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Review

Directed evolution of enzymes for biocatalysis and the life sciences

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Abstract. Engineering the specificity and properties of enzymes and proteins within rapid time frames has become feasible with the advent of directed evolution. In the absence of detailed structural and mechanistic information, new functions can be engineered by introducing and recombining mutations, followed by subsequent testing of each variant for the desired new function. A range of methods are available for mutagenesis, and these can be used to introduce mutations at single sites, targeted regions within a gene or randomly throughout the entire gene. In addition, a number of different methods are available to allow recombination of point mutations or blocks of sequence space with little or no homology. Currently, enzyme engineers are still learning which combinations of selection methods and techniques for mutagenesis and DNA recombination are most efficient. Moreover, deciding where to introduce mutations or where to allow recombination is actively being investigated by combining experimental and computational methods. These techniques are already being successfully used for the creation of novel proteins for biocatalysis and the life sciences.

Key words. Protein engineering; directed evolution; enzymes; enzyme activity; DNA shuffling; error-prone PCR.

Introduction

During the last few years, directed evolution has emerged as the method of choice for engineering functions and properties of enzymes. Before the emergence of this technique, rational engineering, which relied on the gathering of extensive structure-function relationships of enzymes, offered the only possibility for the creation of new enzyme activities, and some notable successes have been reported [1–5] (fig. 1). However, rational redesign is beset by problems, most notably the amount of data that has first to be accumulated on each individual enzyme under study, and because, even now, our understanding of the relationship between enzyme structure and function is limited. In addition, the prediction of the effect of mutations is complicated by the growing realization that enzyme molecules exist in solution as a mixture of structural conformers, and that dynamics play an important role in enzyme function. For example, nuclear magnetic resonance (NMR) spectroscopy has identified residues both proximal and distal to the active site of dihydrofolate reductase (DHFR) that are highly dynamic and that are conserved across 36 diverse species of DHFR. This suggests a key role for these residues in catalysis [6], a fact confirmed by site-directed mutagenesis of these residues [7]. In addition, a network of coupled promoting motions, in the same regions identified by the previous NMR measurements, has been identified in DHFR [8]. It may not be surprising, therefore, that planned effects of rationally designed mutations do not always produce the desired outcome and that directed

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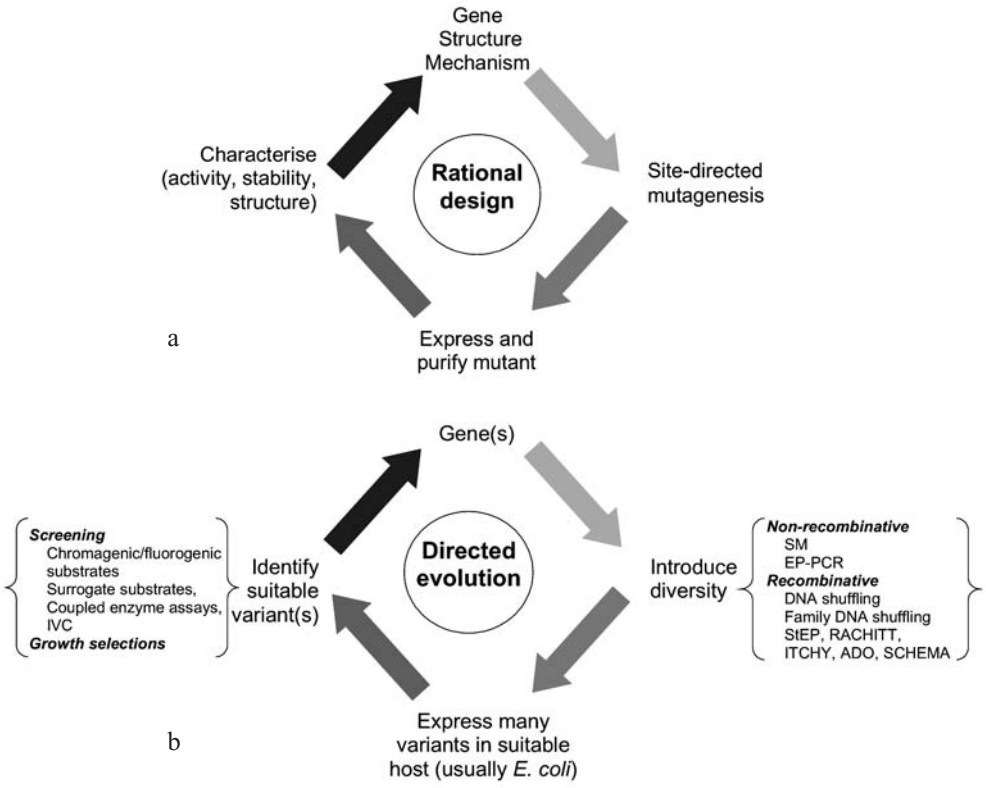

Figure 1. Schematic representations of the processes of rational design and directed evolution. (*a*) The rational redesign cycle requires the appropriate gene and suitable knowledge of the structure and mechanism of the enzyme. Residues are targeted for site-directed mutagenesis and after expression and purification the properties of the new enzyme are assessed. Further residues may be targeted in further rounds of site-directed mutagenesis. (*b*) The directed evolution cycle requires the gene (or genes) of interest, but there is no requirement for a detailed knowledge of structure or function. Diversity may be introduced using a range of methods, and after expression variants with the desired property are selected or screened out of the mixture. Further rounds of directed evolution may be carried out using the first-generation DNA as parent for the second round (see text for abbreviations).

evolution often yields mutations in evolved enzymes which are not directly in the enzyme active site.

By contrast with rational enzyme redesign, directed evolution (fig. 1b) does not rely on the detailed understanding of the relationship between enzyme structure and function. Instead, this technique relies on Darwinian principles of mutation and selection. Enough diversity is created in the starting gene such that an improvement in the desired function or property will be represented in a library of variants. Subsequently, screening or selection methods are used to identify variants with the desired improvements, and these selected variants are used as progeny for the next generation of mutagenesis and selection (fig. 1).

In this review, the range of strategies and screening/selection methods available for directed evolution will be illustrated using a number of key examples from the recent literature. Finally, the success of directed evolution as a tool for the creation of novel enzymes for biocatalysis and the life sciences will be highlighted with a number of recent examples.

