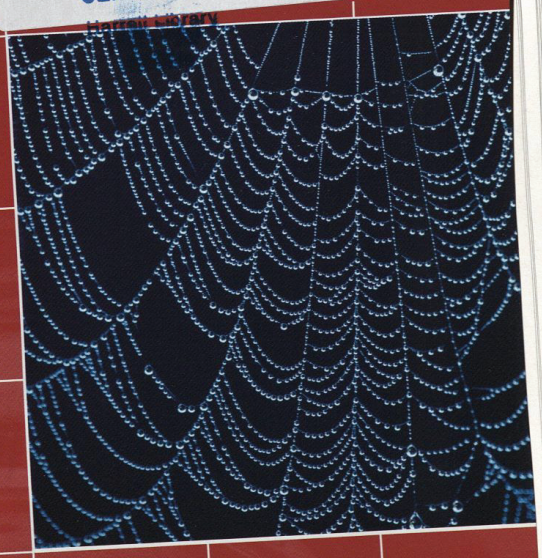
Vol 16 No 4 August 2005

ELSEVIER

Current Opinion in Biotechnology

Martin Rosenberg & Kenneth Timmis, Editors

RECEIVED
SEP 0 9 2005



August 2005

Protein technologies and commercial enzymes

Edited by Bernhard Hauer and Brian K Kay

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Semi-rational approaches to engineering enzyme activity: combining the benefits of directed evolution and rational design

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Many research groups successfully rely on whole-gene random mutagenesis and recombination approaches for the directed evolution of enzymes. Recent advances in enzyme engineering have used a combination of these random methods of directed evolution with elements of rational enzyme modification to successfully by-pass certain limitations of both directed evolution and rational design. Semi-rational approaches that target multiple, specific residues to mutate on the basis of prior structural or functional knowledge create 'smart' libraries that are more likely to yield positive results. Efficient sampling of mutations likely to affect enzyme function has been conducted both experimentally and, on a much greater scale, computationally, with remarkable improvements in substrate selectivity and specificity and in the *de novo* design of enzyme activities within scaffolds of known structure.

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Current Opinion in Biotechnology 2005, **16**:378–384

This review comes from a themed issue on
Protein technologies and commercial enzymes
Edited by Bernhard Hauer and Brian K Kay

Available online 1st July 2005

0958-1669/$ – see front matter
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DOI 10.1016/j.copbio.2005.06.004

Introduction

Enzymes are powerful catalysts that are able to increase reaction rates by up to 17 orders of magnitude [1,2]. Certain enzymes display perfect control over stereochemistry and regioselectivity, while others display a breadth of specificity; either feature may be attractive for industrial and synthetic applications [3]. However, few enzymes naturally catalyze the reactions that chemists need under conditions that are industrially convenient and economically advantageous. Many efforts are currently devoted to the modification of enzyme activities to meet the needs of today's chemists.

Multiple approaches have been developed to allow the identification of mutant enzymes possessing desirable qualities such as increased activity, modified specificity, selectivity or cofactor binding [4]. The earliest approach was rational design, which was used to modify the specificity of enzymes [5–9]. This approach requires an in-depth knowledge of the structural features of the enzyme active site and their contribution to function. The complexity of the structure/function relationship in enzymes has proven to be the factor limiting the general application of rational design. More recently, directed evolution has proven to be a powerful tool for the modification of enzyme activities and has become the most widely used approach. Briefly, directed evolution consists of the low frequency introduction of randomly distributed mutations in a gene of interest, followed by selection of the mutated proteins possessing the desired properties. Recent advances in this area include the application of the immune system 'hypermutation' process, which is conducted entirely in live cells [10]. Directed evolution can enable the relatively rapid engineering of enzymes without requiring an in-depth understanding of structure/function relationships, unlike rational design. The numerous methodologies and successes of directed evolution will not be detailed here as recent reviews have been devoted to the topic [11–15] (also see the article by AA Henry and FE Romesberg in this issue). Because large numbers of mutants must generally be screened to obtain a significant, desired effect on enzyme activity, the main limitation of directed evolution is the necessity of developing a high-throughput screening methodology that allows identification of the desired property under relevant conditions. Not all enzyme activities are readily amenable to developing a high-throughput screening method, nor are all screening methodologies easy to implement at the required scale.

