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Manipulating the Stereoselectivity of Limonene Epoxide Hydrolase by Directed Evolution Based on Iterative Saturation Mutagenesis

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Abstract: Limonene epoxide hydrolase from *Rhodococcus erythropolis* DCL 14 (LEH) is known to be an exceptional epoxide hydrolase (EH) because it has an unusual secondary structure and catalyzes the hydrolysis of epoxides by a rare one-step mechanism in contrast to the usual two-step sequence. From a synthetic organic viewpoint it is unfortunate that LEH shows acceptable stereoselectivity essentially only in the hydrolysis of the natural substrate limonene epoxide, which means that this EH cannot be exploited as a catalyst in asymmetric transformations of other substrates. In the present study, directed evolution using iterative saturation mutagenesis (ISM) has been tested as a means to engineer LEH mutants showing broad substrate scope with high stereoselectivity. By grouping individual residues aligning the binding pocket correctly into randomization sites and performing saturation mutagenesis iteratively using a reduced amino acid alphabet, mutants were obtained which catalyze the desymmetrization of cyclopentene−oxide with stereoselective formation of either the (*R*,*R*)- or the (*S*,*S*)-diol on an optional basis. The mutants prove to be excellent catalysts for the desymmetrization of other *meso*-epoxides and for the hydrolytic kinetic resolution of racemic substrates, without performing new mutagenesis experiments. Since less than 5000 tranformants had to be screened for achieving these results, this study contributes to the generalization of ISM as a fast and reliable method for protein engineering. In order to explain some of the stereoselective consequences of the observed mutations, a simple model based on molecular dynamics simulations has been proposed.

Introduction

Epoxide hydrolases (EHs) are enzymes that can be used as catalysts in the hydrolytic desymmetrization of *meso*-epoxides and in the kinetic resolution of racemic substrates with formation of enantiomerically pure or enriched vicinal diols.[1] In some cases they are attractive alternatives to the powerful Jacobsen salen-cobalt catalysts.[2] Most EHs react by a two-step mechanism in which a short-lived covalent enzyme−ester is formed by nucleophilic attack of an aspartate at one of the two C atoms of the H-bonded and therefore activated epoxide moiety in the rate- and stereochemistry-determining step, followed by rapid hydrolysis of the enzyme−ester intermediate.[1] Limonene epoxide hydrolase (LEH) constitutes one of the rare exceptions,[1] because it catalyzes direct hydrolysis of epoxides by positioning and activating water at the active site, thereby enabling smooth nucleophilic attack with inversion of configuration at the epoxide C atom.[3,4] The crystal structure of LEH does not show the usual α/β-fold found in most EHs but features a curved 6-stranded mixed β-sheet surrounded by four helices, thereby shaping a deep binding pocket.[3b] Further insight into the mechanism has been provided by a recent QM/MM study.[5] In nature, LEH catalyzes the hydrolysis of limonene epoxide (**1**) present in *Rhodococcus erythropolis* DCL14.[3]

Since this substrate is a fairly bulky trisubstituted epoxide, one might expect this EH to be a viable catalyst for a wide variety of structurally different epoxides. Unfortunately, enantioselectivity is generally poor in such cases ($E = 3-11$),[4a]

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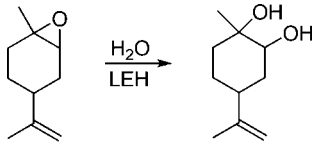
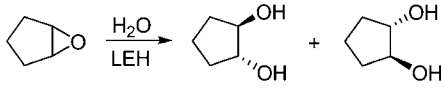


1 **2**

which means that wild-type (WT) LEH can hardly be exploited in synthetic organic chemistry. As an example, it catalyzes the hydrolytic desymmetrization of cyclopentene−oxide (**3**) with poor enantioselectivity (ee = 14% in meager favor of (*R*,*R*)-**4**).



3 (*R*,*R*)-**4** (*S*,*S*)-**4**

Hoping to obtain LEH mutants which catalyze the formation of (*R*,*R*)-**4** and (*S*,S)-**4** on an optional basis with high enantioselectivity, we decided to apply directed evolution.[6,7] Since industrial organic chemists need stereoselective catalysts with a reasonable substrate scope,[8] not just one for the reaction of a single compound, we also planned to test the evolved mutants in the reaction of other substrates without performing additional mutagenesis experiments.

Directed evolution of stereoselective enzymes has emerged as a prolific source of catalysts for asymmetric transformations in synthetic organic chemistry.[6,7] The bottleneck of laboratory evolution is the screening step which is particularly challenging when assaying stereoselectivity.[9] Therefore, we and others have recently stressed "quality, not quantity".[7,10,11] Small mutant libraries of the order of 100−800 members (transformants) can be screened for enantioselectivity within 1 day using medium-throughput ee assays based on standard automated GC or HPLC, but such libraries need to be characterized by superior quality, meaning a high frequency of hits showing pronounced catalyst improvement.[10]

Our contribution to achieving expediency in this endeavor is iterative saturation mutagenesis (ISM), which can be applied to the control of stereoselectivity[7,10] and to the enhancement of thermostability and robustness in hostile organic solvents.[10b] Appropriate sites in an enzyme, labeled A, B, C, D, etc., each comprising one or more amino acid positions, are subjected to saturation mutagenesis with formation of focused libraries. Subsequently, the hits in the libraries are used as templates to perform saturation mutagenesis at the respective other sites, and the process is continued iteratively until the desired degree of catalyst improvement has been achieved (for a 4-site ISM scheme, see Scheme S4 in the Supporting Information). When evolving stereoselectivity and/or substrate scope (rate), the criterion for choosing appropriate sites is based on the Combinatorial Active Site-Saturation Test (CAST),[7,10a,c,d] according to which residues aligning the binding pocket are considered. A further tool in "smart" library creation is the use of reduced amino acid alphabets employing the appropriate codon degeneracy, because this decimates the degree of oversampling .[7,10] In the present study we wanted to test the utility of ISM in the evolution of stereoselective LEH mutants. At the outset of the project, stringent requirements were defined by restricting the size of the mutant libraries to a few thousand transformants, i.e., we wanted to know how far one can get by investing a minimum amount of laboratory work. In contrast to several other EHs,[12] LEH has not been subjected to directed evolution previously.

