J|A|C|S ARTICLES

Published on Web 06/10/2010

Iterative Saturation Mutagenesis Accelerates Laboratory Evolution of Enzyme Stereoselectivity: Rigorous Comparison with Traditional Methods

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Abstract: Efficacy in laboratory evolution of enzymes is currently a pressing issue, making comparative studies of different methods and strategies mandatory. Recent reports indicate that iterative saturation mutagenesis (ISM) provides a means to accelerate directed evolution of stereoselectivity and thermostability, but statistically meaningful comparisons with other methods have not been documented to date. In the present study, the efficacy of ISM has been rigorously tested by applying it to the previously most systematically studied enzyme in directed evolution, the lipase from *Pseudomonas aeruginosa* as a catalyst in the stereoselective hydrolytic kinetic resolution of a chiral ester. Upon screening only 10 000 transformants, unprecedented enantioselectivity was achieved ($E = 594$). ISM proves to be considerably more efficient than all previous systematic efforts utilizing error-prone polymerase chain reaction at different mutation rates, saturation mutagenesis at hot spots, and/or DNA shuffling, pronounced positive epistatic effects being the underlying reason.

Introduction

Directed evolution[1] constitutes a method for engineering essentially any property of enzymes as catalysts in synthetic organic chemistry and biotechnology, including stability[2] and stereoselectivity.[1i,3] The most often used gene mutagenesis methods are error-prone polymerase chain reaction (epPCR), saturation mutagenesis, and DNA shuffling. However, it is not at all clear what the best strategy actually is because statistically meaningful comparisons assessing a broad range of mutagenesis methods and strategies are essentially unknown.[4] Methodology development in the quest to make laboratory evolution more efficient and therefore faster than in the past is currently an important focus of research.[1,5] The challenge is to maximize the quality of mutant libraries, defined in terms of the frequency of superior mutants (hits) in a given library *and* the actual degree

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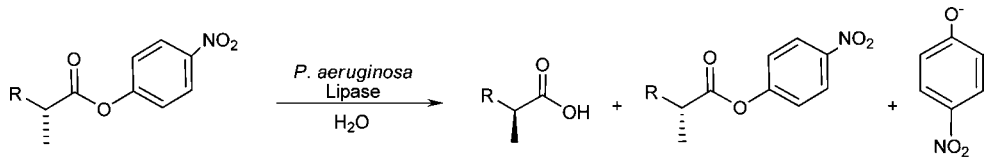
10.1021/ja1030479

Chart 1



rac-**1**
R = $CH_3(CH_2)_6CH_2$-

(*S*)-**2** (*R*)-**1** **3**

of catalyst improvement.[5d] High-quality libraries require less screening effort,[5d] which to this day is the bottleneck of laboratory evolution.[1,6] Motivated by the credo "quality, not quantity",[5a] we have recently developed iterative saturation mutagenesis (ISM), according to which appropriate sites in the protein, comprising one or more amino acid positions, are first randomized with formation of focused libraries.[5d,7] The gene of a given hit then serves as a template for performing saturation mutagenesis at the respective other sites, and the process is repeated until the desired catalyst quality has been achieved. Stereoselectivity,[7a−c] substrate acceptance (rate),[7b] and thermostability[7c] can be handled, the criteria for choosing the proper randomization sites being different according to the catalytic property under study. When addressing stereoselectivity and/or substrate scope, sites aligning the complete binding pocket are considered in a process termed combinatorial active-site saturation test (CAST).[7a,b,8] Rather than arbitrarily targeting a certain site for saturation mutagenesis and then possibly turning to another residue, as we[4h,9] and others have done previously for various purposes,[1,10] iterative CASTing constitutes methodical systematization and is therefore a useful acronym to distinguish it from saturation mutagenesis at alternative (remote) sites. Since only small mutant libraries in the range of 100−3000 transformants are generally required, the screening effort is minimized. However, meaningful assessment relative to other methods is possible only if direct comparisons are made. Here we present the first comprehensive undertaking of this kind, which throws light not only on ISM itself but also on conventional approaches used thus far in directed evolution.[1]

To date, the most thoroughly investigated case of directed evolution concerns the lipase A from *Pseudomonas aeruginosa* (PAL) as a catalyst in the hydrolytic kinetic resolution of the chiral ester *rac*-**1** (Chart 1), an enzyme that we have focused on since the mid-1990s.[4h,9,11,12] Using PAL as the model protein and epPCR as the gene mutagenesis method, we provided proof-of-principle of laboratory evolution of enantioselective enzymes as a potentially prolific source of catalysts for asymmetric transformations.[11] Subsequently we tested a variety of other mutagenesis techniques and strategies in order to evolve enhanced or reversed stereoselectivity, thereby setting the stage for comparing mutagenesis methods. As part of several comprehensive studies involving PAL, various approaches were explored, including four successive rounds of epPCR at low mutation rate,[11] a fifth round,[12] epPCR at high mutation rate,[4h] saturation mutagenesis at hot spots identified by epPCR,[9] saturation mutagenesis at a four-residue site aligning the binding pocket,[4h] and DNA shuffling of various mutants obtained at low and high error-rate epPCR[4h] as well as DNA shuffling with simultaneous saturation mutagenesis at two hot spots.[4h,12] Today these strategies are conventional. Turning from one hot spot to another using saturation mutagenesis or epPCR was part of these exercises, but systematization as in ISM was not considered. Following the screening of about 50 000 transformants, a variant 1H8 was identified showing a selectivity factor of $E = 51$ in favor of (*S*)-**1**, which is a notable increase relative to the performance of wild-type (WT) PAL ($E = 1.1$). It is characterized by six point mutations, Asp20Asn/Ser53Pro/Ser155Met/Leu162Gly/Thr180Ile/Thr234Ser, only Leu162Gly being next to the binding pocket.[4h,12]

Mechanistically, activated serine at position 82 as part of the catalytic triad Asp229/His251/Ser82 adds nucleophilically to the ester carbonyl function with formation of the usual oxyanion in the rate- and stereochemistry-determining step.[13] A theoretical study subsequently revealed enhanced enantioselectivity as being due to a relay mechanism, and also predicted that only two of the six accumulated point mutations are actually necessary for achieving high enantioselectivity, namely Ser53Pro/Leu162Gly,

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which was substantiated experimentally by generating the respective double mutant ($E = 63$).[14] This was a triumph of theory, but it also suggested that the strategies that we were applying, although successful, were far from optimal. Superfluous point mutations accompanied by excessive screening labor are likely to be common in laboratory evolution, although this point is rarely illuminated.[1] In view of the extensive available data regarding the evolution of enantioselective PAL variants,[4h,9,11,12] we surmised that this experimental platform is ideal for testing ISM and consequently for comparing directed evolution methods in a systematic and rigorous manner.