A further limitation of directed evolution relates to the random nature of the mutations introduced: in the case where functional information (from point mutations, random mutagenesis or deduction by sequence alignment) or structural information exists, it would be advantageous to exploit this by concentrating mutations where they might be the most effective [16]. Analysis of enzyme modification results indicates that the majority of mutations that beneficially affect certain enzyme properties (enantioselectivity, substrate specificity and new catalytic activities) are located in or near the active site, and more specifically near residues that are implicated in binding or catalysis [15,17,18•,19]. Other properties (stability and activity) can be improved by mutations either near or far from the active site, although there are many more mutations far from the active site to test out [19].

Table 1

Comparison of approaches for engineering enzyme activity.

	Rational design	Random mutagenesis	Semi-rational design
High-throughput screening or selection method	Not essential	Essential	Advantageous but not essential
Structural and/or functional information	Both essential	Neither essential	Either is sufficient
Sequence space exploration	Low	Moderate, random	Experimental: moderate, targeted Computational: vast, targeted
Probability of obtaining synergistic mutations	Moderate	Low	High

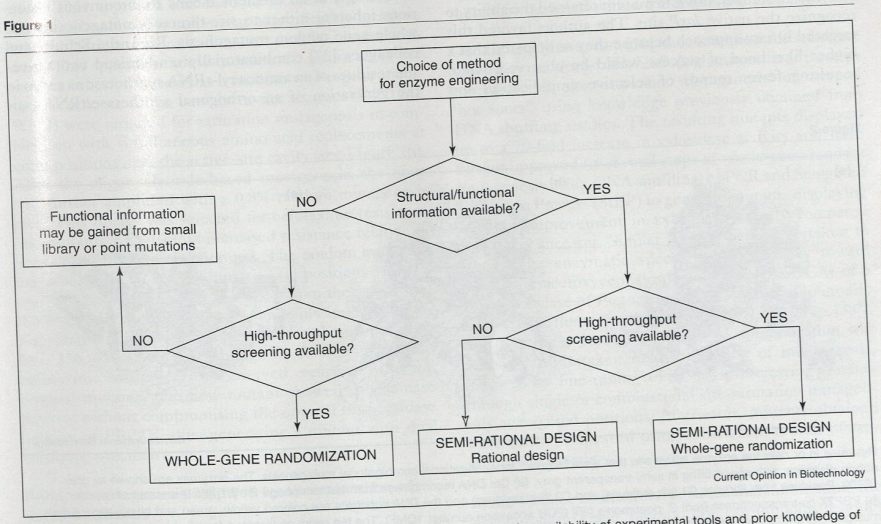
Consequently, saturation mutagenesis (where all 20 natural amino acids are tested at residues in or near the active site) can be coupled to recombination of these mutations in order to increase the likelihood of beneficially modifying the catalytic activity relative to random mutagenesis approaches. This 'semi-rational' approach could be particularly advantageous in instances where no high-throughput screening method is available, based on the argument that a 'smarter' library can be built with the same number of mutants than with a random whole-gene mutagenesis approach [15] (Table 1; Figure 1).

This review provides an overview of recent articles where the semi-rational design of enzyme activities has been successfully demonstrated through the use of combinatorial mutagenesis biased toward the active site, based both on experimental and computational approaches.

Modifying enzyme activity using experimental semi-rational approaches

The experimental combination of rational and random protein engineering approaches has been successfully applied toward the modification of enzyme activities. The following examples have been grouped according to the specific experimental approaches applied, which are guided by the amount and nature of structural and functional information available at the outset of the study.

