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Directed Evolution of Enantioselective Enzymes: An Unconventional Approach to Asymmetric Catalysis in Organic Chemistry

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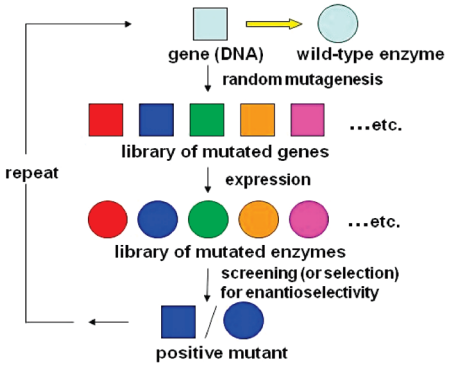

In the mid-1990s, I initiated in my group an unusual kind of project which is perhaps strange for a synthetic organic chemist who was otherwise focusing on the development of chemo-, regio-, and stereoselective organometallic reagents and catalysts. The basic idea was to imitate evolution in Nature in the quest to create enantioselective enzymes for application in synthetic organic chemistry. Rather than relying on design based on the assessment of steric and electronic effects (and on experience and serendipity), which was and still is the usual procedure in research regarding stereoselective transition-metal-based catalysis, the underlying idea was to harness in the laboratory the powerful driving force inherent in Darwinian principles comprising iterative cycles of mutation and selection. In this Perspective, composed on the occasion of the 2009 Arthur C. Cope Award address, I focus on the principles, successes, and future challenges of this unconventional approach to asymmetric catalysis.

1. Introduction

Following the announcement of the spectacular total synthesis of Vitamin B12 by Woodward, Eschenmoser, and co-workers more than three decades ago, some chemists and a few policy makers voiced the opinion that organic chemists were now in the position to synthesize any complex compound, and that the field of synthetic organic chemistry had reached a mature status not requiring any further methodology development. How wrong they were, then and today! By the mid-1990s, a number of truly efficient systems for asymmetric transition metal catalysis had been established, Sharpless oxidations and Noyori reductions being spearhead examples which inspired hundreds of other groups to join forces in these and related efforts. Therefore, the question in my group arose as to why one should attempt yet another completely unrelated approach. Apart from the philosophical side, the answer is obvious: There cannot be such a thing as a universal transition metal catalyst or organocatalyst, which means that the practicing organic chemist is in need of a wide (and ever expanding!) toolbox of catalysts, reagents, and methods, making ecologically and economically viable transformations possible. Thus, synthetic organic chemistry is not just a "service science" for other expanding disciplines, which is important in its own right, but occupies a central and prominent role in science of the 21st century.

Success in asymmetric catalysis depends on mechanistic and theoretical insight, intuition, experience, and perseverance (which is sometimes coupled with serendipity).[1] The new approach that I highlight here is different in nature, but it likewise entails a number of challenges. In 1994, a paper by Pim Stemmer appeared, describing DNA shuffling as a

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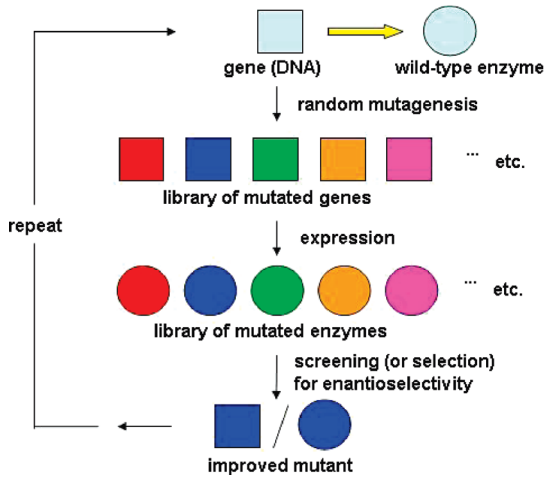


FIGURE 1. General scheme for the directed evolution of enantioselective enzymes.[6]

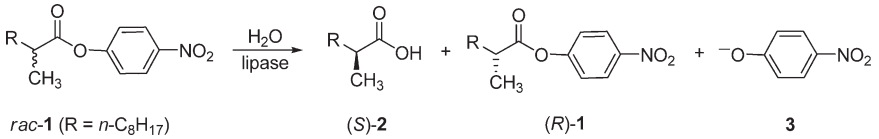
genetic method to increase the activity of a β-lactamase.[2] Crudely speaking, the method involves, as an example, the fragmentation of two homologous genes which are then reassembled enzymatically, a process that imitates sexual evolution in Nature. This seminal report inspired me to think about a fundamentally new approach to asymmetric catalysis, namely, the directed evolution of enantioselective enzymes. We first searched the literature regarding other gene mutagenesis methods available at the time, including error-prone polymerase chain reaction (epPCR) and saturation mutagenesis (see descriptions below).[3] These methods provide libraries of mutant enzymes, the size of which can be varied over a very large range (e.g., from a few hundred to millions). This in itself does not (yet) constitute an evolutionary process, until a given library has been screened for a given protein property (or selection is applied), and the gene of the best mutant (hit) is subjected once more to mutagenesis/screening (selection), thereby exerting evolutionary pressure. This was demonstrated by Hageman in 1986 using a certain type of saturation mutagenesis[4] and in 1993 by Arnold in a ground-breaking study based on several rounds of epPCR,[5] both studies focusing on the improvement of protein stability. Mutations were introduced which lead to new hydrogen bonds or salt bridges on the surface, acting like molecular clamps which stabilize the protein, among other forces. Being an organic chemist, I was interested in enantioselectivity and substrate acceptance, which I thought would be more related to Emil Fischer's lock-and-key principle (or Koshland's induced fit). My group was not certain how well-directed evolution would respond to stereoselectivity, but we nevertheless proposed the general scheme pictured in Figure 1.[6] Accordingly, one starts with a gene that encodes an enzyme showing poor enantioselectivity in a reaction of interest, subjects it to one of the available gene mutagenesis methods, inserts the gene library into a bacterial host, plates the host out on agar plates, and following expression and screening identifies the best enzyme mutant. In order to achieve the desired degree of enantioselectivity, as many cycles as necessary are transversed, each time using the newly mutated gene as a template. This scheme includes the possibility of choosing *R*- or *S*-selectivity on an optional basis, specifically by screening (or selecting) for the respective enantiomer.

