#### **Directed Evolution**

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### Directed Evolution of Enantioselective Enzymes: Iterative Cycles of CASTing for Probing Protein-Sequence Space\*\*

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Directed evolution of enantioselective enzymes as catalysts in synthetic organic chemistry has emerged as a fundamentally new and useful approach to asymmetric catalysis.<sup>[1]</sup> It is based on earlier contributions by molecular biologists, biochemists, and biotechnologists who developed efficient random-gene mutagenesis methods, expression systems, and enzyme assays in the quest to enhance the stability and/or activity of enzymes.<sup>[2]</sup> By passing through at least two cycles of mutagenesis/screening,<sup>[3]</sup> Darwinistic character is introduced which forms the general basis of directed evolution. Therein, we first developed high-throughput ee assays<sup>[4]</sup> and applied them in the evolution of active and enantioselective mutants of lipases,<sup>[5]</sup> monooxygenases,<sup>[6]</sup> and epoxide hydolases.<sup>[7]</sup> Other researchers<sup>[8]</sup> have also contributed to this new area of asymmetric catalysis. Most directed evolution studies either begin with one or more cycles of error-prone polymerase chain reactions (epPCRs)<sup>[9]</sup> to introduce more or less random mutations that cover the whole gene (and thus enzyme) or a recombinant method such as DNA shuffling<sup>[10]</sup> is applied.

More recently, the utility of generating focused libraries of mutants that result from amino acid randomization at defined positions in the enzyme, has started to be explored.<sup>[2,5c,11]</sup> For example, in the case of the directed evolution of enantiose-lective lipases, we generated a focused library through the simultaneous randomization of four amino acids located adjacent to the binding pocket.<sup>[5c]</sup> In the quest to enhance enzyme activity, other groups have created focused libraries through the randomization of two, three, four, or more amino acids near the binding site,<sup>[11]</sup> as in the case of chorismate mutases.<sup>[11a]</sup>

In another study, systematic saturation mutagenesis at every-single position of the Bacillus subtilis lipase, which is composed of 181 amino acids, was considered, thereby generating 181 focused libraries for the purpose of finding enantioselective mutants.<sup>[12]</sup> This idea was developed independently by scientists at Diversa (USA), who were able to produce highly enantioselective nitrilases by this method.<sup>[13]</sup> Such saturation mutagenesis can be restricted to selected positions near the active site,<sup>[14]</sup> thereby lowering the screening effort (although restricting catalyst diversity). Other focused libraries,<sup>[2,11]</sup> such as those of binary patterning,<sup>[11a,b]</sup> are also useful tools. Although these strategies lead to improved enzymes, they are not evolutionary in nature unless at least one further round of mutagenesis/screening follows. In many, if not most, practical cases this may be necessary. Efficiency in scanning larger parts of the proteinsequence space with the generation of high-quality libraries is therefore mandatory.<sup>[15]</sup> It minimizes the undesired formation of inferior (e.g., inactive) enzyme mutants and thus reduces the overall screening effort, which can be substantial in the case of enantioselectivity.<sup>[4]</sup>

Recently, we introduced a practical method in directed evolution called a combinatorial active site saturation test (CAST). CAST was originally developed with the purpose of expanding the range of substrate acceptance of an enzyme.<sup>[16]</sup> Based on the 3D structure of the enzyme, two or three amino acids, whose side chains reside next to the binding pocket, are identified and the respective positions are then randomized simultaneously with the creation of relatively small libraries of mutants. Through the use of this method, it was possible to obtain mutant lipases that catalyze the hydrolysis of esters derived from sterically demanding acids that are normally not at all accepted as substrates.<sup>[16]</sup> CASTing thus means the systematic design and screening of focused libraries around the complete binding pocket. Owing to the fact that two or three amino acids (rather than just one<sup>[12-14]</sup>) are simultaneously randomized at each site, high catalyst diversity is possible, therefore theoretically allowing the possibility of cooperative effects. The method is a useful alternative to epPCR as the starting point of directed evolution studies. However, it is not, in itself, an evolutionary process because only the initial libraries are considered. In our original paper,<sup>[16]</sup> we suggested that, for future work, Darwinistic character, which is central to directed evolution,<sup>[1,2,10,11,15]</sup> can be introduced through the performance of further rounds of mutagenesis/screening. This can be performed by using any



