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Application of directed evolution in the development of enantioselective enzymes*

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Abstract: A novel approach to developing enantioselective enzymes for use in organic chemistry has been devised which is independent of structural or mechanistic aspects. The underlying idea is to combine appropriate methods of random mutagenesis, gene expression, and high-throughput screening for enantioselectivity. If these actions are performed in repetitive cycles, an evolutionary pressure is created that leads to sequential improvements of the enantioselectivity of a given enzyme-catalyzed reaction. The concept is illustrated by an example involving the lipase-catalyzed hydrolytic kinetic resolution of an α-chiral ester, the enantioselectivity increasing from *ee* = 2% (*E* = 1.1) for a wild-type enzyme to *ee* = 90–93% (*E* = 25) for the best mutants.

INTRODUCTION

The stereoselective synthesis of chiral organic compounds is of immense academic and industrial interest [1–4]. It has been estimated that the current world market for chiral fine chemicals such as pharmaceutical or plant protecting agents exceeds USD 80 billion [1]. Asymmetric catalysis is the most efficient way to prepare enantiomerically pure or enriched organic compounds. In doing so, organic chemists have two options, namely to use 1) transition-metal catalysts or 2) biocatalysts. How does one obtain such chiral catalysts? It is accepted that in the case of the development of chiral transition-metal catalysts, which generally involves ligand tuning (Fig. 1a) [2], several factors are crucial to success, namely intuition, experience- and structural information, as well as knowledge of the mechanism of reaction and of the kinetics. Unfortunately, a great deal of trial and error is also necessary [2]. This latter aspect becomes apparent when remembering that more than 2000 chiral phosphorus-containing ligands have been prepared one by one, yet only a handful are highly enantioselective for specific metal-catalyzed reactions and substrates. In the case of biocatalysis [3], the user also insists on high activity and enantioselectivity, which is the reason why enzymes are generally chosen and not other types of biocatalysts. Biologists continue to isolate new enzymes, thereby providing the interested organic chemist with the opportunity to test these biocatalysts with a variety of substrates. High enantiomeric excesses (*ee*) have been observed in a number of cases, and industry is beginning to consider enzyme catalysis more so than in the past [4] in spite of some difficulties [5]. Progress in biotechnological engineering during the last decade has contributed to this development. Nevertheless, one of the problems associated with biocatalysis is the fact that the enzymes are substrate-specific, which means that for a given reaction of interest enantioselectivity may be unacceptably low. *De novo* design in the form of site-specific mutagenesis (Fig. 1b) [6], which can be considered to be analogous to ligand tuning in asymmetric transition-metal catalysis, has not proven to be an easy and generally successful tool in the difficult endeavour of developing enantioselective enzymes for use in organic chemistry.

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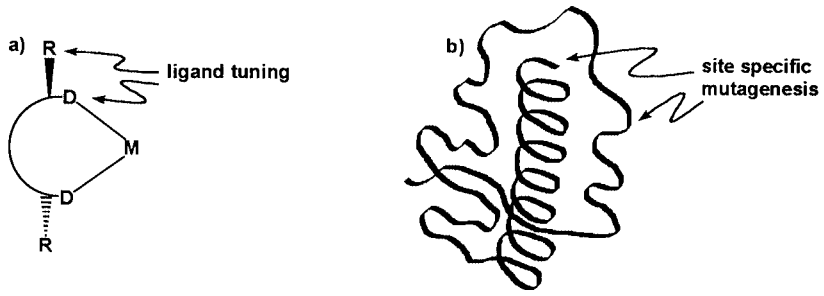


Fig. 1 a) Schematic representation of ligand tuning in the design of a chiral transition-metal (M) catalyst, C_2-symmetry arbitrarily being shown; the arrows symbolize points of potential structural variation and D denote donor atoms. b) Schematic representation of "*de novo* design" of an enantioselective enzyme, the arrows symbolizing the exchange of amino acids on the basis of site specific mutagenesis.

In spite of many difficulties it is important that research in the area of enantioselective transition-metal catalysis and traditional enzyme catalysis for use in organic chemistry be continued. Nevertheless, we have recently developed a radically different approach, which is the subject of this review.

