Divergent Evolution

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Converting an Esterase into an Epoxide Hydrolase**

Helge Jochens, Konstanze Stiba, Christopher Savile, Ryota Fujii, Juin-Guo Yu, Tatsiana Gerassenkov, Romas J. Kazlauskas, and Uwe T. Bornscheuer**

Dedicated to Professor Kalle Hult on the occasion of his 65th birthday

Divergent evolution has created superfamilies of enzymes with the same protein fold, but different catalytic abilities. For example, the α/β-hydrolase superfamily[1] enzymes all catalyze reactions involving a nucleophilic attack: ester,[2] amide,[3] epoxide,[4] and alkyl halide hydrolysis,[5] cyanide addition to aldehydes forming a carbon–carbon bond[6] as well as several others. Many of these enzymes, and especially lipases and esterases, accept a wide range of substrates, each with high stereoselectivity, making them versatile catalysts for organic synthesis.[2,7]

The different catalytic abilities require distinct mechanistic steps, but some of these mechanistic steps may be shared. X-ray crystallography and biochemical studies suggest that the new mechanistic steps require only a few amino acid substitutions, but the amino acid sequences of enzymes within a superfamily differ by hundreds of substitutions and possible insertions and deletions.

Previous reports that involved changing the catalytic activity of an enzyme required different approaches. Changing from hydrolysis of a thioester to hydrolysis of a β-lactam required insertion, deletion, and substitution of loops as well as amino acid substitutions.[8] Changing 4-chlorobenzoyl-CoA dehalogenase to a crotonase activity required eight amino acid substitutions.[9] However, in a few cases, a single amino acid substitution could introduce new catalytic activity: from a racemase to an aldolase, from an esterase to a perhydrolase, from an epimerase to an *o*-succinylbenzoate synthase, and from a decarboxylase to a racemase.[10,11] Researchers have also introduced enzymatic activity into non-catalytic proteins, but these experiments all required extensive substitutions.[12]

Herein, we test the hypothesis that a few amino acid substitutions are sufficient to interconvert the catalytic abilities of different enzymes within the α/β-hydrolase family. Our test case is to convert an esterase from *Pseudomonas fluorescens* (PFE) into an epoxide hydrolase. Mechanistic considerations suggest that two or three amino acid substitutions could convert an esterase mechanism into an epoxide hydrolase mechanism (Scheme 1). Esterases have a Ser-His-Asp catalytic triad,[13] whereas epoxide hydrolases use an Asp-His-Asp triad,[14] so one substitution is needed in the triad. In addition, epoxide hydrolases contain two tyrosines to protonate the epoxide oxygen during catalysis, which are two more substitutions. In one case, only one of these tyrosines was essential to catalysis,[15] so perhaps only one is needed.

To identify the positions of these residues within the PFE we compared the structures and amino acid sequences of six epoxide hydrolases (PDB entries: 2E3J, 2CJP, 1S8O, 1CQZ, 1EHY, and 1Q07) with three esterases (PDB entries: 1VA4, 1P0, and 1ZOI) using clustalw.[17] This comparison (Figure 1) identified the position of the catalytic nucleophile (D94), four possible positions for the two mechanistically important tyrosines (Y125, Y139, Y143, Y195) and three further amino acids that are conserved in epoxide hydrolases but are missing from PFE (P29, H93, K188). The position of one of these tyrosines was identified as being at amino acid 195, but as it was not definitely clear where to introduce the second tyrosine, it was placed at all three alternative positions. Consequently, the following mutants were created by QuikChange site-directed mutagenesis and expressed recombinantly in *E. coli*: **M1**: S94D; **M2**: S94D, F125Y, V195Y; **M3**: S94D, F143Y, V195Y; **M4**: S94D, F125Y, F143Y, V195Y; **M5**: L29P, S94D, F125Y, K188M; **M6**: L29P, S94D, F125Y, V195Y; **M7**: L29P, F93H, S94D, F125Y, V139Y, V195Y. As expected, all mutants showed no esterase activity against *p*-nitrophenyl acetate above background levels ($< 10\,\mathrm{mU\,mg^{-1}}$), as the catalytic nucleophile was replaced by an aspartate. Unfortunately, all these mutants also showed no detectable epoxide hydrolase activity towards *p*-nitrostyrene oxide (see Supporting Information).