Results and Discussion

Initial Saturation Mutagenesis Experiments. LEH is a 149 amino acid (16.5 kDa) enzyme which has been characterized by X-ray crystallography of a sample containing the inhibitor valpromide (2-*n*-propylpentanoic acid amide).[3b] In order to make a sound decision regarding proper CAST sites for saturation mutagenesis, we first performed induced fit docking of the model substrate **3** using the published crystal structure. This procedure led to the identification of eight residues for potential randomization, namely, Met32, Leu35, Leu74, Met78, Ile80, Val83, Leu114, and Ile116 (Figure 1a). The next step required a decision concerning the question of how to group these amino acid positions into appropriate sites. In principle, one could opt for eight single residue sites, a strategy that we successfully applied in the directed evolution of the enoate-reductase YqjM.[10c] Using NNK codon degeneracy (N, adenine/cytosine/guanine/thymine; K, guanine/thymine) encoding all 20 canonical amino acids, this would entail eight randomization libraries, each requiring about 100 transformants to be screened for 95% library coverage assuming the absence of amino acid bias.[10]

In other studies, however, we discovered that randomization at sites composed of more than one amino acid position lead to

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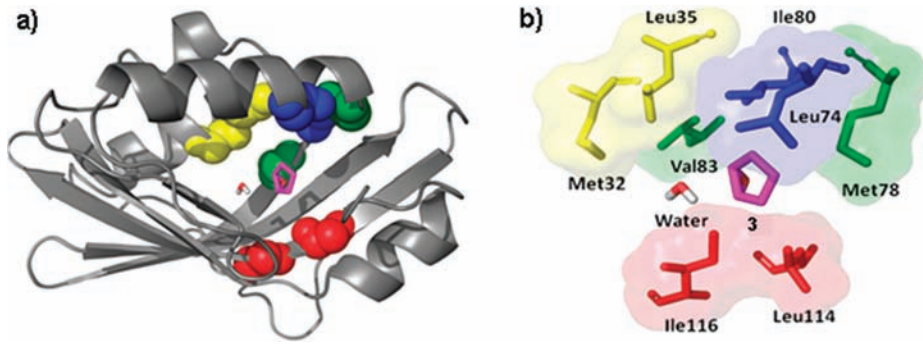

Figure 1. (a) Eight amino acid residues aligning the binding pocket of LEH, the analysis being based on induced fit docking of *meso*-substrate **3** (purple) using the published crystal structure.[3b] (b) Grouping of the eight residues into four randomization sites A (Met32/Leu35), B (Leu74/Ile80), C (Leu114/Ile116), and D (Met78/Val83).

libraries characterized by particularly high quality,[10d,12b] which was traced to the occurrence of cooperative effects operating between the newly introduced amino acids within a given site and between sets of mutations arising from the different sites as the ISM process proceeds. Cooperativity entails epistatic effects which are more than additive, which means that it is the ideal form of epistasis in directed evolution.[10a,d] When choosing single-residue sites in an ISM scheme, cooperative effects are by nature not relevant in the initial randomization libraries[10c] and become possible only in the subsequent mutagenesis generations when point mutations begin to interact with one another. In the present study we decided to group the eight residues (Figure 1a) into four sites, each composed of two amino acid positions, namely, A (Met32/Leu35), B (Leu74/Ile80), C (Leu114/Ile116), and D (Met78/Val83) (Figure 1b).

When choosing NNK codon degeneracy, requiring in each library the screening of about 3000 transformants for 95% coverage,[10a,b] the total screening effort would amount to about 12 000 measurements. Since this does not include any ISM steps and already exceeds the screening limit set by ourselves, we opted for NDT codon degeneracy (D, adenine/guanine/thymine; T, thymine), encoding 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, Gly). They constitute a balanced mixture of building blocks having polar, nonpolar, charged, noncharged, aromatic, and nonaromatic side chains. As pointed out previously, NDT requires for 95% library coverage the screening of only 430 transformants.[10a,b] We settled for 466, which means that the total screening effort regarding the initial four libraries is calculated to involve about 1864 transformants. Following the generation of the four initial libraries at sites A, B, C, and D, we screened for activity using an adapted form[13a] of Reymond's adrenaline test[13b,c] and subsequently applied automated GC in order to obtain the respective ee values.

The performance of the three best mutants in each library is summarized in Table 1, which reveals some remarkable trends. In particular, mutants which improve (*R*,*R*)-selectivity of WT-LEH beyond 14% ee are found only in libraries B and D, whereas mutants displaying reversed enantioselectivity in favor of (*S*,*S*)-**4** occur solely in libraries A and C. There was no compelling reason to expect this. Indeed, in our previous study concerning the enhancement as well as the reversal of enantioselectivity of the enoate-reductase YqjM as a catalyst in the asymmetric reduction of 3-methylcyclohexenone, both (*R*)- and (*S*)-selective mutants were discovered in one and the same randomization library.[10c] The best hits showing the highest degree of enantioselectivity in the present study proved to be double mutants, namely, H139 (Met32Leu/Leu35Phe) from library A, H143 (Leu74Ile/Ile80Cys) from library B, H126 (Leu114Cys/Ile116Val) from library C, and H175 (Met78Phe/Val83Ile) from library D. The biggest change in terms of net $\Delta\Delta G^{\ddagger}$ relative to WT occurs in the case of mutant H126 with reversed enantioselectivity ($0.7 + 4.1 = 4.8$ kJ·mol^{-1}).

