# Enzyme Redesign

Trevor M. Penning*

*Department of Pharmacology, University of Pennsylvania School of Medicine, 135 John Morgan Building, 3620 Hamilton Walk, Philadelphia, Pennsylvania 19104-6084*

Joseph M. Jez

*Structural Biology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037*

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## Contents



## I. Introduction

Enzyme redesign refers to the ability to rationally alter enzyme structure to cause a predicted change in function. This article discusses challenges facing the designer and the available tools in the designer's workshop to achieve this goal. It describes major success stories including changes in substrate specificity, inversion of stereochemistry, engineering new reaction mechanisms into the same active site, and conversion of ligand-binding sites into catalytic centers. These studies affect our ideas of enzyme evolution and raise the issue of whether chemistry (reaction mechanism) or specificity (ligand discrimination) evolved first.

* To whom correspondence should be addressed. Phone: (215) 898-9445. Fax: (215) 573-2236. E-mail: penning@pharm.med.upenn.edu.

## II. Goals and Challenges

The process of enzyme redesign addresses questions of catalysis and ligand recognition; it provides a basis for the applied use of engineered enzymes and presents a number of challenges for the protein engineer. First, is it possible to alter substrate specificity and obtain reasonable catalytic efficiencies ($k_{cat}/K_m$) without altering the overall reaction mechanism? Second, is it possible to change cofactor specificity (e.g., $NADP^+$ versus $NAD^+$)? Third, can redesign invert the stereochemistry of an enzyme-catalyzed reaction at the level of the substrate, product, or cofactor? Modifying the stereochemical outcome of a reaction may have practical benefits, since many enzymes serve as chiral catalysts for synthetic purposes in the chemical and pharmaceutical industry. Fourth, can alteration of an existing active site introduce catalysis of a new chemical reaction? Strategies directed at this aim include diverting covalent enzyme reaction intermediates down new pathways, unmasking and optimizing alternate reactions by site-directed mutagenesis, and mutation of active site residues to introduce new catalytic functions. Fifth, is it possible to alter the structure of a ligand-binding site sufficiently to introduce a catalytic activity into that site when none existed before? The last three goals represent the most difficult protein-engineering problems.

## III. The Designer's Workshop

### A. Choosing a Blueprint

The fundamental question of enzyme redesign is how to change enzyme A into enzyme B? Implicit in

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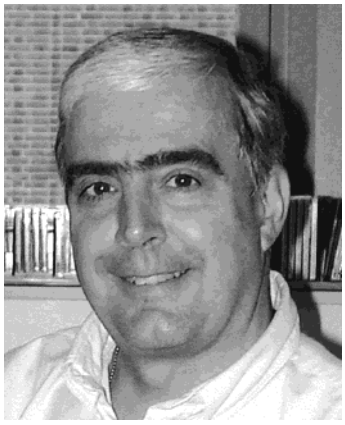
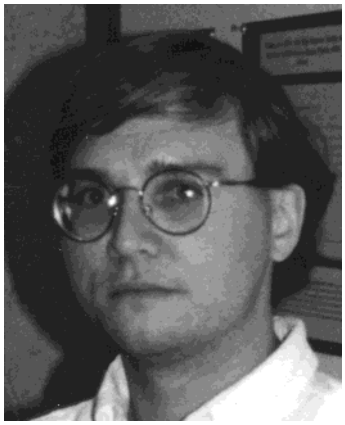


Trevor M. Penning is Professor of Pharmacology, Biochemistry & Biophysics and Obstetrics & Gynecology as well as Associate Dean for Postdoctoral Research Training at the University of Pennsylvania School of Medicine. He received his Ph.D. degree in Biochemistry from Southampton University, U.K., where he studied the mechanism of action of steroid hormones. He then went on to do postdoctoral studies at The Johns Hopkins University School of Medicine under the mentorship of Dr. Paul Talalay. In 1982 he became a faculty member at the University of Pennsylvania. Dr. Penning's research on the enzymology of steroid hormones has made him one of the premier investigators in the world in understanding the mechanism, structure, and specificity of the family of hydroxysteroid dehydrogenases. He has achieved scientific distinction as a world leader in the field of steroid biochemistry as demonstrated by over 90 publications, 50 abstracts, and 5 U.S. patents. He is the recipient of numerous prizes and awards. He received the Albert Ethelbert Ebert Prize and Medal from the American Pharmaceutical Association, he has been a recipient of a Career Development Award from the National Cancer Institute, and he was elected to The Johns Hopkins Society of Scholars in 1998. He serves on the Editorial Advisory Board for the *Biochemical Journal* and is on the Editorial Board of *Steroids*.