Because it was not obvious why the created mutants failed to catalyze the reaction, we applied directed evolution to search for missing key amino acids that correct the imperfect geometry. Starting from mutants **M4**–**M6**, we created a mutant library by a error-prone polymerase chain reaction (epPCR) and selected active variants using a growth assay.[18]

[*] Dr. C. Savile, Dr. R. Fujii, J.-G. Yu, T. Gerassenkov, Prof. R. J. Kazlauskas
University of Minnesota, Department of Biochemistry, Molecular Biology & Biophysics
1479 Gortner Avenue, 174 Gortner Lab, St. Paul, MN 55108 (USA)
Fax: (+1) 612-625-5780
E-mail: rjk@umn.edu
Dipl.-Biochem. H. Jochens, Dr. K. Stiba, Prof. Dr. U. T. Bornscheuer
Institute of Biochemistry, Dept. of Biotechnology & Enzyme Catalysis, University of Greifswald
Felix-Hausdorff-Strasse 4, 17487 Greifswald (Germany)
Fax: (+49) 3834-86-80066
E-mail: uwe.bornscheuer@uni-greifswald.de
Homepage: http://www.chemie.uni-greifswald.de/~biotech

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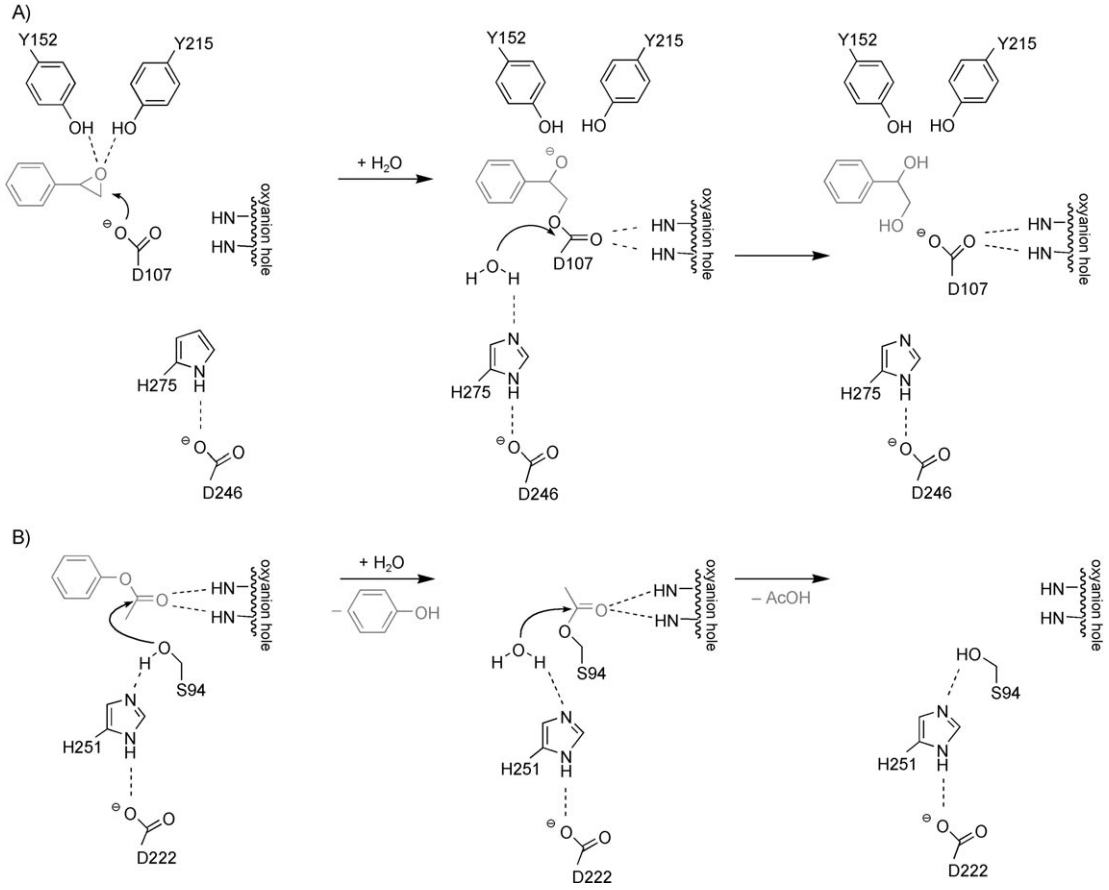