Upon contemplating this scheme, one is perhaps tempted to believe that such an "evolutionary machine" must work, and that nothing can go wrong. This may well be true, but it is really a question of how much experimental effort is needed when attempting to reach a given goal. The state of affairs is similar to the situation in synthetic organic chemistry, as delineated above. Therefore, efficiency stands at the heart of research in directed evolution, which in turn relates to the problem of the (endless) protein sequence space. Consider, for example, a protein composed of 300 amino acids. If only one random amino acid exchange is strived for using the usual amino acid alphabet of 20 members, then application of a simple algorithm predicts 5700 different mutants, but 16 million in the case of two simultaneous substitutions and about 30 billion when three exchanges occur simultaneously.[3] Thus, the challenge inherent in putting the scheme in Figure 1 into practice revolves around two points: (1) choosing the appropriate gene mutagenesis method coupled with devising a strategy for its optimal use in probing protein sequence space as efficiently as possible, and (2) developing high-throughput ee-screening systems for the rapid evaluation of the enantiomeric purity of thousands of samples.[7] In this Perspective, I focus mainly on the former point by highlighting a few of our early and current directed evolution studies and conclude by suggesting future research directions. Comprehensive reviews have recently appeared elsewhere which include all of our work, including the development of high-throughput ee-screening systems, as well as the important contributions of other academic and industrial groups who have joined efforts in the area of directed evolution of enantioselective enzymes.[8]

2. Proof-of-Principle

When we began this project, the first goal was proof-of-principle.[6a] Since we had some experience with enzymes in a previous study regarding sol−gel immobilization of lipases,[9] I thought that this class of enzymes should be used in an exploratory study. Unfortunately, we were unable to obtain the necessary gene (plasmid) of a standard lipase such as CALB from industrial sources, and synthesis of the respective gene was expensive in those days. We also had to learn basic molecular biology and how to perform standard procedures such as PCR-based protocols in the laboratory (which organic chemists learn rapidly!). Fortunately, a colleague at the Biology Department of nearby Ruhr-Universität Bochum, Karl-Erich Jaeger, was willing to collaborate. We chose the enzyme that he had been working on for other reasons, namely, the lipase from *Pseudomonas aeruginosa* (PAL). PAL proved to be our "friend" for some years to come[10] because it served as a model enzyme for testing a variety of different mutagenesis strategies to this day, although it will probably never be used as a catalyst in practical terms due to several less-attractive traits relating to the expression system. The model reaction that we chose (Scheme 1) was the hydrolytic kinetic resolution of *rac*-2-methyldecanoic acid *p*-nitrophenyl ester (*rac*-**1**).[6] The wild-type (WT) PAL catalyzes this reaction with a very low preference for the formation of (*S*)-**2**.

SCHEME 1



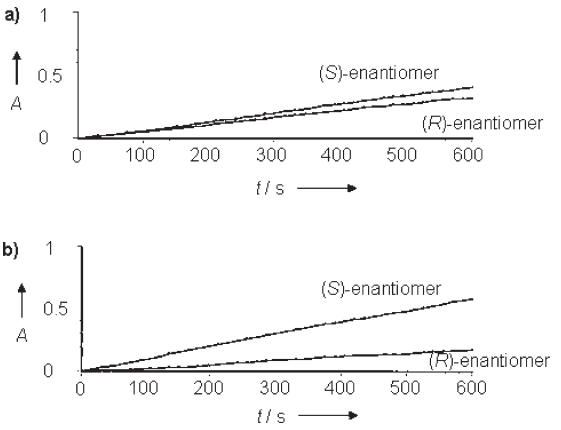


FIGURE 2. Medium-throughput assay for evaluating the enantioselectivity of the hydrolytic kinetic resolution of the lipase-catalyzed hydrolysis of *rac*-**1**.[6] (a) WT lipase from *P. aeruginosa* signaling low enantioselectivity; (b) improved mutant in the first generation indicating improved enantioselectivity.

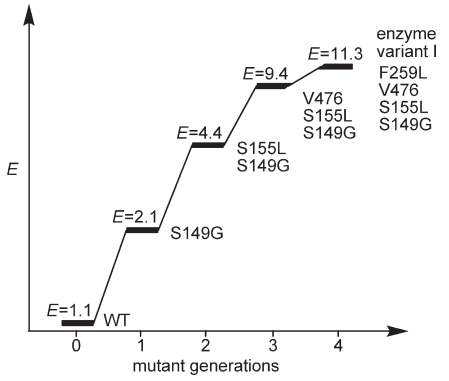


FIGURE 3. Enhanced *E* values of the PAL-catalyzed hydrolysis of *rac*-**1** by cumulative mutations introduced by four rounds of epPCR.[6a]

The first challenge was to implement a medium- or high-throughput screening system for identifying enantioselective PAL mutants, which was not trivial because in those days no such analytical procedures were known.[7] We finally solved the problem by devising the following scheme. Instead of using a racemate, we prepared (*R*)- and (*S*)-**1** by the Evans procedure and used them separately pairwise on 96-microtiter plates by adding a drop of the supernatant of each mutant and monitoring the initial rate of formation of the yellow-colored phenolate (**3**) at 410 nm by a UV/vis plate reader. Figure 2 shows the result of the WT PAL and of an improved mutant. The respective hit was then studied more closely as a catalyst in the actual hydrolytic kinetic resolution of *rac*-**1**, allowing for the determination of the respective selectivity factor *E*. Using this procedure, we were able to test about 400−500 transformants per day, which was later improved to a throughput of about 800 measurements per day. Subsequently, we developed other more efficient methods, including the Mülheim MS-based ee assay employing labeled substrates which in favorable cases allows up to 10 000 exact ee determinations per day.[11] It has been applied industrially in the directed evolution of an enantioselective nitrilase useful in the synthesis of a chiral intermediate needed for synthesizing the cholesterol-lowering therapeutic drug Lipitor.[12]

Upon going through four cycles of epPCR and screening about 2000−3000 mutants at each stage, enantioselectivity was enhanced stepwise, culminating in the best mutant (variant I) with an *E* value of 11 (Figure 3). Although enantioselectivity had not reached a practical degree, meaning a selectivity factor of at least 50, the results were published as proof-of-principle.[6a]

A fifth round of mutagenesis/expression/screening increased the *E* value to about 13, but it became clear to us that a different strategy had to be developed. We tested saturation mutagenesis at the sites where the mutations had occurred, speculating that they are "hot spots".[13] This type of gene mutagenesis means formation of so-called focused libraries formed by randomization at the chosen sites, that is, by the introduction of all of the other 19 possible proteinogenic amino acids.[3] The strategy proved to be successful at some, but not at all, hot spots. Saturation mutagenesis was also tested in a totally different sense, specifically at a sight comprising four amino acid positions next to the binding pocket as suggested by the X-ray structure, theoretically leading to $20^4 = 160\,000$ mutants.[13] Although we did not consider oversampling,[14] which would ensure on statistical grounds that all mutants had actually been evaluated, this focused library did indeed contain some highly improved hits. Nevertheless, at that time, we really "missed the boat" because no attempt at systematization around the complete binding pocket was attempted nor envisioned (see discussion below). DNA shuffling of the evolved mutants did not provide any improved mutants, until genes of mutants generated by epPCR at high mutation rate averaging three amino acid substitutions were shuffled. Finally, a combination of saturation mutagenesis at a previously identified site and DNA shuffling provided the most enantioselective mutant (variant J) displaying a selectivity factor of $E = 51$ in the model reaction, requiring about 50 000 transformants to be screened.[13] These efforts, summarized in Figure 4, consumed several years of research, and it set the stage for generalizing the concept of directed evolution of enantioselective enzymes.