Library construction: choosing the level of diversity

A range of strategies for the introduction of diversity into the starting gene(s) are available, and these can be broadly divided into two classes; (i) non-recombinative and (ii) recombinative methods, and can range from creating libraries with as few as 200 variants to many tens of thousands of variants.

Non-recombinative methods

Non-recombinative methods generally create diversity via point mutation and include the directed substitution of single amino acids, the insertion or deletion of more than one amino acid, for example by cassette mutagenesis, and random mutagenesis across the whole gene. Thus, a variety of methods are available depending on the extent of mutation required. In cases where a high-resolution structure of the target protein with bound substrate or inhibitor is available, residues which contact the substrate can be identified and can be hypothesized to be responsible in varying degrees for the natural reaction specificity. Mu-

tation of these contacting residues to all other 19 amino acids by saturation mutagenesis (sm) can often lead to the identification of variants with significantly altered substrate specificity. For example, Schultz and co-workers used saturation mutagenesis at five positions in the active site of the *Methanococcus jannaschii* tyrosyl transfer RNA (tRNA) synthetase to alter the amino acid specificity so that it accepts only an unnatural amino acid [9]. Using several rounds of positive and negative growth selection, a mutant synthetase was obtained which had a k_{cat}/K_m for the target unnatural amino acid *O*-methyl-L-tyrosine, 100-fold higher than for the natural substrate tyrosine.

A disadvantage of using crystal structures to identify residues thought to be responsible for substrate specificity is that this approach may ignore residues distant from the active site. Directed evolution experiments often produce mutations that do not occur in the active site. For example, following four rounds of error-prone polymerase chain reaction (EP-PCR) and screening, the enantioselectivity of a *Pseudomonas aeruginosa* lipase was increased from 1.1 to 26 (fig. 2) [10]. None of the five mutations in the evolved enzyme were in the substrate binding site. A recent molecular modeling investigation allowed interpretation of these distant 'hotspot' residues and concluded that the mutations create a new binding pocket for the 'unnatural' enantiomer and form a new stabilizing hydrogen bond. However, the contribution of individual mutations in evolved enzymes is not usually determined, and it has been suggested that targeted mutagenesis, followed by recombination, will be more effective than random mutagenesis alone. In fact, using a *Pseudomonas fluorescens* esterase in a model study, Horsman et al. (2003) suggest that random mutagenesis is strongly biased towards residues far from the active site because there are more residues distal to the active site than there are proximal, and that mutagenesis of active site residues will be more effective [11].

After saturation mutagenesis, the next level of complexity involves the introduction of mutations to targeted regions of the gene of interest. For example, Wymer et al. (2001) introduced random mutations into residues 131–168 of the *Escherichia coli* KDPG-aldolase in an attempt to increase the poor activity of the wild-type enzyme towards benzaldehyde. A double mutant was identified which was shown to have a markedly altered substrate specificity profile compared to the wild-type enzyme, albeit with low, but synthetically useful activity. Interestingly, it was shown that the specificity change was due to relocation of the active site lysine residue from one β strand to a neighboring one [12].

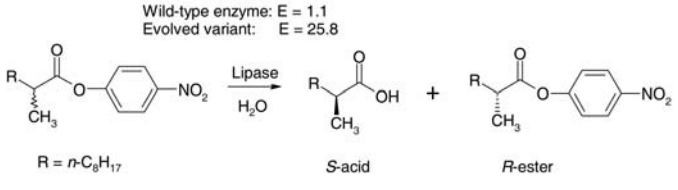

Figure 2. The directed evolution of an enantioselective lipase [10]. The wild-type enzyme shows no significant enantioselectivity for the hydrolysis of 2-methyldecanoic acid *p*-nitrophenyl ester, whereas the evolved variant after four rounds of evolution showed a selectivity factor, *E*, of 25.8 in favor of the *S*-configured acid.

The simplest, and still a popular, method of choice for introducing diversity is EP-PCR. The mutation rate can be adjusted so that, usually, an average of 1–2 amino acid mutations is introduced per gene product [13, 14]. It is generally accepted that using a low mutation rate increases the probability of discovering beneficial mutations, since most random mutations are either neutral or deleterious. However, a study using nucleoside triphosphate analogues [15] to achieve mean mutation rates of 8.2 and 27.2 nucleotide mutations per gene concluded that highly active TEM-1 β-lactamase mutants could be identified [16] even at these high mutation rates. After screening pools of $<1.5\times10^5$ colonies, one clone with three amino acid mutations was found to exhibit a minimum inhibitory concentration for cefotaxime 20,000-fold higher than the wild-type lactamase and with a k_{cat}/K_m ~2400-fold higher than the wild-type enzyme. It was shown that all three mutations in the selected variant were required for the 20,000-fold increase in activity. This mutant compares very well to that obtained via DNA shuffling (see below), whereby $\sim1\times10^9$ colonies were screened [17]. Thus, high mutation rates may be appropriate for certain scaffolds. It has been noted, however, that the number of mutations in the selected variants is far lower than the mean mutation rate of the library, and that those variants with fewer mutations were identified by the selection system [18].

A number of examples attest to the success of EP-PCR with low mutation rates. For example, a cyclodextrin glycosyltransferase from *Bacillus circulans* was evolved to increase the hydrolytic activity 90-fold [19]. Analysis of the mutants identified in this study provided evidence in support of an induced-fit mechanism, whereby binding of sugar acceptors activates the transglycosylation reaction.

