Enhancing the Enantioselectivity of an Epoxide Hydrolase by Directed Evolution

ARTICLE *in* ORGANIC LETTERS · FEBRUARY 2004

Impact Factor: 6.36 · DOI: 10.1021/ol035898m · Source: PubMed

13 AUTHORS, INCLUDING:

Marco Bocola
RWTH Aachen University
65 PUBLICATIONS **1,550** CITATIONS
SEE PROFILE

Michael Arand
University of Zurich
129 PUBLICATIONS **6,717** CITATIONS
SEE PROFILE

Alain Archelas
Aix-Marseille Université
103 PUBLICATIONS **2,848** CITATIONS
SEE PROFILE

ORGANIC LETTERS

2004
Vol. 6, No. 2
177−180

Enhancing the Enantioselectivity of an Epoxide Hydrolase by Directed Evolution

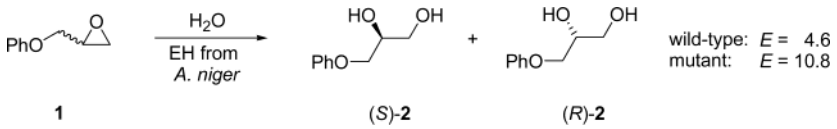
Manfred T. Reetz,*,[†] Claudia Torre,[†] Andreas Eipper,[†] Renate Lohmer,[†] Marcus Hermes,[†] Birgit Brunner,[†] Andrea Maichele,[†] Marco Bocola,[†] Michael Arand,[‡] Annette Cronin,[‡] Yvonne Genzel,[§] Alain Archelas,[§] and Roland Furstoss[§]

Max-Planck-Institut für Kohlenforschung, Kaiser-Wilhelm-Platz 1, 45470 Mülheim/Ruhr, Germany, Institut für Pharmakologie und Toxikologie, Universität Würzburg, Versbacher Strasse 9, 97078 Würzburg, Germany, and Faculté des Sciences de Luminy, UMR CNRS 6111, Case 901, 163 Av. de Luminy, 13288 Marseille Cedex 9, France

reetz@mpi-muelheim.mpg.de

Received September 29, 2003

ABSTRACT



1 **(*S*)-2** **(*R*)-2

The epoxide hydrolase (EH) from *Aspergillus niger*, which shows a selectivity factor of only $E = 4.6$ in the hydrolytic kinetic resolution of glycidyl phenyl ether, has been subjected to directed evolution for the purpose of enhancing enantioselectivity. After only one round of error-prone polymerase chain reaction (epPCR), enantioselectivity was more than doubled ($E = 10.8$). The improved mutant enzyme contains three amino acid exchanges, two of which are spatially far from the catalytically active center.

The hydrolytic kinetic resolution of chiral epoxides can be performed either with synthetic transition metal catalysts (Jacobsen catalysts)[1] or enzymatically using epoxide hydrolases (EH).[2,3] The former option constitutes one of the most efficient and reliable methods for asymmetric transition metal catalysis. The potential of the enzymatic approach was recently increased by the commercialization of three epoxide hydrolases, specifically the EH from *Aspergillus niger* and *Rhodococcus rhodochrous*, as well as human microsomal epoxide hydrolase.[4] These and other EHs have been demonstrated to be highly active and enantioselective catalysts for the hydrolytic kinetic resolution of a variety of epoxides.[2,3] However, the substrate specificity of enzymes limits the range of compounds that can be converted enantioselectively. Moreover, some classes of epoxides such as trans-1,2-disubstituted substrates react with unacceptably low rates, a problem that has also been encountered in Jacobsen chemistry. In principle, enzyme properties can be engineered by "rational design" using site-specific mutagenesis, but in the case of enantioselectivity, this is far from trivial. As a viable alternative, we recently introduced the concept of directed evolution of enantioselective enzymes.[5] This strategy comprises the proper combination of random mutagenesis

[†] Max-Planck-Institut für Kohlenforschung.
[‡] Universität Würzburg.
[§] UMR CNRS 6111.

