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Evolving strategies for enzyme engineering

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Directed evolution is a common technique to engineer enzymes
for a diverse set of applications. Structural information and an
understanding of how proteins respond to mutation and
recombination are being used to develop improved directed
evolution strategies by increasing the probability that mutant
sequences have the desired properties. Strategies that target
mutagenesis to particular regions of a protein or use
recombination to introduce large sequence changes can
complement full-gene random mutagenesis and pave the way
to achieving ever more ambitious enzyme engineering goals.

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Introduction

Enzymes are Nature's catalysts, tremendously accelerating the rates of a wide range of biochemical reactions, often with exquisite specificity. Harnessing enzymes for other purposes usually requires engineering them to improve their activity or stability. One approach to engineering enzymes is to make specific modifications, but this demands a detailed and frequently unattainable understanding of the relationship between sequence and function. Directed evolution bypasses this problem in much the same way as natural evolution, by combining mutation with selection or screening to identify improved variants.

Because it is never possible to test more than an infinitesimal fraction of the vast number of possible protein sequences, it is essential to have a strategy for creating directed evolution sequence libraries that are rich in proteins with the desired enzymatic function. Such libraries can be designed by drawing on our knowledge of how proteins respond to mutation [1–3] and of sequence-structure-function relationships. These libraries themselves in turn generate new information about proteins and protein evolution [4,5]. Here, we review recent successes in the directed evolution of enzymes, with a special focus on how knowledge is incorporated into directed evolution strategies. Other recent reviews describe in detail how these engineered enzymes have been utilized in chemical synthesis [6] and as components of engineered pathways [7,8].

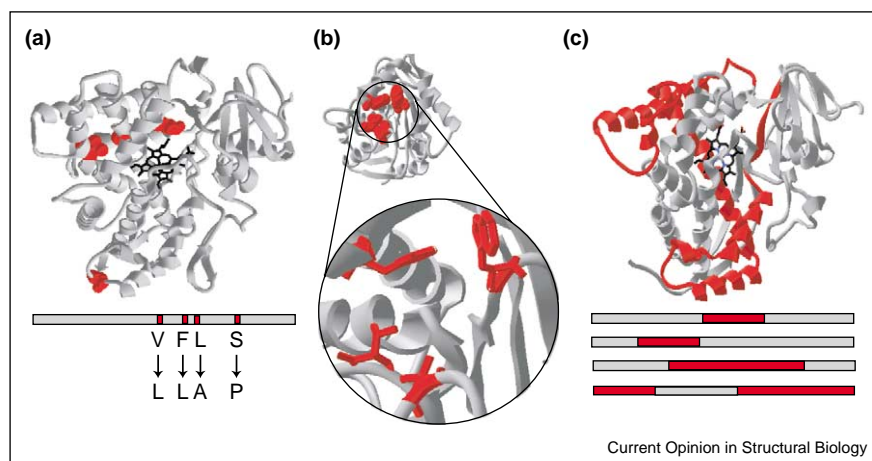## Directed evolution strategies

Directed evolution works when the researcher can find at least one enzyme with improved properties in the sequence library. The most naïve strategy of creating a library of random protein sequences is not useful for most enzyme engineering goals. Although sequences with simple functions such as ATP binding [9] or primitive esterase activity [10] have been isolated from random or hydrophobic-polar patterned sequence libraries, such libraries have yet to yield the complex functional enzymes that are typically the goal of engineering. Indeed, one recent study estimated that the fraction of all sequences that fold into enzymes with a specified biochemical function is as low as 1 in 10^{77} [11].

Most directed evolution strategies involve making relatively small changes to existing enzymes. This takes advantage of the fact that enzymes often have a range of weak promiscuous activities that are quickly improved with just a few mutations [12**] (see also Update). However, only a small number of random mutations can be made at a time, as each new mutation typically inactivates between 30 and 40% of the remaining active proteins [1]. This exponential decline in the fraction of functional proteins is due to the fact that amino acid mutations tend to destabilize a protein's native structure [2]. Below, we consider strategies (Figure 1) for introducing enough sequence diversity to create enzymes with the desired properties.