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one of the conventional methods, such as epPCR or DNA shuffling after CASTing, which may lead to remote effects.<sup>[1,17]</sup> As an alternative, we herein propose iterative cycles of CASTing. Consider, for example, an enzyme for which the CAST analysis requires four randomization sites A, B, C, and D. At each site, two or three amino acid positions are randomized simultaneously depending on the CAST analysis. Following the generation and screening of the four respective initial libraries, the gene of each hit is then used as a template for the second round of CASTing. In each case, only two libraries are necessary, for example, the gene of the best mutant arising from A is used as a template for randomization at sites B and C, etc. (Figure 1). In practice, not all branches of this confined protein-sequence space need to be considered, nor is complete over-sampling absolutely mandatory.



*Figure 1.* Schematic illustration of iterative CASTing involving (as an example) four randomization sites A, B, C, and D: Confined protein-sequence space for evolutionary enzyme optimization (redundancy in some cases is expected).

The concept of CASTing is illustrated herein with the directed evolution of enantioselective mutants of the epoxide hydrolase from *Aspergillus niger* (ANEH)<sup>[18–20]</sup> as catalysts in the hydrolytic kinetic resolution of the glycidyl ether **1** with formation of diols (*R*)- and (*S*)-**2**. The wild-type enzyme (WT-ANEH) catalyzes this reaction with a low selectivity factor (E = 4.6) in favor of (*S*)-**2** [Eq. (1)]. In previous work, the

$$\begin{array}{ccc} & & H_{2}O \\ \hline PhO & & ANEH \end{array} \xrightarrow{HO} & OH \\ \hline PhO & & PhO \end{array} \xrightarrow{OH} + \begin{array}{c} HO & OH \\ \hline PhO & & PhO \end{array} \xrightarrow{OH} + \begin{array}{c} HO & OH \\ \hline PhO & & PhO \end{array}$$
(1)

enantioselectivity in this reaction was increased to E = 10.8 by screening several libraries produced by epPCR at different mutation rates (the number of total clones amounted to 20000).<sup>[7a]</sup> The *ee* screening was performed through the application of our previously described MS-based system<sup>[21]</sup> following a pre-screen<sup>[22]</sup> to identify the active clones. In the present study, we employed an improved expression system in *E. coli*,<sup>[7b]</sup> adapted the MS screen, and used an efficient preselection system for the elimination of inactive clones.<sup>[23]</sup>

The X-ray crystal structure<sup>[24]</sup> of the WT-ANEH reveals, amongst other things, a narrow hydrophobic tunnel as the

substrate binding site.<sup>[25]</sup> The catalytically active triad, the tyrosines 251 and 314 (bind and activate the epoxide through H-bonds), and aspartate 192 (acts as the nucleophile in the rate-determining step) are located in and around the tunnel.<sup>[24]</sup> Inspection of the 3D structure suggests the creation of six CAST libraries<sup>[26]</sup> to be produced separately by randomization at sites 193/195/196 (A), 215/217/219 (B), 329/330 (C), 349/350 (D), 317/318 (E), and 244/245/249 (F) (Figure 2).

We have previously pointed out that for 95% coverage of a CAST library resulting from the simultaneous randomization at two (or three) amino acid positions, an excess of about 3000 (or 98000, respectively) clones needs to be screened.<sup>[16,27]</sup> However, one can settle for a lower degree of over-sampling and still be successful;<sup>[28]</sup> we have made these and other compromises in the present study. Experimentally, we began by subjecting the WT-ANEH to CASTing with generation of CAST libraries at sites A, B, and C, that is, in three separate experiments, positions 193/195/196, 215/217/ 219, and 329/330, respectively, were randomized. As shown in Table 1, no enhancement in enantioselectivity was observed in any of the mutants screened in the libraries that originated from the randomization of sites A or C. In contrast, several markedly improved mutants were discovered in the CAST library produced by randomization at positions 215/217/219 (site B). Therefore, the decision was made to continue the evolutionary process on this branch and not to consider, at this point, CAST libraries that potentially originate by randomization at sites D, E, and F, separately. One of the best mutants created by randomizing at site B (215/217/219) by using the WT gene, variant LW081, characterized by the three mutations L215F, A217N, and R219S, leads to an E value<sup>[29]</sup> of 14. This is similar to the structurally related and equally selective mutant LW080 (Table 1). Three other mutants appear to be more active because they showed higher conversions after shorter reaction times,<sup>[30]</sup> however, they were seen to be less stereoselective.

