

Improved biocatalysts by directed evolution and rational protein design

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The efficient application of biocatalysts requires the availability of suitable enzymes with high activity and stability under process conditions, desired substrate selectivity and high enantioselectivity. However, wild-type enzymes often need to be optimized to fulfill these requirements. Two rather contradictory tools can be used on a molecular level to create tailor-made biocatalysts: directed evolution and rational protein design.

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Abbreviations

E	enantioselectivity
ee	enantiomeric excess
epPCR	error-prone PCR
IGPS	indole-3-glycerol phosphate synthase
PCR	polymerase chain reaction
PRAI	phosphoribosylanthranilate isomerase
SDM	site-directed mutagenesis

Introduction

The application of enzymes — especially in organic synthesis — is now well documented in the literature [1*,2–4]. In the past decade, a considerable number of processes have been commercialized in industry [5*]. Characteristic features of many biocatalysts, such as high chemo-, regio- and stereoselectivity at ambient temperatures, often makes them superior to chemical catalysts. In addition, recent progress in genetic-manipulation techniques enables the large-scale supply of many enzymes at reasonable prices. However, identification of new biocatalysts (for example, by screening of soil samples or strain collections by enrichment cultures) does not always yield suitable enzymes for a given synthetic problem. To overcome this limitation, tailor-made biocatalysts can be created from wild-type enzymes by protein engineering using computer-aided molecular modeling and site-directed mutagenesis, or by directed (molecular) evolution techniques (Figure 1; see also Update).

Rational design usually requires both the availability of the structure of the enzyme and knowledge about the relationships between sequence, structure and mechanism/function, and is therefore very information-intensive. On the other hand, rapid progress in solving protein structures by NMR spectroscopy instead of by X-ray diffraction of crystals and

the enormously increasing number of sequences stored in public data bases have significantly eased access to data and structures. Using molecular modeling, it has been possible to predict how to increase the selectivity, activity and the stability of enzymes, even if there are no structural data available and the structure of a homologous enzyme is used as a model. For details concerning the potential of this method, readers are referred to a recent review [6*]. Depending on the purpose of the mutagenesis, amino acid substitutions are often selected by sequence comparison with homologous sequences. The results have to be carefully interpreted, however, because minor sequence changes by a single point-mutation may cause significant structural disturbance. Thus, comparison of the three-dimensional structures of mutant and wild-type enzymes are necessary to ensure that a single mutation is really site-directed.

In sharp contrast, directed evolution (also called evolutive biotechnology or molecular evolution [7–12]; see also Update) involves either a random mutagenesis of the gene encoding the catalyst (e.g. by error-prone PCR) or recombination of gene fragments (e.g. derived from DNase degradation, the staggered extension process or random priming recombination). Libraries thus created are then usually assayed using high-throughput technologies to identify improved variants.