Scheme 1. Mechanistically essential amino acid residues in *Agrobacterium radiobacter* epoxide hydrolase (EchA).[15] A) Formation and liberation of the alkyl enzyme intermediate derived from styrene oxide as substrate and *Pseudomonas fluorescens* esterase (PFE).[16] B) Formation and liberation of the acetyl enzyme intermediate derived from phenyl acetate as substrate.

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1VA4: LKEVTLVGFSMGGGDVARYIARHGS . . . LKATVDCVTAFAE-------TDFRPDMAK . . .
1Z0I: IQGAVHVGHSTGGGEVVRYMARHPE . . . AKAHYDGIVAFSQ-------TDFTEDLKG . . .
1P0I: PKSVTLFGESAGAASVSLHLLSPGS . . . LKIFFPGVSEFGKESILFHYTDWVDDQRP . . .

1EHY: IEKAYVVGHDFAAIVLHKFIR-KYS . . . IHGGFNYYRANIRPDAALWTDLDHTMSDL . . .
1CQZ: IPQAVFIGHDWAGVMVWNMAL-FYP . . . FRGPLNWYRN-TERNWKWSCKGLGRKILV . . .
1Q07: GSGYIIQGGDIGSFVGRLLGVGFDA . . . FPRAIHTYRE----TTPTASAPNGATMLQ . . .
2E3J: AEQAFVVGHDWGAPVAWTFAW-LHP . . . FGGPLSFYHN-IDNDWHDLADQQGKPLTP . . .
2CJP: EEKVFVVAHDWGALIAWHLCL-FRP . . . FTGAVNYYRA-LPINWELTAPWTGAQVKV . . .
1S8O: LSQAVFIGHDWGGMLVWYMAL-FYP . . . FRGPLNWYRN-MERNWKWACKSLGRKILI . . .
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Figure 1. Part of the comparison of the structure and amino acid sequences of three esterases (PFE = 1VA4) with six different epoxide hydrolases (EH; for PDB codes, see text). The catalytic aspartate (D) and one of the mechanistically important tyrosines (Y) of the epoxide hydrolases and the catalytic serine (S) of the esterases are highlighted.

Although we identified eight clones out of 25000 that grew under these conditions, in the subsequent HPLC validation, no activity against *p*-nitrostyrene oxide was detected.

A further structural comparison of the six epoxide hydrolases showed that a loop with 20 amino acids at the supposed entrance to the active site[16] differs in PFE (Figure 2). In the epoxide hydrolases, this loop lies to one side of the active site, but in PFE, this loop blocks the entrance. The loop may also position the mechanistically important Y152 (position 139 in PFE) as it is the last residue of this loop.