The mutant gene that corresponds to enzyme LW081 was then used as a template for a second cycle of CASTing, specifically by (arbitrarily) focusing on positions 329/330 (site C). Substantial improvements in enantioselectivity were observed with mutant LW086 (E=21) being the best enzyme (Table 1). Mutant LW086 is characterized by two additional mutational changes of M329P and L330Y. Before continuing the ascent in enantioselectivity, it was of interest to perform CASTing with one of the mutant genes that encodes an enzyme displaying enhanced activity<sup>[30]</sup> but not improved enantioselectivity. For this purpose, gene LW037 (Table 1) was subjected to randomization at positions 329/330 with the creation of a new library. However, no substantially improved mutants were found (Table 1).

We then refocused our attention on mutant LW086 and used the corresponding gene in another cycle of CASTing. This time, positions 349/350 (site D) were chosen for simultaneous randomization. The best mutant was LW123 with one new mutational change C350V (E=24). At this point, two different decisions for further evolutionary optimization appeared plausible: use of the same gene (LW086) for randomization at the other sites of the enzyme (A, B, C, E,

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**Figure 2.** CASTing of the epoxide hydrolase from *Aspergillus niger* (ANEH) based on the X-ray crystal structure of the WT.<sup>[24]</sup> Top: Defined randomization sites A–E; Bottom: Top view of tunnel-like binding pocket showing sites A–E (blue) and the catalytically active Asp192 (red).

and/or F), or continuation of optimization with LW123. Herein we chose the latter, and created two new CAST libraries by using the LW123 gene as the template and focusing on sites E and F. This led to mutants LW126 (E = 49) and LW144 (E = 35) as shown in Table 1 and Figure 3.

Upon subjection of the gene LW126 to two separate and independent rounds of CASTing at positions 193/195/196 (site A) and 244/245/249 (site F), no substantial improvements in enantioselectivity were observed. Parallel to these efforts and before examining other positions, such as those defined by B, C, or D, gene LW144 was used as the template for CASTing in a different branch of the protein-sequence space. Randomization at positions 317/318 (site E) resulted in the identification of the, to date, most enantioselective mutant LW202, which shows a selectivity factor of  $E = 115 \pm 10$  (Table 1 and Figure 3). This corresponds to a 25-fold increase in enantioselectivity relative to the WT, which therefore shows that iterative CASTing is an efficient way to engineer the ANEH tunnel. Sequencing revealed the presence of two new mutations, T317W and T318V. This means that the best enzyme variant (LW202) is characterized by a total of nine mutational changes (L215F, A217N, R219S, L249Y, T317W, T318V, M329P, L330Y, and C350V). At this stage we refrain from speculating about the origin of enhanced enantioselectivity. Before any sound conclusions can be made, isolation of mutant LW202 in its pure form is necessary, as are kinetic and detailed theoretical studies. Moreover, the question whether all nine mutational changes in the best mutant LW202 are actually necessary for high enantioselectivity needs to be addressed systematically. An initial experiment points to a



*Figure 3.* Iterative CASTing in the evolution of enantioselective epoxide hydrolases as catalysts in the hydrolytic kinetic resolution of *rac*-1.