Results and Discussion

Strategies for Choosing ISM Sites. It is well known that lipases harboring esters as substrates have "two" binding pockets, one for the acid and the other for the alcohol part of the substrate, catalytically active serine as part of the triad Asp/His/Ser lying between them.[15] In the present case, the activated hydroxy function of Ser82 in the catalytic triad Asp229/His251/Ser82 adds nucleophilically to the carbonyl function of the ester *rac*-**1** with formation of the respective short-lived oxyanion. Since the mutagenesis/screening experiments were performed with PAL using the hydrolytic kinetic resolution of the ester *rac*-**1** having the stereogenic center in the acid part, we focused solely on the acid-binding pocket in order to select appropriate residues for saturation mutagenesis. Guided by the analysis of the crystal structure of PAL (PDB code 1ex9),[13] we selected six amino acid residues, Met16, Leu17, Leu159, Leu162, Leu231, and Val232, for saturation mutagenesis (Figure 1). The distance values of the α-C-atom of all these six residues to the α-C-atom of the acid moiety (C4) from the phosphonate inhibitor bound in the crystal structure of PAL, corresponding to the stereocenter C2 of substrate **1** bound as the oxyanion, are as follows: Met16, 5.3 Å; Leu17, 8.4 Å; Leu159, 8.5 Å; Leu162, 6.5 Å; Leu231, 7.0 Å; and Val232, 7.3 Å.

When deciding on how to group these six single residues into CAST sites for saturation mutagenesis, the problem of oversampling needs to be considered.[5d,7c,16] The six amino acid positions could be classified, for example, as six single-residue randomization sites. Each saturation mutagenesis library would then require, in the case of NNK codon degeneracy encoding all 20 canonical amino acids, the screening of about 100 transformants for 95% library coverage (N, adenine/cytosine/guanine/thymine; K, guanine/thymine). Alternatively, it is possible to consider, for example, three, two-residue CAST sites, each requiring an oversampling of about 3000 transformants for similar library coverage. These values were calculated using the CASTER computer aid[7c] which is based on previous algorithms assuming the absence of amino acid bias.[16b] Grouping into three, two-residue sites involves a greater screening effort in the case of the initial libraries, but the situation reverses in the subsequent iterative steps if the complete ISM scheme is fully investigated (Table 1). In the case of a reduced amino acid alphabet using, for example, DNT codon degeneracy (D, adenine/guanine/thymine; T, thymine) encoding 11 amino acids (Ala, Asn, Asp, Cys, Gly, Ile, Phe, Ser, Thr, Tyr, and Val) or NDT encoding 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, and Gly), the screening effort is sharply reduced in both cases,[7c] but again double-residue sites appear to have a statistical advantage over single-residue analogues in an ISM scheme. Moreover, we speculated that in such a grouping cooperative (not just additive) effects could operate between the two point mutations within each set and also between sets of mutations in the subsequent iterative steps.[17] In contrast, cooperative effects are not possible in the initial libraries when choosing single-residue sites. In the present study we therefore opted for three sites, each comprising two amino acid positions, namely A (Met16/Leu17), B (Leu159/Leu162), and C (Leu231/Val232), shown in Figure 1, which also features catalytically active Ser82 as well as lid 1 and lid 2.[18]

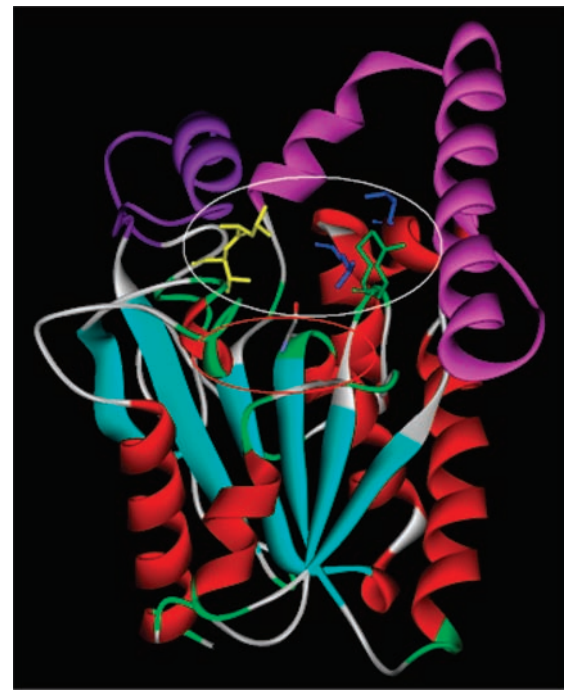

Figure 1. Schematic representation of amino acid residues considered for saturation mutagenesis, based on the X-ray structure of WT-PAL:[13] sites A (Met16/Leu17, green), B (Leu159/Leu162, blue), and C (Leu231/Val232, yellow) around the active site Ser82 (stick representation in gray and red) in the acid-binding pocket (white circle). The red circle marks the alcohol-binding pocket, in the case at hand harboring the *p*-nitrophenyl moiety of *rac*-**1**. At the top of picture, helix and loop in pink (right, Asp113-Leu156) and purple (left, Pro203-Asn228) represent lid 1 and lid 2, respectively.[18]

Generation of Mutants with High Stereoselectivity. When considering an ISM system consisting of three sites, each site being visited once in a given upward pathway, a total of 15 saturation mutagenesis libraries defining six different pathways are theoretically possible (Table 1 and Figure 2a). A systematic search provides a means to identify the best pathway. In the present case we used NNK codon degeneracy and screened about 3000 transformants in each of the three initial libraries.