Five of the six point mutations in the best mutant (variant X) proved to be on the surface of the enzyme, remote from the active center. The point mutation next to the binding pocket alone failed to induce high enantioselectivity. This came as a surprise because it was the first report of distal mutations influencing enantioselectivity, which is usually

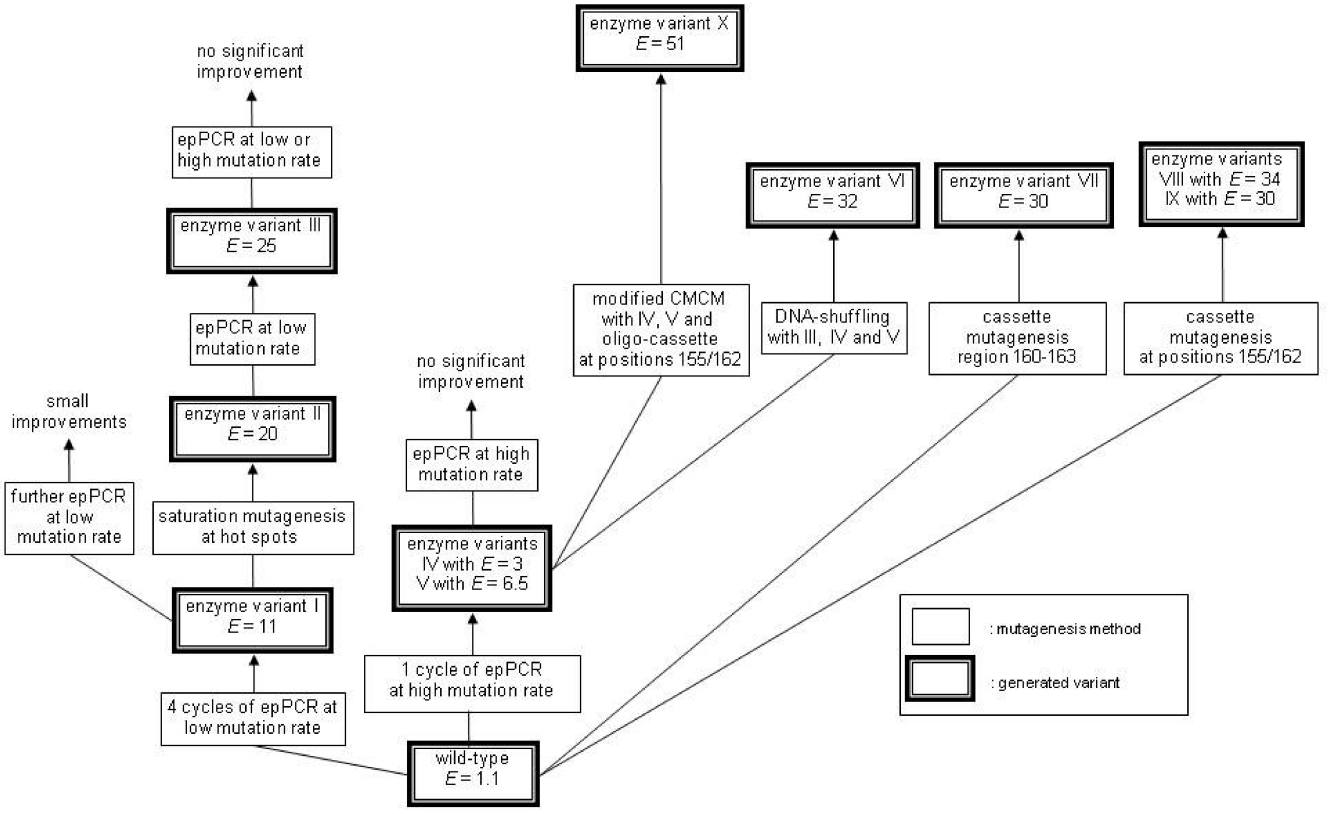


FIGURE 4. Schematic summary of the directed evolution of enantioselective lipase variants originating from the WT PAL used as catalysts in the hydrolytic kinetic resolution of ester *rac*-**1**.[13] CMCM = combinatorial multiple-cassette mutagenesis.

associated with the binding pocket. A detailed theoretical study based on MM/QM not only provided a viable explanation on a molecular level (relay effect) but also led to the prediction that only two of the mutations are actually necessary.[15] This prompted us to prepare the respective double mutant by conventional site-specific mutagenesis and to test it in the model reaction. Indeed, it turned out to be even better (E = 63), which is certainly a triumph of theory.[15b] Learning from directed evolution also deepens our knowledge of how enzymes function on a molecular level. However, the results were also disturbing because they signaled that our mutagenesis strategies, although successful, were far from optimal.

During the first half of the present decade, we nevertheless continued to apply the strategies outlined in Figure 4, among others in the directed evolution of enantioselective monooxygenases[16] (see section 3 below). Moreover, a number of other academic and industrial groups applied these and related protocols in order to evolve enantioselective enzymes for use in synthetic organic chemistry and in biotechnology.[8] These include not only further lipases but also stereoselective aldolases, monoamine oxidases, benzoylformate decarboxylases, halohydrin dehalogenases, phosphotriesterases, epoxide hydrolyses, nitrilases, hydantoinases, and esterases (the reader is referred to recent overviews).[8] Inspite of these successes, *it became clear that methodology development was necessary in order make possible fast and efficient directed evolution of enantioselective and thermostable enzymes*. Our own contributions in this important endeavor are outlined in section 4; others are covered by general reviews on directed evolution.[3]

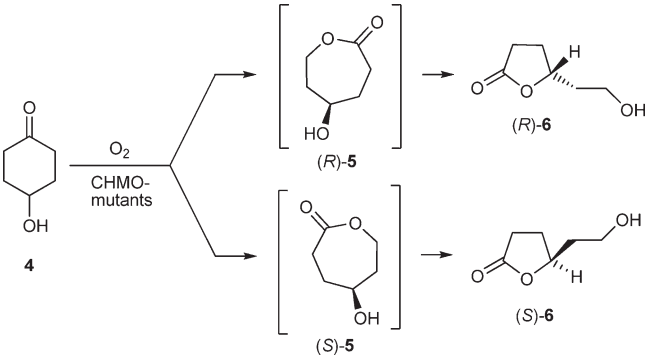
3. Initial Approaches to the Directed Evolution of Enantioselective Baeyer−Villigerases