EP-PCR at low mutation rate suffers from one drawback: there is an inherent bias introduced since on average only 5.6 amino acids per codon can be accessed given the substitution of a single nucleotide [20]. In addition, the inherent bias of polymerases further reduces the diversity that can be accessed by EP-PCR. These problems can be overcome by the use of gene site saturation mutagenesis (GSSM) [21]. GSSM is a method that uses sets of degenerate primers to introduce all 19 amino acid substitutions at every position of the gene to produce every possible single amino acid mutant. This method has been used to produce every single amino acid variant of a 330-amino

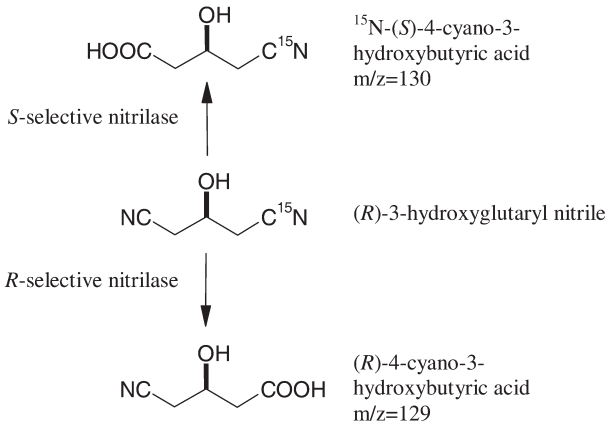

Figure 3. Mass spectrometric method for identifying enantioselective nitrilase variants [21]. Specifically [^{15}N] labeled 3-hydroxyglutaryl nitrile was incubated with the variants of nitrilase produced during directed evolution, and an *R*-selective nitrilase that would operate at high concentration of the substrate (*R*)-3-hydroxyglutaryl nitrile was selected on the basis of the mass of the product produced. Only an *R*-selective nitrilase hydrolyzes the isotopically labeled nitrile to cause loss of the ^{15}N. *S*-selective variants will hydrolyze the non-^{15}N-labeled nitrile group, and ^{15}N will be retained in the product; thus enantioselectivity of variants may be assessed by mass spectrometry of the enzyme-catalyzed reaction products.

acid nitrilase, which were subsequently screened for improved enantioselectivity towards the hydrolysis of cyano-3-hydroxybutyric acid using a ^{15}N-labeled analogue and mass spectrometry (fig. 3). This approach led to the identification of variants at 17 different positions. Secondary characterization showed that residues Ala190 and Phe191 were mutational hotspots. The same technique was used to increase the thermal stability of a microbial haloalkane dehalogenase [22]. Subsequent recombination of the single site mutations improved the half-life at 55 °C from 11 min for the parental enzyme to 29,000 min and the melting temperature of the final variant was increased 8 °C.

In addition, the use of an engineered DNA polymerase, Mutazyme (Stratagene, La Jolla, CA), that has a reversed mutational bias compared to *Taq* polymerase, in combination with *Taq* polymerase, may result in the production of unbiased libraries [18]. Mutazyme has been used in the directed evolution of a β-glucuronidase towards increased β-galactosidase activity; however, a direct comparison of the resultant library to that produced by *Taq* was not made [23].

Recombinative methods

Despite the important and growing use of non-recombinant methods for variant library production, the most significant changes in enzyme function have been created using recombinative methods, and DNA shuffling is still the most popular method of recombining DNA, whether homologous genes from different sources are being recombined, or for the recombination of point mutations. Briefly, the original DNA shuffling technique [17, 24] involves the controlled fragmentation of the source DNA using DNase I, followed by a primer-less, reassembly PCR reaction, which gradually produces full-length recombined sequences. Finally, the small amount of full-length gene present in the reassembly reaction is amplified by a standard PCR reaction in the presence of flanking primers.

In a recent example of the use of DNA shuffling, the *E. coli* β-glucuronidase gene was subjected to three rounds of DNA shuffling and screening (fig. 4). A mutant was isolated which catalyzed the hydrolysis of a β-galactoside 500 times more efficiently than the wild-type enzyme, with a 52 million-fold inversion in specificity [25]. Site-directed mutagenesis, and subsequent kinetic analysis of the mutant proteins, showed that the four mutations found in the evolved enzyme were synergistic and that specialized enzymes can be evolved from broad specificity ancestors.

It has also been shown that DNA shuffling of as few as two homologous sequences can be sufficient to generate novel enzyme specificities [26]. Two highly homologous triazine hydrolases which differ at only nine amino acid positions were shuffled. Approximately 1600 variants were then screened against a library of 15 triazine substrates, and variants with up to 150-fold higher activities were identified, as well as variants that could hydrolyze five out of eight triazines that were not substrates for either parental enzyme (fig. 5). These novel enzymes were

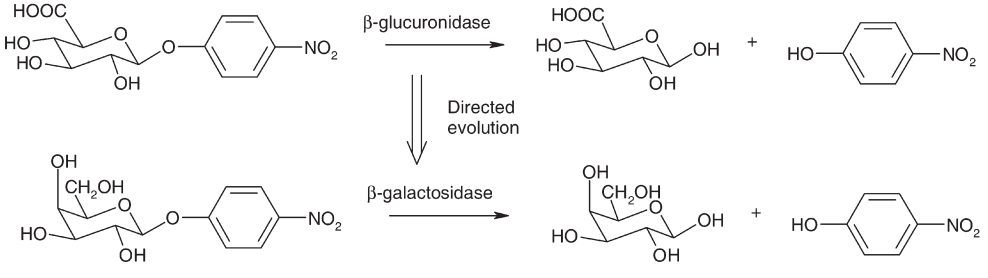

Figure 4. Conversion of a β-glucuronidase into a β-galactosidase by directed evolution [25].

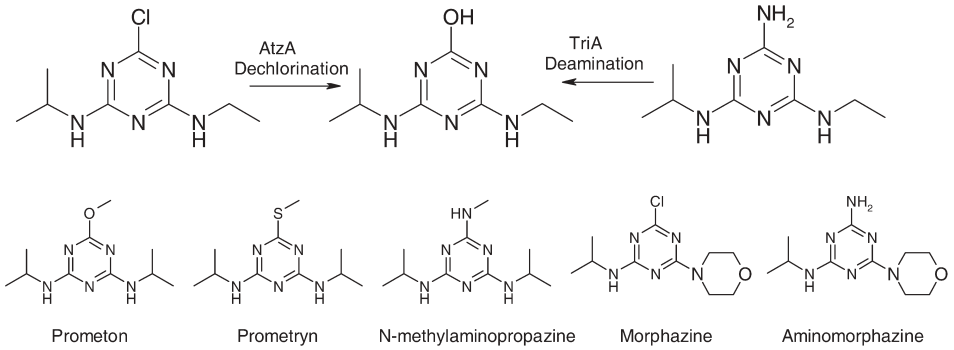

Figure 5. The hydrolysis of atrazine and aminoatrazine by wild-type AtzA and TriA enzymes, and the structures of the five triazines that were not substrates for the wild-type enzymes but for which activity was found after directed evolution of new catalysts [26].

discovered even though all 512 possible permutations were probably not screened.

