Designed evolution of enzymatic properties

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By providing a simple and reliable route to enzyme improvement, directed evolution has emerged as a key technology for enzyme engineering and biocatalysis. Recent advances include the evolution of a novel catalytic activity using the α/β barrel scaffold, evolution of a cofactor-free monooxygenase, and the engineering of regulatable enzymes. New screening systems for enantioselectivity and protein solubility, and the continuing stream of new methods for creating enzyme libraries further extend evolution's reach.

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Abbreviations

BLIP	β-lactamase-inhibitory protein
CAT	chloramphenicol acetyltransferase
ee	enantiomeric excess
GFP	green fluorescent protein
GUS	β-glucuronidase
HRP	horseradish peroxidase
IGPS	indole-3-glycerol-phosphate synthase
ITCHY	incremental truncation for the creation of hybrid enzymes
MAb	monoclonal antibody
***p*NB E**	*p*-nitrobenzyl esterase
PRAI	phosphoribosylanthranilate isomerase
PSA	prostate-specific antigen
StEP	staggered extension process

Introduction

Enzymes are fascinating catalysts, enviable for their ability to catalyze reactions with high specificity (often enantio- or regio-selectively) under mild conditions. Any number of their properties may need to be altered, however, when biocatalysis is considered for a specific application. Directed evolution offers a way to optimize enzymes rapidly and in the absence of structural or mechanistic information [1–3].

Directed evolution experiments implement a simple, iterative Darwinian optimization algorithm. Molecular diversity is typically created by random mutagenesis and/or recombination of a target gene [4–7] or a family of related genes [8]. Improved variants are identified in a screen (or selection) that accurately reflects the properties of interest. The gene(s) encoding those improved enzymes are used as parents for the next round of evolution. This review covers progress made during the past year in the use of evolutionary methods to improve or alter enzymatic properties as well as new contributions to the technology and methods.

Oxidative enzymes

The desire to eliminate the need for a cofactor and thereby create a single-enzyme hydroxylation catalyst led Joo *et al.* [9••] to direct the evolution of cytochrome $P450_{cam}$ to function via the 'peroxide shunt' pathway. (In this pathway, the electrons required at distinct steps of the P450 catalytic cycle are derived from peroxide instead of NAD[P]H.) $P450_{cam}$ variants with ~20-fold improvements in naphthalene hydroxylation over the wild-type enzyme were identified in just one round of random mutagenesis and recombination of improved clones. It may be possible to use this approach to generate other efficient P450 hydroxylation catalysts that do not require external cofactor, thereby greatly simplifying their use in organic synthesis [10].

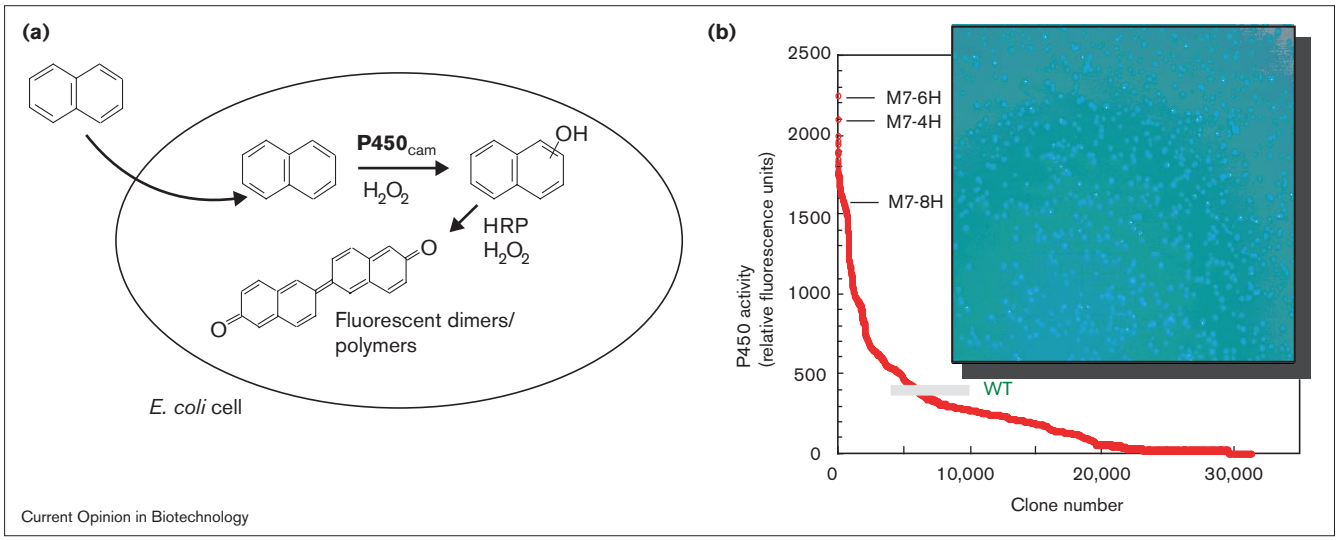
A generally useful high-throughput screen was developed in this study: naphthols generated by the $P450_{cam}$ variants were oxidatively coupled by intracellularly coexpressed horseradish peroxidase (HRP), converted into fluorescent compounds *in vivo*, and quantified by fluorescence digital imaging [9••,11] (Figure 1). Solution-phase screening is also possible using a conventional, or a recently developed microfabricated, fluorescence-activated cell sorter (FACS) [12•]. Libraries of a wide variety of aromatic hydroxylation catalysts may be screened using this assay system [11].