An important partial oxidation reaction in synthetic organic chemistry is the Baeyer−Villiger (BV) reaction of ketones using stoichiometric amounts of H_2O_2, per-acids, or alkylhydroperoxides with formation of esters or lactones.[17] Acids, bases, and transition metal complexes are known to catalyze these C−C activating transformations, asymmetric versions being possible with chiral transition metal complexes[18] or organocatalysts.[19] However, acceptable degrees of enantioselectivity are possible only when strained cyclobutanone derivatives are used as starting materials for the reaction. In contrast, biocatalysis using flavin-dependent enzymes such as cyclohexanone monooxygenases (CHMOs) is considerably more attractive, excellent enantioselectivities being achieved with a fairly broad range of substrates undergoing oxidative desymmetrizaton or kinetic resolution.[20] However, limitations exist in that many potential substrates of interest are either not accepted or show poor enantioselectivity, in addition to other problems. In enzymatic BV reactions, dioxygen (air) reacts with the enzyme-bound flavin (FAD) in reduced form to provide an intermediate FAD-hydroperoxide, which in the deprotonated form adds nucleophilically to the ketone with formation of the Criegee intermediate followed by σ-bond migration.[21] One oxygen atom from O_2 is transferred to

the ketone, while the other one ends up in water. Therefore, the oxidized flavin has to be recycled by reduction induced by the cofactor NADPH. Although practical NADPH regeneration systems are available,[22] whole cells have been used traditionally in BV reactions.[20,21] The major reason for this choice has to do with the fact that such useful Baeyer–Villigerases as CHMOs or cyclopentanone monooxygenases (CPMOs) are not very stable, which makes the in vitro use of isolated enzymes difficult and far from practical. The inherent instability of many Baeyer–Villigerases may be the reason why these enzymes have not been exploited in industrial applications, even in whole cell systems, even though upscaling has been shown to be successful in several cases.[23]

Some years ago, we initiated a study regarding the directed evolution of enantioselective Baeyer–Villigerases, the CHMO from *Acinetobacter* sp. NCIMB serving as the enzyme and 4-hydroxycyclohexanone (**4**) as the model compound in a desymmetrization reaction (Scheme 2).[16a] In this case, the immediate product **5**, formed in the rate-determining step, rearranges to the thermodynamically more stable lactone **6**. The WT CHMO accepts this substrate, but enantioselectivity is poor (ee = 9% in favor of (*R*)-**6**).

SCHEME 2



At the time of the mutagenesis experiments, no structural information whatsoever was available of any Baeyer–Villigerase. Thus, the data coming from previous mechanistic studies[21] simply allowed us to formulate a general scheme as shown in Figure 5.

We first applied epPCR at medium error rate and screened 10 000 transformants, a process that led to the identification of improved *R*- and *S*-selective mutants (Table 1). Several of the improved *R*-selective mutants were then subjected to a second round of epPCR, which provided a variant showing an ee value of up to 90%.

Systematization in the form of further rounds of epPCR experiments was not strived for at this stage, but the *S*-selective mutant 1-K2-F5, characterized by a single point mutation, Phe432Ser, attracted our attention because it induced reversal of enantioselectivity (Table 1, last entry). We tested the mutant in the desymmetrization of a number of structurally different ketones and discovered that it is has a notably wide substrate scope (Table 2).[24] Thus, although mutant 1-K2-F5 was evolved for a single substrate, it is in fact an excellent catalyst for other structurally different compounds, as well. The traditional credo in directed evolution,[3] "you get what you screen for", can be extended by the corollary "*you may get more than what you screen for*." Indeed, this possibility has not been examined systematically in many directed evolution studies, yet it is important from the viewpoint of organic chemists. Along these lines, it is interesting to note that in a different project regarding CHMO as a catalyst in the sulfoxidation of prochiral thio-ethers such as **7** (Scheme 3) with formation of chiral sulfoxides such as **8** the screening process (by another co-worker) led once more to the identification of the same mutant 1-K2-F5! It leads to unusually high enantioselectivity (with ee >98% in favor of (*R*)-sulfoxides), while other mutants allow for complete reversal of enantioselectivity (ee >97% in favor of (*S*)-sulfoxides).[16b]

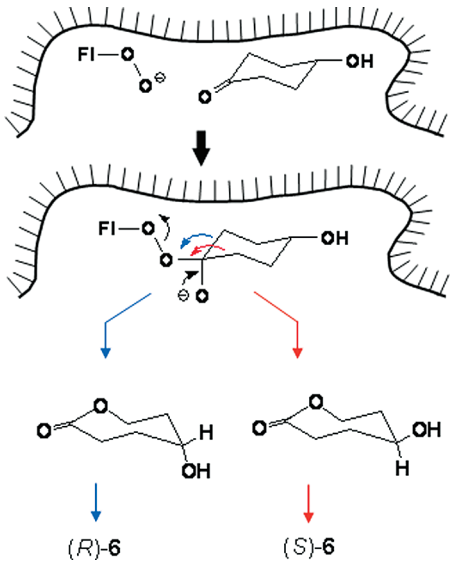


FIGURE 5. Scheme illustrating the CHMO-catalyzed Baeyer–Villiger reaction of 4-hydroxycyclohexanone (Fl = flavin).[16a] Migration of the enantiotopic σ-bond indicated by the blue arrow leads to (*R*)-**6**, whereas migration of the other σ-bond (red arrow) initiates the formation of (*S*)-**6**.

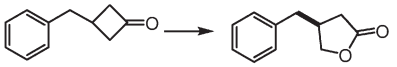
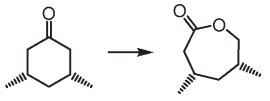
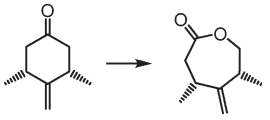
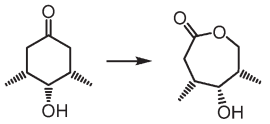
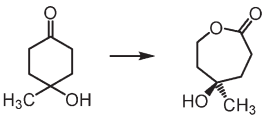
TABLE 1. Altered CHMO Mutants Identified in the First Round of epPCR (reaction time = 24 h; 23–25 °C; > 95% conversion)[a]

mutant	amino acid exchanges	favored enantiomer of **6**	ee (%)
wild-type (WT)		*R*	9
1-C2-B7	Phe432Tyr/Lys500Arg	*R*	34
1-F1-F5	Leu143Phe	*R*	40
1-E12-B5	Phe432Ile	*R*	49
1-H7-F4	Leu426Pro/Ala541Val	*R*	54
1-H3-C9	Leu220Gln/Pro428Ser, Thr433Ala	*S*	18
1-F4-B9	Asp41Asn/Phe505Tyr	*S*	46
1-K6-G2	Lys78Glu/Phe432Ser	*S*	78
1-K2-F5	Phe432Ser	*S*	79

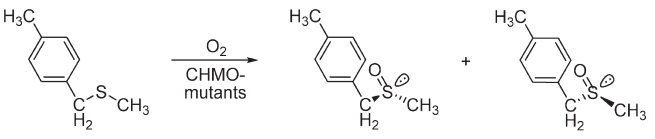
[a] In the second epPCR cycle using 1-K2-F5 (Phe432Ser), enantioselectivity rose to 90% ee.[16a]

This research calls for several additional comments. While yields and enantioselectivities are excellent, application of the evolved mutants requires the use of whole cells. Although whole cell technology is often preferred in industry,[25] and indeed such processes have been up-scaled for Baeyer–Villigerases,[23] organic chemists are generally not trained to perform such experiments, nor do normal

TABLE 2. Oxidative Desymmetrization Using the CHMO Mutant 1-K2-F5 (Phe432Ser)[24]