Figure 1



Selection of the preferred experimental approach for enzyme engineering based on the availability of experimental tools and prior knowledge of structure and function. Rational design, semi-rational design or whole-gene randomization each refer to multiple methodologies, as outlined in the text. The enzyme engineering approach that may have the greatest potential for success is in upper case letters, while alternative approaches are in lower case letters.

Corteva Exhibit 2038 - Page 2 of 5
Bayer v. Corteva - IPR2023-01038

Targeted randomization of defined residues based on structural knowledge

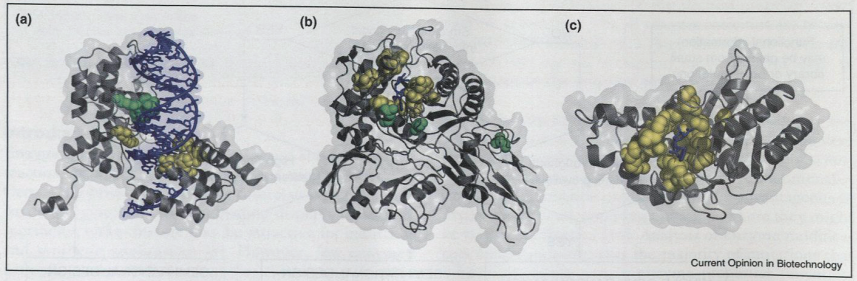
Structural information, when available, can allow one to target specific residues in direct contact with the substrate or near the active-site cavity for mutagenesis, alone or in combination. Although the choice of the positions to mutate remains rational in most cases, the choice of amino acids to be encoded can be broad; furthermore, the simultaneous randomization at targeted positions may result in synergistic effects [20] that could not have been predicted by mutating positions individually.

Santoro and Schultz [21•] have used targeted randomization to modify the substrate specificity of a Cre DNA recombinase from bacteriophage P1 that recognizes DNA sequences known as *loxP* sites. The researchers used structural information of the Cre–DNA complex to target two regions of the Cre recombinase in contact with *loxP* base pairs suspected to be important for recombination activity. By creating two distinct libraries (C1 and C2; see Figure 2a) of five and six simultaneously randomized residues in contact with variants of *loxP* sites (library size $\sim 10^8$) and by subjecting the *E. coli*-transformed libraries to a powerful recombination assay of fluorescent reporter proteins sorted by FACS (fluorescence-activated cell sorting), they isolated a mutant that efficiently recombined a new *loxP* site not recognized by the wild-type enzyme. Moreover, this Cre mutant retained the ability to recognize the native *loxP* site. The authors favored this targeted library approach because they anticipated that a higher likelihood of success would be observed, while requiring fewer rounds of selective amplification and mutagenesis compared with random mutagenesis of the whole gene.

Similarly, in a successful attempt to investigate the detoxification role of epoxide hydrolases, Rui *et al.* [22] used a combination of rational design and saturation mutagenesis at targeted active-site positions to expand the substrate range of an epoxide hydrolase from *Agrobacterium radiobacter* AD1 (EchA) to include chlorinated epoxides. On the basis of a careful investigation of the active site by structural comparison to related enzymes, residues F108, C248, I219 and I111 (in single-letter amino acid code), in the vicinity of the catalytic triad, were separately randomized by saturation mutagenesis. As mutant C248I showed a slight increase in rate of *cis*-1,2-dichloroethylene (*cis*-DCE) mineralization (2.7-fold increase with respect to the wild type), it was used as a template for successive rounds of saturation mutagenesis targeting residues F108 and I219. The approach generated the triple active-site area mutant F108L-I219L-C248L, which displayed a 10-fold enhancement in *cis*-DCE mineralization relative to wild-type EchA.