Table 1. Selected Hits from Initial Randomization Libraries at Sites A, B, C, and D

	mutant	mutations	conv. (%)	ee (%)	abs. conf.	$-\Delta\Delta G^{\ddagger}$ (kJ·mol^{-1})[a]
		WT LEH	72	14	(*R*,*R*)	0.7
library A	H139	Met32Leu/Leu35Phe	79	24	(*S*,*S*)	1.2
	H33	Met32Leu/Leu35Cys	78	16	(*S*,*S*)	0.8
	H35	Met32Leu/Leu35Val	78	10	(*S*,*S*)	0.5
library B	H142	Leu74Val/Ile80Val	67	53	(*R*,*R*)	2.9
	H51	Leu74Ile/Ile80Val	75	58	(*R*,*R*)	3.3
	H143	Leu74Ile/Ile80Cys	75	66	(*R*,*R*)	3.9
library C	H22	Leu114Ile/Ile116Val	74	50	(*S*,*S*)	2.7
	H23	Leu114Val/Ile116Val	72	60	(*S*,*S*)	3.4
	H126	Leu114Cys/I116Val	72	68	(*S*,*S*)	4.1
library D	H175	Met78Phe/Val83Ile	82	29	(*R*,*R*)	1.5
	H176	Met78Ile/Val83Ile	80	13	(*R*,*R*)	0.6
	H177	Met78Val/Val83Ile	68	7	(*R*,*R*)	0.3

[a] Energies calculated relative to ee = 0%.

Deconvolution Experiments. In order to identify the nature of epistatic interactions operating between the respective point mutations in each double mutant originating in the initial libraries, systematic deconvolution experiments were performed by generating and testing the respective two single mutants. By comparing the relevant ee values and the respective $\Delta\Delta G^{\ddagger}$ data (Table 2), it can be seen that cooperative effects are operating in two of the four mutants, namely, H139 from library A and H126 from library C. For example, in the case of the double-mutant H126 showing an ee value of 68% (*S*,*S*) corresponding to $\Delta\Delta G^{\ddagger} = -4.1$ kJ·mol^{-1}, the single mutants Leu114Cys and Ile116Val lead to enantioselectivities of only 4% ee and 38% ee, corresponding to $\Delta\Delta G^{\ddagger}$ values of -0.2 and -2.0 kJ·mol^{-1}, respectively. Noteworthy are also the results of deconvoluting the (*S*,*S*)-selective double-mutant H139 (Met32Leu/Leu35Phe). The respective single mutants Met32Leu and Leu35Phe are both (*R*,*R*)-selective! *This stands in sharp contrast to the traditional expectation of protein engineers.* Going the other way, i.e., combining two point mutations each correlating with a certain improved enzyme property is generally believed to result in an enhancement of that particular catalytic parameter. Indeed, in

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Table 2. Results of Deconvoluting the Double Mutants H139, H143, H126, and H175, the Model Reaction Being the Hydrolytic Desymmetrization of epoxide **3**

mutant	mutations	conv. (%)	ee (%)	abs. conf	$-\Delta\Delta G^{\ddagger}$ (kJ·mol^{-1})[a]
H139	Met32Leu/Leu35Phe	79	24	(*S*,*S*)	1.2
H1	Met32Leu	76	14	(*R*,*R*)	0.7
H130	Leu35Phe	75	2	(*R*,*R*)	0.1
H143	Leu74Ile/Ile80Cys	75	66	(*R*,*R*)	3.9
H6	Leu74Ile	80	28	(*R*,*R*)	1.4
H136	Ile80Cys	75	50	(*R*,*R*)	2.7
H126	Leu114Cys/Ile116Val	72	68	(*S*,*S*)	4.1
H17	Leu114Cys	82	4	(*S*,*S*)	0.2
H10	I1e116Val	63	38	(*S*,*S*)	2.0
H175	Met78Phe/Val83Ile	82	29	(*R*,*R*)	1.5
H137	Met78Phe	78	25	(*R*,*R*)	1.3
H138	Val83Ile	78	18	(*R*,*R*)	0.9

[a] Energies calculated relative to ee = 0%.

an earlier study regarding substrate acceptance (rate) of a lipase in the hydrolysis of bulky esters, we combined point mutations introduced earlier, which resulted in a further enhancement of rate.[6g] Relevant is the report by Bornscheuer and co-workers, who showed that upon deconvoluting an evolved double mutant of an esterase with enhanced (*S*)-selectivity, one of the respective single mutants displayed the expected (*S*)-selectivity but the other proved to be (*R*)-selective.[14] Our case is even more drastic, which shows that such cooperative effects can hardly be predicted by present theory. In the case of mutant H143 from library B, essentially full additivity pertains, whereas the interaction between the two point mutations in the case of mutant H175 from library D proved to be somewhat less than additive but not antagonistic (Table 2).

Iterative Rounds of Saturation Mutagenesis. We then had to make decisions regarding further evolutionary optimization based on ISM. A complete 4-site ISM scheme leading to diol **4** with a given absolute configuration would entail 24 pathways and 64 randomization libraries (S4, Supporting Information). Thus, for both (*R*,*R*)- and (*S*,*S*)-selectivity a total of 48 pathways and 128 randomization libraries are relevant. In the present study the goal was not to test all theoretically possible pathways but to see how far one gets by exploring a severely limited number of options. We therefore decided to restrict ISM to three sites and to explore only two pathways, one directed toward (*R*,*R*)-selectivity and the other toward (*S*,*S*)-selectivity.