HRP, a highly glycosylated enzyme with four disulfide bonds, is notorious for its inability to fold properly and, therefore, be functionally expressed in recombinant hosts that are suitable for protein library construction and screening, such as *Escherichia coli* and *Saccharomyces cerevisiae*. Random mutagenesis and screening were used to identify HRP variants that are expressed in active form in *S. cerevisiae* at a level 40-times higher than wild-type [13]. These expression levels make it now feasible to tailor HRP for use in chemical synthesis or diagnostics [14].

Evolving protein solubility and expression

Two promising selections for protein solubility and expression have been developed [15,16•]. In one method, the protein of interest is expressed as an amino-terminal fusion with chloramphenicol acetyltransferase (CAT) [15]. It was shown that CAT fusions to insoluble proteins conferred significantly lower chloramphenicol resistance compared to fusions with soluble proteins. This property makes it possible to select for protein variants with increased solubility. A soluble mutant of HIV integrase fused to CAT was selected out of a large pool of insoluble wild-type constructs by plating the expression hosts on high levels of chloramphenicol. In the second method, the protein of interest is expressed as an amino-terminal fusion to green fluorescent protein (GFP) [16•]. Using

Figure 1



Screen for $P450_{cam}$ aromatic hydroxylation activity using horseradish peroxidase (HRP) [9••]. **(a)** Naphthalene is taken up by the *E. coli* cell where it is hydroxylated by $P450_{cam}$. The resulting naphthol is oxidatively coupled by intracellularly coexpressed HRP and converted into fluorescent polymers, which remain associated with the cell. **(b)** Representative results of screening the first $P450_{cam}$ random mutant library using fluorescence digital imaging (inset shows image of a section of an agar plate). Naphthalene hydroxylation activities, as measured by the total fluorescence generated during a fixed incubation period, are plotted in descending order. The range of total fluorescence of clones expressing wild-type $P450_{cam}$ is indicated with gray bar. Confirmed higher activity mutants are labeled.

various test cytosolic proteins, it was shown that the fluorescence of the GFP fusions was related to the productive folding of the upstream protein. The utility of the GFP folding reporter was further demonstrated by directed evolution of protein folding. After several rounds of DNA shuffling and backcrossing with the wild-type sequence, highly soluble variants of proteins (the bullfrog H-subunit of ferritin and the Cys33→Thr mutant of gene V protein) that normally misfold and aggregate when expressed in *E. coli* were identified.

The great advantage of these methods over mutagenesis and functional screening (as was done for expression of HRP [13]) is that there is no need for a specific functional screen. The disadvantage is that there is no functional screen — function does not necessarily accompany acquisition of solubility or folding.

Enantioselectivity

By inverting the enantioselectivity of a key enzyme in a multi-enzyme pathway, May *et al.* [17•] improved a process for the production of L-methionine (L-Met) in *E. coli*. Through random mutagenesis, saturation mutagenesis and screening, the enantioselectivity of a D-hydantoinase was inverted (from enantiomeric excess $ee_D = 40\%$ to $ee_L = 20\%$) and its total activity was increased fivefold. Introduction of the evolved L-hydantoinase into a recombinant whole-cell catalyst increased productivity for L-Met and decreased accumulation of an undesired intermediate, compared to cells with the wild-type pathway. It is worth noting that a single amino acid substitution was sufficient to invert the hydantoinase enantioselectivity. Highly D-selective variants ($ee_D = 90\%$) containing a single amino acid substitution were also found.