Substrate	ee (%)
	94
	99
	91
	97
	78
	96
	> 99
	> 99
	> 99
	99

SCHEME 3



7 (*R*)-**8** (ee = 98.7%) (*S*)-**8**

chemical laboratories contain the required special equipment. The logical way out of this dilemma would be an in vitro process, in which the isolated enzyme in conjunction with an NADPH regeneration system[22] is used. Unfortunately, this has not proven to be possible in practical teerms, simply because the enzymes in isolated form are too unstable. We were therefore struck by the announcement of Fraaije and Janssen that they had discovered, on the basis of genome mining, a thermostable Baeyer–Villigerase, which they dubbed phenylacetone monooxygenase (PAMO).[26] Unfortunately, only phenylacetone and a few other linear phenyl-substituted ketones react with reasonable rates. We therefore decided to embark on a project concerning protein engineering of PAMO, the goal being to widen substrate scope with concomitant control of enantioselectivity and retainment of robustness. Since the X-ray structure of PAMO had become available in 2005,[27] the first regarding a Baeyer–Villigerase, which also helped us to interpret our previous results of CHMO on the basis of a homology model (Figure 6), we first attempted "rational design". Mechanistically, Mattevi and Fraaije had postulated on the basis of the X-ray structure that Arg337 stabilizes the Criegee intermediate by hydrogen bonding.[27] Unlike CHMO (which has a reasonably broad substrate spectrum),[20] PAMO has an additional "bulge" in the loop aligning the active site, specifically the extra residues 441–443. We speculated that this makes the binding pocket smaller than in CHMO, thereby leading to a narrow substrate scope. Consequently, focused deletions were considered, induced by conventional site-specific mutagenesis.[28] Some of the mutants with truncated bulges displayed a slightly improved substrate scope, but only 2-phenylcyclohexanone derivatives were accepted. At that point, we shifted our efforts to methodology development, in general, and returned to the unsolved problem of narrow substrate acceptance at a later stage (section 4).

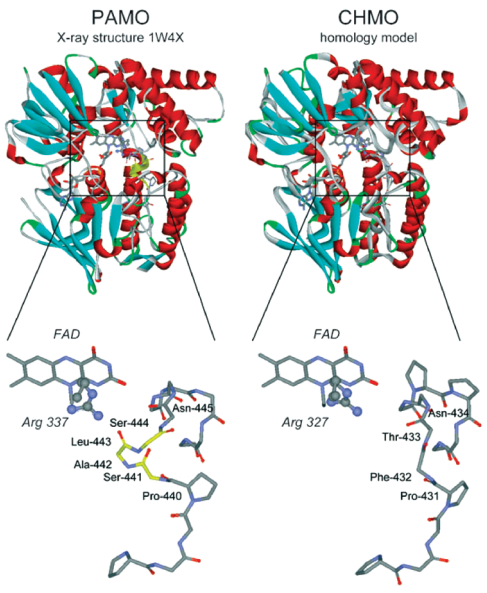


FIGURE 6. Comparison of the crystal structure 1W4X of PAMO[27] (left) and the homology model[28] of CHMO (right) with 40.3% sequence identity. The upper part shows the overall fold, and the lower part is a zoom into the active site showing the FAD cofactor as solid sticks and the catalytic arginine in ball and stick model. The yellow color highlights the presence of two additional amino acids in the arginine-stabilizing loop of PAMO compared with CHMO drawn as a backbone representation.

4. Methodology Development in Directed Evolution

By 2004, the concept of directed evolution of enantioselective enzymes appeared to be reasonably well-established, with all studies in our lab and in other groups relying on epPCR, DNA shuffling, and occasionally on saturation mutagenesis at select sites in the enzyme, as delineated above.[8] However, we were not fully content with the results because signs had appeared at various points of our research that suggested an inefficient character inherent in our mutagenesis strategies. Indeed, researchers active in the field of directed evolution, in general, were beginning to call for the development of methods that ensure library quality, not quantity.[29] We recently defined library quality in terms of the degree of enzyme improvement *and* frequency of hits.[14e] This means that quality needs to be viewed in terms of the screening effort, which is the traditional bottleneck in directed evolution.

Our contribution to methodology development in directed evolution is iterative saturation mutagenesis (ISM).[30,31] First, a criterion needs to be defined which allows the experimenter to make an appropriate choice as to the sites at which saturation mutagenesis is to be performed. The sites, designated as A, B, C, etc. can be composed of one, two, three, or more amino acid positions. Once this choice has been made, randomization using the QuikChange[32] protocol of Stratagene or some other saturation mutagenesis procedure such as our improved version[33] is performed with formation of the respective mutant libraries, which are subsequently screened for the catalytic property of interest (substrate acceptance, enantioselectivity, thermostability). The genes of the respective hits are then used as templates for saturation mutagenesis at the other sites. The general scheme of this strategy is shown in Figure 7, featuring the case of four sites A, B, C, and D in which each site is visited only once in the respective evolutionary process.[30,31] Our most recent work has shown that it is not necessary to explore all of the pathways.

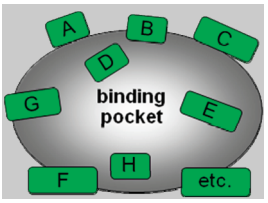


FIGURE 8. General scheme for CASTing. The sites A, B, C, etc. align the binding pocket and can be composed of one or more amino acid positions.

Crucial to success is the optimal choice of the randomization sites. When evolving substrate acceptance and/or enantioselectivity, the combinatorial active-site saturation test (CAST) was developed,[34] according to which all sites harboring amino acids next to the binding pocket are considered (Figure 8). This is simply a systematization of focused library generation, previously used by us[13] and subsequently by others[3,8] at select positions in the quest to enhance enantioselectivity. When evolving thermostability, we developed a different criterion. In this case, those sites that are characterized by the highest B factors are chosen (B-FIT method).[35] B-FIT has proven to be quite successful, leading to unprecedented degrees of thermostabilization, a method that has been highlighted elsewhere.[31,35b]

Sometimes the initial CASTing libraries already contain sufficiently improved variants,[34] but the real power of the approach lies in the iterative procedure. The first example of iterative CASTing concerns the hydrolytic kinetic resolution

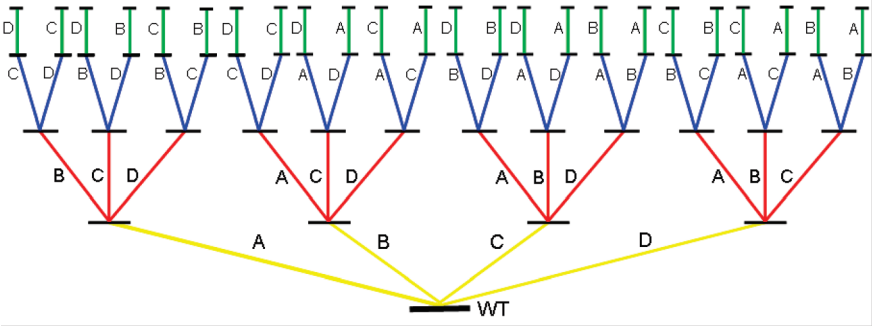
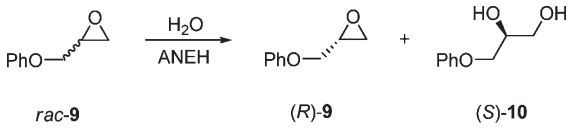


FIGURE 7. ISM employing four sites A, B, C, and D, each site in a given upward pathway being visited only once.[30,31]

of *rac*-**9** (Scheme 4) catalyzed by the epoxide hydrolase from *Aspergillus niger* (ANEH).[30] The WT ANEH displays a slight preference for (*S*)-**10** ($E = 4.6$).