Table 1: Results of CASTing (I) and iterative CASTing (II).<sup>[a]</sup>

	Random- ization sites	Template	Mutant	t <sub>r</sub> [min] <sup>[b]</sup>	Conversion [%]	<i>ee</i> <sub>p</sub> [%] <sup>[c]</sup>	ee <sub>s</sub> [%] <sup>[d]</sup>	Newly introduced mutations			E <sup>[e]</sup>	No. mutants screened	% active mutants
I	А	No substantial improvements										4000	38
			LW081	60	34	80	44	L215F	A217N	R219S	14)		
	В	WT-ANEH	LW080	60	34	81	45	L215F	A217N	R219T	14		
			LW037	10	42	53	40			R219T	5	2200	25
			LW041	10	34	61	32		A217P	R219H	6		
			LW044	10	44	49	39			R219M	4 J		
	С	No substantial improvements								500	44		
II			1W086	60	40	84	60		M329P	1330Y	21)		
		LW081	LW102	60	31	86	41		M32K	L330T	19		
	C D	LW037 LW086	LW094	60	71	37	97		M329T	L330O	8 (	2000	87
			LW096	60	78	27	100		M329S	L330P	8		
			1W123	60	50	84	72			C350V	24)		
			LW122	60	27	85	27		L349V	C350V	16	2200	6
			LW125	60	32	82	37		L349V		16		
	E	LW123	LW126	60	47	89	86			T318N	49)		
			LW127	60	56	74	99		T317F	T318N	34	1600	66
			LW142	30	61	59	100		T317I	T318L	35		
	F		LW144	60	47	87	81			L249Y	35	1300	9
	А	118/2.26			No	substantial	improvem	ents				3100	4
	F	LW126			No	substantial	improvem	ents				1600	27
	E	LW144	LW202	60	48	95	94		T317W	T318V	>100	2000	63

[a] CASTing performed as a means to evolve enantioselective epoxide hydrolases as catalysts in the hydrolytic kinetic resolution of *rac*-1. [b]  $t_r$  = reaction time. [c]  $ee_p$  = enantioselectivity of the product. [d]  $ee_s$  = enantiopurity of the substrate. [e] E = enantioselectivity of the reaction.

substantial cooperative effect in at least one case. This concerns mutation C350V, introduced in mutant LW086, which raises the *E* value from 21 to (only) 24 (Figure 3; Table 1). We discovered that upon reintroduction of cysteine at position 350 in the final mutant LW202, enantioselectivity drops sharply from E = 115 to E = 60. This means that mutation C350V introduced in the early phase of the evolutionary process plays a significant role in the final mutant LW202. The mechanism of this cooperative effect needs to be studied in the future.

The present results were obtained by screening a total of only 20000 clones. This is about the same number of clones previously screened when using epPCR (at different mutation rates) as the method for library formation,<sup>[7a]</sup> yet the results of the two approaches are dramatically different (E = 115 versus E = 10.8). In iterative CASTing, the lower numbers of screened mutants are linked with higher numbers of libraries which need to be created. However, the latter is not the bottleneck, because a library of ANEH mutants can be generated in one day. It is likely that if iterative CASTing were to be performed more extensively to cover more of the focused branches of the confined protein-sequence space (Figure 1), even more hits would be discovered. For practical purposes this is not necessary, although we plan to do so for theoretical reasons. We anticipate that iterative CASTing<sup>[31]</sup> may also be useful when attempting to evolve other enzyme properties such as enhanced activity and/or broadened substrate acceptance.<sup>[16,32]</sup>

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- For reviews of directed evolution of enantioselective enzymes, see: a) M. T. Reetz, *Proc. Natl. Acad. Sci. USA* 2004, *101*, 5716– 5722; b) M. T. Reetz in *Methods in Enzymology, Vol. 388* (Eds.: D. E. Robertson, J. P. Noel), Elsevier, San Diego, CA, 2004, pp. 238–256; c) M. T. Reetz, *Tetrahedron* 2002, *58*, 6595–6602; d) See also N. J. Turner, *Trends Biotechnol.* 2003, *21*, 474–478.
- [2] For reviews of directed evolution, see: a) Methods in Molecular Biology, Vol. 230 (Eds.: F. H. Arnold, G. Georgiou), Humana, Totowa, NJ, 2003; b) Directed Molecular Evolution of Proteins (or How to Improve Enzymes for Biocatalysis), (Eds.: S. Brakmann, K. Johnsson), Wiley-VCH, Weinheim, 2002; c) Enzyme Functionality-Design, Engineering, and Screening, (Eds.: A. Svendsen), Marcel Dekker, New York, 2004; d) Evolutionary Methods in Biotechnology (Clever Tricks for Directed Evolution), (Ed.: S. Brakmann, A. Schwienhorst), Wiley-VCH, Weinheim, 2004; For a historical account of directed evolution which includes the early contributions of Manfred Eigen, see: e) A. Koltermann, U. Kettling, Biophys. Chem. 1997, 66, 159– 177.
- [3] One of the first reports regarding more than one cycle of mutagenesis/selection leading to evolutionary pressure was published in the mid 1980s, although this crucial idea was not generalized until the 1990s: see reference [2] and H. Liao, T. McKenzie, R. Hagemann, *Proc. Natl. Acad. Sci. USA* 1986, 83, 576-580.
- [4] For reviews of high-throughput ee assays, see: a) M. T. Reetz, Angew. Chem. 2001, 113, 292-320; Angew. Chem. Int. Ed. 2001, 40, 284-310; b) M. T. Reetz, in Enzyme Functionality-Design, Engineering, and Screening (Ed.: A. Svendsen), Marcel Dekker, New York, 2004, pp. 559-598; c) M. T. Reetz, in Evolutionary Methods in Biotechnology (Eds.: S. Brakmann, A. Schwienhorst), Wiley-VCH, Weinheim, 2004, pp. 113-141; d) M. T.