Significant studies on the directed evolution of enantioselective catalysts are still scarce [5], one reason being the lack of generally applicable high-throughput screening methods. Two new methods for monitoring enantioselectivity have been reported [18,19]. Both are able to determine exact ee values (within 5–10% of the true ee) and are based on electrospray ionization mass spectrometry (ESI-MS). One system employs isotopically labeled *pseudo*-enantiomeric substrates [18]. The second analyzes chiral compounds of varying structures after derivatization with a suitable mass-tagged chiral agent [19]. Finally, the feasibility of screening for enantioselectivity by time-resolved IR-thermographic detection has been demonstrated [20]. This method, however, is not yet quantitative. There are as yet no reports of these methods being applied to enzyme evolution.

Stability and activity in unusual environments

Two properties often reported to be mutually incompatible, thermostability and catalytic activity at low temperature, were enhanced simultaneously in the psychrophilic subtilisin S41 by using a combination of directed evolution methods (error-prone PCR, saturation mutagenesis, staggered extension process [StEP] recombination, and screening for initial activity and residual activity after incubation at an elevated temperature) [21,22•]. The half-life ($t_{1/2}$) of the best S41 variant at 60°C was 500 times that of wild type and its catalytic efficiency was increased over a

wide temperature range (10–60°C). The evolved S41 contained only seven amino acid substitutions, a tiny fraction of the 30–80% sequence differences that typically separate psychrophilic enzymes from their mesophilic and thermophilic homologs. This study clearly outlines the advantage of directed evolution in being able to distinguish what is physically possible from what is biologically relevant, a distinction that will greatly aid our understanding of how proteins adapt to new environmental challenges.

The Stevens' laboratory has determined the crystal structures of wild-type *p*-nitrobenzyl esterase (*p*NB E) and its laboratory-evolved thermophilic counterpart at high resolution [23]. The thermophilic *p*NB E, an eighth generation variant containing 13 amino acid substitutions, has a 17°C higher melting temperature (T_m) and approximately fourfold increased activity at room temperature relative to wild type [24,25]. The crystal structures reveal that the thermostabilizing mutations serve to stabilize flexible loops and to collectively reorganize the active-site cavity. Helix stabilization, altered core packing and introduction of surface salt bridges are also observed. Mutations exert their influence on the *p*NB E structure over large distances. Some stabilizing interactions depend on the structural effects of previous mutations and therefore could not have been designed directly into the wild-type background. Further biophysical characterization of the lineage of thermostable *p*NB E variants included a tryptophan phosphorescence study of how the flexibility of the enzyme changed during its evolution [25]. Increases in stability were often accompanied by decreases in flexibility, that is, longer tryptophan phosphorescence lifetimes. In contrast, catalytic activity and flexibility of core residues showed no correlation.

In an interesting contrast to the efforts described above to evolve a thermophilic esterase free from the constraints of biological function, Merz *et al.* [26•] used a genetic selection in *E. coli* to isolate variants of a thermophilic indoleglycerol phosphate synthase that allowed the cells to grow faster at 37°C. The selected variants all exhibited higher turnover rates (k_{cat}), which were accompanied by greatly decreased affinity for both the substrate and product and decreased thermostability relative to wild type, which demonstrated that turnover rate was the selected property that led to higher fitness. With no direct pressure to maintain thermostability, the enzymes became less stable while they acquired higher turnover. The selected enzymes were also more flexible than wild type.

Ellington and co-workers [27•] sought to increase the resistance to glutaraldehyde of β-glucuronidase (GUS) in order to extend its use as a reporter in gene expression studies. After one round of error-prone PCR and two rounds of DNA shuffling, a GUS mutant was isolated with catalytic activity resistant to ~80-fold higher levels of glutaraldehyde than wild type. Even though glutaraldehyde is known to modify and crosslink lysine residues in proteins, only one of the amino acid substitutions in the octuple mutant GUS occurred at a lysine. The remaining seven mutations mapped onto the surface of the protein near lysine residues, implying that the surface chemistry of the enzyme has evolved to either accept or avoid glutaraldehyde modifications. It was also demonstrated that, in contrast to wild type, the evolved GUS could be used as a cell-lineage tracer in a model organism (*Xenopus* embryos).