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Table 1. Statistical Consequences as a Function of Grouping Single CAST Residues into Randomization Sites

type of grouping	no. of pathways in complete ISM scheme	no. of libraries in complete ISM scheme	total number of transformants screened in complete ISM scheme for 95% coverage	
			using NNK codon	using NDT or DNT codon
6 single-residue sites	720	1956	183 864 (94 per lib.)	66 504 (34 per lib.)
3 double-residue sites	6	15	45 990 (3066 per lib.)	6450 (430 per lib.)
2 triple-residue sites	2	4	392 652 (98 163 per lib.)	20 700 (5175 per lib.)
1 six-residue site	1	1	3.21×10^9	8.95×10^6

Medium-throughput screening (700 transformants/day) was performed using our original *ee*-assay in which a UV/vis plate reader monitors the initial reaction rates of (*R*)- and (*S*)-**1** separately, the relative slopes serving as an indicator for the occurrence of a hit having enhanced enantioselectivity[11] (Figure S1, Supporting Information). Whereas the libraries generated at sites A and C failed to contain significantly improved variants, the library at site B was found to harbor several hits, the best one being variant 1F8 with a single mutation Leu162Asn, showing a selectivity factor of $E = 8$ in favor of (*S*)-**1**. The results of a kinetic study of mutant 1F8 and of some other prominent mutants arising from randomization at sites B and C are summarized in Table 2.

We then utilized the gene of variant 1F8 as a template for performing two separate second-round randomization experiments at the other two sites, A (Met16/Leu17) and C (Leu231/Val232), respectively, despite the fact that the initial libraries generated thereat had not provided any significantly improved single or double mutants in terms of enhanced enantioselectivity. In order to decrease the screening effort, we considered a reduced amino acid alphabet on the basis of NDT or DNT codon degeneracy (Table 1), a strategy that we had previously developed in order to increase the efficacy of directed evolution based on saturation mutagenesis.[5d] Since both sites harbor leucine, we opted for DNT codon degeneracy for a simple reason: DNT does not encode leucine, thereby reducing the amount of "starting" 1F8 variants in the respective libraries and increasing the number of new mutants in the screened library. It is known that in any saturation mutagenesis library, WT or "starting" mutants always appear, which reduces quality.[5d,7a] In the present case of a two-residue site, about 430 transformants need to be screened for 95% library coverage[5d,7] (Table 1), and we settled for 500. The best hit (1B2) originated from the second-round library at site A, containing two new mutations, Met16Ala/Leu17Phe. Mutant 1B2 is thus characterized by three-point mutations Met16Ala/Leu17Phe/Leu162Asn. It proved to be so enantioselective that application of the Sih equation[19] for determining the *E*-value is no longer meaningful. Kinetic characterization revealed a selectivity factor of $E = 594$ (*S*) in the model transformation involving (*S*)- and (*R*)-**1** (Table 2). Upon targeting site C using the gene of 1F8 as the template, no significantly improved mutants were found. At this point, screening amounted to the evaluation of a total of only 10 000 transformants, yet unprecedented (*S*)-enantioselectivity had evolved. The pathways considered thus far are shown schematically in Figure 2. From a practical viewpoint, further mutagenesis, e.g., using 1B2 as a template to randomize at site C, was not necessary.

Kinetic Studies. Enhancing the enantioselectivity of a kinetic resolution can come about by increasing the reaction rate of one enantiomer, or by decreasing the rate of the mirror-image compound, or both. Kinetic data regarding the hydrolytic reactions of (*S*)-**1** and (*R*)-**1** were obtained separately using purified WT-PAL, the previously obtained best mutant 1H8 with six mutations,[4h] and the two newly evolved mutants of the present study, 1F8 (Leu162Asn) and 1B2 (Met16Ala/Leu17Phe/Leu162Asn), respectively. The results summarized in Table 2 point to a remarkable effect, namely that in the case of the best mutant of the present study (1B2), the rate of reaction k_{cat} of the (*S*)-**1** has been increased dramatically (37-fold), a slight decrease (5.6-fold) in reaction rate of the (*R*)-enantiomer contributing additionally to the high overall enantioselectivity ($E = 594$). Together these effects lead to a perfect kinetic resolution.

Unveiling the Reason for the Efficacy of ISM. It is clear that the sum of the single mutation Leu162Asn (site B) obtained in the initial randomization experiment and the two mutations

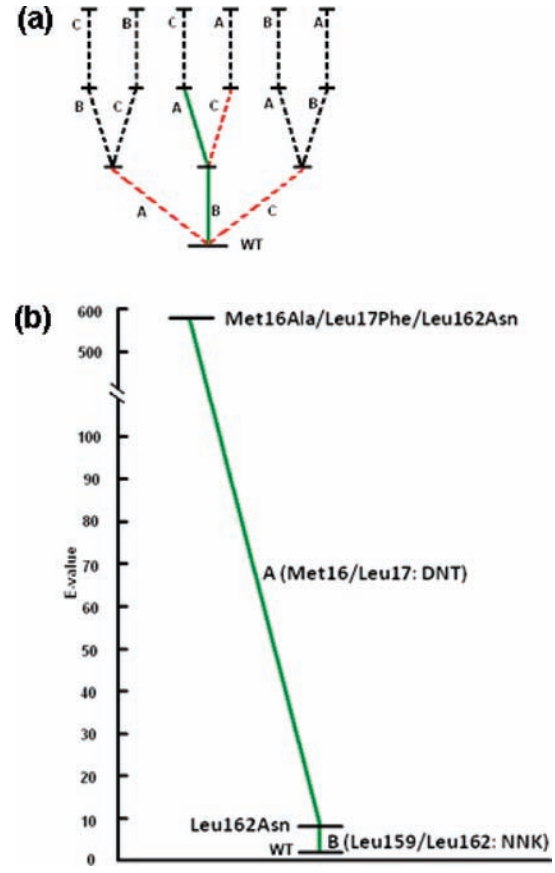

Figure 2. (a) Scheme of an ISM system composed of three sites, A, B, and C. Solid green connections define the best pathway in the present PAL study, dotted red lines indicating saturation mutagenesis which failed to provide significantly improved PAL mutants and dotted black lines denoting unexplored saturation mutagenesis experiments. (b) Real-scale scheme of the best pathway, WT→B→A, leading to the best mutant 1B2 characterized by mutations Met16Ala/Leu17Phe/Leu162Asn and showing a selectivity factor of $E = 594$ (*S*) in the hydrolytic kinetic resolution of *rac*-**1**.