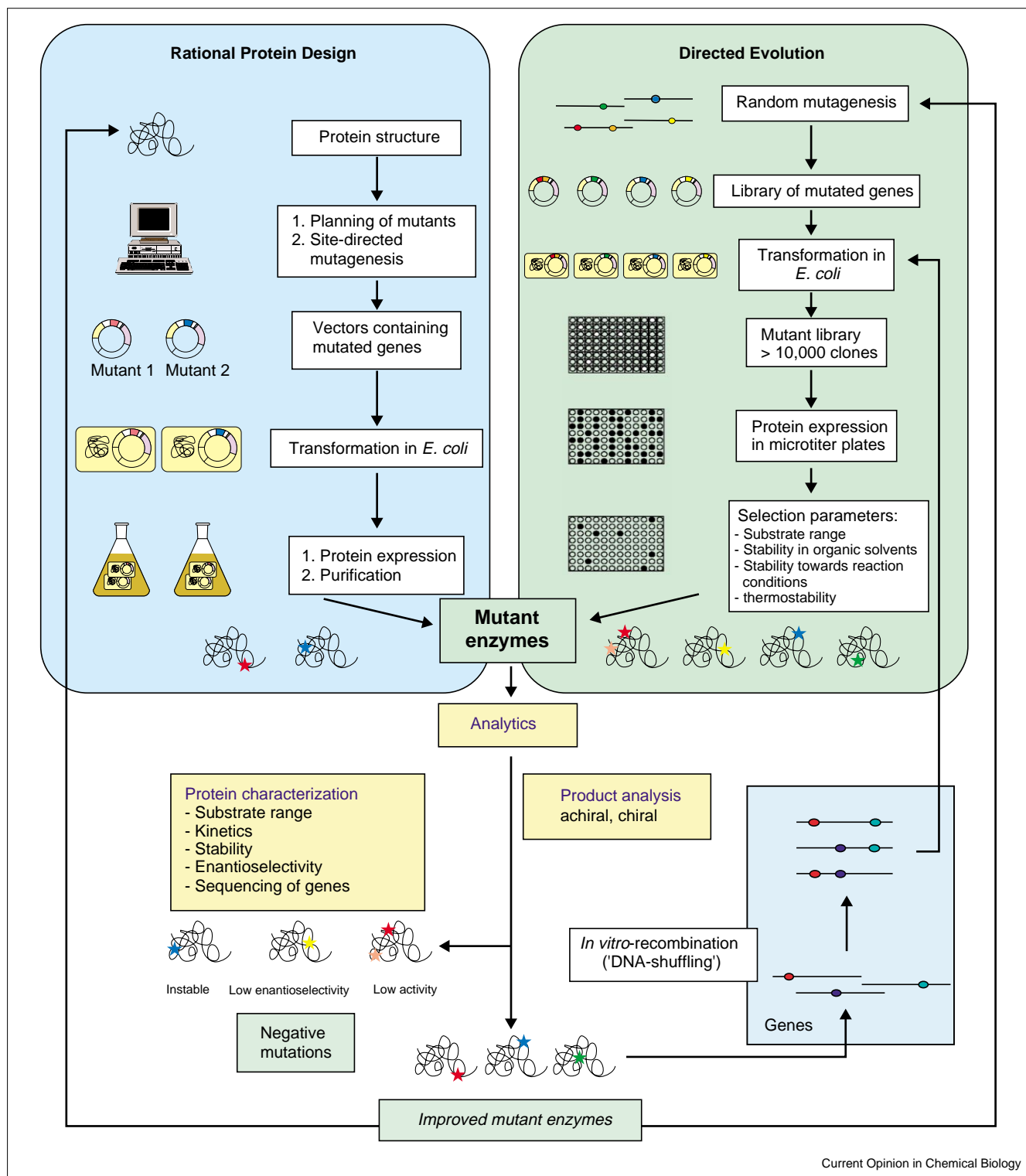
For both approaches to protein engineering, the gene(s) encoding the enzyme(s) of interest, a suitable (usually microbial) expression system, and a sensitive detection system are prerequisites.

In this review, we present successful examples of the creation of suitable biocatalysts, concentrating on those published since 1999. Methods for the generation of mutant libraries as well as principles of screening or selection are out of the scope of this article and readers are referred to the references given above.

Directed evolution

Mutants of an esterase from *Pseudomonas fluorescens* produced by directed evolution using the mutator strain *Epicurian coli* XL1-Red were assayed for altered substrate specificity using a combination of screening and selection ([13*]; see also Update). Key to the identification of improved variants acting on a sterically hindered 3-hydroxy ester was an agar-plate assay system based on pH indicators that give a change in color upon hydrolysis of the ethyl ester. Parallel assaying of replica-plated colonies on agar plates supplemented with the glycerol derivative of the 3-hydroxy ester was used to refine the identification, because only *E. coli* colonies producing active esterases had access