At this stage we were particularly interested in improving reversed enantioselectiviy of mutant H126 (68% ee in favor of (*S*,*S*)-**4**) originating from library C. Its gene was used as a template for saturation mutagenesis at two other sites. We opted for sites A and B, although the initial randomization round at site B had provided only (*R*,*R*)-selective mutants. Consequently, two pathways C → B → A and C → A → B were explored experimentally (Figure 2).

It can be seen that pathway C → B → A ends with the evolution of mutant H178 having an ee value of 93% (*S*,*S*), whereas C → A → B provides mutant H150 with 92% ee (*S*,*S*) (Figure 2). Although the two ee values are essentially identical, the point mutations accumulated in the respective second and third generations are quite different: Mutant H178 (Leu114Cys/Ile116Val/*Met32Cys/Ile80Phe*) versus mutant H150 (Leu114Cys/Ile116Val/*Met32Leu/Leu35Phe/Leu74Phe/Ile80Val*). It is also interesting to note that in pathway C → A → B leading to mutant

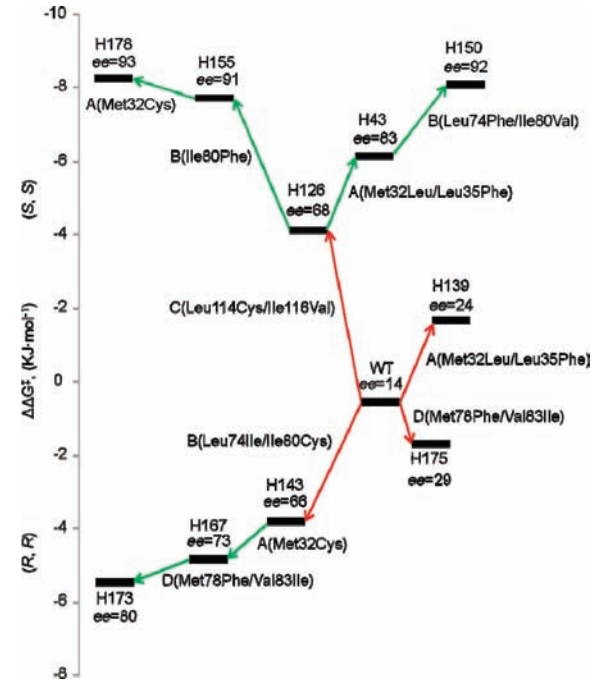

Figure 2. Multiple pathways explored for inverting and improving the enantioselectivity of LEH as a catalyst in the desymmetrization of cyclopentene oxide (**3**). Red arrows denote the results of generating and screening the initial libraries at sites A, B, C, and D; green arrows highlight the results of a restricted set of ISM experiments.

H150, saturation mutagenesis at site A in the second mutagenesis step results in the simultaneous introduction of two point mutations, Met32Leu/Leu35Phe, which are identical to those introduced in the initial randomization library at site A. There is no reason to expect this, although in the initial rounds of saturation mutagenesis this was one of the two cases displaying cooperative effects. In contrast, the last randomization experiment in pathway C → B → A at site A provides a single new point mutation Met32Cys. Taken together, the two pathways leading to 92−93% ee in favor of (*S*,*S*)-**4** required a total screening effort of only 2300 transformants.

In the quest to improve the (*R*,*R*)-selectivity of WT LEH, only a select number of ISM experiments were performed (Figure 2). The gene of improved mutant H143 obtained in the initial saturation mutagenesis library at site B was used as a template to perform randomization at site A. This provided mutant H167 characterized by ee = 73%, which was improved to 80% ee by continuing ISM at site D (Figure 2). It is noteworthy that in the final ISM round at site D of this (*R*,*R*)-selective pathway B → A → D, the same two point mutations (Met78Phe/Val83Ile) occur as in the initial library D. Apparently, no other combination is more effective. The (*R*,*R*)-selective pathway B → A → D required the screening of about 1400 transformants, which means that the total screening effort in Figure 2 amounted to less than 4700 transformants.

Thermostability Studies. The introduction of point mutations in an enzyme, for whatever purpose, may lead to a decrease in thermostability.[6,7] In order to test this in the case of the best mutants H150, H173, and H178, the respective T_{50}[15] values were measured, the temperatures at which 50% residual activity occurs following a heat treatment of 15 min.[10b,15] Whereas T_{50}[15] for WT LEH was found to be 49.5 °C, which is in line with literature reports,[4] the mutants showed slightly lower values: H150 (47.0 °C), H173 (47.0 °C), H178 (48.0 °C). The thermal

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Table 3. Hydrolytic Desymmetrization of *meso*-Epoxides Using LEH Mutants Specifically Evolved for the Reaction of Substrate **3**

	3		**5**		**7**		**9**		**11**	
mutant	% *ee*	specific activity[a]	% ee	specific activity[a]	% ee	specific activity[a]	% ee	specific activity[a]	% ee	specific activity[a]
WT	14 (*R*,*R*)	23	0	74	19 (*S*,*S*)	30	5 (*S*,*S*)	146	93 (*R*,*R*)	264
H150	92 (*S*,*S*)	13	96 (*S*,*S*)	67	93 (*S*,*S*)	26	86 (*S*,*S*)	nd[b]	95 (*R*,*R*)	nd[b]
H178	93 (*S*,*S*)	20	97 (*S*,*S*)	197	98 (*S*,*S*)	65	93 (*S*,*S*)	127	63 (*R*,*R*)	187
H173	80 (*R*,*R*)	24	90 (*R*,*R*)	123	77 (*R*,*R*)	73	83 (*R*,*R*)	144	99 (*R*,*R*)	297

[a] Specific activity: mmol/min/mg protein. [b] nd: not determined.