In addition to simple DNA shuffling, a number of more intricate methods for recombination have been described. Staggered extension process (StEP) uses a simple PCR reaction with very short elongation times; recombination occurs where partially elongated strands melt and anneal to a new template, producing a crossover [27]. Random chimeragenesis on transient templates (RACHITT) [28] is similar to DNA shuffling but requires many more experimental steps; however the method does produce a much larger number of crossovers than basic DNA shuffling. Synthetic shuffling [29] and assembly of designed oligonucleotides (ADO) [30] are two reported methods of recombination which make use of entirely synthetic oligonucleotides that result in the production of full-length genes with defined crossover points and composition. Both techniques result in significantly increased recombination frequency and have been validated experimentally.

A number of methods are also becoming available for the recombination of non-homologous genes. For example, Incremental Truncation for the Creation of HYbrid enzymes (ITCHY) involves the direct ligation of truncated N- and C-terminal fragments of two genes, removing the requirement of homology [31]. However, crossovers occur at random positions, and the initial products only contain a single crossover. DNA shuffling of the ITCHY products can generate products with multiple crossovers, and plasmid systems are available for selection of only in-frame ligation products [32, 33]. O'Maille et al. (2003) used extensive protein structure and sequence information to design oligonucleotides that were used to amplify defined segments from each parent that were then assembled by overlap PCR, thus crossovers occurred at specifically designed points [34]. A computational algorithm (SCHEMA) has also been developed which identifies optimal crossover points using structural information to identify sites with minimal interaction with the rest of the protein [35]. Application of this algorithm, subsequent shuffling at the computed sites and screening of two distantly related lactamases validated the approach for the construction of highly hybridized, folded chimeras [36].

These more recent methodologies, which undoubtedly add to our protein engineering toolbox, are, however, rather more complicated in execution than the original DNA shuffling method or EP-PCR. Consequently, the choice of method will depend on a tradeoff between the length of time allowed for protocol development and library construction, and the expected gain in function as a result. The combination of DNA shuffling, EP-PCR and saturation mutagenesis remains the most popular method of variant library construction.

Screening methodologies

Even with new methods for the creation of diversity in variant libraries and the firmly established methods of saturation mutagenesis and DNA shuffling, the screening or selection of those variants with the new desired or improved activity remains the most critical step in directed evolution experiments. While we cannot here review all of the possible screening/selection methods (for recent reviews see [37, 38]), a number of recent examples will be used to illustrate the diversity of methods available.

Functional genetic selections where the transformed organism can only survive if the desired activity is present, or above background levels, are very efficient methods for searching for a protein function that confers a survival advantage to the host organism. However, many applications of directed evolution, for example, identifying improved functions such as thermo- or solvent stability, are usually impossible using selection methods, although, under certain special conditions, they may be achieved [39]. Thus, while genetic selections have been used to great effect, many researchers concentrate on developing screening protocols, whereby every member of the library is individually assayed for function, in contrast to

selection where only the survivors of the selection process remain. This necessitates the move of the screen into high-throughput methodologies, and the use of chromogenic substrates remains popular. For example, by shuffling together two phytoene desaturases from two different microbial sources and expressing the shuffled enzymes along with the rest of the carotenoid pathway, Schmidt-Dannert et al. (2000) have evolved a desaturase that can introduce six double bonds into the carotenoid product. Such products are pink in color, whereas carotenoids with fewer double bonds are yellow. Thus, the direct visual screening of colonies expressing the artificial pathways allowed quick identification of hits, which were verified in a secondary screen using high-performance liquid chromatography (HPLC) [40].

Where products of the desired reaction are not directly colored or fluorescent, traditional assays for purified enzymes can be scaled down for use in 96-well microtitre plates. Thus, colonies can be grown directly in microtitre plates and lysed if necessary. Often, a rapid primary screen, which is used to eliminate those clones with zero activity, is combined with a more sensitive secondary screen. For example, in the selection of proteases with high activity at room temperature, enzymes with high thermo- and solvent stability and useful pH dependence were selected from a library of hybrid proteases, constructed by DNA shuffling of 26 protease genes [41], which was first screened for general activity by the formation of clearing zones when microcultures were grown on medium containing skimmed milk. Those clones showing activity were then regrown and assayed using a fluorogenic substrate. This strategy successfully identified variants that were more active than the parent enzymes for each property, and also variants with novel combinations of the parental properties.

While existing screening methodologies have proved successful, the development of new techniques for searching protein libraries will be vital for the directed evolution of certain enzymes. In vitro compartmentalisation (IVC) is a recently designed system for the selection of DNA methyltransferases, as well as other enzymes [42, 43]. In IVC, the linkage between genotype and phenotype is achieved by compartmentalizing single genes in the aqueous compartments of a water-in-oil emulsion (fig. 6). Briefly, an in vitro transcription/translation mixture and a library of mutant methyltransferase genes, each containing a restriction-methylation (RM) site, were dispersed to form a water-in-oil emulsion, where, on average, each compartment contains only a single mutant gene. Thus, following transcription/translation, in each droplet any active methyltransferase methylates the enclosed RM site, thus protecting the DNA from restriction digestion. After the emulsion is broken and the aqueous compartments combined, digestion with the cognate restriction enzyme digests the genes that encode inactive methyltransferases and subsequently allow the survival of those genes that encode active enzymes. These are then amplified by PCR and submitted to further rounds of selection. This system has been used to isolate a variant of *Hae*III methyltransferase that methylates a novel target site with a 670-fold improvement in k_{cat}/K_m, resulting in a variant with a higher catalytic efficiency than the wild-type enzyme with the natural target site [44]. It remains to be seen whether this ingenious system can be applied to other challenging enzymes.