Figure 1



Comparison of rational protein design and directed evolution. During rational protein design, mutants are planned on the basis of their protein structure and then prepared by SDM. After transformation in the host organism (e.g. *E. coli*), the variant is expressed, purified and analyzed for desired properties. Directed evolution starts with the preparation of mutant gene libraries by random mutagenesis, which are then expressed in the host

organism. Protein libraries are usually screened in microtiter plates using a range of selection parameters. Protein characterization and product analysis sort out desired and negative mutations. *In vitro* recombination by DNA shuffling, for example, can be used for further improvements. Both protein engineering approaches can be repeated or combined until biocatalysts with desired properties are generated.

to the carbon source glycerol, thus leading to enhanced growth and, in turn, larger colonies. In another example, a growth selection based on adipyl-leucine or adipyl-serine was used to identify acylase mutants acting on adipic acid instead of glutaric acid sidechains in cephalosporins (CF Sio, JM van der Laan, AM Riemens, WJ Quax, personal communication). Random mutagenesis of the α -subunit, then growth selection of active variants followed by saturation mutagenesis identified three mutants showing decreased V_{\max}/K_M values for glutaryl substrates and higher values towards the adipyl analog.

Another alternative for selection is the use of phage display [14,15] to select for mutants with catalytic activity. Anchoring the substrate to the phage in such a way that the product remains bound too, might enable capturing of active catalysts [16*,17].

In an excellent contribution, Fersht and co-workers [18**] evolved phosphoribosylanthranilate isomerase (PRAI) activity from the α/β -barrel scaffold of indole-3-glycerol phosphate synthase (IGPS). Their success provides a striking example for testing the 'conserved scaffold' hypothesis of enzyme evolution. Reetz and co-workers [19,20] considerably increased the enantioselectivity (E) of a lipase from *Pseudomonas aeruginosa* PAO1 towards 2-methyldecanoate by screening mutant libraries using optically pure (*R*)- or (*S*)-*p*-nitrophenyl ester. Further improvement of the best mutant (E ~11, 81% enantiomeric excess [*ee*]) by saturation mutagenesis led to E ~26 for a mutant bearing five amino acid substitutions. The recently solved structure of this lipase [21] suggests that the increased enantioselectivity is caused by increasing the flexibility of distinct loops of the enzyme; however, none of the mutations are located near the binding pocket.

In a similar approach, the enantioselectivity of an esterase from *P. fluorescens* was increased from E = 3.5 to E = 5.2–6.6 in a single round of mutation [22]. Here, resorufin esters of (*R*)- or (*S*)-3-phenylbutyric acid were used for the determination of E values, enabling measurement of fluorescence in microtiter plates and thereby avoiding problems with interfering compounds present in the culture medium.

In both examples, the mutants screened for might be 'adapted' to the bulky chromophore ester, rather than the 'true' ester used in organic synthesis applications (i.e. an acetate of a racemic alcohol). To overcome this problem, Reetz and co-workers developed more sophisticated methods using isotopically labelled acetates [23*] or capillary electrophoresis [24**]. The latter method allows accurate *ee* determination of amines with a throughput of at least 7000 samples per day.

Digital-image screening was used by Arnold's group [25*] to identify P450_{cam} variants showing enhanced activity in naphthalene hydroxylations, in the absence of the cofactor NADPH, via a 'peroxide shunt' pathway. Co-expression of

P450_{cam} with horseradish peroxidase from *E. coli* converted hydroxylation products into fluorescent products amenable by digital screening. In another example, a triple mutant of P450 BM-3 obtained by directed evolution was found to hydroxylate indole, producing indigo and indirubin [26]. Interestingly, the authors screened for P450 variants with altered chain-length specificity, but by chance discovered variants hydroxylating indole.

Recently, Arnold and co-workers [27*] also reported the inversion of enantioselectivity of a hydantoinase, from D-selectivity (40% *ee*) to moderate L-preference (20% *ee* at 30% conversion) by a combination of error-prone PCR (epPCR) and saturation mutagenesis. Only one amino acid substitution was sufficient to invert enantioselectivity. Thus, production of L-methionine from D,L-5-(2-methylthioethyl)hydantoin in a whole-cell system of recombinant *E. coli*, also containing a L-carbamoylase and a racemase, at high conversion became feasible. The substrate specificity of a peroxidase from *Saccharomyces cerevisiae* towards guaiacol was increased 300-fold by means of DNA shuffling [28*].