Angew. Chem. Int. Ed. 2006, 45, 1236-1241

## Communications

Reetz, in *Methods in Molecular Biology, Vol. 230* (Eds.: F. H. Arnold, D. Georgiou), Humana Press, Totowa, NJ, **2003**, pp. 259–282; e) M. T. Reetz, in *Methods in Molecular Biology, Vol. 230* (Eds.: F. H. Arnold, G. Georgiou), Humana Press, Totowa, NJ, **2003**, pp. 283–290; f) D. Wahler, J.-L. Reymond, *Curr. Opin. Biotechnol.* **2001**, *12*, 535–544.

- [5] a) M. T. Reetz, A. Zonta, K. Schimossek, K. Liebeton, K.-E. Jaeger, Angew. Chem. 1997, 109, 2961–2963; Angew. Chem. Int. Ed. Engl. 1997, 36, 2830–2832; b) K. Liebeton, A. Zonta, K. Schimossek, M. Nardini, D. Lang, B. W. Dijkstra, M. T. Reetz, K.-E. Jaeger, Chem. Biol. 2000, 7, 709–718; c) M. T. Reetz, S. Wilensek, D. Zha, K.-E. Jaeger, Angew. Chem. 2001, 113, 3701–3703; Angew. Chem. Int. Ed. 2001, 40, 3589–3591; d) D. Zha, S. Wilensek, M. Hermes, K.-E. Jaeger, M. T. Reetz, Chem. Commun. 2001, 2664–2665.
- [6] a) M. T. Reetz, B. Brunner, T. Schneider, F. Schulz, C. M. Clouthier, M. M. Kayser, *Angew. Chem.* 2004, *116*, 4167–4170; *Angew. Chem. Int. Ed.* 2004, *43*, 4075–4078; b) M. T. Reetz, F. Daligault, B. Brunner, H. Hinrichs, A. Deege, *Angew. Chem.* 2004, *116*, 4170–4173; *Angew. Chem. Int. Ed.* 2004, *43*, 4078–4081.
- [7] a) M. T. Reetz, C. Torre, A. Eipper, R. Lohmer, M. Hermes, B. Brunner, A. Maichele, M. Bocola, M. Arand, A. Cronin, Y. Genzel, A. Archelas, R. Furstoss, *Org. Lett.* 2004, *6*, 177–180; For an improved expression system of ANEH in *E. coli*, see: b) F. Cedrone, S. Niel, S. Roca, T. Bhatnagar, N. Ait-Abdelkader, C. Torre, H. Krumm, A. Maichele, M. T. Reetz, J. C. Baratti, *Biocatal. Biotransform.* 2003, *21*, 357–364.
- [8] For further examples of enhanced enantioselectivity (evolutionary process was not involved in all cases), see: a) Aminotransferases: G. W. Matcham, A. R. S. Bowen, Chim. Oggi 1996, 14, 20-24; b) Esterases: U.T. Bornscheuer, J. Altenbuchner, H. H. Meyer, Biotechnol. Bioeng. 1998, 58, 554-559; c) Hydantoinases: O. May, P. T. Nguyen, F. H. Arnold, Nat. Biotechnol. 2000, 18, 317-320; d) Phosphotriesterases: M. Chen-Goodspeed, M. A. Sogorb, F. Wu, S.-B. Hong, F. M. Raushel, Biochemistry 2001, 40, 1325-1331; e) M. Chen-Goodspeed, M. A. Sogorb, F. Wu, F. M. Raushel, Biochemistry 2001, 40, 1332-1339; f) Monoamine oxidases: M. Alexeeva, A. Enright, M. J. Dawson, M. Mahmoudian, N. J. Turner, Angew. Chem. 2002, 114, 3309-3312; Angew. Chem. Int. Ed. 2002, 41, 3177-3180; g) Lipases: Y. Koga, K. Kato, H. Nakano, T. Yamane, J. Mol. Biol. 2003, 331, 585-592; h) Aldolases: M. Wada, C.