Evolution of a novel activity

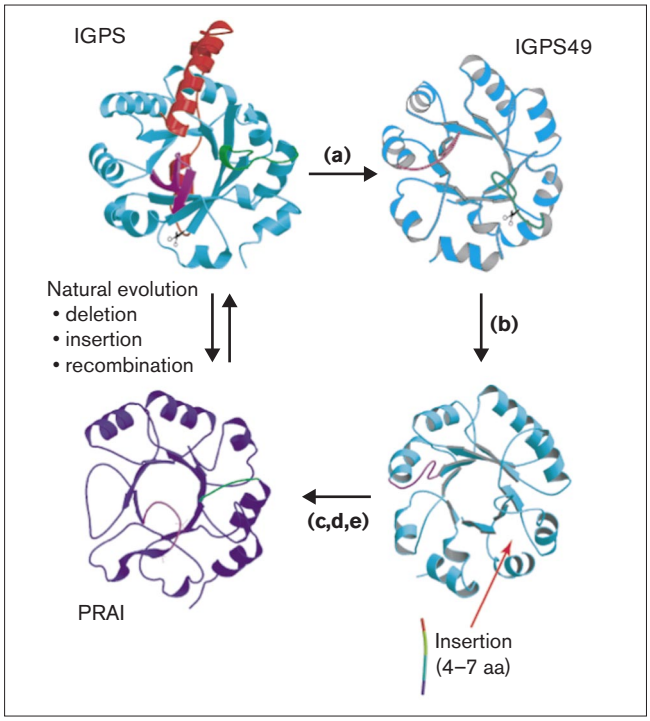
Using a combination of rational design and directed evolution methods, Fersht and co-workers [28••] provided a fine example of evolution of a novel function in the scaffold of an α/β-barrel protein by switching the activity of indole-3-glycerol-phosphate synthase (IGPS) to that of phosphoribosylanthranilate isomerase (PRAI). Substrate-binding residues and catalytic residues occur in structurally distinct regions in IGPS and PRAI, and these enzymes share a common ligand, carboxyphenylamino-1′-deoxyribulose-5′-phosphate, which is the substrate of IGPS and the product of PRAI.

As outlined in Figure 2, the strategy for interconverting these two enzymes consisted of identifying and targeting the regions in the IGPS scaffold that are significantly different from those in PRAI; 48 residues from the amino-terminus of IGPS were deleted, the β1α1 active-site loop was shortened by deleting 15 residues and inserting randomized sequences of 4–7 residues, and the β6α6 active-site loop on the combined mixture of the β1α1 libraries was modified by introducing a PRAI consensus sequence and an aspartic acid residue at position 184 to act as an active site general base. Members of this library showed weak or no ability to complement an *E. coli* PRAI-deficient strain. Two rounds of DNA shuffling and StEP recombination of selected clones, however, yielded a variant that had sixfold higher catalytic activity than wild-type PRAI and no IGPS activity. The evolved PRAI showed 28% amino acid sequence identity to PRAI and 90% to IGPS. Here, directed evolution was able to build upon the suboptimal rationally designed starting points to create a fully functional new enzyme. This work exemplifies the power of marrying rational and evolutionary design principles.

Engineering allosteric interactions

Fastrez and co-workers [29•] reported the engineering of a regulatable TEM-1 β-lactamase. Random peptide sequences of three to seven residues were inserted into solvent-exposed loops around the enzyme active site to create recognition sites, and the antibiotic resistance provided by β-lactamase was used for *in vivo* selection of active clones. Biopanning of the active libraries on immobilized monoclonal antibodies (MAbs) against the prostate-specific antigen (PSA) yielded enzyme variants whose activity was, in most cases, strongly inhibited upon MAb binding. In a competitive assay in which presence of PSA increased β-lactamase activity through binding to MAbs, PSA could be detected at a minimal concentration of 10 nM. As no information about the natural epitope is

Figure 2



Design strategy for building phosphoribosylanthranilate isomerase (PRAI) activity into the indole-3-glycerol-phosphate synthase (IGPS) scaffold. Top left, initial IGPS scaffold. Extra amino-terminal residues are red, active-site loops β1α1 and β6α6 are green and magenta, respectively. Bottom left, PRAI scaffold. **(a)** Deletion of 48 residues (red structure) from the amino terminus of IGPS yielding IGPS49. **(b)** Deletion of 15 amino acids corresponding to loop β1α1 and replacement with randomized sequences of 4–7 residues. **(c)** Introduction of an aspartic acid residue at position 184 and replacement of loop β6α6 by a PRAI consensus sequence. **(d)** *In vivo* selection of variants by complementation of an *E. coli* PRAI-deficient strain. **(e)** DNA shuffling of improved clones and selection to improve the new PRAI function of the scaffold. Reproduced from [28••] with permission.

required, this method may be used to generate molecular sensors for a wide variety of analytes.