SCHEME 4



rac-**9** (*R*)-**9** (*S*)-**10**

With the help of the X-ray structure of WT ANEH,[36] six CAST sites A, B, C, D, E, and F harboring either two or three amino acid positions were chosen for saturation mutagenesis using the usual NNK codon degeneracy[14e] encoding all 20 proteinogenic amino acids. The best hit was found to originate from the library generated at site B ($E = 14$), which was then used as the start of an otherwise arbitrarily chosen pathway B → C → D → F → E.[30] Following the five-step iterative procedure in which five *sets* of mutations accumulated, a highly enantioselective mutant LW202 was identified, showing a selectivity factor of $E = 115$. The five sets of mutations add up to nine amino acid exchanges (Figure 9).[30] The overall procedure required the screening of only 20 000 transformants, which happens to be about the same number required in our earlier epPCR-based study of the same enzyme and identical substrate, but which had provided a selectivity factor of only $E = 11$.[37] Thus, we concluded that iterative CASTing leads to the creation of "smart" libraries of mutants, which was later corroborated by other studies in our group and in other laboratories.[38]

These results inspired us to pose three questions: (1) What is the source of enhanced enantioselectivity on a molecular level? (2) Are there pathways other than B → C → D → F → E that also lead to highly enantioselective ANEH mutants? (3) Using the five sets of mutations observed experimentally, is it possible to construct a fitness landscape comprising 5! = 120 pathways leading from WT ANEH to the specific mutant LW202, and if so, how many of the 120 trajectories are energetically favorable in that no local minima occur? The

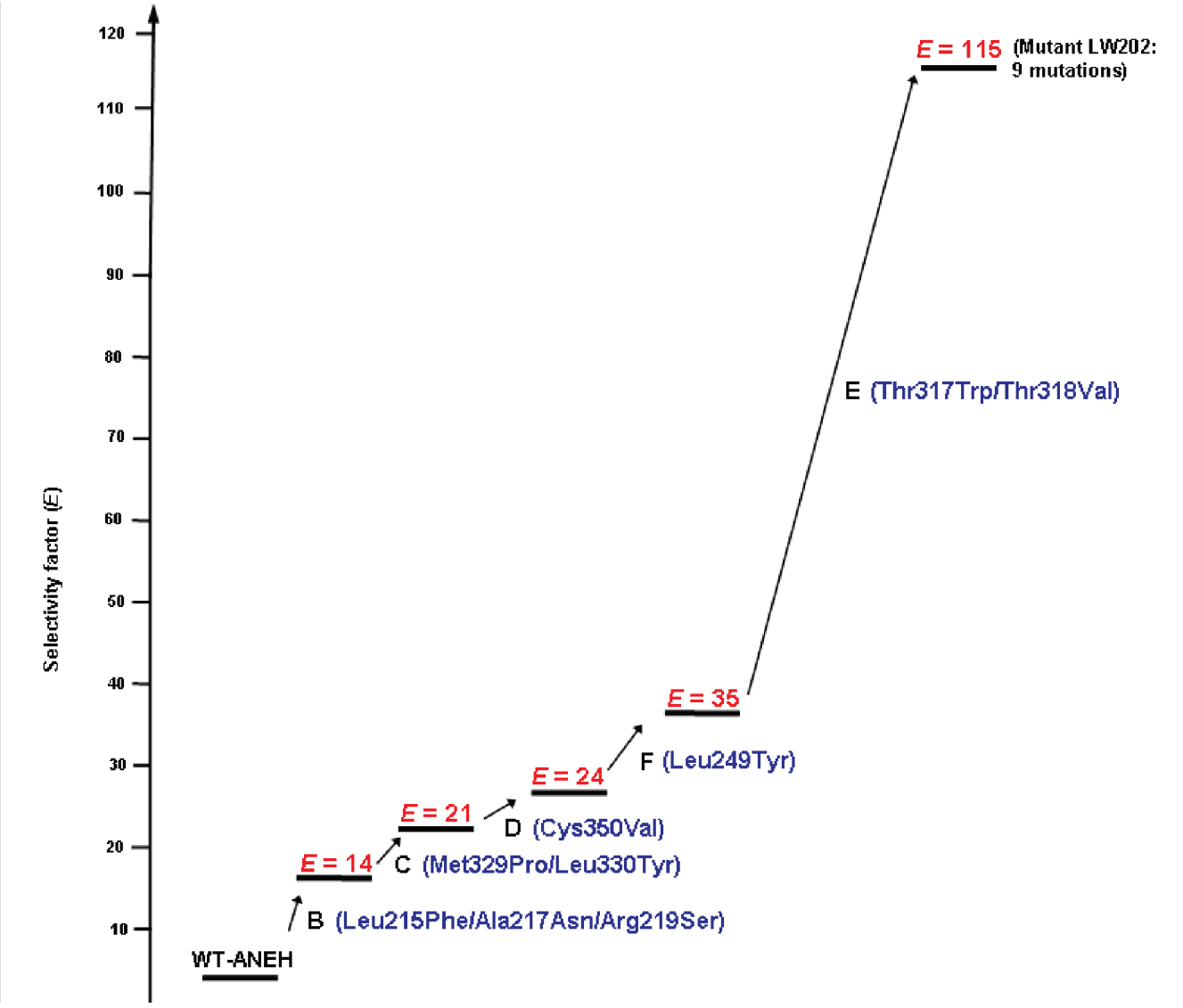


FIGURE 9. Iterative CASTing in the enhancement of enantioselectivity of the hydrolytic kinetic resolution of (*rac*)-**9** catalyzed by ANEH variants.[30]

last question has recently been answered by applying a special deconvolution strategy,[39] which led to the conclusion that about 50% of the 120 pathways in the restricted protein sequence space lead to mutant LW202, not just B → C → D → F → E! The experimentally constructed fitness landscape is shown in Figure 10. Moreover, it was possible to assess experimentally the epistatic interactions between the sets of mutations along all pathways in terms of additive, partially additive, cooperative, and antagonistic effects. The reader is referred to the original paper describing all details, which support the notion that iterative CASTing constitutes an unusually efficient strategy in directed evolution.[39] The answer to question (2) requires a different experimental platform since new amino acid exchanges are allowed, which is an ongoing project.