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Table 2. Kinetic Study of Various Mutants of Wild-Type *P. aeruginosa* Lipase (WT-PAL) Obtained from Iterative Saturation Mutagenesis, Deconvolution Study of Best Mutant (1B2), and Conventional Methods, (*S*)-**1** and (*R*)-**1** Serving Separately as Substrates

				(*S*)-**1**			(*R*)-**1**			
entry	mutant	site of ISM mutation	rounds of saturation mutagenesis	K_m ($\times 10^{-4}$ M)	k_{cat} ($\times 10^{-3}$ s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	K_m ($\times 10^{-4}$ M)	k_{cat} ($\times 10^{-3}$ s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	*E*
1	WT-PAL			8.6	37.4	43.5	7.9	29.9	37.8	1.1(*S*)
2	Leu162Gly (2C11)	B	1st round	3.4	3.2	9.4	1.9	0.3	1.6	5.9(*S*)
3	Leu162Ile (2H9)	B	1st round	9.4	13.6	14.5	1.2	0.3	2.5	5.8(*S*)
4	Leu162Thr (19F12)	B	1st round	13.2	167	126	9.7	30.1	31.0	4.1(S)
5	Leu162Asn (1F8)	B	1st round	5.6	42.1	75.2	7.9	7.4	9.4	8.0(*S*)
6	Leu231Ile/Val232Cys (1C7)	C	1st round	2.9	0.4	1.4	5.0	0.8	1.6	1.1(*R*)
7	Met16Ala/Leu17Phe/Leu162Asn (1B2)	B/A	2nd round	3.4	1374	4041	7.9	5.4	6.8	594(*S*)
8	Met16Ala/Leu17Phe	mutant of deconvolution study		22.0	16.1	7.3	20.6	5.7	2.8	2.6(*S*)
9	Met16Ala/Leu162Asn	mutant of deconvolution study		3.9	1.4	3.6	9.5	1.5	1.6	2.2(*S*)
10	Leu17Phe/Leu162Asn	mutant of deconvolution study		6.9	1.0	1.4	7.5	1.0	1.3	1.1(*S*)
11	Asp20Asn/Ser53Pro/Ser155Met/ Leu162Gly/Thr180Ile/Thr234Ser (1H8)	best mutant from conventional method of enzyme evolution[12]		10.1	38.6	38.2	62.6	5.5	0.9	42.4(*S*)

Met16Ala/Leu17Phe (site A) accumulated in the iterative step induces a dramatic increase in stereoselectivity. Since a double mutant corresponding to Met16Ala/Leu17Phe was not found in the initial saturation mutagenesis library at site A, it is likely that strong cooperative effects operating between the mutations are involved. In order to validate this, deconvolution experiments were performed by generating appropriate mutants using site-directed mutagenesis. For this purpose variants Met16Ala/Leu17Phe, Met16Ala/Leu162Asn, and Leu17Phe/Leu162Asn were tested in kinetic experiments using (*S*)-**1** and (*R*)-**1** (Table 2). It can be seen that the epistatic interaction of mutation Leu162Asn with the set of two mutations Met16Ala/Leu17Phe results in a dramatic cooperative effect, the two respective variants having selectivity factors *E* of only 8.0(*S*) and 2.6(*S*), respectively. Together they orchestrate unprecedented enantioselectivity amounting to $E = 594$ (*S*). Moreover, letting the observed point mutation at position 162 interact individually with those at positions 16 and 17 in the form of variants Met16Ala/Leu162Asn ($E = 2.2$) and Leu17Phe/Leu162Asn ($E = 1.1$), respectively, actually reduces enantioselectivity relative to the single mutant Leu162Asn ($E = 8.0$). We conclude that the decision to group the originally identified six single amino acid positions into a set of three, two-residue sites is indeed optimal, and that a grouping defined by six single-residue sites would not have been successful in the present system. However, this does not mean that the process of grouping into single-residue sites always ends up in failure.[7b]

Substrate Scope of Best Mutant Met16Ala/Leu17Phe/Leu162Asn. From the viewpoint of organic chemists or biotechnologists, an enzyme variant evolved for a specific substrate should also be a viable catalyst for a set of structurally different compounds, first and foremost in terms of activity.[3] We therefore tested the best mutant, 1B2, as a catalyst in the hydrolytic kinetic resolution of racemic compounds **4**−**11** (Chart 2). Since the actual directed evolution experiments were performed using *rac*-**1** in the attempt to increase enantioselectivity, there was no stringent reason to expect wide substrate acceptance. Remarkably, enhanced activity relative to WT-PAL was observed in all cases, demonstrating that substrate scope has been widened (Table 3). For example, in the case of substrate **4**, k_{cat}/K_m characterizing the reaction of the preferred (*S*)-enantiomer increases by a factor of 5×10^3 upon going from WT-PAL to

Chart 2

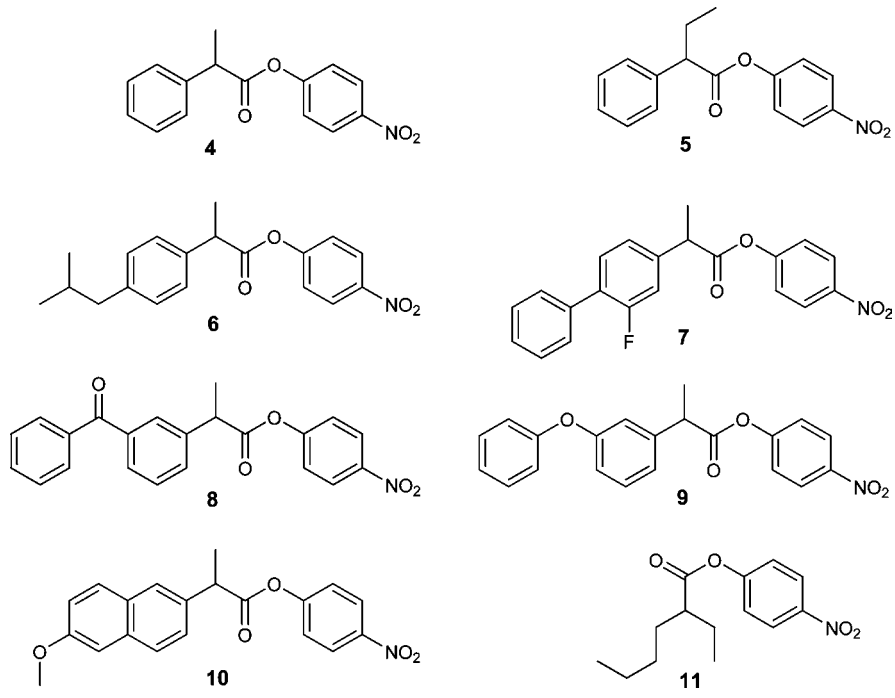

Table 3. Altered Substrate Scope (Rate) and Enhanced Enantioselectivity upon Going from WT-PAL to ISM-Evolved Mutant 1B2 (Met16Ala/Leu17Phe/Leu162Asn), Measured by Kinetics