-C. Hsu, D. Franke, M. Mitchell, A. Heine, I. Wilson, C.-H. Wong, Bioorg. Med. Chem. 2003, 11, 2091-2098; i) Epoxide hydrolases: B. van Loo, J. H. L. Spelberg, J. Kingma, T. Sonke, M. G. Wubbolts, D. B. Janssen, Chem. Biol. 2004, 11, 981-990; j) L. Rui, L. Cao, W. Chen, K. F. Reardon, T. K. Wood, Appl. Environ. Microbiol 2005, 71, 3995-4003.
- [9] a) D. W. Leung, E. Chen, D. V. Goeddel, *Technique* 1989, *1*, 11–15; b) R. C. Cadwell, G. F. Joyce, *PCR Methods Appl.* 1992, *2*, 28–33; c) T. Eggert, M. T. Reetz, K.-E. Jaeger, in *Enzyme Functionality–Design, Engineering, and Screening* (Ed.: A. Svendsen), Marcel Dekker, New York, 2004, pp. 375–390; d) For an illuminating study concerning high-error-rate epPCR, see: D. A. Drummond, B. L. Iverson, G. Georgiou, F. H. Arnold, *J. Mol. Biol.* 2005, *350*, 806–816.
- [10] For a review of DNA shuffling, see: K. A. Powell, S. W. Ramer, S. B. del Cardayré, W. P. C. Stemmer, M. B. Tobin, P. F. Longchamp, G. W. Huisman, *Angew. Chem.* 2001, 113, 4068–4080; *Angew. Chem. Int. Ed.* 2001, 40, 3948–3959.
- [11] For examples of focused libraries, see: references [2,5c,12–15] and a) S. V. Taylor, P. Kast, D. Hilvert, *Angew. Chem.* 2001, *113*, 3408–3436; *Angew. Chem. Int. Ed.* 2001, *40*, 3310–3335; b) H. Xiong, B. L. Buckwalter, H. M. Shieh, M. H. Hecht, *Proc. Natl. Acad. Sci. USA* 1995, *92*, 6349–6353; c) J. U. Bowie, R. T. Sauer, *Proc. Natl. Acad. Sci. USA* 1989, *86*, 2152–2156; d) Y. Koga, K.

Kato, H. Nakano, T. Yamane, J. Mol. Biol. 2003, 331, 585-592: e) J. A. Wells, M. Vasser, D. B. Powers, Gene 1985, 34, 315-323; f) J. D. Stevenson, S. J. Benkovic, J. Chem. Soc. Perkin Trans. 2 2002, 1483-1493; g) M. Wilming, A. Iffland, P. Tafelmeyer, C. Arrivoli, C. Saudan, K. Johnsson, ChemBioChem 2002, 3, 1097-1104; h) A. Juillerat, T. Gronemeyer, A. Keppler, S. Gendreizig, H. Pick, H. Vogel, K. Johnsson, Chem. Biol. 2003, 10, 313-317; i) L. B. Evnin, J. R. Vásquez, C. S. Craik, Proc. Natl. Acad. Sci. USA 1990, 87, 6659-6663; j) A. R. Schmitzer, F. Lépine, J. N. Pelletier, Protein Eng. Des. Sel. 2004, 17, 809-819; k) J. Yang, Y. Koga, H. Nakano, T. Yamane, Protein Eng. 2002, 15, 147-152; 1) M. S. Warren, S. J. Benkovic, Protein Eng. 1997, 10, 63-68; m) T. Yano, S. Oue, H. Kagamiyana, Proc. Natl. Acad. Sci. USA 1998, 95, 5511-5515; n) E. M. Gabor, D. B. Janssen, Protein Eng. Des. Sel. 2004, 17, 571-579; o) D. K. Dube, L. A. Loeb, Biochemistry 1989, 28, 5703-5707.