Several other groups [23–26] have also successfully conducted simultaneous or successive targeted randomization of multiple active-site positions based on structural knowledge, as an efficient means to circumvent limitations inherent either to site-directed mutagenesis or to whole-gene random mutagenesis. Recently, Schultz and colleagues [27] combinatorially randomized two active-site residues of an aminoacyl-tRNA synthetase as a step in the generation of an orthogonal synthetase/tRNA pair

Figure 2



Residues in or near the enzyme active site that were targeted for semi-rational combinatorial mutagenesis. The enzymes are shown as grey ribbon diagrams with space-filling in semi-transparent gray. **(a)** Cre DNA recombinase from bacteriophage P1 [21] (PDB accession number 1CRX). Residues from libraries C1 (six residues) and C2 (five residues) and the DNA substrate are colored yellow, green and blue, respectively. **(b)** PBP2X DD-transpeptidase from *S. pneumonia* [30] (PDB accession number 1QMF). The ten residues targeted for combinatorial site-directed mutagenesis are colored yellow and the three subsequent unforeseen beneficial mutations obtained by epPCR are colored green. Cefuroxime is acylated at the active-site residue Ser337 and is colored blue. **(c)** TEM-1 β-lactamase from *E. coli* [43] (PDB accession number 1FQG). The 19 active-site residues targeted for *in silico* mutagenesis using PDA are colored yellow. The benzylpenicillin antibiotic is acylated at the active-site residue Ser70 and is colored blue. Figure generated with Pymol Release 0.98 (http://www.pymol.org).

that efficiently and selectively incorporates an unnatural amino acid into proteins.

In some cases, the structural information available is not sufficient to rationally select residues to be randomized. Thus, molecular modelling studies have been successfully used to identify residues most likely to be in contact with substrate molecules. Our group used this approach to perform combinatorial targeted mutagenesis of all 16 principal active-site residues of type II R67 dihydrofolate reductase, allowing the selection of new, highly modified active-site environments with activity at least as great as the native enzyme [28]. Similarly, the substrate specificity of *Bacillus stearothermophilus* SD1 D-hydantoinase was modified by a semi-rational mutagenesis approach targeting residues chosen on the basis of previous modelling studies [29].

Simultaneous random mutagenesis and site-saturation of defined residues

Based on evolutionary protein-fold similarity between two related enzyme families, Peimbert and Segovia [30] introduced a β-lactamase activity into PBP2X DD-transpeptidase from *Streptococcus pneumonia* through a combination of error-prone polymerase chain reaction (epPCR) and saturation mutagenesis at targeted positions. This approach was used to increase the odds of obtaining beneficial mutations at unpredictable locations in combination with mutations at carefully chosen residues. This approach should increase the return using the same number of mutants as a random whole-gene mutagenesis scheme. Thus, two active-site residues (F450 and W374) were targeted for saturation mutagenesis in combination with simultaneous amino acid replacements at eight positions near the active-site cavity (see Figure 2b). After the oligonucleotide-based mutagenesis, the gene was further amplified with a 0.8% random mutagenesis rate. This library was selected for cefotaxime resistance and mutants with 10-fold increased resistance relative to wild-type PBP2X were obtained. The random mutagenesis resulted in additional mutations at positions 312, 452 and 554, both proximal to and distal from the active site. To assess the impact of certain randomly inserted mutations, saturation mutagenesis was undertaken at positions 312, 336, 450 and 452. Although no further increase in cefotaxime resistance was observed compared with the original mutants, one new mutant showed β-lactamase activity without compromising the original DD-peptidase activity of PBP2X, thus generating a mutant with dual substrate specificity.

Random mutagenesis followed by site-saturation of defined residues

The structural or functional information necessary to make rational choices for the residues to mutate is not always available. To circumvent this limitation, several research groups have efficiently undertaken rounds of whole-gene randomization to provide 'leads' (i.e. residues identified as potentially advantageous when mutated) in conjunction with fine-tuning of the 'lead mutants' through site-saturation mutagenesis of the identified residues.