EVOLUTION IN THE TEST TUBE

Evolution is at the heart of biology. It therefore seemed worthwhile to try to simulate the basic principles of evolution in the quest to create enantioselective catalysts in the laboratory. In collaboration with biologist K.-E. Jaeger we have devised an approach to the development of chiral catalysts for use in organic synthesis which is independent of structural or mechanistic factors (Fig. 2) [7].

The underlying concept is to employ "evolution in the test tube" (*in vitro* evolution) [7] which had previously been used to improve the thermal stability and activity of enzymes [8–10]. We speculated that if the enantioselectivity (% *ee*) of a reaction of interest, A→B, is poor using a given enzyme isolated in nature (wild-type), then directed evolution can be used to enhance stereoselectivity to an acceptable level. In the worst case the *ee*-value is near zero for the wild-type enzyme. As a first step in the overall process, the natural gene (DNA segment) which encodes for the particular enzyme is subjected to random mutagenesis such as error-prone polymerase chain reaction (ep PCR) [8,9], DNA shuffling

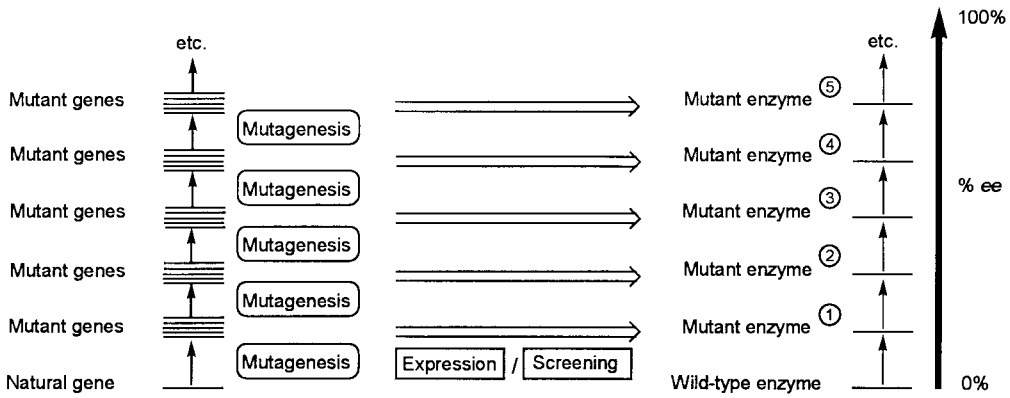
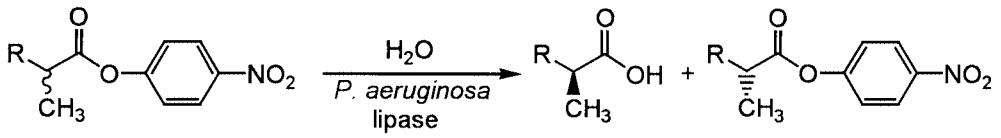


Fig. 2 Evolution in the test tube as a means to create enantioselective enzymes.

[10], or other modern methods [11]. The library of mutant genes is then inserted into a suitable bacterial host (expression system). With the help of a robotic system the bacterial colonies are finally transferred into the wells of microtiter plates (e.g., 96-format), and following the addition of nutrient broth the individual bacterial colonies are allowed to grow, each producing a mutant enzyme in the respective supernatant. Using robotics and the proper screening system, the best mutant displaying the highest enantioselectivity is identified [7]. At this stage the process has the character of combinatorial asymmetric catalysis, similar to research in transition-metal catalysis [12]. However, then the decisive step is undertaken. The corresponding mutant gene is subjected to another round of random mutagenesis, and the process is repeated as often as necessary. Thus, the idea of repetitive cycles of random mutagenesis goes far beyond combinatorial catalysis due to the evolutionary character. The inferior enzymes and genes are discarded, and the genetic information of the best gene encoding for the most enantioselective enzyme is passed onto the next generation. Thus, the overall process can be viewed as directed evolution of enantioselective enzymes. Depending upon whether one screens for (*R*)- or (*S*)-selective variants of a particular enzyme, the direction (sense) of enantioselectivity can, in principle, be stipulated by the experimentator.