A different approach was described for the creation of allosteric sites on GFP [30]. TEM-1 β-lactamase and β-lactamase-inhibitory protein (BLIP) were used as the model protein–ligand system in this study. β-Lactamase was inserted into a solvent-exposed loop of GFP, and this fusion protein was subjected to two rounds of error-prone PCR. Binding of BLIP to β-lactamase was expected to stabilize the conformation of the inserted domain and lead to increased GFP fluorescence in positive clones. Visual screening for colonies with enhanced fluorescence in the presence of BLIP yielded a BLIP-sensitive double mutant with both mutations in the β-lactamase region. *In vitro* characterization of the variant fusion protein confirmed that it was indeed acting as a BLIP sensor. A wide variety of 'allosteric GFP biosensors' may be constructed by this method.

Enzyme evolution by DNA family shuffling

DNA family shuffling, or evolution by *in vitro* recombination of naturally occurring homologous genes [8], has been used to improve a variety of enzyme properties [31•,32,33]. Herpes simplex virus thymidine kinase (HSV TK) is a promising target for gene therapy because of its ability to phosphorylate thymidine analogs not accepted by its human counterpart [34]. Stemmer and co-workers [31•] recombined the two most closely related HSV TK genes (78% DNA identity) to identify chimeras with enhanced ability to phosphorylate zidovudine (AZT). Recursive rounds of shuffling yielded two clones that sensitized *E. coli* to 32-fold less AZT compared with the most active parent. Both evolved chimeras exhibited a reduced K_M for AZT as well as relaxed specificity for thymidine.

Often in real-world applications, combinations of properties that may be achieved are as important as the improvements in individual properties. Ness *et al.* [35•] shuffled 26 subtilisin genes (56–99% identical at the DNA sequence level) to create a library of chimeras that were screened for four specific properties. Chimeras possessing all combinations of properties of the individual parents were identified, as were chimeras that were significantly improved over any of the parental enzymes for each single property. Suboptimal parents were found to contribute sequence elements to several improved clones.

Incremental truncation and nonhomologous recombination

Ostermeier *et al.* [36] introduced a new combinatorial approach to protein engineering termed incremental truncation. This methodology was first successfully applied to identify bisection points that would allow the conversion of a monomeric enzyme (*E. coli* glycinamide ribonucleotide formyltransferase [PurN]) into its functional heterodimer. An extension of this method, termed incremental truncation for the creation of hybrid enzymes (ITCHY), was developed to produce functional interspecies hybrids in a manner independent of DNA sequence homology [37•,38]. The method was tested on PurN and its human counterpart GART, which have only 50% identity at the DNA level [37•]. ITCHY libraries were created between the amino-terminus of PurN and the carboxy-terminus of GART by progressive truncation of the coding sequences with exonuclease III and ligation of the products. The most active PurN–GART hybrid identified by ITCHY showed ~500-fold reduced activity compared to wild-type PurN.

The ability of ITCHY and DNA shuffling to create functional hybrids was then compared. The shuffled library had a higher frequency of positives but was not very diverse: only single crossover hybrids were identified with all crossovers occurring in a region of high DNA homology. In contrast, functional hybrids identified by ITCHY (which are single crossover hybrids by necessity) had a

more diverse set of fusion points, including fusions in regions of nonhomology. The ITCHY method in combination with DNA shuffling offers the possibility to recombine nonhomologous genes [38]. The advantages of nonhomologous recombination to evolution *in vitro* need to be investigated further.

Yet more DNA recombination methods

Kikuchi *et al.* [33] reported a method for family shuffling in which they replaced the DNase I fragmentation step by DNA digestion with restriction enzymes in an effort to reduce the background from parental sequences. Using the restriction enzymes dramatically increased the frequency of chimera formation between two genes that share 84% identity at the DNA sequence level (from 1% to almost 100%). Digestion of each of the parental genes with several sets of restriction enzymes followed by digest reassembly in two steps results in the formation of more diverse chimeric structures.

Volkov *et al.* [39] describe a simple method, termed heteroduplex recombination, which creates libraries of chimeric DNA sequences derived from two homologous parental sequences. A heteroduplex formed *in vitro* is used to transform bacterial cells where repair of regions of non-identity in the heteroduplex creates a library of new sequences composed of elements from each parent. Heteroduplex recombination generates multiple crossovers and should be particularly useful for recombining large genes or entire operons. A convenient test system for evaluating the performance of DNA recombination methods was described in which recombination between truncated variants of GFP reconstructs the full-length gene and restores its characteristic fluorescence.

Conclusions

Directed evolution is being used to solve ever more difficult enzyme engineering problems. Future work needs to include enzymes of potential practical significance but which are ill-behaved: membrane-bound enzymes or those that require cofactors or other complex accessory proteins. Innovative strategies for generating diversity and high-throughput screening technologies will make it possible to evolve multifunctional enzymes and even whole biosynthetic pathways.

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