(1) Jacobsen, E. N. *Acc. Chem. Res.* **2000**, *33*, 421−431.
(2) (a) Faber, K. *Biotransformations in Organic Chemistry*, 3rd ed.; Springer: Berlin, 1997. (b) Drauz, K.; Waldmann, H. *Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook*, 2nd ed.; VCH: Weinheim, 2002; Vol. I−III.
(3) (a) Archer, I. V. J. *Tetrahedron* **1997**, *53*, 15617−15662. (b) Orru, R. V. A.; Faber, K. *Curr. Opin. Chem. Biol.* **1999**, *3*, 16−21. (c) Steinreiber, A.; Mayer, S. F.; Faber, K. *Synthesis* **2001**, 2035−2039. (d) Archelas, A.; Furstoss, R. *Curr. Opin. Chem. Biol.* **2001**, *5*, 112−119. (e) Archelas, A.; Furstoss, R. *Trends Biotechnol.* **1998**, *16*, 108−116. (f) Bellucci, G.; Chiappe, C.; Cordoni, A.; Ingrosso, G. *Tetrahedron Lett.* **1996**, *37*, 9089−9092. (g) Arand, M.; Müller, F.; Mecky, A.; Hinz, W.; Urban, P.; Pompon, D.; Kellner, R.; Oesch, F. *Biochem. J.* **1999**, *337*, 37−43. (h) Arand, M.; Wagner, H.; Oesch, F. *J. Biol. Chem.* **1996**, *271*, 4223−4229.

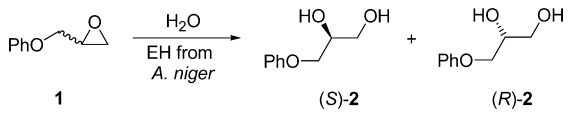
(4) Commercially available from Fluka/Aldrich/Sigma.

10.1021/ol035898m CCC: $27.50
Published on Web 12/20/2003

methods and protein expression[6] coupled with efficient high-throughput screening.[7] As a first example, a highly enantioselective lipase variant was evolved as a catalyst in the hydrolytic kinetic resolution of a chiral ester by applying several cycles of error-prone polymerase chain reaction (epPCR), saturation mutagenesis, and DNA shuffling.[5] It was shown that amino acid substitutions at positions far away from the catalytically active site affect enantioselectivity,[5e] a surprising phenomenon that was subsequently explained by a novel relay mechanism.[8] On the basis of these positive results, we have started to apply the concept of directed evolution of enantioselective enzymes to EHs.[9]

The hydrolytic kinetic resolution of glycidyl phenyl ether (**1**) with formation of diol **2** was known to be catalyzed by the EH from *A. niger*, although with low enantioselectivity (Scheme 1).[10] The selectivity factor *E* (reflecting the relative

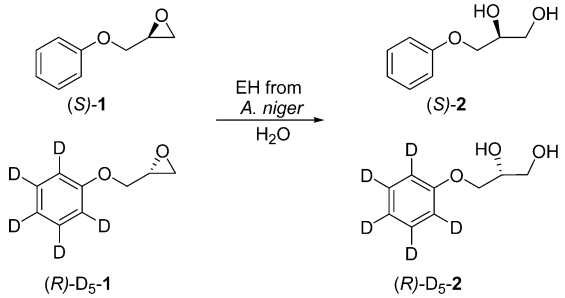
Scheme 1



rate of the reaction of the two enantiomers) amounts to only 4.6, in slight favor of the (*S*)-product. We therefore chose this as a model system to test the possibility of improving enantioselectivity.