mutant	K_m ($\times 10^{-4}$ M)	k_{cat} ($\times 10^{-3}$ s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	K_m ($\times 10^{-4}$ M)	k_{cat} ($\times 10^{-3}$ s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	*E*
		(*S*)-**4**			(*R*)-**4**		
WT	8.1	0.2	0.25				
1B2	17.2	161	93.6	28.2	8.5	3.0	31(*S*)
		(*S*)-**5**			(*R*)-**5**		
WT	2.3	0.1	0.4	6.6	0.1	0.2	2.0(*R*)
1B2	25.0	239	95.6	10.1	0.9	0.9	106(*S*)
		(*S*)-**6**			(*R*)-**6**		
WT	1.4	0.2	1.4	4.4	0.4	0.9	1.6(*S*)
1B2	23.8	2949	1239	59.1	134	22.7	55(*S*)

mutant 1B2. Thus, when addressing substrate acceptance (rate), ISM appears to be far more effective than our previous strategy based on combining point mutations obtained by saturation mutagenesis at two different sites.[8b] We prefer not to speculate on the reason for this finding. Moreover, the kinetic studies of *rac*-**4**, *rac*-**5**, and *rac*-**6** using variant 1B2 also showed that good to excellent stereoselectivity was achieved in favor of the (*S*)-substrates, the *E*-values amounting to 31, 106, and 55, respectively (Table 3). In the case of the other substrates, moderate but still respectable enantioselectivity resulted, the selectivity factors being derived by application of the Sih equation:[19] *rac*-**7**, $E = 12$; *rac*-**8**, $E = 17$; *rac*-**9**, $E = 23$; *rac*-**10**, $E = 20$; and *rac*-**11**, $E = 18$, all in favor of the respective (*S*)-enantiomer.

Unveiling the Source of Enhanced Enantioselectivity. In order to gain some insight regarding the source of enhanced reaction rate and enantioselectivity of the superior mutant 1B2 (Met16Ala/Leu17Phe/Leu162Asn), molecular modeling and molecular dynamics (MD) simulations were performed, similar to those reported previously for the originally evolved mutant 1H8 having six point mutations.[14] As before, the crystal structure of WT-PAL[13] was utilized as a starting point, and amino acid mutations were introduced using the modeling suite YASARA.[20] The substrates (*R*)- and (*S*)-**1** were introduced as tetrahedral intermediates (oxyanions) covalently bound to catalytically active Ser82.

One important result is the realization that additional space at the binding pocket in mutant 1B2 is created by the point mutation Leu162Asn, thereby inducing significant rate enhancement of the reaction of the model compound and the additional bulky substrates (Figure 3). Accordingly, the side chain of Asn162 was found in a rotamer in which it reaches away from the active site by undergoing an H-bond relationship with Ser158 and His83 near the surface of the enzyme. In effect, Asn162 can then be considered to be a much smaller residue than Leu162, which is characterized by a fairly space-filling hydrophobic isobutyl side chain pointing into the binding pocket of WT-PAL. In the case of the best earlier mutant, 1H8 ($E = 51$),[4h] having six point mutations, a space-providing effect was also induced by a mutational change at the same amino acid position, although the molecular basis of the particular point mutation observed, Leu162Gly, is obviously different.[14] Point mutation Met16Ala in the best variant, 1B2, also contributes to the enlargement of the binding pocket, but it is the cooperativity acting between the three point mutations Met16Ala/Leu17Phe/Leu162Asn which is responsible for the overall effect regarding increased activity and enhanced enantioselectivity. Interestingly, the third point mutation in variant 1B2, namely Leu17Phe, leads to an energetically favorable interaction of the phenyl group with the *p*-nitrophenyl moiety of the substrate.

The creation of more space does not necessarily need to correlate with higher reaction rate. Indeed, the reshaping of the binding pocket induces a second effect which is crucial, namely additional stabilization of the oxyanion beyond the normal stabilizing interactions in traditional oxyanion formation. This provides additional stabilization of the transition state of the rate- and stereochemistry-determining step, a phenomenon which is possible with the favored (*S*)-**1** but not with the disfavored (*R*)-enantiomer. The effect comes about by additional H-bonding between His83 and the oxyanion originating from (*S*)-**1** (Figure 4). In the case of the disfavored substrate (*R*)-**1**, severe steric clash between the α-methyl group at the stereogenic center and the imidazole moiety of His83 occurs, preventing any gain in stabilization. Moreover, the simulation in the case of (*R*)-**1** points to a twisted binding mode which results in a less productive conformer, because now the usual oxyanion stabilization arising from His251 is largely disrupted. This model is in full accord

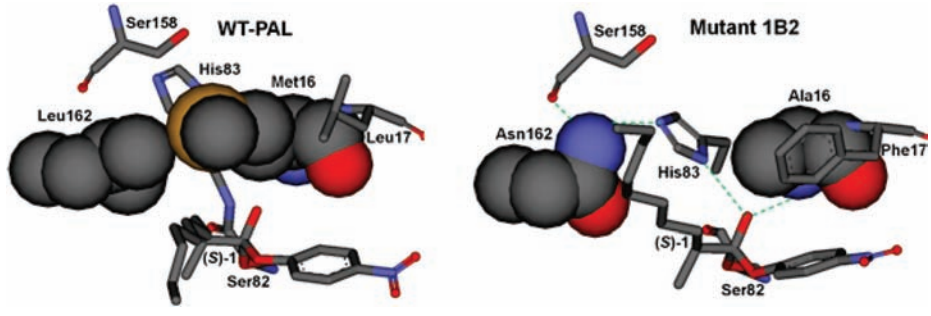

Figure 3. Comparison of the acid-binding pocket of WT-PAL (left) and model of the best mutant, 1B2 (right), with bound (*S*)-**1** to active site Ser82. The strong cooperative effect between mutations Met16Ala/Leu17Phe/Leu162Asn leads to the creation of a larger binding pocket and thereby to the accommodation of the long aliphatic arm of (*S*)-**1** (right) better than WT-PAL (left). In mutant 1B2 (right), a new extended H-bond network indicated by green lines occurs between Asn162, Ser158, His83, carbonyl oxygen of (*S*)-**1**, and Ala16, the side chain of Asn162 pointing away from the acid-binding pocket.