It is often assumed that improving a biocatalyst in one direction affects other desired enzyme characteristics. It has been demonstrated, however, that it is possible to increase the thermostability of a cold-adapted protease to 60°C while maintaining high activity at 10°C [29,30*]. The best psychrophilic subtilisin S41 variant contained only seven amino acid substitutions resembling only a tiny fraction of the usual 30–80% sequence difference found between psychrophilic enzymes and mesophilic counterparts.

In an excellent contribution, researchers at Maxygen (USA) and Novo Nordisk (Denmark) simultaneously screened for four properties — activity at 23°C, thermostability, organic-solvent tolerance and pH-profile — in a library of family-shuffled subtilisins, and reported variants with considerably improved characteristics for all parameters [31**].

Instead of improving one specific biocatalyst, the engineering of entire metabolic pathways by means of directed evolution is also feasible. The first example has recently been demonstrated in which phytoene desaturases and lycopene cyclases were shuffled in the context of a carotenoid biosynthetic pathway assembled from different bacterial species [32**]. Molecular breeding based on mixing genes and *in vitro* evolution using *E. coli* as host, resulted in the generation of new products (e.g. fully conjugated and cyclic carotenoids).

Rational protein design

Rational protein design by site-directed mutagenesis (SDM) is still a very effective strategy to elaborate improved enzymes. Useful strategies such as the reinforcement of a promiscuous reaction, change of enzyme mechanism, substrate specificity, cofactor specificity enantioselectivity, and stability, as well as the elucidation of enzyme mechanisms have been reported. Comprehensive overviews can be found in a number of reviews [33–35].

Rational protein design has successfully been used for stabilization of enzymes towards thermostability and oxidation. Examples of successful strategies to enhance thermostability are the removal of asparagine residues in α -amylase [36], the introduction of more rigid structural elements such as proline into α -amylase [37] and D-xylose isomerase [38], or disulfide bridges to stabilize hen lysozyme [39]. The introduction of additional hydrophobic contacts was shown to stabilize 3-isopropylmalate dehydrogenase [40] and formate dehydrogenase from *Pseudomonas* sp. [41]. Factors influencing thermostability have recently been elucidated by a structural comparison of various enzymes from mesophilic and thermophilic organisms [42].

To increase stability towards oxidation, removal of cysteine and methionine residues exhibited positive effects in the case of formate dehydrogenase [43], (*R*)-3-hydroxybutyrate dehydrogenase [44], and D-amino acid oxidase [45].

In contrast to directed evolution, successful examples of improved and inverted enantioselectivity of enzymes by SDM are rare. The effects of certain mutations are more difficult to predict compared with those from efforts focusing on altering other catalytic properties. A very good example is the inversion of the enantioselectivity of vanillyl-alcohol oxidase by two point mutations, which switched the enzyme from (*R*)- to (*S*)-specificity (80% *ee*) with respect to the formation of 1-(4'-hydroxyphenyl)ethanol by hydroxylation of 4-ethyl-ethanol [46*]. Another successful example has been reported by Schmid and co-workers [47,48*], who investigated the molecular bases of stereoselectivity of two different lipases by SDM based on molecular modeling studies, and thereby generated mutants with altered stereoselectivity compared with that of the wild-type enzyme.