A prerequisite for the directed evolution of enantioselective enzymes is the availability of an efficient expression system and an appropriate high-throughput ee assay.[5,7] An expression system for the EH from *A. niger* based on the construct pGEF Asp-EH in *E. coli* BL21(DE3) was previously described.[11] Sufficient quantities of this recombinant wild-type EH, which allows for commercialization, has also been achieved by using a heterologous overexpression within another *A. niger* strain.[4,12] Moreover, in preliminary work, we developed an ESI-MS-based high-throughput ee assay[13] based on the use of a 1:1 mixture of (*S*)-**1** and the pseudo-enantiomer (*R*)-D_5-**1** (Scheme 2). Since the two starting compounds and the

Scheme 2



two products (*S*)-**2**/(*R*)-D_5-**2** differ by five mass units, their respective ratios can be determined by ESI-MS. To reduce the extent of ESI-MS screening, a pretest[14] for epoxide hydrolase activity was routinely applied, which is based on the known reaction of an epoxide with 4-*p*-nitrobenzylpyridine leading to the rapid formation of a blue dye. It has been parallelized to function efficiently on mictrotiter plates.[14] Thus, reaction mixtures in the wells of microtiter plates (96- or 384-well format) in which a blue color is not formed contain active EH mutants. These mutants were then investigated for enantioselectivity using the ESI-MS assay.

Several libraries of mutant EHs were prepared by applying epPCR under various conditions and transforming into *E. coli* BL21(DE3). Single transformants were cultured in liquid media in a 96-well format, and the pretest for activity was performed using bacterial cultures and the racemic epoxide **1**. Of the first 20 000 clones that were screened, approximately 20% showed appreciable activity as revealed by the pretest. The active mutants were then screened by our ESI-MS-based ee assay leading to the identification of several dozen mutants with improved enantioselectivity.[9] Most of these exhibited selectivity factors ranging between 5 and 7, but several had distinctly higher *E*-values. The latter mutants were subsequently studied in laboratory scale reactions, the enantiopurity being determined by traditional HPLC analysis using chirally modified columns. The *E*-values were subsequently derived using the formula of Sih[15] (Table 1).

The most selective EH variant (IS002B1) displays an *E*-value of 10.8 in favor of (*S*)-**2**. Thus, enantioselectivity has been more than doubled with respect to the wild-type enzyme. This mutant, which also appears to be quite active, is characterized by three amino acid exchanges, A217V, K332E, and A390E. In 3 out of 11 cases under investigation, plasmid DNA could not be recovered. This not only prevented the determination of the respective amino acid exchanges (Table 1) but also prohibited their use in subsequent rounds of directed evolution.

(5) (a) Reetz, M. T.; Zonta, A.; Schimossek, K.; Liebeton, K.; Jaeger, K.-E. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2830−2832. (b) Zha, D.; Wilensek, S.; Hermes, M.; Jaeger, K.-E.; Reetz, M. T. *Chem. Commun. (Cambridge)* **2001**, 2664−2665. (c) Reetz, M. T.; Wilensek, S.; Zha, D.; Jaeger, K.-E. *Angew. Chem., Int. Ed.* **2001**, *40*, 3589−3591. (d) Reetz, M. T. *Pure Appl. Chem.* **2000**, *72*, 1615−1622. (e) Reetz, M. T. *Tetrahedron* **2002**, *58*, 6595−6602.

(6) (a) Arnold, F. H. *Nature* **2001**, *409*, 253−257. (b) Powell, K. A.; Ramer, S. W.; del Cardayré, S. B.; Stemmer, W. P. C.; Tobin, M. B.; Longchamp, P. F.; Huisman, G. W. *Angew. Chem., Int. Ed.* **2001**, *40*, 3948−3959. (c) Brakmann, S.; Johnsson, K. *Directed Molecular Evolution of Proteins (or How to Improve Enzymes for Biocatalysis)*; Wiley-VCH: Weinheim, Germany, 2002. (d) Arnold, F. H.; Georgiou, G. *Directed Enzyme Evolution: Screening and Selection Methods*; Humana Press: Totowa, NJ, 2003; Vol. 230.

(7) Reetz, M. T. *Angew. Chem., Int. Ed.* **2001**, *40*, 284−310.