To test the role of the loop, the whole loop in the PFE mutant **M7** (containing mutations L29P, F93H, S94D, F125Y, V139Y, V195Y) was replaced by the corresponding element of the EchA by a PCR-based method based (EchA numbering: P132 to Y152). The resulting chimera (**M8**) could be expressed in *E. coli* as a soluble enzyme by coexpression of chaperones (see the Supporting Information). Protein (ca. 10 mg) was isolated after His-tag purification and desalting. This chimera catalyzed the slow hydrolysis of *p*-nitrostyrene oxide (Figure 3) with an initial activity of 9 mU mg^{-1} and a turnover number of 0.01 s^{-1} at a 50 μM substrate concentration. The catalytic activity differed from batch to batch and some samples showed no activity. In spite of extensive experimentation (see the Supporting Information), we have not found a satisfactory explanation. We hypothesize that the mutations hinder protein folding, and subtle changes in experimental conditions change the amount of properly folded enzyme. We could not measure K_M and V_{max} values

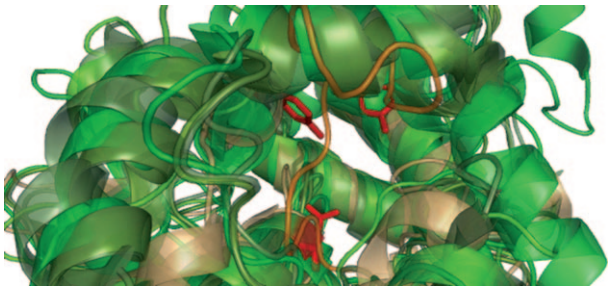

Figure 2. Multiple structural alignment of six epoxide hydrolases (green) with PFE (light orange) showing that a loop (A120–V139) present in PFE (dark orange) possibly blocks the entrance to the active site (red sticks).

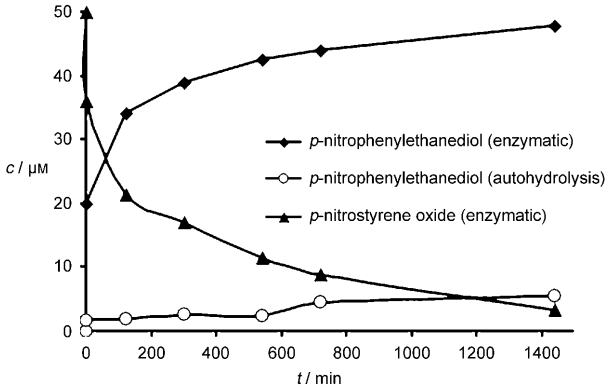

Figure 3. Hydrolysis of *p*-nitrostyrene oxide catalyzed by loop mutant **M8**. The protein concentration was 0.5 mg mL^{-} (16.7 μM), so complete conversion corresponds to approximately three substrate molecules hydrolyzed per enzyme molecule.

owing to substrate inhibition (see the Supporting Information). This inhibition was significant even at concentrations of 50 μM, and lower substrate concentrations were below the detection limit. The loop mutant showed high enantioselectivity ($E > 100$) for the (*R*)-enantiomer of *p*-nitrostyrene oxide (Figure 4). The template epoxide hydrolase, EchA, also favors the (*R*)-enantiomer. We also detected low background activity in crude *E. coli* cell extract (0.06 mU mg^{-1}), but this reaction favored the (*S*)-enantiomer. The His-tag purification used to prepare the loop mutant removed this contaminating activity.

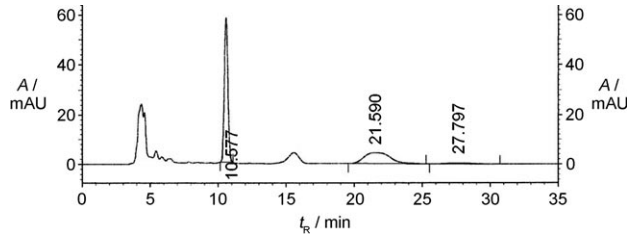

Figure 4. HPLC chromatogram for the conversion of *p*-nitrostyrene oxide by loop mutant **M8**. 50 μL of a prepared sample ($c = 50$ μM) was measured on a HPLC using Chiracel OD-H column (for sample preparation, see the Supporting Information). (*R,S*)-*p*-Nitrostyrene oxide ($t_R = 10.577$ min), (*R*)-*p*-nitrophenylethanediol ($t_R = 21.590$ min), (*S*)-*p*-nitrophenylethanediol ($t_R = 27.797$ min).