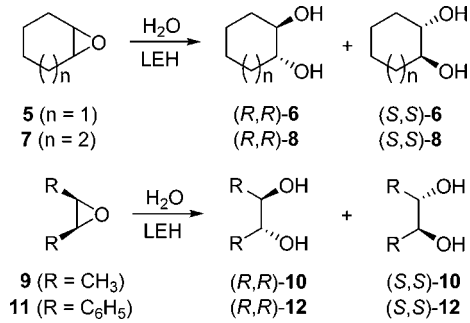
Table 4. Hydrolytic Kinetic Resolution of Epoxides *rac*-**13** and *rac*-**15** Using WT LEH and Mutants H173 and H178 as Catalysts

		rac-**13**				*rac*-**15**			
entry	mutant	ee_p (%)	conv. (%)	*E* value	specific activity[a]	ee_p (%)	conv. (%)	*E* value	specific activity[a]
1	WT	37	33	2.6 (*R*)	267	40	36	2.8 (*R*)	32
2	H173	35	26	2.3 (*R*)	292	92	30	36 (*R*)	45
3	H178	92	31	32 (*R*)	223	91	43	44 (*S*)	137

[a] Specific activity: (mmol/min/mg protein).

stability profiles are basically consistent with those of circular dichroism (see Supporting Information). Thus, there is some but no significant trade off in going from WT LEH to the evolved stereoselective mutants.

Investigation of Substrate Scope and Activity. In an effort to see how the three best mutants H150, H173, and H178 perform as catalysts in the hydrolytic desymmetrization of other *meso*-configurated epoxides, compounds **5**, **7**, **9**, and **11** were tested as substrates. Table 3 shows that substrate scope of these mutants is remarkably broad, because activity, improved enantioselectivity, and inverted enantioselectivity are in most cases even higher than in the reaction of the model compound **3** which was actually used in the evolution experiments. With the exception of substrate **11**, WT-LEH is consistently a poor catalyst (Table 3). Cases of higher enantioselectivity than in the model reaction are mutant H173 as a catalyst in the desymmetrization of **5** (97% ee in favor of (*S*,*S*)-**6**) and variant H178 as a catalyst for inverting stereoselectivity (90% ee in favor of (*R*,*R*)-**6**). Mutant H173 leads to an inversion of configuration, except for the case of epoxide **11**, which is currently difficult to rationalize. WT LEH and the mutants generally display similar activity. All of the intermediate mutants along the ISM scheme (Figure 2) were likewise tested as catalysts in the hydrolytic desymmetrization reactions, likewise resulting in high enantioselectivity with respect to improvement and inversion as seen by the respective "ISM schemes", although no additional mutagenesis experiments were performed (see Supporting Information).



It is informative to compare these results with the catalytic profiles of other EHs as catalysts in the same hydrolytic reactions. In the desymmetrization of *meso*-substrates **3**, **5**, and**11** using the EH from *Rhodotorula glutinis*, high enantioselectivity can be achieved but only in favor of the (*R*,*R*)-products (**3**, ≥ 98% ee; **5**, 90% ee; **9**, 90% ee).[16] Other epoxide hydrolases identified by gene mining show slightly higher enantioselectivity than the mutants of the present study but again only in favor of the (*R*,*R*)-products.[17] Single-site saturation mutagenesis of the EH from *Agrobacterium radiobacter* resulted in the improvement of enantioselectivity in the desymmetrization of **9** favoring (*R*,*R*)-**10** (ee = 99%), but reversal of stereoselectivity was not reported.[18] Synthetic Jacobsen-type salen-cobalt catalysts in ring-opening reactions of *meso*-epoxides have the enormous advantage that different nucleophiles such as azide (>95% ee)[2a] or benzoate (65−93% ee)[2c] can be employed with formation of compounds having high enantiomeric enrichment, the absolute configuration of the catalyst ligand determining the absolute configuration of the products.[2] In the original Jacobsen system involving monomeric chiral Co catalysts, water was used as the nucleophile in the hydrolytic kinetic resolution of monosubstituted epoxides with very high selectivity factors often exceeding $E = 100$,[2b] but desymmetrization of *meso*-epoxides under such conditions was not reported. Subsequently, the analogous oligomeric complexes were used in the hydrolytic desymmetrization of *meso*-epoxide **5** (ee = 94%) being the only example.[2e,f] Later it was found that such complexes catalyze the hydrolytic desymmetrization of epoxides **3** (98% ee) and **9** (87% ee).[2g]

It was also of interest to test structurally very different substrates such as monosubstituted epoxides *rac*-**13** and *rac*-**15**, this time in hydrolytic kinetic resolution. As in the previous examples, WT LEH proved to be a poor catalyst, the selectivity factor *E* amounting to only 2.6 and 2.8, respectively, both in slight favor of the respective (*R*)-diols. Table 4 shows that notable improvements in (*R*)-selectivity are observed for both substrates ($E = 32$ and 36, respectively) and that in the case of