(8) Bocola, M.; Otte, N.; Jaeger, K.-E.; Reetz, M. T.; Thiel, W. *ChemBioChem*, in press.

(9) Torre, C. Dissertation, Ruhr-Universität Bochum, Bochum, Germany, 2003.

(10) Genzel, Y.; Archelas, A.; Furstoss, R. Unpublished results.

(11) Arand, M.; Hemmer, H.; Dürk, H.; Baratti, J.; Archelas, A.; Furstoss, R.; Oesch, F. *Biochem. J.* **1999**, *344*, 273−280.

(12) Visser, J.; Archelas, A.; Arand, M.; Furstoss, R. Unpublished results.

(13) Schrader, W.; Eipper, A.; Pugh, D. J.; Reetz, M. T. *Can. J. Chem.* **2002**, *80*, 626−632.

(14) Zocher, F.; Enzelberger, M. M.; Bornscheuer, U. T.; Hauer, B.; Schmid, R. D. *Anal. Chim. Acta* **1999**, *391*, 345−351.

(15) Chen, C.-S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294−7299.

Table 1. Improved Variants of the EH from *Aspergillus niger* as Catalysts in the Hydrolytic Kinetic Resolution of Epoxide **1**

variant	ee_p (*S*) [%]	conversion [%] (reaction time [min])	*E*	amino acid exchanges
IS002B1	74	39 (2)	10.8	A217V, K332E, A390E
IV001G2	73	24 (5)	8.1	not determined
IY001B1	69	35 (30)	7.7	not determined
IR003B1	69	31 (5)	7.4	R219G
IT001A1	66	38 (30)	7.2	F84L, M245L
II001G7	68	30 (30)	6.8	not determined
IB001C2	64	38 (30)	6.6	F340Y
IE001H6	65	34 (45)	6.4	A327V
IS001H8	67	23 (30)	6.2	A327V
IL001D4	62	30 (3)	5.5	P222S
WT	56	33 (45)	4.6	

The X-ray crystal structure of the wild-type EH from *A. niger* reveals a dimer comprised of identical subunits.[16] Each monomeric unit contains the catalytic triad (aspartate, aspartate, histidine) typical for most EHs.[2,3] Aspartate 192 attacks the epoxide nucleophilically with formation of a glycol-monoester intermediate that is hydrolyzed in a second step. Figure 1 shows the known three-dimensional structure

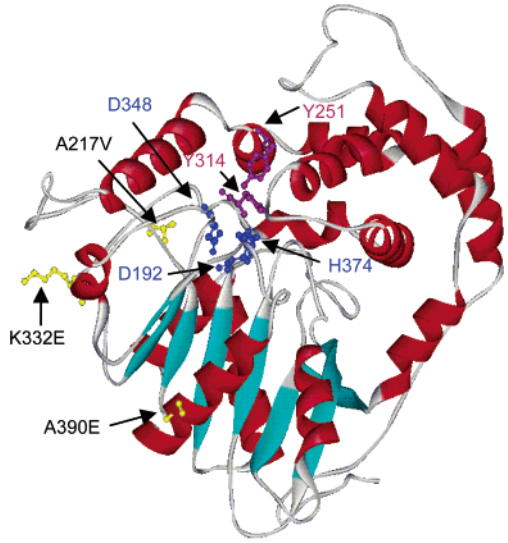


Figure 1. Crystal structure[16] of the monomer of the wild-type EH from *Aspergillus niger* showing the catalytic triad (blue), the two tyrosines that activate the epoxide by H-bonding (purple), and the three amino acid substitutions (yellow).