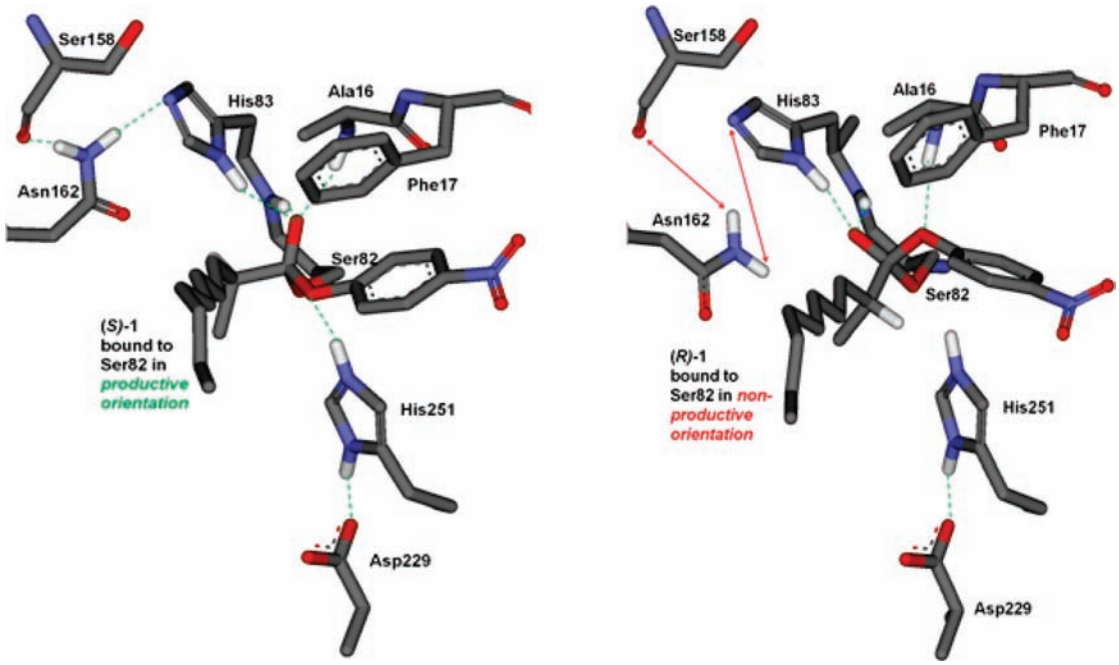

Figure 4. Comparison of oxyanion-stabilizing H-bonds (indicated by green lines) in mutant 1B2 in a productive binding mode for (*S*)-**1** (left) and in a nonproductive orientation as found for the less reactive (*R*)-**1** (right). In the nonproductive orientation, the catalytic H-bond toward His251 from the catalytic triad is lost, and the negatively charged oxyanion is stabilized by only two H-bonds. Due to disruption of the H-bond network in nonproductive binding mode (right), Asn162 is found in different conformers, floating toward the substrate binding space (red double-headed arrows) and occupying space thereat.

with the results of the kinetic study (Table 2). Indeed, the calculated difference in stabilization energy of the two enantiomers amounts to about 3 kcal/mol. Finally, we speculated that water could also bind in the newly created pocket, thereby increasing the difference in energy. Although a stable minimum structure was identified, it was found that, during the simulation, water moves around and is less frequently observed near the oxyanion than His83.

Conclusions

More than 15 years of research in directed evolution of enzymes have shown that some degree of catalyst improvement can always be achieved, irrespective of the applied gene mutagenesis method. This record reflects the power of laboratory evolution as a protein engineering method, yet the pressing issue currently concerns efficacy.[1−5] It is not only industrial biotechnology that needs methods for rapid directed evolution enabling reliable timeframes for the discovery and production of effective catalysts for new tasks. The challenge is of a fundamental nature, requiring basic research. Our contribution in this mission is iterative saturation mutagenesis (ISM), which we previously illustrated in the evolution of stereoselectivity of an epoxide hydrolase[7a] and of an enoate reductase[7b] and in thermostabilization of a lipase,[7c] but it was not clear how general this approach is and especially how it compares with other directed evolution methods.

In order to subject ISM to rigorous comparison with other directed evolution approaches, and at the same time to extend its application, we revisited in the present study the previously most intensively investigated enzyme in this respect, namely the lipase from *Pseudomonas aeruginosa* (PAL) as a catalyst in the hydrolytic kinetic resolution of the chiral ester *rac*-**1**.[4h,9,11,12] As already delineated, previous efforts based on standard approaches using epPCR at different mutation rates, various forms of saturation mutagenesis, and DNA shuffling resulted in a selectivity factor of $E = 51$, the overall effort requiring the screening of about 50 000 transformants.[12] In sharp contrast, ISM as applied in the present study has provided a much better mutant having dramatically improved enantioselectivity ($E = 594$), while requiring a highly reduced screening effort of only 10 000 transformants. Deconvolution experiments showed that superfluous mutations do not occur when applying ISM, in contrast to the original method based on a combination of epPCR, saturation mutagenesis, and DNA shuffling approaches,[14b] and that the method is characterized by the occurrence of strong cooperative effects acting between point mutations and sets of mutations. We have observed this effect in a previous ISM study concerning the enhancement of enantioselectivity of an epoxide hydrolase,[17] and we conclude that positive epistasis of this kind is the best prerequisite for high-quality libraries in directed evolution. In the present case, additivity alone would not have resulted in a significant increase in stereoselectivity. Finally, the best mutant proved to be a viable catalyst in the hydrolysis of a set of bulky chiral esters not accepted by WT-PAL, enantioselectivity being respectable to excellent ($E = 12-106$). Thus, without performing any additional mutagenesis experiments, it is possible to "get more than what you screen for".[7b] Since we did not focus on substrate scope in the actual mutagenesis experiments, these findings should be considered as being fortuitous. However, ISM can, in fact, be applied in order to address the issue of substrate acceptance (rate) directly.[7b] Recently, this was demonstrated once more by Bäckvall and co-workers, who applied ISM in the quest to engineer enantioselectivity and substrate scope of lipase A from *Candida antarctica* (CALA).[21b]