- [12] S. A. Funke, A. Eipper, M. T. Reetz, N. Otte, W. Thiel, G. van Pouderoyen, B. W. Dijkstra, K.-E. Jaeger, T. Eggert, *Biocatal. Biotransform.* 2003, 21, 67–73.
- [13] G. DeSantis, K. Wong, B. Farwell, K. Chatman, Z. Zhu, G. Tomlinson, H. Huang, X. Tan, L. Bibbs, P. Chen, K. Kretz, M. J. Burk, *J. Am. Chem. Soc.* **2003**, *125*, 11476–11477.
- [14] a) G. P. Horsman, A. M. F. Liu, E. Henke, U. T. Bornscheuer, R. J. Kazlauskas, *Chem. Eur. J.* 2003, *9*, 1933–1939; b) S. Park, K. L. Morley, G. P. Horsman, M. Holmquist, K. Hult, R. J. Kazlauskas, *Chem. Biol.* 2005, *12*, 45–54; c) R. Kazlauskas, *Chem. Listy* 2003, *67*, 329–337.
- [15] S. Lutz, W. M. Patrick, Curr. Opin. Biotechnol. 2004, 15, 291– 297.
- [16] M. T. Reetz, M. Bocola, J. D. Carballeira, D. Zha, A. Vogel, Angew. Chem. 2005, 117, 4264–4268; Angew. Chem. Int. Ed. 2005, 44, 4192–4196.
- [17] For examples of remote effects, [14] see: a) M. Bocola, N. Otte, K.-E. Jaeger, M. T. Reetz, W. Thiel, ChemBioChem 2004, 5, 214-223; b) S. Oue, A. Okamoto, T. Yano, H. Kagamiyama, J. Biol. Chem. 1999, 274, 2344-2349; c) A. Iffland, P. Tafelmeyer, C. Saudan, J. Johnsson, Biochemistry 2000, 39, 10790-10798; d) M. Kumar, K. K. Kannan, M. V. Hosur, N. S. Bhavesh, A. Chatterjee, R. Mittal, R. V. Hosur, Biochem. Biophys. Res. Commun. 2002, 294, 395-401; e) P. K. Agarwal, S. R. Billeter, P. T. R. Rajagopalan, S. J. Benkovic, S. Hammes-Schiffer, Proc. Natl. Acad. Sci. USA 2002, 99, 2794-2799; f) H. Zhao, F. H. Arnold, Protein Eng. 1999, 12, 47-53; g) L. K. Jackson, J. Baldwin, R. Akella, E. J. Goldsmith, M. A. Phillips, Biochemistry 2004, 43, 12990-12999; h) B. Lingen, D. Kolter-Jung, P. Dünkelmann, R. Feldmann, J. Grötzinger, M. Pohl, M. Müller, ChemBioChem 2003, 4, 721-726; i) S. McQ. Gould, D. S. Tawfik, Biochemistry 2005, 44, 5444-5452; j) V. G. H. Eijsink, S. Gåseidnes, T. V. Borchert, B. van den Burg, Biomol. Eng. 2005, 22, 21-30.
- [18] a) C. Morisseau, A. Archelas, C. Guitton, D. Faucher, R. Furstoss, J. C. Baratti, *Eur. J. Biochem.* 1999, 263, 386–395;
  b) K. M. Manoj, A. Archelas, J. C. Baratti, R. Furstoss, *Tetrahedron* 2001, 57, 695–701;
  c) M. Arand, H. Hemmer, H. Dürk, J. Baratti, A. Archelas, R. Furstoss, F. Oesch, *Biochem. J.* 1999, 344, 273–280.
- [19] For reviews of epoxide hydrolases, see: a) K. Faber, R. V. A. Orru in *Enzyme Catalysis in Organic Synthesis* (Eds.: K. Drauz, H. Waldmann), Wiley-VCH, Weinheim, **2002**, pp. 579–608; b) C. Morisseau, B. D. Hammock, *Annu. Rev. Pharmacol. Toxicol.* **2005**, 45, 311–333; c) A. Archelas, R. Furstoss, *Curr. Opin. Chem. Biol.* **2001**, 5, 112–119.
- [20] For other important work on the directed evolution of enantioselective epoxide hydrolases, see references [8i,j].
- [21] a) M. T. Reetz, M. H. Becker, H.-W. Klein, D. Stöckigt, Angew. Chem. 1999, 111, 1872–1875; Angew. Chem. Int. Ed. 1999, 38, 1758–1761; W. Schrader, A. Eipper, D. J. Pugh, M. T. Reetz, Can. J. Chem. 2002, 80, 626–632.