Random mutagenesis

The most straightforward strategy for library construction is to randomly mutate the full gene of an enzyme with a function close to the desired function. This approach requires no structural or mechanistic information, and can uncover unexpected beneficial mutations. Using sequential rounds of error-prone PCR to make an average of a few mutations per gene, followed by screening or

Figure 1

Random mutagenesis, targeted mutagenesis and recombination are three strategies for producing sequence libraries for directed evolution. **(a)** Random mutagenesis introduces amino acid substitutions throughout the protein and can uncover beneficial mutations distant from the active site. The red residues in the structure at top show four mutations uncovered by random mutagenesis that enhanced the activity of mammalian cytochrome P450 2B1 on several substrates [15]. The bar below shows the distribution of mutations in the primary sequence. **(b)** Targeted mutagenesis uses structural information to select a small number of amino acids for randomization and is a good method for making multiple active site mutations. The red residues were targeted for mutagenesis to increase the enantioselectivity of *Pseudomonas fluorescens* esterase [26•]. **(c)** Recombination of protein sequences can introduce large numbers of simultaneous sequence changes. The structure shows a chimera of two bacterial cytochrome P450 sequences (fragments from different parents are shown in red and gray) that exhibits an altered functional spectrum [48]. Chimeras are created by recombining fragments of different protein sequences, indicated by the bars. The PDB structures in the figure are 1SUO, 1VA4 and 2HPD, respectively.

selection for improved variants, is effective for a wide range of engineering goals. The creation of enantioselective catalysts from an enzyme whose structure is unknown is one such application. A single round of error-prone PCR produced several dozen cyclohexane monooxygenases with *R* or *S* selectivity [13•], and a second round of random mutagenesis and screening generated an enzyme that catalyzed the reaction with an enantiomeric excess of 90%. Error-prone PCR was used to enhance the selectivity of a cytosine deaminase designed for use in killing tumor cells [14]. Cytochromes P450, which are important for the metabolism of drugs and other xenobiotics, have been engineered with multiple rounds of error-prone PCR for increased activity on several biologically active compounds [15,16]. Many of the beneficial mutations found by random mutagenesis of these P450s are distant from the active site and would not have been predicted by current 'rational' design methods.

Beneficial mutations found by random mutagenesis can be combined by DNA shuffling. A study with β-glucuronidase showed that beneficial mutations drive each other to extinction during recursive random mutagenesis, but that this problem can be eliminated by DNA shuffling [17]. Random mutagenesis and DNA shuffling were used to isolate *Taq* polymerase mutants with expanded substrate repertoires [18]. A similar approach was taken to engineer a pectin lyase enzyme with a melting temperature of 73 °C for use in cotton fabric processing [19]. All

pectin lyase single mutants were constructed and the 12 most thermostable ones were identified. Enzymes with all combinations of these 12 single mutants were then screened for thermostability. The best single mutant showed an increase in the melting temperature of about 3 °C, but the best combined mutant was 16 °C more stable.

Random mutagenesis can also uncover additional beneficial mutations in rationally designed enzymes. The Withers laboratory [20] used two rounds of error-prone PCR and screening to enhance the oligosaccharide synthesis activity of a rationally designed glycosynthase by 27-fold. Several of the beneficial mutations were distant from the active site. Similarly, a yeast three-hybrid selection identified glycosynthase mutants with a total of four amino acid substitutions distant from the active site [21]. Hellinga and co-workers [22] used a computational design algorithm to engineer triose phosphate isomerase activity into a ribose-binding protein. The designed enzyme enhanced the reaction rate by more than five orders of magnitude, and a single round of error-prone PCR provided surface mutations that doubled the enzyme's catalytic rate and allowed it to support cell growth on glycerol.

Targeted mutagenesis
Some engineering goals, such as dramatically altering an enzyme's specificity or regioselectivity, may require mul-

tiple mutations of the active site. Such combinations of mutations are difficult to uncover with full-gene random mutagenesis, because libraries with small numbers of mutations contain multiple active site mutations at low frequency, whereas libraries with large numbers of mutations will contain mostly inactive sequences. Additionally, single nucleotide changes to a codon typically access only about six of the nineteen possible amino acid substitutions. These problems can be circumvented by targeting mutagenesis to a subset of residues deemed likely to affect the desired function. This strategy requires structural or biochemical data in order to choose just a small subset of positions, as the number of possible sequences increases exponentially with the number of residues that are being mutated simultaneously.

Using a high-resolution crystal structure to target mutagenesis to three active site residues, Hill *et al.* [23] created a triple mutant of phosphotriesterase with a rate enhancement of three orders of magnitude for the degradation of organic triesters such as those used in chemical warfare agents. Crucially, two of the corresponding single mutants did not increase activity and so would not have been identified if they had been explored one at a time.