Compared with factors influencing stereospecificity and enantioselectivity, the prediction of molecular factors affecting catalytic activity are easier to predict. In the case of pyruvate decarboxylase from *Zymomonas mobilis*, mutation of Trp392, a bulky residue in the substrate-binding channel, increased the carboligase side-reaction of the enzyme by a factor of six, without negatively influencing the stability and the enantioselectivity [49]. Sterical principles were also valid in the case of PepC, which is an aminopeptidase, because of four carboxy-terminal residues interacting with the active site. Removal of the carboxy-terminal tetrapeptide converted the aminopeptidase into an oligopeptidase ([50]; see also Update). The substrate range of P450_{cam} was extended from camphor to polycyclic aromatic hydrocarbons by mutation of two aromatic residues (Phe87 and Tyr96) in the substrate-access channel [51]. On the basis of a homology model, various mutants of PER-1 β -lactamase have been generated with improved activity towards different β -lactam-based antibiotics [52]. By point mutation of Gln27 — a residue located in a cap closing the back of the active site — the catalytic properties of an *Aspergillus fumigatus* phytase were successfully optimized [53]. The sidechain of this non-conserved residue is in close contact with the substrate during

catalysis. Thus, mutation of glutamine to leucine or isoleucine prevents the formation of a hydrogen bond between the substrate and the sidechain. Alteration of substrate specificity was achieved by SDM of human leukocyte 5-lipoxygenase, yielding a 15-lipoxygenating biocatalyst [54*].

An example for the generation of a new enzymatic activity by protein design has been reported by the Christen group [55], who obtained a dicarboxylic amino acid β -lyase, an enzyme not found in nature, by SDM of a tyrosine phenol-lyase.

Combination of both methods

An impressive example of the use of directed evolution and rational protein design was shown by researchers at Novo Nordisk (Bagsvaerd, Denmark). They targeted a heme peroxidase from *Coprinus cinereus* to be used as a dye-transfer inhibitor in laundry detergent [56**]. Screening for improved stability was performed by measuring residual activity after incubation under conditions mimicking those in a washing machine (e.g. pH 10.5, 50°C, 5–10 mM peroxide). Mutants were obtained by epPCR (out of 64,000) as well as by rational design. Surprisingly, for both methods sequencing of the best variants identified position Glu239 to be crucial for success. Saturation mutagenesis showed that replacement with glycine — as predicted by computer-modeling — gave the best performance. Subsequent *in vivo* shuffling led to dramatic improvements, yielding a mutant with 174 times the thermal stability and 100 times the oxidative stability of the wild-type peroxidase.

Another example of the successful combination of both mutagenesis strategies is the improvement of the thermal stability of a 3-isopropylmalate dehydrogenase variant from *Bacillus subtilis*, which was obtained by a triple mutation using SDM followed by epPCR. The latter mutagenesis identified two further stabilizing point-mutations, which were then combined using SDM [57].

Conclusions

Recent work in the area of protein engineering, summarised in this article, shows that rational design and directed evolution are both applicable to creating desired mutant enzymes, although the positions of mutations often differ considerably. For rational protein design, those amino acid residues that appear 'logical' to the researcher examining the three-dimensional structure are usually modified (i.e. they are close to the active site, the binding pocket etc.). In sharp contrast, sequencing of variants obtained by directed evolution followed by their structural analysis very often reveals that mutations are far away from the place where the reaction takes place (as was shown for improved enantioselectivity [19] and altered substrate specificity [13*] of hydrolases). First examples from careful analysis of three-dimensional structures of mutants and wild types help to identify how 'irrational' mutations affect the catalytic properties of a biocatalyst [58**,59**]. Further work will surely help to increase our understanding of structure/function relationships and will see more combinations of both protein engineering tools.

Update

Recent work has shown that the chain-length specificity of P450 BM-3 can be modulated by rational design [60]. In contrast to the wild-type, a variant bearing five mutations accepted a *p*-nitrophenoxystyrene (C8-pNCA), whilst the activity towards C10- and C12-pNCA remained unchanged.

Phospholipase activity was introduced into a *Staphylococcus aureus* lipase by directed evolution using epPCR and gene shuffling [61]. The best variant contained six mutations and displayed a 11.6-fold increase in phospholipase activity and a 11.5-fold increased phospholipase : lipase ratio compared to the wild type. A very brief opinion on the same subject as this review compares mutational and selective mechanisms and basic requirements of rational design and directed evolution [62]. Also, two reviews summarizing recent achievements and future prospects in the field of directed evolution were recently published [63,64].

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