of the monomer,[16] displaying the active site, the two tyrosines that bind and activate the epoxide via H-bonding and the three mutations of the most enantioselective EH-variant (IS002B1). It can be seen that two mutations (K332E and A390E) occur at positions remote from the active center, whereas A217V is somewhat closer. The side chain of the amino acid at position 217 is located about 9 Å from the nucleophilic active site D192. Molecular modeling (MOLOC MD calculations) of the wild-type EH with both enantiomers of **1** shows that the side chain of residue 217 is in van der Waals contact with the phenyl-ring of the substrate. A preliminary model suggests that the larger valine side chain at residue 217 disfavors the (*R*)-substrate due to steric interaction. The role of the remote substitutions K332E and A390E is more difficult to reveal, requiring more extensive theoretical investigations. Rare cases of remote effects with respect to enzyme stability and/or activity were reported previously by other groups.[17]

In summary, the present results show that it is possible to apply the methods of directed evolution in the quest to develop epoxide hydrolases having enhanced enantioselectivity.[18] Further rounds of epPCR, saturation mutagenesis, and DNA shuffling now need to be applied. However, before doing so, a problem that hampers systematic optimization of the present reaction or of the kinetic resolution of other more difficult substrates needs to be resolved, namely, our observation that the expression system is genetically unstable. Whereas this is of little concern when producing large quantities of single enzyme variants, it causes difficulties when performing directed evolution. Thus, our immediate efforts are now directed toward improving the expression system.[19]

(16) (a) Zou, J. Y.; Hallberg, B. M.; Bergfors, T.; Oesch, F.; Arand, M.; Mowbray, S. L.; Jones, T. A. *Structure (London)* **2000**, *8*, 111−122. (b) http://www.pdb.ccdc.cam.ac.uk/pdb.

(17) (a) Zhao, H.; Arnold, F. H. *Prot. Eng.* **1999**, *12*, 47−53. (b) Arnold, F. H.; Wintrode, P. L.; Miyazaki, K.; Gershenson, A. *Trends Biochem. Sci.* **2001**, *26*, 100−106. (c) Iffland, A.; Tafelmeyer, P.; Saudan, C.; Johnsson, K. *Biochem.* **2000**, *39*, 10790−10798. (d) Kumar, M.; Kannan, K. K.; Hosur, M. V.; Bhavesh, N. S.; Chatterjee, A.; Mittal, R.; Hosur, R. V. *Biochem. Biophys. Res. Commun.* **2002**, *294*, 395−401. (e) Agarwal, P. K.; Billeter, S. R.; Rajagopalan, P. T. R.; Benkovic, S. J.; Hammes-Schiffer, S. *Proc. Natl. Acad. Sci.* **2002**, *99*, 2794−2799.

(18) Other cases of improving enzyme enantioselectivity or substrate acceptance by directed evolution:[5] (a) Fong, S.; Machajewski, T. D.; Mak, C. C.; Wong, C.-H. *Chem. Biol.* **2000**, *7*, 873−883. (b) May, O.; Nguyen, P. T.; Arnold, F. H. *Nat. Biotechnol.* **2000**, *18*, 317−320. (c) Henke, E.; Bornscheuer, U. T. *Biol. Chem.* **1999**, *380*, 1029−1033. (d) Horsman, G. P.; Liu, A. M. F.; Henke, E.; Bornscheuer, U. T.; Kazlauskas, R. J. *Chem. Eur. J.* **2003**, *9*, 1933−1939. (e) DeSantis, G.; Wong, K.; Farwell, B.; Chatman, K.; Zhu, Z.; Tomlinson, G.; Huang, H.; Tan, X.; Bibbs, L.; Chen, P.; Kretz, K.; Burk, M. J. *J. Am. Chem. Soc.* **2003**, *125*, 11476−11477.

Acknowledgment. Support from the European Community (Project QLRT-2000-00426) and the Fonds der Chemischen Industrie is gratefully acknowledged.

(19) Cedrone, F.; Niel, S.; Roca, S.; Bhatnagar, T.; Ait-Abdelkader, N.; Torre, C.; Krumm, H.; Maichele, A.; Reetz, M. T.; Baratti, J. C. *Biocatal. Biotransform.* Submitted.

Supporting Information Available: Details of mutagenesis methods, cloning, growth, and expression and type of screening. This material is available free of charge via the Internet at http://pubs.acs.org.

OL035898M