The problem of inverting the enantioselectivity of a lipase offers an interesting comparison between full-gene random mutagenesis and targeted mutagenesis. Reetz and co-workers [24] used several rounds of full-gene random mutagenesis and DNA shuffling to invert the enantioselectivity of a lipase of unknown structure from *S* to *R*. Another lipase was engineered for the same goal by simultaneous mutation of four active site residues [25]. A single round of this targeted mutagenesis uncovered two mutants with enantioselectivities comparable to those of the enzymes created by several rounds of full-gene mutagenesis. These two mutants each also unexpectedly contained a substitution at another location, although it is not known if these mutations were beneficial or neutral. Kazlauskas and co-workers [26●] compared targeted mutagenesis with full-gene random mutagenesis for enhancing the enantioselectivity of an esterase. They concluded that beneficial mutations can be found both in and distant from the active site, but that active site mutations tended to be more effective.

A variety of other enzymes have recently been engineered by targeted mutagenesis. Mutating three active site residues of penicillin acylase created six variants with improved activity, five of which were triple mutants [27]. Juillerat *et al.* [28] targeted four active site residues to engineer an *O*6-alkylguanine-DNA alkyltransferase for the efficient *in vivo* labeling of fusion proteins. They developed a selection system that allowed them to examine over 20,000 mutants and found that the best variants were triple mutants, suggesting the importance of simultaneously exploring multiple mutations.

Novel DNA and RNA polymerases have also been engineered by targeted mutagenesis. Chelliserrykattil and Ellington [29] mutated four amino acids in RNA polymerase to engineer the enzyme to transcribe 2′-*O*-methyl RNA. Using a screen that selected variants that generated more RNA, they identified several mutants that incorporated nucleotides modified at the 2′ position. Fa *et al.* [30] used targeted mutagenesis to engineer a DNA polymerase to specifically incorporate 2′-*O*-methyl ribonucleoside triphosphates by mutating six amino acids and selecting improved variants using phage display. Targeted mutagenesis of two active site residues was used to engineer a thioredoxin protein to replace the disulfide bond formation system in *Escherichia coli* [31].

Schultz and co-workers have created tRNA synthetases that charge orthogonal tRNAs with non-natural amino acids by targeting mutagenesis to five or six amino acids involved in substrate recognition. They then performed a positive selection for recognition of the non-natural amino acid and a negative selection against recognition of other amino acids [32]. In the past year, this technique has been used to engineer synthetases for the incorporation of *p*-iodo-L-phenylalanine into proteins for structure determination [33], glycosylated amino acids for the synthesis of glycoproteins [34] and a photocaged cysteine that can be deprotected upon UV irradiation [35].

The best mutants discovered by targeted mutagenesis almost always contain multiple mutations. These mutations are often beneficial as single mutants, but evidence is accumulating that at least some of them are beneficial only in combination [23]. A possible reason for these synergistic effects is that multiple active site mutations can cause significant structural changes of the active site, as has been noted for tRNA synthetases [32]. In this case, targeted mutagenesis strategies that simultaneously mutate multiple residues offer an approach to uncovering coupled mutations that cannot be found using random mutagenesis or saturation mutagenesis of single residues.

Recombination

Recombining structurally similar proteins can access larger degrees of sequence change than random mutagenesis [3]. Family shuffling recombines natural proteins with high sequence identity without any need for structural information and can lead to significant improvements in activity. Family shuffling of two different microbial aryl dioxygenase operons generated an enzyme with fourfold enhanced activity for use in a short semi-synthetic route to the high-value flavor compound strawberry furanone [36]. Dioxygenase proteins have also been engineered to degrade environmentally damaging polychlorinated biphenyl (PCB) compounds by family shuffling [37]. Family shuffling of four mammalian serum paraoxonase genes created paraoxonases that could be expressed in *E. coli* [38]. The sequence changes in the

more soluble variants were numerous and subtle, for instance, changing hydrophobic residues to other hydrophobic residues. One of the soluble variants was used to solve the X-ray crystal structure of these important proteins [39]. Random mutagenesis was combined with family shuffling to increase the activity of an enzyme with glyphosate *N*-acetyltransferase activity 10,000-fold, allowing it to confer resistance to the herbicide glyphosate on *Arabidopsis*, tobacco, maize and *E. coli* [40••]. The information derived from sequence alignments of protein families can also be incorporated into directed evolution by building libraries in which positions are mutated to those residues that occur most frequently in multiple sequence alignments. Amin *et al.* [41•] used two rounds of this approach to increase the melting temperature of *Enterobacter cloacae* β-lactamase by 9 °C.