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Table 4. List of Forward Primers

name of primer	sequence of primer	name of library/mutant generated
WT_LibA_NNK_F	CTGGCCCACGGC**NNKNNK**GGCTTCGACAACATC	library A of WT-PAL
WT_LibB_NNK_F	TCACTGGGCTCG**NNK**GAGTCG**NNK**AACAGCGAGGGTGCC	library B of WT-PAL
WT_LibC_NNK_F	GCCAACGACGGC**NNKNNK**GGCACCTGCAGTTCGC	library C of WT-PAL
1F8_LibA_DNT_F	CTGGCCCACGGC**DNTDNT**GGCTTCGACAAC	library A of mutant 1F8
1F8_LibC_DNT_F	CACCGCCAACGACGGC**DNTDNT**GGCACCTGCAGTTCG	library C of mutant 1F8
Met16Ala/Leu17Phe_F	TCACTGGGCTCGCTGGAGTCG**CTG**AACAGCGAGGGTGCC	mutant Met16Ala/Leu17Phe using plasmid of mutant 1B2
Met16Ala/Leu162Asn_F	CTGGCCCACGGCGCG**CTC**GGCTTCGACAACATC	mutant Met16Ala/Leu162Asn using plasmid of mutant 1B2
Leu17Phe/Leu162Asn_F	CTGGCCCACGGC**ATG**TTTGGCTTCGACAACATC	mutant Leu17Phe/Leu162Asn using plasmid of mutant 1B2

We conclude that ISM in the form of iterative CASTing is a viable method to address the challenging problem of narrow substrate scope and/or poor stereoselectivity of enzymes, traditional limitations that are often encountered in white biotechnology. When faced with the problem of how to group individual CAST residues into ISM sites, we recommend two- or three-residue sites in combination with a reduced amino acid alphabet, because this maximizes the probability of cooperative epistatic interactions along evolutionary pathways. Recently, other groups likewise confronted with the numbers problem in laboratory evolution[5d] have applied ISM in the form of CASTing successfully for a variety of purposes, including enhancing and inverting the enantioselectivity of the lipase from *Candida antarctica* A,[21] expanding substrate scope and enhancing enantioselectivity of an arylmalonate decarboxylase,[22] introducing promiscuous reactivity in an amidohydrolase with the evolution of phosphotriesterase activity,[23] inverting the stereoselectivity of the esterase from *Burkholderia gladioli*,[24] turning a cellobiose- into a lactose-phosphorylase,[25] altering coenzyme binding specificity of a xylose reductase (NADH versus NADPH),[26] and modulating lignin biosynthesis for better utilization of plants in paper-making, biofuel production, and agriculture.[27] Unfortunately, in those studies, comparisons with other methods of laboratory evolution were not made. Nevertheless, in all cases, only small libraries were needed. ISM is a knowledge-driven approach which requires structural data, be it for addressing stereoselectivity/substrate scope or thermostability. When such information is lacking, the limitation of ISM becomes apparent.

Finally, whereas CASTing[5d,7,8] constitutes systematization of saturation mutagenesis at sites aligning the binding pocket and is therefore based on previous work regarding focused libraries,[4h,10] it is also possible to choose alternative randomization sites rationally, e.g., at second-sphere residues[28] or at remote residues which can be expected to induce allosteric effects in the absence of effectors.[29] The utility of ISM in such cases has yet to be explored.

Experimental Section

General. All chiral *p*-nitrophenyl esters (**1**−**11**) were synthesized in our laboratory by the standard procedure.[8b]

Plasmid Preparation. Designed pUCPCL6AN plasmid, containing wild-type *LipA* gene of *Pseudomonas aeruginosa* PAO1,[13] is a source of expression of lipase in expression host lipase-negative *P. aeruginosa* PABST7.1. Plasmids isolated from *P. aeruginosa* PABST7.1 are unmethylated[30,31] and are transformed into chemical competent cells of *Escherichia coli* DH5α[32] for methylation. Plasmid isolated from *E. coli* is used as template for PCR, which can easily be digested by restriction endonuclease *Dpn*I (New England Biolabs) after PCR to remove the template DNA. QIAprep spin miniprep kit (Qiagen) was used for plasmid preparation from cells of *P. aeruginosa* PABST7.1 and *E. coli* DH5 α.

Culture Conditions of Bacterial Strains. LB agar and 2x Luria−Bertani (LB) broth supplemented with carbenicillin (100 μg/mL) and tetracycline (50 μg/mL) were used to grow the pUCPCL6AN containing *P. aeruginosa* PABST7.1, while LB agar and LB broth supplemented with only carbenicillin (100 μg/mL) were used to culture the pUCPCL6AN containing *E. coli* DH5α. *P. aeruginosa* PABST7.1 can grow in the presence of tetracycline-containing LB. The incubation temperature of *P. aeruginosa* PABST7.1 and *E. coli* DH5α on LB agar plate was 37 °C, while in broth it was 30 and 37 °C, respectively, with shaking at 250 rpm (for culture in 100−500 mL Erlenmeyer flask) or 800 rpm (for deep-well plates).