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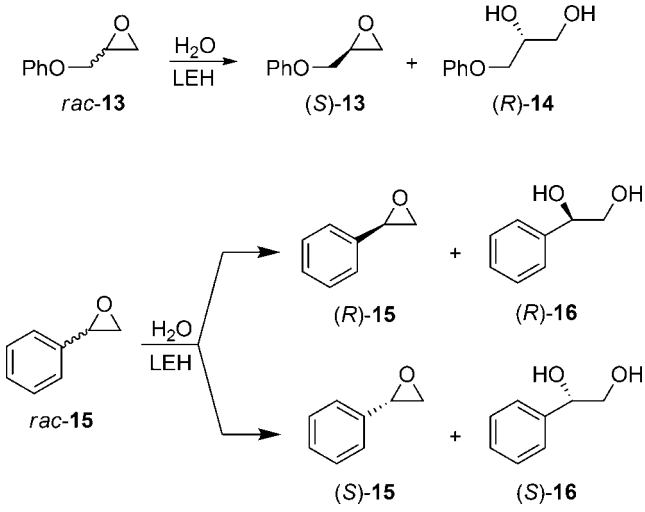
rac-**15** reversal of enantioselectivity is possible on an optional basis by using mutant H178 ($E = 44$ in favor of (*S*)-**8**). These results are of theoretical interest, but from a synthetic point of view they are not particularly significant because Jacobsen's catalysts are clearly superior in such asymmetric transformations,[2b] as are some WT EHs[1] or mutants.[12b,19] For example, although the EH from *Aspergillus niger* (ANEH) is a poor catalyst in the hydrolytic kinetic resolution of *rac*-**13** ($E = 4.6$ (*S*)),[12b] a mutant evolved by ISM proved to be highly enantioselective ($E = 115$ (*S*)),[12b] which was also efficient in the reaction of other racemic monosubstituted epoxides.[19] Directed evolution of the EH from *Agrobacterium radiobacter* as a catalyst in the hydrolytic kinetic resolution of *rac*-**15** improved enantioselectivity from $E = 16$ (WT) to $E = 38$ (best mutant favoring (*R*)-**16**).[20]

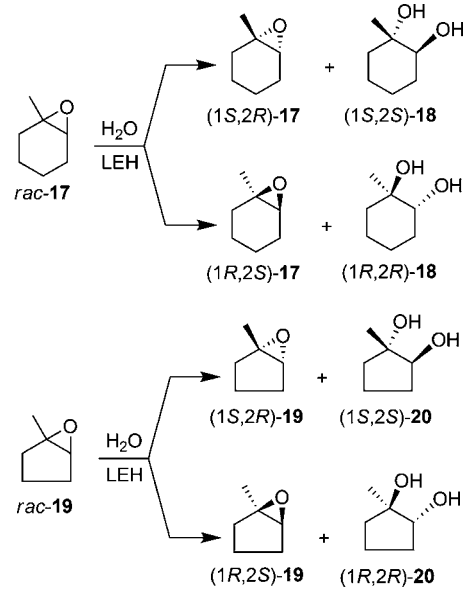


Testing Stereoconvergency of WT LEH and Evolved Mutants in Hydrolytic Reactions of Other Racemic Substrates. It has been reported that WT LEH displays stereoconvergent behavior in the hydrolytic reaction of a mixture of limonene oxide diastereomers, (1*S*,2*R*,4*R*) and (1*R*,2*S*,4*R*), both leading to the (1*S*,2*S*,4*R*)-limonene diol in a sequential manner.[4] From a synthetic organic viewpoint, enantioconvergency entails interesting perspectives, especially when a racemate leads to a single enantiomer at >95% conversion. This has been observed for some EHs[21] and for a mutant evolved by ISM for this purpose.[22] In the present study we tested WT LEH and the mutants, specifically evolved for the desymmetrization of *meso*-substrate **3**, as catalysts in the stereoconvergent transformation of epoxides *rac*-**17** and *rac*-**19.** Table 5 reveals poor catalytic behavior of WT LEH in these reactions. At complete conversion of *rac*-**17**, very limited stereoconvergency was observed in favor of (1*S*,2*S*)-**18** (ee = 19%), while in the reaction of *rac*-**19** essentially racemic product resulted. In contrast, some of the LEH mutants proved to be markedly improved catalysts, although in no case truly practical results were achieved. Even the best ee value did not exceed 63% (Table 5, entries 2−4). However, both (1*R*,2*R*)- and (1*S*,2*S*)-selective mutants are now available for partially stereoconvergent transformations of both substrates *rac*-**17** and *rac*-**19**, setting the stage for future optimization using the tools of directed evolution. Some of the intermediate mutants evolved for the hydrolytic desymmetrization of **3** display similar catalytic profiles (see Supporting Information).

Table 5. Test for Possible Stereoconvergent Catalysis Using WT LEH and Mutants in the Hydrolytic Reaction of *rac*-**17** and *rac*-**19** Leading to Diols **18** and **20**, respectively

		18			20	
entry	mutant	conv. (%)[a]	% ee	specific activity[b]	conv. (%)[a]	% *ee*
1	WT LEH	>99	19 (1*S*,2*S*)	2573	99	2 (1*S*,2*S*)
2	H150	>99	63 (1*S*,2*S*)	nd[c]	98	28 (1*S*,2*S*)
3	H173	>99	50 (1*R*,2*R*)	2291	98	25 (1*R*,2*R*)
4	H178	>99	55 (1*S*,2*S*)	2197	99	51 (1*S*,2*S*)

[a] Conversion at reaction time of 12 h. [b] Specific activity (mmol/min/mg protein). [c] Specific activity not determined.