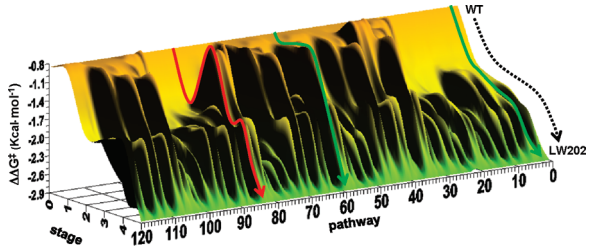


FIGURE 10. Energy profile of the two types of pathways leading from the WT to the mutant LW202: Energetically favored (green) as in the original B → C → D → F → E (pathway 2) or D → C → F → E → B (pathway 60) and disfavored (red) as in E → C → F → D → B (pathway 84).

The first question regarding the factors contributing to the dramatic enhancement of enantioselectivity has recently been treated in detail.[40] Kinetic data, molecular dynamics (MD) simulations, molecular modeling, inhibition experiments, and X-ray structural work uncovered the underlying factors at each evolutionary stage. Indeed, this is the first time that the X-ray structure of an enantioselective mutant generated by directed evolution has been obtained. Only the essential features of our model are reiterated here. The mechanism of ANEH catalysis was known to involve binding and activation of the epoxide by hydrogen bonding between two tyrosines and the epoxide O atom, followed by rate-determining nucleophilic attack by a nearby aspartate with formation of a covalent ester enzyme intermediate, which is hydrolyzed in a rapid second step.[41] Our mechanistic and structural studies unambiguously show that in the WT ANEH both enantiomers bind and react similarly, whereas in the case of the best mutant LW202, the rate of the disfavored enantiomer is reduced significantly.[40]

The reason for this becomes apparent upon comparing the X-ray structures of the WT ANEH[36] and that of LW202,[40] in conjunction with MD calculations and molecular modeling. *It is the first time that the 3D structure of an evolved enantioselective mutant enzyme has been obtained.* The X-ray structures of WT ANEH and LW202 have the identical fold and can hardly be distinguished, but the respective binding pockets are dramatically different. For the disfavored enantiomer, it is impossible for the substrate to bind and be activated by the two tyrosines *and* at the same time be positioned optimally for nucleophilic attack by the aspartate to occur. The distance between the nucleophilic aspartate O

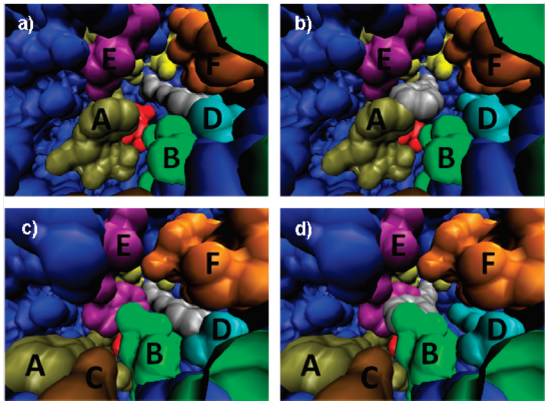


FIGURE 11. Docking of the substrate **9** (gray) in the binding pocket of WT ANEH and mutant LW202 determined by X-ray analysis (colored areas marked A−F refer to CAST sites). (a) Binding pocket of WT ANEH in which slightly favored (*S*)-**9** has been docked; (b) binding pocket of WT ANEH in which slightly disfavored (*R*)-**9** has been docked; (c) binding pocket of best mutant LW202 in which highly favored (*S*)-**9** has been docked; (d) binding pocket of best mutant LW202 in which the highly disfavored (*R*)-**9** has been docked; in all cases, the respective X-ray structural data were used.[40]

atom and the epoxide C atom is simply too long (5.8 Å) for smooth rate-determining reaction to occur. Figure 11 shows the results of the X-ray structure analyses of the WT ANEH and mutant LW202 in which (*R*)- and (*S*)-**9** have been modeled into the respective binding pockets.[40] It can be seen that not only are the shapes of the two binding pockets very different, but the disfavored (*R*)-**9**, forming H bonds with the two tyrosines, results in severe steric clashes (Figure 11d). This model not only illuminates the source of enhanced enantioselectivity on a molecular level but also deepens our understanding of the details of how the enzyme functions. Learning from directed evolution thus fulfills several purposes.[15,40]

Returning to the main subject of this section, methodology development in the form of ISM has proven to be unusually productive, delivering high-quality libraries in all cases tested so far. For example, the B-FIT method has been applied successfully to the thermostabilization of a lipase.[35] In other work, CASTing has been applied in the quest to increase the range of substrate acceptance and enantioselectivity of the thermostable Baeyer−Villigerase PAMO by a notable extent.[42] In unpublished work, which is particularly useful for comparing the "old" strategies that we used until about 2004 with the new ones based on ISM, we returned to our original project regarding the directed evolution of the lipase PAL and the hydrolytic kinetic resolution of the model compound *rac*-**1** (section 2). Using only two cycles of CASTing at two different positions in the enzyme, a mutant showing an *E* value of more than 500 was identified, although only 3400 transformants had to be screened![43] This compares well with the results of our earlier studies regarding the same system but employing epPCR and DNA shuffling, which required 50 000 transformants (reactions), leading to an *E* value of only 51. The dramatic difference in screening effort and experimental result speaks for ISM as a method which delivers truly high-quality mutant libraries. CASTing has also been utilized by us[34] and by others[38] to increase

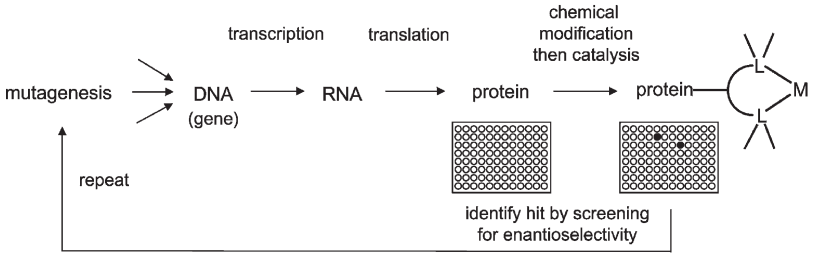


FIGURE 12. Concept of directed evolution of hybrid catalysts showing the flow of genetic information from the gene to transition metal hybrid catalysts.

enantioselectivity of other enzymes and of hybrid enzymes[44] (see section 5). The use of reduced amino acid alphabets[14e,45] adds yet another dimension to all procedures based on ISM because it provides a means to handle the numbers problem in directed evolution effectively.