The family shuffling protocol relies on regions of sequence identity to create crossovers that recombine the sequences of related proteins. This protocol is therefore limited to proteins with more than 70–75% identity, because libraries created from more diverged sequences tend to yield mostly parent sequences. A variety of methods have been developed to avoid this problem in the recombination of divergent sequences by using mismatched PCR primer pairs [42–45] or the assembly of oligonucleotides [46].

The use of structural information can enrich the fraction of folded proteins in recombination libraries. Choosing crossover sites that preserve residue–residue interactions in the new chimeras has been shown to be helpful in predicting whether β-lactamase [47] and cytochrome P450 [48] chimeras retain function or fold. FAMCLASH [49] is an energy function that incorporates chemical and sequence knowledge to predict the relative activities of chimeras. Experimental data on the efficacy of this energy function are still sparse, as it has been applied only to single-crossover libraries of dihydrofolate reductase. The RASPP algorithm [50] can be used in combination with any pairwise energy function to determine the best crossover locations for constructing libraries. Recombination libraries themselves can also be used to gather information about the structural and functional modularity of proteins [51,52].

Although the studies described above demonstrate that recombining highly diverged but homologous sequences can produce libraries of diverse folded sequences, so far there has been little work to test whether it is also a useful method for discovering new functions. A tantalizing hint is that four out of fourteen chimeras of two cytochrome P450 proteins with 64% sequence identity show new product profiles [48]. Further work is necessary on this topic.

Non-homologous recombination that combines fragments of unrelated proteins is another way to introduce large sequence changes. A new methodology was used to recombine the non-homologous chorismate mutase and fumarase proteins [53]. However, only 0.0005% of the resulting sequences retained chorismate mutase activity and most of these sequences were nearly full-length chorismate mutase proteins with small fragments of fumarase near the termini. This illustrates the potential difficulty of recombining non-homologous proteins without using additional structural information.

A striking application of non-homologous recombination is Ostermeier and co-workers' creation of a protein that combines the activity of a β-lactamase with the maltose-induced conformational change of maltose-binding protein. In one experiment, they randomly inserted the lactamase sequence into the maltose-binding protein and screened for mutants with enhanced lactamase activity in the presence of maltose [54••]. In a second experiment, they first circularly permuted the lactamase sequence and then randomly inserted it into the maltose-binding protein [55]. Interestingly, the majority of the maltose-inducible lactamases consisted of β-lactamase inserted into regions of the maltose-binding protein that had been previously determined to be permissive to insertions, suggesting that the efficiency of this technique could be further improved by including structural information.

Conclusions

Directed evolution is now an established method to engineer enzymes for a wide range of uses. Full-gene random mutagenesis continues to be a straightforward and powerful tool, and studies using this approach repeatedly illustrate that beneficial mutations can occur at unexpected sites. Targeted mutagenesis and recombination can extend directed evolution to the engineering of enzyme properties that require more than a few uncoupled changes in a protein's sequence (which are easily obtained by sequential rounds of random mutagenesis and screening). The increasing incorporation of structural and chemical knowledge will undoubtedly enhance the utility of these methods. The growing use of rational design in conjunction with directed evolution offers the exciting promise of generating libraries containing a high frequency of sequences with the desired functional properties.

Update

Recent work has emphasized the tendency of directed evolution to improve weak promiscuous functions by broadening specificity, as discussed in [12••]. Gould and Tawfik [56] used random mutagenesis and DNA shuffling to enhance the promiscuous esterase activity of carbonic anhydrase by a factor of 40, while leaving the native activity largely unchanged. The alternative goal of narrowing specificity was achieved in the engineering of proteases by simultaneously selecting for cleavage of an

Ala–Arg bond while selecting against cleavage of the Arg–Arg bond preferred by the original enzyme using fluorescence-assisted cell sorting (FACS) [57•]. The evolved enzyme exhibited three-million-fold selectivity for the new peptide bond over the original one, demonstrating that directed evolution can create more specific enzymes when an appropriate counterselection is used to reduce the undesired activity. In this case, a single amino acid substitution effected the change in specificity. We anticipate that many other applications will require multiple substitutions to achieve similar results.

Acknowledgements

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