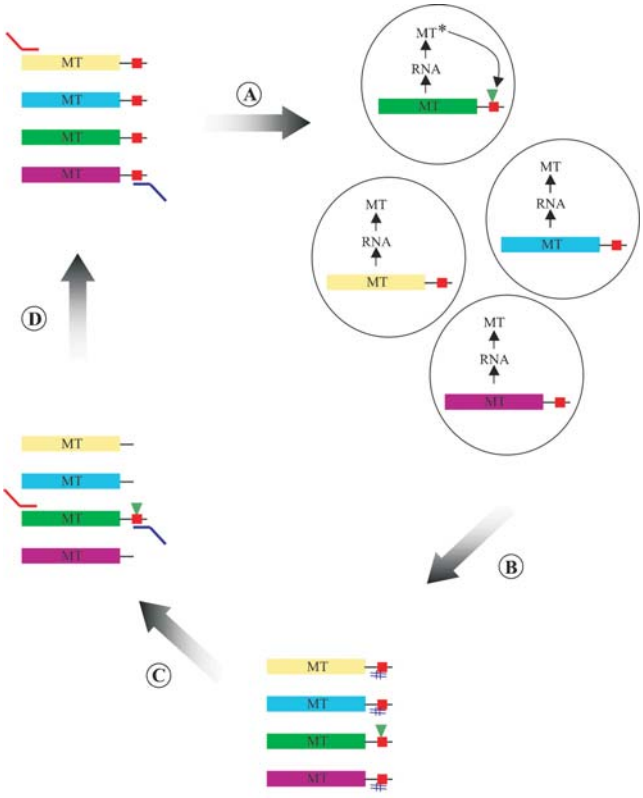

Figure 6. In vitro compartmentalization to create a novel DNA methyltransferase. (*A*) A library of variant methyltransferase genes (MT) with a flanking sequence containing a restriction/methylation (RM) site (red square) is compartmentalized by emulsion formation together with an in vitro transcription/translation mixture such that each droplet contains only one copy of the gene. (*B*) The MT genes are transcribed and translated. Only those variant genes (green) which give rise to active methyltransferases (MT*) result in the addition of a methyl group (green triangle) to the RM site. (*C*) After breaking the emulsion, the mixture is treated with the appropriate restriction enzyme. Only the methylated RM site remains intact. (*D*) PCR amplification using flanking primers is used to amplify the gene. Since one primer binds across the RM site, only those genes with intact RM sites (and hence active MTs) are amplified ready for further rounds of enhancement and screening. Adapted from [44].

Creation of novel biocatalysts by directed evolution

Carbon-carbon bond formation

Carbon-carbon bond formation is a crucial reaction in organic chemistry. Aldolases have proven useful biocatalysts for C-C bond formation; however, the substrate specificity of naturally occurring aldolases has limited their potential use. A number of recent publications have successfully illustrated the applicability of directed evolution to this group of enzymes.

The stereochemistry of carbon-carbon bond formation catalyzed by dihydroxyacetone (DHAP)-dependent aldolases is precisely controlled by the enzyme. A rational engineering attempt to alter the stereochemistry of the bond-forming reaction catalyzed by fructose-1,6-bisphosphate aldolase using multiple sequence alignment and site-directed mutagenesis highlighted the subtleties of enzyme redesign needed [45]. Thus, efforts were redirected to a directed evolution approach. Three rounds of DNA shuffling and screening for the cleavage of a diastereoisomeric substrate resulted in the identification of a variant tagatose-1,6-bisphosphate aldolase with an 80-fold improvement in k_{cat}/K_m towards the unnatural substrate fructose-1,6-bisphosphate, resulting in a 100-fold change in stereospecificity (fig. 7) [46]. ^{31}P NMR spectroscopy was used to show that the >99 : <1 preference of the wild-type enzyme for the formation of tagatose-1,6-bisphosphate had been switched to a 4:1 preference for the diastereoisomer fructose-1,6-bisphosphate, highlighting the feasibility of engineering even the diastereoselectivity of the enzyme reaction.

The substrate specificity of 2-keto-3-deoxy-6-phosphonogluconate (KDPG) aldolase has also been altered by directed evolution. The wild-type enzyme only poorly accepts non-phosphorylated substrate analogues, and this dependency severely limits the synthetic utility of KDPG-aldolase. After three rounds of EP-PCR and DNA shuffling, a variant was obtained with a 70-fold improvement in k_{cat}/K_m towards the non-phosphorylated substrate 2-keto-3-deoxy-6-gluconate, resulting in a 600-fold change in substrate specificity [47]. The mutant also showed increased activity towards L-glyceraldehyde, which is not a substrate for the wild-type enzyme.

Sialic acid aldolase catalyses the reversible aldol condensation of pyruvate and *N*-acetyl-D-mannosamine to produce *N*-acetyl-D-neuraminic acid (D-sialic acid). Sialic acids are essential components of complex oligosaccharides which play pivotal roles in a number of biological processes, including development, activation, aging, oncogenesis, cell adhesion and in host-pathogen and