5. The Concept of Directed Evolution of Hybrid Catalysts

In view of the many different classes of enzymes catalyzing a wide variety of reaction types,[25] directed evolution of enantioselectivity holds significant promises for synthetic organic chemistry.[8] Indeed, biocatalysts for enantioselective hydrolyses, oxidations, reductions, and C−C bond forming reactions have already been obtained in this way. However, enzymes cannot catalyze a huge number of highly useful synthetic transformations known to be possible by transition metal catalysis,[1] for example, hydroformylation, olefin metathesis, allylic substitution, etc. In order to address this issue, I proposed in 2001/2002 the concept of directed evolution of hybrid catalysts.[44,46] It was known since the early work of Whitesides, Kaiser, and others that it is possible to anchor a synthetic catalyst to the binding pocket of an enzyme (or in general to a protein).[47] This provides in each system a single catalyst because only the WT enzyme (protein) was used. The degree of enantioselectivity is then a matter of pure serendipity. However, catalyst tuning is possible by applying directed evolution, fully analogous to the normal forms of this type of protein engineering. Figure 12 illustrates the general concept.[46]

Putting the scheme shown in Figure 12 into practice involves challenges that I had originally underestimated. First, a very efficient expression system is required because more protein in each well of the microtiter plates is necessary than in conventional directed evolution. This is because enzymes are generally much more active than synthetic catalysts. Moreover, bioconjugation with introduction of the transition metal/ligand moieties has to be nearly quantitative, and a simple purification procedure has to be implemented. Nevertheless, this approach to asymmetric transition metal catalysis may prove to be of interest, especially if the experimenter succeeds in increasing the *rate* of the synthetic reaction considerably relative to the ligand/metal catalyst alone in the absence of the protein environment. Recalling Pauling's hypothesis that the transition states of enzyme-catalyzed reactions are stabilized by the protein environment by the additivity of many small interactions, and assuming a similar phenomenon for the hybrid catalysts, optimism is in order. Basically, there are three ways to anchor a metal, as illustrated in Figure 13. Covalent and noncovalent anchoring has

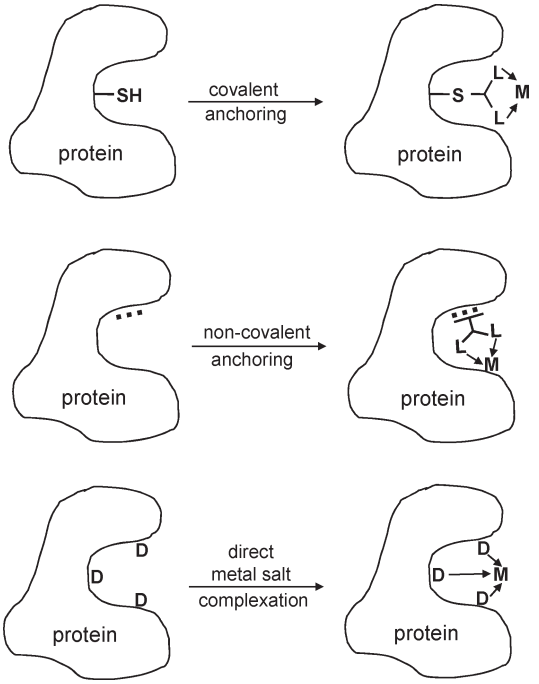


FIGURE 13. Three strategies for introducing transition metals (M) site-specifically in protein hosts.

been achieved in a number of earlier studies, and we have for the first time implemented directed evolution in the case of the noncovalent alternative.[44] It involves the Whitesides system of a biotinylated diphosphine/Rh complex anchoring to streptavidin,[48] a system in which we applied iterative CASTing leading in three steps to an improvement in the ee value of a hydrogenation reaction from 23 to 65% ee. However, this is only a proof-of-principle study, and no one in his right mind would want to utilize this system. Reactions other than the already excellent Noyori BINAP-based catalyst systems would be of greater interest. It should be mentioned that Ward has applied chemical optimization (spacer length between biotin and diphosphine ligand) as well as site-specific mutagenesis in order to obtain high enantioselectivity in the Whitesides system.[48c]

The third method outlined in Figure 13 concerns the design of a metal binding site in a protein using donor capacities of appropriate amino acids. This would circumvent bioconjugation. My group has recently designed such a binding site in a robust and thermostable protein, opening the way for further research. I admit that the hybrid catalyst concept is at a purely academic stage at this time.

6. Conclusions and Perspectives

Space does not permit our other past and present projects regarding the directed evolution of enantioselective enzymes as catalysts in organic chemistry to be highlighted here, nor can the contribution of other groups in this area be considered in detail. The reader is referred to recent reviews.[3,8] I conclude with the following general statements:

(a) Directed evolution of enantioselective enzymes constitutes a fundamentally new approach to asymmetric catalysis.

(b) Our early strategies based on epPCR, DNA shuffling, and select saturation mutagenesis proved to be successful in our lab and in other groups.

(c) The new method based on iterative saturation mutagenesis (ISM) in the embodiment of CASTing for broader substrate acceptance and/or enantioselectivity and B-FIT for protein thermostabilization allows for faster and more efficient directed evolution, provided structural data are available in this knowledge-driven approach.

A few additional comments are in order. The ISM-based toolbox, hopefully useful for molecular biologists, biotechnologists, or organic chemists interested in directed evolution, is actually more diverse than superficially apparent. Again, the crucial issue is the quality of mutant libraries because this relates to the screening problem which is the bottleneck of laboratory evolution. The quality of ISM libraries can be improved further by utilizing reduced amino acid libraries on the basis of appropriate codon degeneracies.[14e,31,42,45] For example, we have recently shown that the quality of two 5000-membered saturation mutagenesis libraries produced by considering NDT codon degeneracy (encoding 12 amino acids) versus the conventional NNK codon degeneracy (encoding all 20 amino acids) is quite different in terms of the frequency and quality of hits.[14e] The NDT library proved to be of dramatically higher quality. Along a different line, we have discovered that extending the concept of CASTing to include second sphere residues not directly aligning the binding pocket can be very rewarding.[49] Since the ISM approaches are knowledge-driven, which also defines the limitation of the respective strategies, the question arises whether computational tools can be added. One possibility is to utilize genetic algorithms in guiding directed evolution, thereby reducing the screening effort.[50]

In addition to developing further ways to probe protein sequence space efficiently with formation of higher-quality libraries, as in ISM applications, the development of better screening (or selection) systems constitutes another important endeavor. Multiplexing GC and HPLC as described recently by Trapp promises to revolutionize chromatographic procedures because it allows for a considerably higher throughput.[51] Implementing this technology in directed evolution is a current project in collaboration with Oliver Trapp. Taken together with improved mutagenesis strategies, my hope is that future directed evolution studies will not only proceed considerably faster than in the past, the usual necessity to invest time in devising screening systems for each new project and in acquiring expensive robotic equipment to run the respective assays will also no longer pertain. Along a different line, the development of selection instead of screening systems for enantioselectivity remains a challenge, inspite of some progress in this fascinating research area.[52]

The control of stereoselectivity by directed evolution may find applications in areas beyond the creation of enzymes as catalysts in synthetic organic chemistry. Pathway engineering as a method to exploit the cell as a "factory" for producing chiral natural products, already a complementary approach to modern natural products synthesis,[53] may profit from the methods that have been developed in directed evolution. Perhaps in the future it will be possible to alter the stereochemistry of such biochemical transformations in a practical manner useful for organic chemistry.

Acknowledgment. I wish to thank all of my present and past co-workers active in the area of directed evolution. Without their enthusiasm and creativity, we would not be where we are today. Thanks is due to Jürgen Diemer for providing the art work shown on the cover. I also thank the Max-Planck-Society, the Deutsche Forschungsgemeinschaft, the EU, the FCI, and Bayer AG for financial support.

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