Using such a cyclical random/targeted approach, Reetz and collaborators greatly improved the enantioselectivity *(E)* of *Pseudomonas aeruginosa* lipases toward a *p*-nitrophenyl ester in favour of the (2*S*) enantiomer (reviewed in [31••]). Initial improvement from $E = 1.1$ (wild-type enzyme) to $E = 11.3$ was achieved after four rounds of epPCR. Using the mutational knowledge obtained by epPCR, they conducted saturation mutagenesis at given mutational 'hot spots' of the lipase, obtaining $E = 20$. Subsequent rounds of epPCR increased E to 25. In addition, they developed a modified version of Stemmer's combinatorial multiple-cassette mutagenesis method [32] to mutate a defined region of the lipase (residues 160–163) as well as several 'hot spots' (residues 155 and 162). This semi-rational method yielded their most highly enantioselective lipase variant to date ($E = 51$). Other groups have recently demonstrated the power of similar semi-rational approaches toward the evolution of enantioselective enzymes. As a result of the multiple random mutagenesis steps, this approach has also generated many mutations far from the active-site cavity in addition to mutations designed within the active-site cavity [33–35].

Geddie and Matsumura [36] have modified the substrate specificity of *Escherichia coli* β-glucuronidase through combinatorial site-saturation mutagenesis of several 'hot spots', using knowledge previously obtained from DNA shuffling studies. The resulting mutants displayed up to a 70-fold increase in xylosidase activity and were further improved by several steps of whole-gene random mutagenesis using DNA shuffling, epPCR and Staggered Extension Process (StEP) to generate mutants displaying 100-fold improvement in xylosidase activity compared with their ancestor. Similar studies were undertaken to modify the enzymatic specificity and activity toward 7-aminodesacetoxycephalosporanic acid (7-ADCA) of a glutaryl acylase of *Pseudomonas* SY-77 [37] and to modify and improve fluorescent proteins from *Discosoma* sp. [38•]. In all of these studies, whole-gene randomization was generally undertaken in a first round of mutagenesis, followed by 'fine-tuning' of the most interesting mutants through single or combinatorial site-saturation mutagenesis at targeted positions. Afterwards, mutants obtained were further improved using subsequent rounds of random mutagenesis.

In the absence of structural or functional information, the 'Evolutionary Trace' method [39] can pinpoint residues to mutate. This method entails correlating evolutionary variations within a gene of interest with divergences in the phylogenetic tree of that sequence family. This has

Corteva Exhibit 2038 - Page 3 of 5
Bayer v. Corteva - IPR2023-01038

been shown to reveal the relative functional importance of residues and to identify functional sites [40]. A recent review by Minshull *et al.* [41] provides a highly comprehensive overview of this and other approaches for identifying residues that may be functionally relevant.

These recent examples demonstrate the power of structure-based semi-rational approaches targeting active-site residues in enzyme engineering by enhancing our capacity for rational design, while exploiting the advantageous aspects of random mutagenesis. We can thus cycle between light sampling of randomly distributed mutations and saturation mutagenesis at a limited number of positions likely to affect the property under study, increasing the likelihood of identifying beneficial, cooperative effects with respect to enzyme catalysis.

Semi-rational and combinatorial design using computational approaches

Although semi-rational, combinatorial mutagenesis directed at active-site residues limits the number of variants generated relative to available sequence space and theoretically focuses on an area of sequence space where improvements have a greater likelihood of occurring, it can nonetheless generate greater numbers of mutants than can be screened. Indeed, combinatorial randomization of only five residues generates a library of 20^5 possibilities (3.2×10^6 mutants), too large a number for manual screening. Thus, to increase the power of semi-rational and combinatorial modification of enzyme activities, computational methods have been developed based on protein design algorithms. These methods can either perform a virtual screening of a vast library or can be applied to the design of enzyme active sites.