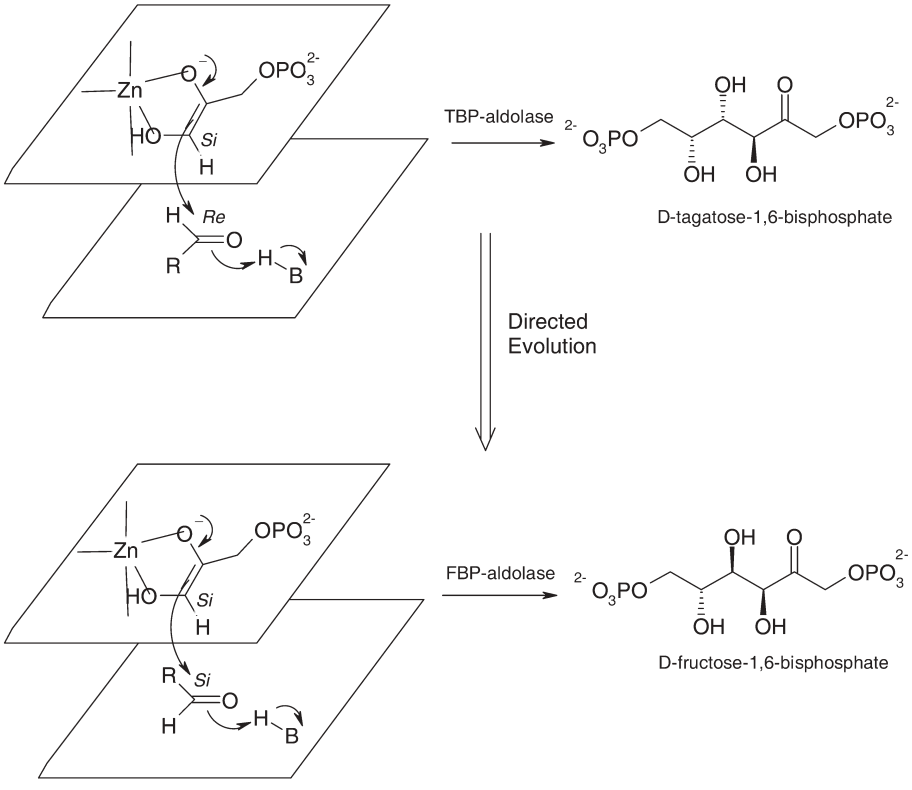

Figure 7. Directed evolution to alter the stereochemistry of the aldolase reaction [46]. Variants of the tagatose bisphosphate aldolase were selected that carry out the fructose bisphosphate aldolase reaction in which the *Si* face of the enediolate intermediate attacks the *Si* face of the aldehyde substrate rather than the *Re* face as found in the wild-type enzyme.

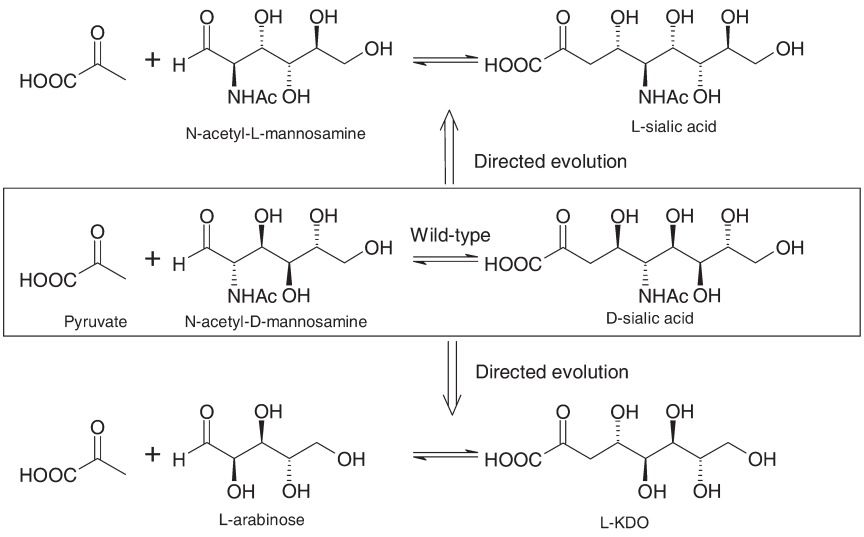

Figure 8. The improvement of the catalytic activity of sialic acid aldolase towards *N*-acetyl-L-mannosamine and L-arabinose by directed evolution [48].

host-parasite interactions. EP-PCR and saturation mutagenesis were used to improve the catalytic activity of sialic acid aldolase towards enantiomeric substrates such as *N*-acetyl-L-mannosamine and L-arabinose to provide access to L-sialic acid and L-KDO, respectively (fig. 8) [48]. Although the target substrate for improvement was L-sialic acid, activity of the wild-type enzyme towards this enantiomer was too low for improvement of activity to be detected. Thus, a surrogate substrate, L-KDO, was chosen for screening, in the hope that active mutants would also be active with L-sialic acid. After a combination of EP-PCR, DNA shuffling and saturation mutagenesis, a mutant was obtained which showed a complete inversion of enantioselectivity. The most improved variant showed a 7.4-fold increase in catalytic efficiency (k_{cat}/K_m) towards L-KDO with a 2.3-fold decrease in efficiency with D-KDO. The turnover number for L-KDO was higher than that for D-KDO, leading to a reversal of enantioselectivity. Unfortunately, activity of the mutant towards L-sialic acid was lower than that for the wild-type enzyme and could not be determined, again proving the maxim 'you get what you screen for' [49]. Mutations in the evolved aldolase were found outside the active site of the enzyme, and a preliminary crystal structure showed no significant difference between the wild-type and mutant enzyme.

Creation of a broad specificity amine oxidase

It is becoming increasingly important to create not only biocatalysts with changes in stereoselectivity and specificity, but also enzymes with broader specificity than naturally occurring enzymes. Directed evolution has recently been used to provide a new route for the deracemization of chiral amines (fig. 9). It has been shown that multiple cycles of enzyme-catalyzed oxidation, followed by non-selective reduction, can be used for the deracemization of a range of α-amino acids in good yield and optical purity. The availability of an enantioselective amine oxidase suitable for the repeated oxidation-reduction cycles was critical to the general applicability of this approach. The wild-type amine oxidase from *Aspergillus niger* was previously shown to have high activity towards simple aliphatic amines but much lower activity towards benzylamine. It was reasoned that this enzyme would be a good starting point for the directed evolution of an amine oxidase with improved enantioselectivity and catalytic activity. Random variants of the oxidase were generated using the *E. coli* mutator strain XL1-Red [50], and approximately 150,000 colonies were screened using a simple colorimetric screen. This led to the identification of a clone with a clear improvement in selectivity and activity towards L-α-methylbenzylamine. After partial purification and characterization, this mutant was shown to have a k_{cat} 47-fold higher towards the target amine compared to the wild-type enzyme, and was 5.8-fold more se-

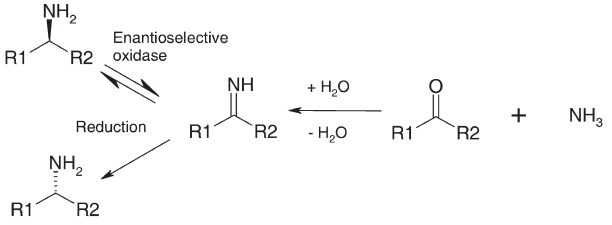

Figure 9. The deracemization of chiral amines using a cyclic sequence of enantioselective oxidation using an amine oxidase and a non-selective reducing agent [50].

lective towards the L-enantiomer. Subsequently, it was shown that the variant, which possessed only a single amino acid change, displayed a very different substrate specificity than the wild-type enzyme [51]. While the wild-type amine oxidase displayed better activity towards simple achiral amines such as amylamine and benzylamine, the variant shows highest activity towards substrates containing a primary amine group flanked by a methyl group and a branched alkyl/aryl group. With all substrates tested, the *S* enantiomer was the preferred substrate.