Library Generation, Storage, Screening, and Sequencing. Saturation mutagenesis libraries were created at sites A (Met16/Leu17), B (Leu159/Leu162), and C (Leu231/Val232) using forward primers (Table 4, see below) and a silent reverse primer (GGAGCCGGTGAGCGTGGGTCTCGCGG, 3750−3725 bp of pUCPCL6AN) according to an improved PCR-based method for creating saturation mutagenesis libraries.[33] PCR reactions were performed with KOD Hot Start polymerase (Novagen, USA) using these primers and WT-PAL plasmid (pUCPCL6AN isolated from *E. coli* DH5α) as the template DNA. PCR conditions used were 98 °C, 10 min; five cycles of (96 °C, 2 min; 60 °C, 1 min; 72 °C, 4 min) for the generation of megaprimer; 20 cycles of (98 °C, 1 min; 68 °C, 10 min); and final extension at 72 °C, 14 min. The PCR products were treated with *Dpn*I to digest the parent plasmid (2 h at 37 °C with 10 units of *Dpn*I × two times) and purified with PCR purification kit (QIAquick, Qiagen, Germany). The purified PCR product was electroporated into freshly prepared *P. aeruginosa* electrocompetent

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cells, made by the method of Enderle and Farwell,[34] and selected for antibiotics resistance on LB agar carbenicillin (100 μg/mL) and tetracycline (50 μg/mL) plates. Colonies appeared in 24−36 h and were picked by toothpicks, transferred to preculture media, 900 mL of 2x LB broth medium containing carbenicillin (100 μg/mL) and tetracycline (50 μg/mL) in a 96-square-well storage plate (capacity, 2.2 mL; Thermo Scientific, UK), and incubated at 30 °C under shaking of 800 rpm. Preculture and 70% glycerol were mixed in 1:1 volume to make 200 μL in a 96-round-well glycerol stock storage plate (NUNC; Thermo-Fisher Scientific, Denmark) and stored at −80 °C. A fresh 900 μL of 2x LB broth was inoculated from 25 μL preculture by replication program of liquid handling robotics (TECAN, Switzerland), allowed to grow at 30 °C for 5 h, induced by 0.1 mM isopropyl β-thiogalactopyranoside (IPTG), and allowed to express lipase for 5 h more. The plates were centrifuged at 4000 rpm for 60 min, and then the supernatant was used for lipase assay by UV/vis screening method[21] using (*S*)-**1** and (*R*)-**1** separately. Positive hits were sequenced by Eurofins Medigenomix GmbH, Germany.

Site-Specific Mutagenesis. The above-described PCR protocol, *Dpn*I digestion, transformation, and screening methods were used to generate mutants (Met16Ala/Leu17Phe, Met16Ala/Leu162Asn, and Leu17Phe/Leu162Asn) of the deconvolution study using the plasmid of mutant 1B2 (isolated from *E. coli* DH5alfa) as template DNA and three different forward primers (Table 4) along with reverse primer.

Kinetic Measurements. WT-PAL and mutants were purified using the reported method,[35] with minor modifications. The purity of the enzyme was tested by SDS−PAGE, and the concentration of the purified enzyme was estimated by the Bradford method (Bio-Rad protein assay kit). To access the enantioselectivity of WT-PAL and mutants, both enantiomers of *p*-nitrophenyl esters of **1**, **4**, **5**, and **6** were used separately at various concentration ranges, their hydrolysis was monitored at 25 °C in potassium phosphate buffer (50 mM, pH 7.5, 1.0% Triton X-100), and the values of K_m and k_{cat} were derived from the corresponding Michaelis−Menten plots. *E*-factors of WT-PAL and mutants were calculated as $(k_{cat}/K_m)_S/(k_{cat}/K_m)_R$ or $(k_{cat}/K_m)_R/(k_{cat}/K_m)_S$ (Tables 2 and 3).

Reaction and Analysis Conditions for Determining the Conversion and Enantioselectivity of Substrates *rac*-7−11. The reactions were performed as follows: Potassium phosphate buffer (750 μL, 50 mM, pH 7.5, 1.0% Triton X100) and substrates *rac*-**7**−**11** in acetonitrile (100 μL, 10 mg/mL) were added to the purified enzymes (150 μL equiv to 11−15 μg of protein).The reaction mixture was shaken at 800 rpm for 90 min at 30 °C and was then acidified by adding 10% HCl (20 μL). The acidified solution was extracted twice with 400 μL of methyl *tert*-butyl ether (MTBE). The extracted organic phase was collected in a vial and analyzed by HPLC. The analyses of the enantioselective conversion of substrates **7**−**11** were performed by using a Chiralpak AD-3 chiral column, 150 mm × 4.6 mm i.d. Conditions: mobile phase, *n*-heptane/2-propanole/TFA = 98:2:0.1; flow, 1 mL/min; temperature profile, 298 K; detection, UV at 254 nm. GC analysis was performed as described previously.[8b]

Molecular Modeling and MD Simulation. The molecular modeling suite YASARA-structure version 9.5.4[20] was employed, utilizing the AMBER03[36] force field for the protein and the general amber force field[37] (GAFF) for the ligands throughout this study. The partial charges for the ligands and the covalently bound tetrahedral intermediate were computed using the AM1/BCC procedure[38] as implemented in YASARA-structure.[20] The starting point for molecular modeling was the crystal structure of WT-PAL[13] (PDB 1ex9). The substrates (*R*)- and (*S*)-**1** were introduced as tetrahedral intermediates (oxyanions) covalently bound to catalytically active Ser82 γ-O.

All structures were solvated in a water box and neutralized.[39] The structures were initially minimized using steepest descent and the simulating annealing procedures. All mutations were introduced sequentially using YASARA-structure. MD simulations were carried out at 300 K over 2.5 ns in an NPT ensemble using PME.[40] The first 500 ps was treated as the equilibration phase. All simulations were performed individually for both enantiomers using various starting geometries and rotamers for the mutated residues. The 2.5 ns MD trajectories were sampled every 2.5 ps, resulting in 100 simulation frames per run, which were evaluated and additionally minimized to derive statistical averages and properties of the corresponding local minima. Finally, the energy of the bound tetrahedral intermediate was calculated for every simulation frame, and the distances for all hydrogen bonds stabilizing the oxyanion were also computed.

Acknowledgment. We thank Marcus Hermes for the synthesis of all chiral *p*-nitrophenyl esters (**1**, **4**−**11**), J. Rosentreter for GC analyses, and A. Deege for HPLC analyses. This work was financed by the DFG (Schwerpunkt 1170) and the FCI.

Supporting Information Available: Medium-throughput assay[11] for evaluating mutants as catalysts in the hydrolytic kinetic resolution of *rac*-**1**; complete refs 30 and 33. This material is available free of charge via the Internet at http://pubs.acs.org.

JA1030479

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