The first method is the computational screening of mutant sequences of a virtual library. Computational screening can screen libraries of 10^{80} variants [42], allowing for a first layer of virtual screening to eliminate mutations inconsistent with the protein fold. This is an emerging area in protein design and has seldom yet been applied specifically to enzyme design. However, its resounding success and great promise merit discussion herein. Hayes *et al.* [43•] developed a strategy for the computational screening of large libraries called Protein Design Automation (PDA), for prediction of the optimal sequence that can adopt a desired fold. PDA was used for prescreening large, virtual libraries of mutants (10^{23}), thus decreasing the sequence space of interest by many orders of magnitude. PDA allows all, or a rationally defined set of residues, to change. The optimal sequence is chosen based on its lowest conformational energy and is used to identify other near-optimal sequences through Monte Carlo simulated annealing. The mutations that occur most frequently define the library to be experimentally screened. Using PDA, Hayes and colleagues pinpointed 19 residues of interest in TEM-1 β-lactamase (see Figure 2c), generated *in silico* 7×10^{23} combinatorial mutants of these residues, and chose cut-offs to define the library of roughly 200 000 lowest-energy mutants that were then generated experimentally by mutagenesis and recombination. By selection against the antibiotic cefotaxime, the authors identified a mutant harbouring six mutations in the vicinity of the active site, with a 1280-fold increase in resistance to cefotaxime that was attributed to an increase in relative substrate specificity toward cefotaxime versus ampicillin.

The advantage of PDA is that it samples a vast sequence diversity and allows for multiple mutations to be identified simultaneously, which is particularly beneficial when the effect of multiple mutations is synergistic (non-additive) [20,44]. Furthermore, PDA generates mutations at the level of the amino acid sequence rather than at the level of the nucleotide sequence. Thus, there is no bias against mutations requiring two or three nucleotide modifications, contrary to the important bias that exists in standard random mutagenesis methods. This approach vastly increases explorable sequence space, but has not been specifically designed for improvement of enzyme activity.

The second computational method for semi-rational and combinatorial design, which has been specifically applied to the design of enzyme active sites, was developed by Hellinga and co-workers [45••]. This ground-breaking work, involving the prediction of mutations that are necessary for the introduction of catalytically active sites in non-catalytic protein scaffolds, allowed the authors to achieve the most dramatic success in computational enzyme design to date. In their report, Dwyer *et al.* [45••] converted a non-catalytic ribose-binding protein (RBP) into an analog of triose phosphate isomerase (TIM). Their protein design algorithm first predicted mutations at the ribose-binding area to allow binding of the TIM substrate, dihydroxyacetone phosphate (DHAP) [46]. The algorithm then positioned a set of TIM catalytic residues in the new DHAP-binding pocket. Finally, 14 virtual constructs were experimentally generated and tested for TIM activity. Seven of the mutants possessed TIM activity greater than the background reaction. One of the seven designs was particularly active and through further improvements by computational design and random mutagenesis, allowed the generation of TIM analogs from a non-catalytic protein. The computational design allowed for the definition of 13 to 21 mutations, depending on the TIM analog, that introduce the necessary catalytic residues as well as residues forming a stereochemically complementary substrate-binding cavity. The most active TIM analogs displayed a 10^5–10^6-fold increase in reaction rate over background, which is the largest increase in reaction rate for a rationally designed enzyme to date [47–49], and was sufficiently active to support growth of TIM-deficient *E. coli*. An important contributing factor to the success of this

work results from their superior treatment of electrostatic interactions in a hetereogeneous protein environment [50]. Although this formidable achievement required prior knowledge of the precise chemical and steric requirements of the target activity, it opens the door to the broader creation of desirable catalytic activities within stable and well-behaved frameworks.

Conclusions

Targeted, combinatorial semi-rational mutagenesis is proving highly effective for improving enzyme activities, as it readily allows the creation of neighbouring mutations, of multiple simultaneous mutations and of mutations requiring multiple nucleotide substitutions. This could be particularly advantageous in the modification of enzyme activities, as active-site mutations are frequently coupled and have synergistic effects [20,44]. Important developments in computational methodologies promise to vastly increase the searchable sequence space. Taken with a judicious choice of experimental input, semi-rational mutagenesis permits the experimenter to focus mutations in areas more likely to yield 'lead' results. These methodologies pave the way to exciting areas of enzyme research including efficient modification of existing activities, the development of new activities within existing frameworks, as well as the evolution of 'promiscuous' catalytic activities allowing their efficient exploitation [51,52].

Acknowledgements

The authors thank Romas J Kazlauskas for his careful reading of the manuscript and his helpful comments.