The experimental results indicate that the epoxide hydrolase loop is essential for the newly created activity, but at the molecular level, the reason is uncertain. One possibility is that the esterase loop favors a nonproductive substrate orientation. The esterase substrate, *p*-nitrophenyl acetate, and epoxide hydrolase substrate, *p*-nitrostyrene oxide, most likely orient themselves slightly differently in the active site. A correct *p*-nitrophenyl group orientation for the esterase substrate orientation may be nonproductive for the epoxide hydrolase substrate. The observed substrate inhibition by *p*-nitrostyrene oxide supports the notion that nonproductive orientations of the substrate at the active site are possible. A second possible role for the epoxide hydrolase loop is some required motion. The X-ray structure of EchA gave a non-interpretable electron density for amino acids G138 to H148, suggesting that this region is flexible.[19] The third possibility is that the epoxide hydrolase loop corrects the orientation of Y139. Mutant **M7** contained the Y139 mutation, but did not show epoxide hydrolase activity. The new loop most likely changes the orientation of this amino acid.

Changing just the obvious residues (serine to aspartate, introduction of two tyrosines) was not sufficient, but an additional substitution of a loop conferred epoxide hydrolase activity into the esterase scaffold. The specific activity we measured at substrate concentrations near the detection limit was only 800-fold less than the activity of the template, which is a true epoxide hydrolase. This catalytic activity is too low for practical use, but demonstrates that the principle of interconversion of enzyme activities within the α/β-hydrolase family is possible, and thus opens opportunities to extend catalysis to new, non-natural reactions.

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[1] D. L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, *Protein Eng.* **1992**, *5*, 197.
[2] a) R. J. Kazlauskas, U. T. Bornscheuer in *Biotechnology, Vol. 8a*, 2nd ed (Eds.: H. J. Rehm, G. Reed, A. Pühler, P. J. W. Stadler, D. R. Kelly), Wiley-VCH, Weinheim, **1998**, p. 37; b) M. Cygler, J. D. Schrag, J. L. Sussman, M. Harel, I. Silman, M. K. Gentry, B. P. Doctor, *Protein Sci.* **1993**, *2*, 366.
[3] P. Carter, J. A. Wells, *Nature* **1988**, *332*, 564.
[4] a) M. Arand, A. Cronin, M. Adamska, F. Oesch, *Methods Enzymol.* **2005**, *400*, 569; b) A. Archelas, R. Furstoss, *Trends Biotechnol.* **1998**, *16*, 108; c) K. Faber, M. Mischitz, W. Kroutil, *Acta Chem. Scand.* **1996**, *50*, 249.
[5] a) S. Barth, M. Fischer, R. D. Schmid, J. Pleiss, *Bioinformatics* **2004**, *20*, 2845; b) D. B. Janssen, *Curr. Opin. Chem. Biol.* **2004**, *8*, 150; c) S. Fetzner, F. Lingens, *Microbiol. Rev.* **1994**, *58*, 641; d) P. E. Swanson, *Curr. Opin. Biotechnol.* **1999**, *10*, 365.
[6] a) D. V. Johnson, A. A. Zabelinskaja-Mackova, H. Griengl, *Curr. Opin. Chem. Biol.* **2000**, *4*, 103; b) F. Effenberger, S. Förster, H. Wajant, *Curr. Opin. Biotechnol.* **2000**, *11*, 532; c) M. Schmidt, H. Griengl, in *Biocatalysis—from discovery to application, Vol. 200* (Ed.: W.-D. Fessner), Springer, Berlin, **1999**, pp. 193.