FIRST CASE OF DIRECTED EVOLUTION OF AN ENANTIOSELECTIVE ENZYME

We initially studied the kinetic resolution [13] of the lipase-catalyzed hydrolysis of the chiral ester **1** in which a maximum of 50% conversion is aimed for [7]. Lipases are enzymes that catalyze the hydrolysis of esters [14], the reverse reaction in organic solvents also being possible [15]. The particular enzyme used in our case was the bacterial lipase from *Pseudomonas aeruginosa*, which showed an *ee*-value of only 5% in favor of the (*S*)-acid **2** at 50% conversion.



rac-**1** (R = *n*-C_8H_{17}) **2** (*R*)-**1**

The first step in directed evolution is the consideration of the mutagenesis frequency, which has to do with the problem of exploring protein sequence space. The lipase from *Pseudomonas aeruginosa* has 285 amino acids [14]. Complete randomization would result in 20^{285} different mutant enzymes, which is more than the mass of the universe, even if only one molecule of each enzyme were to be produced [7,11,16]. The other extreme entails the minimum amount of structural change, namely the substitution of a single amino acid per molecule of enzyme by one of the other 19 naturally occurring amino acids. In this case, the library of mutants would theoretically have 5415 members [7,11]. However, when using epPCR as the random mutagenesis method, a library of 5000–6000 members is not expected to contain all theoretically possible permutations [16]. This is because the genetic code is degenerate. If two amino acids are exchanged per enzyme molecule ($M = 2$), then the number of mutant enzymes increases dramatically (about 14 million!) [7,11,16]. In the case of $M = 3$, it is more than 52 billion.

$N = 19\text{M} \cdot 285!/[(285 - M)! \cdot M!] = 5415$, where M = number of amino acid substitutions per enzyme molecule.

We therefore initially chose a low mutagenesis rate so as to induce an average of only one amino acid exchange per enzyme molecule. Indeed, our experience in this area has shown that such relatively low mutagenesis frequencies constitute a successful strategy. Thus, in the case of the kinetic resolution

of the ester **1**, epPCR was adjusted to cause about 1–2 base substitutions per 1000 base pairs of the gene, resulting in an average of one amino acid exchange. Typically, 2000–3000 mutants per generation were screened [7]. Following expression in *E. coli*/*P. aeruginosa*, a screening system based on the UV/Vis absorption of the liberated *p*-nitrophenolate at 410 nm was employed. In doing so, a racemate was not used as is customary in a preparative-scale kinetic resolution. Rather, the experiments on a 96-well microtiter plate were performed in such a way that the optically active (*R*)- and (*S*)-esters were allowed to react pairwise separately, allowing 48 mutant enzymes to be screened within a few minutes. The approximate relative rates of the two reactions as a function of the particular mutant enzyme is thus readily obtained, allowing the identification of the most enantioselective mutant.

In each round of mutagenesis, about 2000 clones were screened, and several improved variants were consistently detected, which were then studied in lab-scale reactions in order to obtain exact *ee*-values and data for the selectivity factor *E* [7] (sometimes designated as the *s*-factor [13]). Originally, about 400 mutant enzymes could be screened per day [7], but it was later possible to assay 1000–1200 variants using the same screening system based on robotics [16]. As a consequence of the first round of mutagenesis and screening, a mutant displaying an *ee*-value of 31% was identified ($E = 2.1$). The corresponding mutant gene was then subjected once more to mutagenesis, and the process was repeated several times. The results after four generations of mutants led to an *ee*-value of 81%, the selectivity factor being $E = 11.3$ (Fig. 3).

These remarkable results constitute proof of principle. Nevertheless, a selectivity factor of $E = 11.3$ cannot be viewed as industrially viable. Thus, a fifth round of mutagenesis was performed, and indeed the usual library of about 2000 mutants contained slightly improved variants. In spite of this advancement it became clear to us that we needed to develop methods that allow for even more efficient ways to explore protein sequence space with respect to enantioselectivity [16,17]. Accordingly, amino acid sequence determinations of the mutants were carried out as a first step. For example, the best mutants of the first three generations, namely P1B01-E4, P2B08-H3, and P3B13-D10 turned out to have the following amino acid substitutions (Fig. 4).