Some experiments regarding the hydrolytic kinetic resolution were also performed in the case of *rac*-**17**. WT LEH is only slightly (1*S*,2*S*) selective ($E = 4$; specific activity, 2573 mmol/min/mg protein), mutant H178 increasing this to $E = 13$ (specific activity,2197 mmol/min/mg protein), and H173 inducing inversion of enantioselctivity ($E = 8$) in favor of (1*R*,2*R*)-**18**. This suggests that ISM should be applied directly to substrates of this kind. In the case of trisubstituted epoxides, Jacobsen catalysts fail.[2]

Scale-Up in the Hydrolytic Desymmetrization of *meso*-Epoxides. Having in hand several enantioselective LEH mutants as catalysts in the hydrolytic desymmetrization of *meso*-epoxides and kinetic resolution of racemic substrates, we considered scale up for practical applications. For illustrative purposes, mutants

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Table 6. Scale Up of the Hydrolytic Desymmetrization of Selected *meso*-epoxides Using Mutants H173 and H178 as Catalysts

entry	mutants	substrate	substrate loading (mg)	conv. (%)[a]	% ee of diol	yield (%)
1	H178	**5**	300	>99	97 (*S*,*S*)	90
2	H178	**7**	150	>99	98 (*S*,*S*)	86
3	H173	**11**	200	15	>99 (*R*,*R*)	91

[a] Reaction time: 15 h.

H178 and H173 were chosen as catalysts in the desymmetrization of *meso*-substrates **5**, **7**, and **9**. Accordingly, 0.5 g (wet cell weight) of fresh cells was used in each 10 mL reaction mixture containing 150−300 mg of epoxide (Table 6). Although there is room for further activity improvement in the case of the sterically demanding diphenyl-substituted epoxide **11** (Table 6, entry 3), the present results document the synthetic utility of these LEH mutants.

Unveiling the Source of Inverted Enantioselectivity. The mechanism of WT LEH involves activation of the substrate by H-bonding of Asp101 to the epoxide O atom, followed by nucleophilic attack of water which is activated and properly positioned by Tyr53, Asp132, and Asn55.[3,4] It was of particular interest to uncover the source of inverted enantioselectivity induced by mutant H178 in the hydrolytic desymmetrization of cyclopentene-oxide (**3**), because this entails the largest degree of stereoselectivity change observed in the present study. As an initial step in this direction, induced fit docking[23] and molecular dynamics (MD) simulations using the Schrödinger package[24] were performed (6 ns) for WT LEH and mutant H178, respectively. As the crucial parameter we considered the distance, *d*, between the attacking O atom of activated water and the two possible enantiotopic C atoms of the bound substrate undergoing nucleophilic substitution with inversion of configuration and formation of the *trans*-diol **4**. Preferred attack is to be expected at the C atom closest to the activated water. As a corollary, the larger the difference in the two distances, the higher the enantioselection. Figure 3a and 3b reveals the reshaping of the binding pocket, featuring the residues that are mutated. In the case of WT LEH, the two distances are not much different, 3.2 versus 3.4 Å (Figure 3c), in line with the observed direction and low degree of enantioselectivity in favor of (*R*,*R*)-**4**. In sharp contrast, the mutational changes in variant H178 cause the repositioning of **3** so that now the other enantiotopic C atom is considerably closer to the attacking water, 3.6 versus 5.2 Å (Figure 3d). Close inspection of the results shows that the larger phenyl group in mutation Ile80Phe "pushes" the substrate down so that now the other C atom of the epoxide moiety is closer to the activated water, a process that is spatially made possible by the other three point mutations Met32Cys/Leu114Cys/Ile116Val. The respective volume change is in line with this model (Supporting Information). Distance alone is not sufficient to describe the ideal positioning of the activated water, because the trajectory of nucleophilic attack needs to be considered. As Table 7 shows, angles describing the trajectory of the favored nucleophilic attack is in fact larger than that of the disfavored mode, as expected. This analysis can be related to a near-attack-conformation as postulated by Bruice in an MD study of another EH.[25]

Table 7. Results of MD Simulations and Docking Experiments Using Epoxide **3**[a]

mutant	distance (Angstroms) from water O atom to C_R of **3**	distance (Angstroms) from water O atom to C_S of **3**	angle (deg) epoxy O atom/C_R/water O atom	angle (deg) epoxy O atom/C_S/water O atom
WT	3.4	3.2	116.4	133.9
H173	3.5	3.1	112.9	140.9
H178	3.6	5.2	146.6	107.5

[a] Note that attack at enantiotopic C-atom C_R occurs with inversion of configuration and leads to the (*S*,*S*)-product, while attack at C_S provides (*R*,*R*)-**4**.

Conclusions and Perspectives

Limonene epoxide hydrolase (LEH) is an unusual EH because it does not exert its catalytic effect by the usual two-step mechanism in which an aspartate undergoes nucleophilic

