of our laboratory evolution study can be found in the generally recognized importance of deracemization reactions (Strauss et al., 1999), in the lack of synthetic enantioconvergent catalysts for the preparation of enantiopure diols, in the usual requirement for a rather complex bi-enzymatic approach using selected and complementary EHs for enantioconvergent processes (Lee and Shuler, 2007), and in reports of rare examples of enantioconvergent wild-type EHs (Pedragosa-Moreau et al., 1996; Monterde et al., 2004; Hwang et al., 2008a; Kotik et al., 2010). One goal of this project was to establish the necessary screening techniques for the *in vitro* evolution of an EH towards an enantioconvergent biocatalyst. As the target enzyme we chose a non-enantioconvergent EH, which is the most difficult starting position for evolving enantioconvergence. The described methodology is generally applicable to the directed evolution of EHs towards enantioconvergence with *rac*-**1** or *para*-chlorostyrene oxide (*rac*-**3**) as the substrate.

2. Materials and methods

2.1. Chemicals and reagents

Benzyl glycidyl ether, 4-(4-nitrobenzyl)pyridine, ethanolamine, racemic styrene oxide (**1**) and *para*-chlorostyrene oxide (**3**), and the pure enantiomers of **1** were purchased from Sigma–Aldrich. Epoxycyclooctane and phenyl-1,2-ethanediol (**2**) were supplied by Merck. The growth media components were from Oxoid Ltd. Protein concentrations were determined using the Protein Assay kit of Bio-Rad Laboratories, Inc.

2.2. Library construction

Libraries were constructed by PCR using either the Phusion site-directed mutagenesis kit (Finnzymes Oy) or the QuickChange Lightning site-directed mutagenesis kit (Agilent Technologies) according to the manufacturers' instructions. The primers used for mutagenesis are shown in Table 1. The pEPHA1 plasmid (5.7 kb), which harbors the EH-encoding cDNA from *A. niger* M200 (Kotik et al., 2007), was used as the template. *Escherichia coli* BL21-Gold cells (Agilent Technologies) were transformed with the mutagenized plasmid and the resulting colonies were transferred into 96-well plates containing 200 μL of LB medium with ampicillin (150 μg mL^{-1}) and glucose (0.5%). The cultures were grown overnight at 29 °C with shaking, and these master plates were stored at −80 °C.

2.3. Activity screening

An aliquot of 20 μL was transferred from a master plate to another 96-well plate containing 150 μL of LB medium with ampicillin (150 μg mL^{-1}) and 500 μM isopropyl β-D-thiogalactopyranoside for EH-overexpression. The expression plates were incubated overnight at 29 °C on a shaker. Twenty microliters of 200 mM phosphate buffer (pH 7.5, containing 1 mM EDTA and 2 mM 2-mercaptoethanol) and 20 μL of epoxide substrate (63 mM racemic benzyl glycidyl ether in ethanol) were added to each well. After incubation at 29 °C for ∼10 h on a shaker, 135 μL of 4-(4-nitrobenzyl)pyridine solution (120 mM, dissolved in 1-pentanol) was added and the contents of each well was mixed. The plate was incubated at room temperature for 1–2 h without shaking, then 20 μL of ethanolamine solution (1.3 M in acetone) was added. A blue color appeared in those wells containing the