[7] a) U. T. Bornscheuer, R. J. Kazlauskas, *Hydrolases in Organic Synthesis—Regio- and Stereoselective Biotransformations*, 2nd ed., Wiley-VCH, Weinheim, **2006**; b) R. D. Schmid, R. Verger, *Angew. Chem.* **1998**, *110*, 1694; *Angew. Chem. Int. Ed.* **1998**, *37*, 1608.
[8] H. S. Park, S. H. Nam, J. K. Lee, C. N. Yoon, B. Mannervik, S. J. Benkovic, H. S. Kim, *Science* **2006**, *311*, 535.
[9] H. Xiang, L. Luo, K. L. Taylor, D. Dunaway-Mariano, *Biochemistry* **1999**, *38*, 7638.
[10] a) F. P. Seebeck, D. Hilvert, *J. Am. Chem. Soc.* **2003**, *125*, 10158; b) D. M. Z. Schmidt, E. C. Mundorff, M. Dojka, E. Bermudez, J. E. Ness, S. Govindarajan, P. C. Babbitt, J. Minshull, J. A. Gerlt, *Biochemistry* **2003**, *42*, 8387.
[11] a) P. Bernhardt, K. Hult, R. J. Kazlauskas, *Angew. Chem.* **2005**, *117*, 2802; *Angew. Chem. Int. Ed.* **2005**, *44*, 2742; b) Y. Terao, K. Miyamoto, H. Ohta, *Chem. Commun.* **2006**, 3600.
[12] a) D. Röthlisberger, O. Khersonsky, A. M. Wollacott, L. Jiang, J. DeChancie, J. Betker, J. L. Gallaher, E. A. Althoff, A. Zanghellini, O. Dym, S. Albeck, K. N. Houk, D. S. Tawfik, D. Baker, *Nature* **2008**, *453*, 190; b) B. Seelig, J. W. Szostak, *Nature* **2007**, *448*, 828; c) L. Jiang, E. A. Althoff, F. R. Clemente, L. Doyle, D. Röthlisberger, A. Zanghellini, J. L. Gallaher, J. L. Betker, F. Tanaka, C. F. Barbas III, D. Hilvert, K. N. Houk, B. L. Stoddard, D. Baker, *Science* **2008**, *319*, 1387.
[13] V. K. Ward, B. C. Bonning, T. Huang, T. Shiotsuki, V. N. Griffeth, B. D. Hammock, *Int. J. Biochem.* **1992**, *12*, 1933.
[14] R. Rink, M. Fennema, M. Smids, U. Dehmel, D. B. Janssen, *J. Biol. Chem.* **1997**, *272*, 14650.
[15] a) B. van Loo, J. H. L. Spelberg, J. Kingma, T. Sonke, M. G. Wubbolts, D. B. Janssen, *Chem. Biol.* **2004**, *11*, 981; b) R. Rink, J. H. L. Spelberg. R. J. Pieters, J. Kingma, M. Nardini, R. M. Kellogg, B. W. Dijkstra, D. B. Janssen, *J. Am. Chem. Soc.* **1999**, *121*, 7417.
[16] a) J. D. Cheeseman, A. Tocilj, S. Park, J. D. Schrag, R. J. Kazlauskas, *Acta Crystallogr. Sect. D* **2004**, *60*, 1237; b) N. Krebsfänger, F. Zocher, J. Altenbuchner, U. T. Bornscheuer, *Enzyme Microb. Technol.* **1998**, *22*, 641; c) G. P. Horsman, A. M. F. Liu, E. Henke, U. T. Bornscheuer, R. J. Kazlauskas, *Chem. Eur. J.* **2003**, *9*, 1933.
[17] npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page = npsa_clustalw.html.
[18] Mutant libraries were copied on agar plates that contained glycidol butyrate. Active clones should then generate glycerol butyrate, which serves as a carbon source for *E. coli* on the minimal media, thus allowing selection for active epoxide hydrolase variants.
[19] M. Nardini, I. S. Ridder, H. J. Rozeboom, K. H. Kalk, R. Rink, D. B. Janssen, B. Dijkstra, *J. Biol. Chem.* **1999**, *274*, 14579.