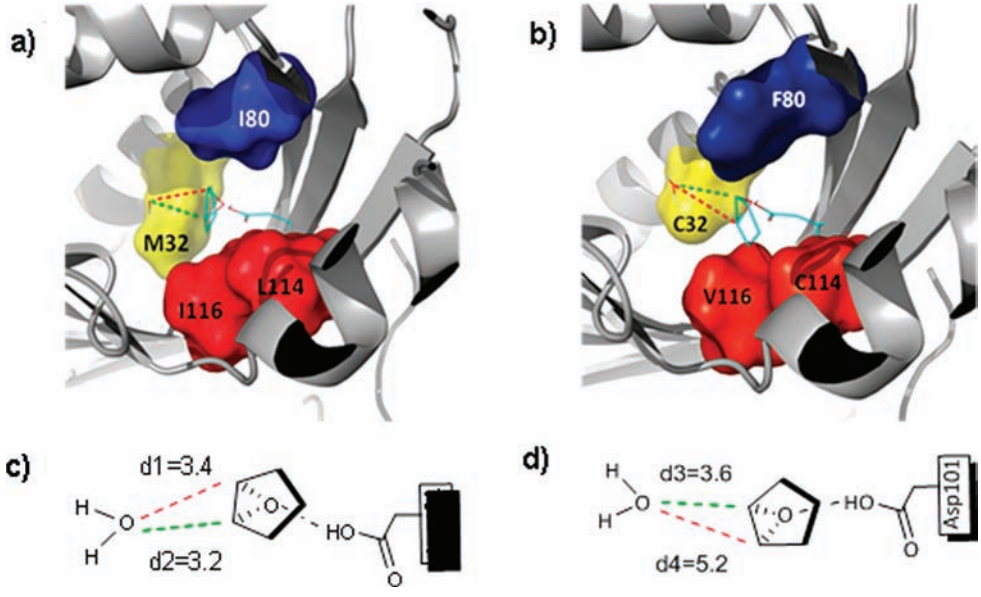

Figure 3. Repositioning of substrate **3** in the reshaped binding pocket of mutants relative to WT LEH as indicated by MD simulations. (a) Binding pocket of (*R*,*R*)-selective WT LEH featuring the residues which undergo mutational change when going to (*S*,*S*)-selective mutant H178. (b) Reshaped binding pocket of mutant H178. (c) Close-up view of the binding pocket of WT LEH featuring the residues which undergo mutational change when going to mutant H178 and the distances d1 and d2 (in Angstroms) to the activated water. (d) Close-up view of the binding pocket of mutant H178 showing mutational changes and the respective distances d3 and d4 (in Angstroms) to the activated water.

reaction at the H-bond-activated substrate in the rate- and stereochemistry-determining step but rather via direct nucleophilic attack by properly positioned and activated water. The natural role of LEH, which has been characterized by X-ray crystallography,[3b] is the hydrolytic conversion of limonene oxide present in *Rhodococcus erythropolis* DCL14 with formation of the respective limonene diol.[3] Unfortunately, WT LEH is not suited for application in synthetic organic chemistry or biotechnology, because substrate scope in terms of acceptable levels of stereoselectivity is highly restricted.[4] We have therefore undertaken the first directed evolution study of LEH. The purpose was not only to manipulate stereoselectivity for a model compound (cyclopentene-oxide) and for a defined set of substrates in hydrolytic desymmetrization and kinetic resolution for practical applications. We also wanted to test the previously developed strategy of protein engineering based on iterative saturation mutagenesis (ISM) in a difficult case. Efficacy in directed evolution means maximizing the degree of catalyst improvement while minimizing the screening effort by generating high-quality libraries.[7,10,11] Keeping these points in mind, we specifically restricted the number of screened transformants in the Darwinian process to less than 5000. A crucial additional tool proved to be the use of a reduced amino acid alphabet based on NDT codon degeneracy encoding 12 instead of the usual 20 canonical amino acids, because this reduces the screening effort drastically. The results of the present study show once more that ISM is indeed a highly efficient tool in accelerated directed evolution. We also provide a guideline for grouping single amino acid residues into optimal randomization sites. As a general recommendation, sites comprising two amino acid positions are to be preferred over single-residue sites.

Using cyclopentene-oxide (**3**) as the model substrate for hydrolytic desymmetrization, with WT LEH displaying an ee value of only 14% (*R,R*), two out of 48 (2×24) theoretically possible ISM pathways were arbitrarily explored for achieving both improved (*R,R)*-selectivity and inverted (*S,S*)-selectivity. One pathway favors the formation of the corresponding (*S,S*)-diol (**4**) (ee = 93%) with inverted stereoselectivity, the other optionally providing access to the enantiomeric product (*R,R*)-**4** with improved enantioselectivity (ee = 80%). Moreover, the evolved mutants were subsequently tested successfully in the hydrolytic desymmetrization of other *meso*-epoxides and in the kinetic resolution of structurally very different racemic substrates, enantioselectivity again being substantial in contrast to WT LEH (ee up to 99%). These efforts did not require any additional mutagenesis experiments, which means once more that in directed evolution "you may get more than what you screen for".[10c] Thermostability of the best mutants proved to be similar to that of WT LEH, which means that no notable compromise regarding this important property was made in the process of genetic optimization. Another noteworthy result of the present study concerns the surprising finding that the point mutations of two single mutants (Met32Leu and Leu35Phe), each being (*R,R*)-selective, when combined in the form of the respective double mutant (Met32Leu/Leu35Phe), induces (*S,S*)-selectivity. This shows that the traditional way of viewing mutational effects as being additive in a given direction should be treated with caution.

Unveiling the source of enhanced stereoselectivity of an evolved mutant enzyme requires investigations which include kinetics, inhibition experiments, crystal structures, and extensive MD simulations as well as induced docking experiments.[19] Such a detailed study still needs to be performed for the LEH mutants. Reported herein are only induced docking experiments and MD simulations of the reaction of cyclopentene-oxide, which indicate that the distances, *d*, between activated water and the two possible C atoms of *meso*-epoxides moiety being attacked nucleophilically are similar when using WT LEH having low enantioselectivity but quite different in the case of the best (*S,S*)-selective mutant. The predicted (preferred) attack corresponds to the shorter distance and explains the observed switch in the direction and degree of enantioselectivity. However, this is a crude model, and other factors may well be involved as well.

ISM constitutes a rational approach to directed evolution, because it is a combination of knowledge-based design and randomization, requiring only small mutant libraries. Recently, it has been applied in the quest to evolve such protein properties as enhanced stereoselectivity and activity, and altered cofactor binding of a number of different enzymes, including enzyme promiscuity and metabolic pathway engineering.[26]

Acknowledgment. We thank Professor Michael Arand (ETH Zürich) for providing the plasmid of pGEF-LEH.

Supporting Information Available: Experimental details and data regarding MD simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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