#### 1240 www.angewandte.org

- [22] For information on pre-screens for EH activity, see: a) F. Zocher, M. M. Enzelberger, U. T. Bornscheuer, B. Hauer, R. D. Schmid, *Anal. Chim. Acta* 1999, 391, 345–351; b) D. Wahler, J.-L. Reymond, *Angew. Chem.* 2002, 114, 1277–1280; *Angew. Chem. Int. Ed.* 2002, 41, 1229–1232; c) K. Doderer, R. D. Schmid, *Biotechnol. Lett.* 2004, 26, 835–839.
- [23] Herein, we employed an efficient selection system based on bacterial (*E. coli*) growth. The latter is directly related to the presence of active epoxide hydrolases which catalyze the detoxicating hydrolysis of epoxide substrates such as 1: M. T. Reetz, L.-W. Wang, *Comb. Chem. High Throughput Screening*, in press.
- [24] a) J. Y. Zou, B. M. Hallberg, T. Bergfors, F. Oesch, M. Arand, S. L. Mowbray, T. A. Jones, *Structure* 2000, 8, 111–122; b) http:// www.pdb.ccdc.cam.ac.uk/pdb.
- [25] For theoretical analyses of other enzymes with narrow tunnellike binding pockets, some of which were re-engineered, see: reference [1a]; a) J. Schmitt, S. Brocca, R. D. Schmid, J. Pleiss, *Protein Eng.* 2002, 15, 595–601; b) H. Henriksson, J. Ståhlberg, R. Isaksson, G. Pettersson, *FEBS Lett.* 1996, 390, 339–344; c) M. Holmquist, F. Haeffner, T. Norin, K. Hult, *Protein Sci.* 1996, 5, 83–88.
- [26] Based on the X-ray crystal structure of ANEH (see reference [24]), the nonresolved loop from postions 320 to 328 over the binding site was constructed by molecular modeling. The CAST libraries were then defined by using the completed protein model with substrate 1 in the binding pocket.
- [27] For the application of mathematical expressions useful in the construction, description, and evaluation of protein libraries, see: a) A. D. Bosley, M. Ostermeier, *Biomol. Eng.* 2005, 22, 57–61; b) W. M. Patrick, A. E. Firth, J. M. Blackburn, *Protein Eng.* 2003, *16*, 451–457; c) W. M. Patrick, A. E. Firth, *Biomol. Eng.* 2005, 22, 105–112.
- [28] In our previous lipase project (reference [5c]), we created a focused library of mutants through the simultaneous randomization of amino acid positions 160/161/162/163 by using appropriate cassette mutagenesis. For a 95% sequence space coverage, this would require the screening of about three million clones (see references [16,27]). Although only 5000 were actually evaluated, markedly improved mutants were in fact found: The selectivity factor in the kinetic resolution of a chiral ester increased from E = 1.2 (WT) to 30 (mutant) (reference [5c]).
- [29] All E values were derived through the application of the formula of Sih et al: C.-S. Chen, Y. Fujimoto, G. Girdaukas, C. J. Sih, J. Am. Chem. Soc. 1982, 104, 7294–7299.
- [30] The conclusion regarding relative activity is only qualitative and is uncertain because it is based on the assumption of equal expression levels for the various mutants. A sound conclusion can only be made following a kinetic study of the isolated and purified mutants (which we are currently performing).
- [31] A different type of evolutionary process can be envisioned through the combination of the mutations of two (or more) enzyme variants identified in the initial CAST libraries.
- [32] For a review of practical asymmetric catalysis emphasizing the need to develop systems showing broad substrate scope, see: M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Keßeler, R. Stürmer, T. Zelinski, *Angew. Chem.* 2004, *116*, 806–843; *Angew. Chem. Int. Ed.* 2004, *43*, 788–824.