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Corteva Exhibit 2038 - Page 4 of 5
Bayer v. Corteva - IPR2023-01038

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Understanding enzyme action on immobilised substrates

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With increasing interest in automated synthesis and screening protocols, solid supported chemistry and biochemistry are attractive technologies. Studies with surface-immobilised substrates have been carried out to analyse enzyme accessibility, kinetics and thermodynamics. Several interesting new methods have been developed to monitor enzyme action on substrates attached to a solid phase such as polymer beads glass or gold surfaces. These include fluorescence measurements, MALDI-TOF mass spectrometry, and the use of quartz crystal microbalances to measure weight changes of immobilised molecules directly on the surface. Approaches that allow spatial resolution in single beads have also been reported. The ability of enzymes to reach the inside of beads is becoming better characterised and new supports have been developed that allow improved accessibility. The equilibrium position of reactions on the solid surface can be substantially shifted compared with reactions in solution, and this can be usefully exploited using hydrolases in reverse. Research is also starting to tackle the way in which kinetics are modified when the substrates are surface immobilised.

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Current Opinion in Biotechnology 2005, **16**:385–392

This review comes from a themed issue on
Protein technologies and commercial enzymes
Edited by Bernhard Hauer and Brian K Kay

Available online 6th July 2005

0958-1669/$ – see front matter
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DOI 10.1016/j.copbio.2005.06.006

Introduction

Traditionally, enzyme action is studied with both catalyst and substrate in solution. The use of immobilised enzymes is also quite familiar, particularly in light of their applications as practical biocatalysts. In this case, the dissolved substrate molecules must diffuse to find the enzyme attached to a solid phase. This review is concerned with a third type of system, in which the substrate moieties are attached to a solid phase. Now the dissolved enzyme molecules must diffuse to the sites where the substrate is bound. A comparison is illustrated in Figure 1.

Several enzymes have evolved naturally to attack solid-phase substrate groups, such as cellulases and lysozymes. These will have special features aiding them to function this way, such as separate surface-binding domains. Enzymes specially adapted to work on solid-phase substrates will not be dealt with here, but recent reviews can be found [1–3].

More recently, enzymes that are normally used with dissolved substrates have been used to catalyse reactions on substrates attached to a solid phase, such as a polymer bead or a modified glass or gold surface. There are several motivations for studying such systems. With increasing interest in automated synthesis and screening protocols, solid supported chemistry and biochemistry are attractive technologies. Thus, enzymes are useful for selected steps in solid-phase chemical synthesis. Their selectivity and ability to act under mild conditions may be exploited in the removal of protecting groups [4], cleavage of linkers at the end of a synthesis [5], or to give selectivity in a particular coupling step [6•,7••,8–12]. There are several reports of on-bead screening of enzyme substrates made by combinatorial solid-phase synthesis [13]. More generally, enzyme assays can conveniently be carried out with solid-phase substrates [14•,15•,16–22], especially on microarrays for the rapid identification of substrates or inhibitors in parallel. There might also be relevance to many processes *in vivo*, in which substrates are attached to surfaces in living cells and tissues [23,24]. The attack on immobilised substrates by enzymes normally acting in solution may be significant, especially in various disease states such as arthritis [25].

Most published studies have concentrated on exploring the scope of applications in synthesis or screening. Rates and/or yields of biocatalytic reactions on solid-phase supports are generally disappointing compared with reactions in solution, as some authors have noted. (Yield can sometimes, but not always, be improved by using a large excess of enzyme.) Such low rates and yields have prompted more detailed fundamental studies of the kinetics and thermodynamics of biocatalytic reactions on immobilised substrates. These studies form the core topic of the present review, which discusses several issues: how to monitor enzyme catalysis on solid phase; how to define and measure kinetic and binding constants when the substrate is attached to a solid phase; how accessible is the substrate to the enzyme; and how will

Corteva Exhibit 2038 - Page 5 of 5
Bayer v. Corteva - IPR2023-01038