In vitro glycorandomization and directed evolution

Glycosylated natural products are of significant medicinal and clinical value as proven therapeutics. Often, the sugars attached to these metabolites determine their biological activity, and consequently alteration of these sugars is an intensive area of research. Recently, in vitro glycorandomization (IVG) has emerged as a useful tool for the synthesis of glycosylated products [52, 53]. This approach uses the nucleotidyl transferases and glycosyl transferases to attach sugar precursors to natural product scaffolds. The substrate specificity of these enzymes may, however, limit their use for IVG. Recently, protein engineering has been used to enhance the promiscuity of these enzymes for IVG. Sugar phosphates are the starting materials for the IVG process, and therefore access to a range of sugar phosphates contributes to the efficiency of IVG. Galactokinases catalyse the formation of α-D-galactose-1-phosphate from D-galactose and ATP. However, the substrate specificity of galactokinase from various sources is severely restricted, and this limits their general use in IVG for the production of a randomized sugar phosphate library. To overcome this bottleneck, the *E. coli* galactokinase GalK was subjected to directed evolution to enhance its promiscuity towards monosaccharides, with the aim of identifying variants with improved activity towards L-sugars and those with altered substituents at C-6. Appropriate sugars were pooled in several sets and screened simultaneously for activity using a general assay for reducing sugars, enabling multi-dimensional screening of sequence space. Following a single round of EP-PCR and screening, a mutant *E. coli* galactokinase, with the single amino acid change Y371H, was found with substantially extended substrate specificity [54]. The variant showed increased activity towards both D- and L-sugars, which were not substrates with the wild-type enzyme, while retaining high activity with the natural substrate D-galactose. As we have seen for a number of other enzymes created by directed evolution, the position of the critical mutation (Y371H) in the variant GalK is far from the deduced active site of the enzyme.

The use of (α/β)8-barrel proteins in directed evolution

In addition to the intelligent selection of mutagenesis and screening methods for directed evolution, educated choices can be made as to the scaffold to be evolved. As such, the $(\alpha/\beta)_8$-barrel proteins are likely targets as scaffolds for the creation of new enzyme activities since approximately 10% of all known enzymes are $(\alpha/\beta)_8$-barrels, or have a $(\alpha/\beta)_8$-barrel domain [55, 56], and this fold has exceptional catalytic efficiency and functional versatility, covering all the primary classes of reaction except the ligases.

The approximate partition of residues responsible for catalysis and binding in the $(\alpha/\beta)_8$ fold offer the exciting prospect to engineer new activities by changing catalysis while retaining binding, or changing binding while retaining catalysis [57–59]. Indeed, a number of recent publications attest to the adaptability of $(\alpha/\beta)_8$-barrels by directed evolution. HisA and phosphoribosylanthranilate isomerase (TrpF) both catalyze the irreversible isomerization of an aminoaldose into an aminoketose in the biosynthesis of the amino acids histidine and tryptophan, respectively. Despite the lack of detectable amino acid sequence identity, both enzymes adopt the $(\alpha/\beta)_8$-barrel fold. Following random mutagenesis and selection, several HisA variants were identified that catalyzed the TrpF reaction [60]. One of these mutants possessed both HisA and TrpF activity. Site-directed mutagenesis showed that a single amino acid change was sufficient to interconvert the substrate specificity from HisA to that of TrpF. These data showed that HisA and TrpF might have evolved from a common ancestor of broader substrate specificity. In another example, the dihydrodipicolinate synthase (DHDPS) activity of sialic acid aldolase was enhanced [61]. A single designed amino acid substitution, L142R, enhanced the DHDPS k_{cat}/K_m 19-fold.

More recently, the functional plasticity of the mechanistically diverse $(\alpha/\beta)_8$-barrel enolase superfamily was demonstrated via the use of directed evolution and site-directed mutagenesis (fig. 10) [57]. The D297G mutant of *E. coli* L-Ala-D/L-Glu epimerase (AEE) was designed by structure comparison to the *E. coli o*-succinylbenzoate synthase (OSBS). In addition, the E323G mutant of the muconate lactonizing enzyme II from *Pseudomonas* (MLE) was identified after a round of DNA shuffling and growth selection. Neither AEE nor MLE catalyze the OSBS reaction. However, both single mutants showed significant OSBS activity as well as reduced activity of the progenitor reactions. This unique example showed that single amino acid mutations on the $(\alpha/\beta)_8$-barrel scaffold can both modify the substrate specificity and the mechanism of the catalyzed reaction.

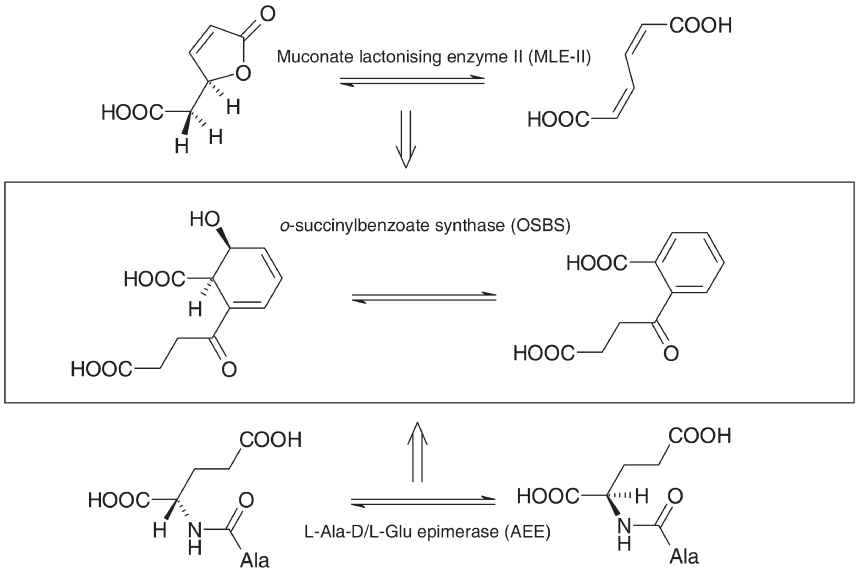

Figure 10. The plasticity of the $(\alpha/\beta)_8$ barrel enolase superfamily. The wild-type L-Ala-D/L-Glu epimerase (AEE) normally catalyzes a 1,1-proton transfer reaction, while the muconate lactonizing enzyme II (MLE-II) catalyzes a cycloisomerization. Neither enzyme has any β-elimination/dehydration activity as is found in *o*-succinylbenzoate synthase (OSBS) activity. OSBS activity has been created in both the AEE and MLE-II frameworks by design or directed evolution [57].