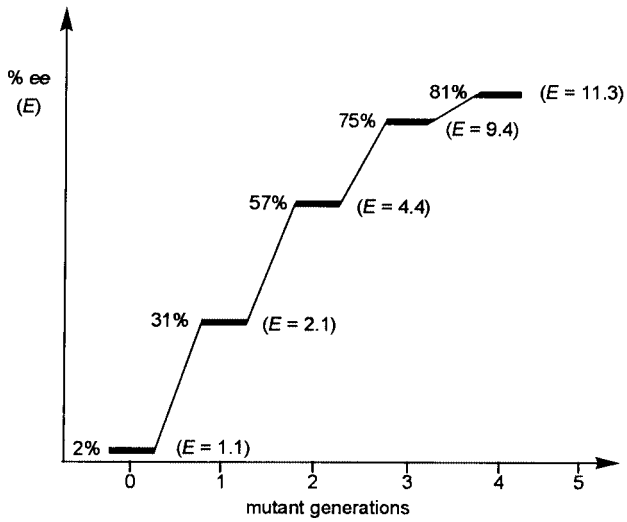


Fig. 3 Increasing the enantioselectivity of the lipase-catalyzed hydrolysis of the model ester **1**.

Best mutant of the first generation (E = 2.1):

P1B01-E4: S149G ($Ser_{149}\rightarrow Gly_{149}$)

Best mutant of the second generation (E = 4.4):

P2B08-H3: S149G ($Ser_{149}\rightarrow Gly_{149}$), S155L ($Ser_{155}\rightarrow Leu_{155}$)

Best mutant of the third generation (E = 9.4):

P3B13-D10: S149G($Ser_{149}\rightarrow Gly_{149}$), S155L ($Ser_{155}\rightarrow Leu_{155}$), V47G ($Val_{47}\rightarrow Gly_{47}$)

Fig. 4 Amino acid substitutions in the first three rounds of mutagenesis (best mutant enzymes in each case).

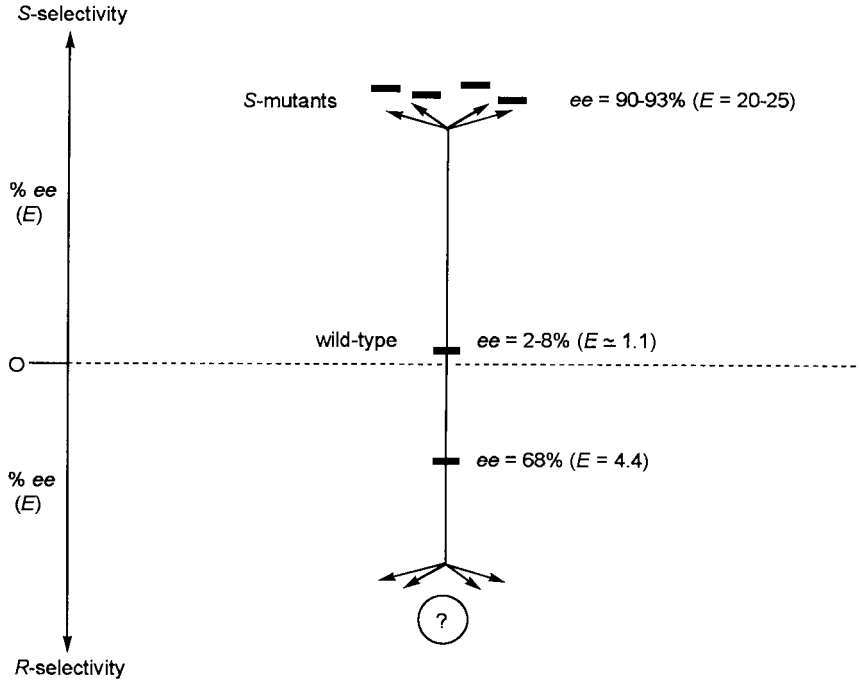


Fig. 5 Optional (*S*)- or (*R*)-selectivity in the lipase-catalyzed reaction of ester **1**.