Novel applications of directed evolution in the life sciences

A large number of methods are now available for the creation of genetic diversity and for screening libraries of mutants for desired activities, and a number of these tools and their use for the creation of novel biocatalysts have been described here. In addition, directed evolution is being increasingly applied to more complex biological design problems that test our limits of molecular engineering.

Engineered protein disulfide formation pathway

The power of directed evolution to solve complex molecular design problems was recently demonstrated by the creation of an engineered protein disulfide formation pathway [62]. In *E. coli*, the proteins DsbA and DsbB are responsible for the formation of disulfide bonds in secreted proteins. In the absence of DsbB, strains of *E. coli* are non-motile because the essential disulfide of the flagella component FlgI cannot be formed. Thus, creation of a new pathway for disulfide formation restores motility, allowing a simple screening method for an evolutionary design process. Two residues known to confer the redox properties of thioredoxin were mutated by saturation mutagenesis, and the subsequent variants expressed in *E. coli* with the leader peptide of the *E. coli* trimethylamine *N*-oxide reductase. Expression of the thioredoxin fusion in an *E. coli dsb*B strain resulted in non-motile cells. However, after screening 4000 colonies for motility, several thioredoxin variants were identified that conferred cell motility. Interestingly, the active variants were shown to have acquired a [2Fe–2S] cluster bridged by two polypeptide chains, the wild-type thioredoxin being monomeric [62].

Genetic circuits

Systems as complex as genetic circuits can also be optimized by directed evolution [63]. Simple man-made genetic circuits hold much promise for biotechnology and for the study of more complex genetic networks in nature. However, the design and implementation of even simple genetic circuits represents a huge challenge for the bioengineer, since many parameters such as protein-DNA interactions, messenger RNA (mRNA) and protein stabilities all need to be optimized simultaneously. Yokobayashi et al. [64] showed that directed evolution can be used to fine-tune components of a simple, rationally designed genetic circuit. The circuit comprised three genes (fig. 11). The *lac*I gene is expressed from the P_{lacIq} promoter, and the product LacI represses the P_{lac} promoter. The P_{lac} pro-

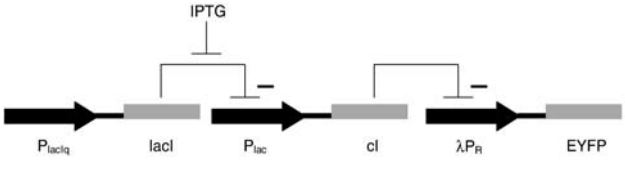

Figure 11. Interactions between the three genes of a simple genetic circuit. Adapted from [67].

moter in turn controls expression of the *cI* gene, the product of which, CI, represses the P_R promoter. The P_R promoter controls production of the enhanced yellow fluorescent protein (EYFP). Thus, increasing IPTG concentration should result in a transition from fluorescence 'on' to non- fluorescence 'off'. However, the leaky nature of the P_{lac} promoter leads to a signal mismatch between the gates, leading to a non-functional circuit, and no EYFP was expressed at any IPTG concentration. It is the fine-tuning of this interaction that was targeted by directed evolution. An earlier, successful, rational attempt to improve gate matching in the circuit involved the labor-intensive process of mutating the RBS upstream of the *cI* gene. Yokobayashi et al. targeted random mutations using EP-PCR to the *cI* gene itself. The difficulty in predicting the effects of mutations in proteins precluded this approach earlier. Functional devices were identified via a two-tier screening strategy. Colonies were first grown in the absence of IPTG, and the fluorescent 'on' colonies were grown in the presence of IPTG. Those colonies that were non-fluorescent or 'off' were then selected for further analysis. These evolved genetic circuits exhibited a sigmoidal fluorescence response to IPTG concentration, illustrating the transition between on and off. The DNA sequences of the evolved variants showed adjustments in protein-DNA and protein-protein interactions had occurred to achieve matching of the genetic circuit. These mutations would have been almost impossible to predict ab initio.

Improving solubility

In addition to the creation of new networks and pathways, directed evolution can be used to provide access to new pharmaceutical targets otherwise elusive to investigation. For example, paraoxonases (PONs) are a group of closely related enzymes that catalyze the hydrolysis of a range of esters, lactones and organophosphates, including Sarin and Soman. In addition, PONs are involved in the prevention of atherosclerosis. PONs therefore represent an attractive group of enzymes for study. Structural and mechanistic studies have, however, been hampered because PONs are not functionally expressed in *E. coli*. Directed evolution was used to achieve functional expression of PONs in *E. coli* [65]. After three rounds of family DNA shuffling and colorimetric screening on agar plates, PON variants were obtained with 40-fold higher activity and 2000-fold shift in substrate specificity. This work demonstrated the potential for directed evolution to solve complex molecular problems to create enzymes that may have use for degrading hazardous organophosphates and the prevention of atherosclerosis. In fact, the crystal structure of this mutant has now been solved, enabling a description of the active site and mechanism of divergence of specificity in the PON family [66].

Concluding remarks and future directions

It has already been established that a wide range of enzyme specificities and functions can be modified by directed evolution. However, as protein and biological engineers become more adventurous, completely new enzyme activities and binding properties will fall with their remit. The success of such efforts will be dependent on the intelligent choice of starting scaffold and the development of novel screening strategies for new activities. Ultimately, discovering how enzymes adapt in nature, such as recruitment of $(\alpha/\beta)_8$-barrels in metabolic pathways, coupled with information obtained from directed evolution experiments with new ideas concerning protein dynamics and motion, will provide valuable lessons to help design new enzyme activities by creating and searching sequence space more effectively. Applying these systems to complex interacting networks such as metabolic pathways and genetic circuits will become feasible.

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