At this stage it is tempting to utilize this type to data in a conventional way, i.e., to define structure/selectivity relations and to continue with site-directed mutagenesis. However, we chose to use the data in a completely different way, specifically with the aim of developing an efficient method to explore protein sequence space. Thus, we drew the following conclusions [16,17]:

1. The process of random mutagenesis/screening identifies sensitive positions ("hot spots") in the enzyme which are responsible for improved enantioselectivity.
2. Such positions are correct, but the particular amino acid identified may not be optimal.
3. Saturation mutagenesis at the hot spots can be expected to generate improved mutants.

Rather than continuing with epPCR in further cycles of random mutagenesis, we decided to utilize appropriate combinations of various types of mutagenesis [16,17]. Saturation mutagenesis is a molecular biological method with which mutations at a given position of an enzyme can be introduced

[11], a small library of only 300–400 mutants being necessary to ensure that all of the remaining 19 amino acids have been introduced. Upon applying this strategy at one of the hot spots (e.g., at position 155), it was discovered that phenylalanine (F) is the best amino acid (i.e., mutants having phenylalanine at position 155 lead to the highest degrees of enantioselectivity). For example, saturation mutagenesis using the best gene in the third generation led to the identification of a mutant enzyme which showed a selectivity factor of $E = 20$. Thereafter, epPCR was applied again, which resulted in $E = 25$! Clearly, the combination of mutagenesis methods, namely epPCR and saturation mutagenesis, constitutes an efficient method to explore protein sequence space with respect to enantioselectivity. Similar experiments using other mutants and positions were also successful. Thus, a small family of enzymes was created, all showing *E*-values of 20–25 and *ee*-values of 90–93% for the model reaction [16,17]. These efforts are ongoing.

A different aspect concerns higher mutagenesis frequency. We discovered that upon doubling the mutagenesis frequency, so as to cause an average of two mutations per enzyme molecule, even better results are obtained. In the first round of mutagenesis a mutant was found displaying an *ee*-value of 68% ($E = 4$) [18]. It will be interesting to see if this change in strategy is always the method of choice. Moreover, it was possible to invert the sense of enantioselectivity of the model reaction. In just two rounds of mutagenesis, an *R*-selective mutant was obtained ($E = 4.4$; *ee* = 68%). Efforts are continuing to increase *R*-selectivity even more. At present these studies can be summarized by Fig. 5.

STRUCTURE/SELECTIVITY RELATIONS

The method we describe here is a rational way to create enantioselective enzymes for use in organic chemistry, specifically because it does not rely on uncertain predictions arising from some imperfect theory. Rather than going the conventional way, namely theoretical prediction/site specific mutagenesis/enantioselective reaction, in a sense we reverse the process. By means of random mutagenesis/screening, enantioselective enzymes are produced that are then sequenced in order to define the structural changes responsible for improved enantioselectivity, and on the basis of this data an understanding of structure/selectivity evolves. This is a powerful method to learn how enzymes function, especially in view of the fact that enantioselectivity is an extremely sensitive probe.

In the present case, this type of study has not been completed, but already some remarkable features are emerging [17]. All of the sensitive positions (hot spots) occur in flexible regions (loops), generally on the surface of the enzyme and not near the catalytically active center. Many of the newly introduced amino acids are glycine, which suggests increased conformational flexibility. Although remote amino acid substitutions have been shown previously to affect stability and activity [9], this is the first time that enantioselectivity has been demonstrated to be governed by amino acid substitutions far removed from the catalytically active center. It appears that there is an optimum with respect to conformational flexibility in the quest to create the most enantioselective enzymes.

CONCLUSIONS

Directed evolution of enantioselective enzymes constitutes a radically new approach to the development of chiral catalysts for use in organic chemistry. Following our initial studies, several other applications of this concept have been described in the literature [19]. It is independent of structural or mechanistic aspects. Of course, once highly enantioselective enzymes are evolved, it is of great interest to characterize them structurally in hope of illuminating the source of enantioselectivity.

The evolutionary approach entails other types of challenges as well, including such questions as 1) how to explore protein space more efficiently with respect to enantioselectivity, 2) how to develop further high-throughput screening or selection systems [20,21], and 3) how to apply these ideas to other enzymes and substrates. If these questions can be answered satisfactorily, then a general and truly

rational way to create enantioselective catalysts to fit the needs of organic chemists will emerge. We hope that more research groups will join this quest.

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