Joseph M. Jez was born on April 27, 1969 in Fairbanks, AK. He received his B.S. degree in Biochemistry from Penn State University (1992) and his Ph.D. degree in Biochemistry & Molecular Biophysics from the University of Pennsylvania (1998). As a graduate student he examined the determinants of steroid recognition in mammalian aldo–keto reductases and engineered the catalytic mechanism of a hydroxysteroid dehydrogenase into a carbon–carbon double bond reductase. His interests in studying and engineering enzyme reaction mechanisms lead him to the Salk Institute's Structural Biology Laboratory for postdoctoral training with Professor Joe Noel. There, using both X-ray crystallography and protein engineering methods, his current research focuses on understanding and manipulating the structure–function relationships of type III polyketide synthases. In his spare time, he frequents the desert climbing mecca of Joshua Tree.

the concept of design is the existence of a blueprint. For protein engineers, this often refers to the availability of structural and sequence information and is information intensive. The more precise this information, the more likely the approach will succeed. Three-dimensional structures obtained by either X-ray crystallography or NMR methods are ideal starting templates. In their absence, the designer can use the sequence of the targeted enzyme to construct a homology model from a related three-dimensional structure. If these options are not available, sequence search programs, such as BLAST (www.ncbi.nlm.nih.gov), can identify known signature motifs or focus attention on regions of interest by revealing amino acid sequence conservation and divergence. In many cases, mining structural and sequence databases, for example, the TIM-barrel database at argo.urv.es/~pujadas/TIM, the Protein Kinase Resource at www.sdsc.edu/kinase, or the Structural Classification of Protein site at scop.mrc-lmb.cam.ac.uk/scop, permits the experimenter to draw the best blueprint for a given protein family. Furthermore, powerful computer algorithms such as TESS[1] (TEmplate Search and Superimposition) can search the Protein Data Bank (PDB; www.rcsb.org/pdb) for substructural three-dimensional motifs. For example, this program has identified non-serine protease structures containing the Ser-His-Asp active site geometry of chymotrypsin.[1] In addition, the PROCAT database (www.biochem.ucl.ac.uk/bsm/PROCAT/PROCAT.html) generated by TESS provides a library of active site geometries that can be used as design templates. No matter what the final goal of the redesign process is, a continually expanding repertoire of tools exists to achieve that goal.

## B. Point Mutagenesis

Following identification of a target region by structural and sequence comparisons, testing of the design blueprint begins. Site-directed mutagenesis using oligonucleotides is the most powerful and widely used approach to introduce point mutations into the starting protein.[2] Two main methods currently are used (Figure 1). In the two-round polymerase chain reaction (PCR) method, the first round of PCR produces two double-stranded cDNA products. One product contains the desired mutation at the 5′-end, and the other product contains the desired mutation at the 3′-end. Heteroduplex formation occurs after denaturation and annealing, but the second round of PCR completes and amplifies only the heteroduplex with 3′-recessed ends resulting in a double-stranded cDNA containing the desired mutation. In the second method,[3−4] forward and reverse primers containing the desired mutation directly amplify from a plasmid template containing the gene target. *Dpn* methylase destroys the parental plasmid leaving behind the PCR-created nicked vector which contains the mutant gene ready for transformation into an *Escherichia coli* (*E. coli*) host. Since both methods are error-prone, sequencing of the PCR products ensures fidelity of the new gene product.

## C. Domain Swapping

Often alteration of larger segments of protein sequence succeeds in altering enzymatic activity when single- or multiple-point mutations fail. Generally, this approach works best when working with

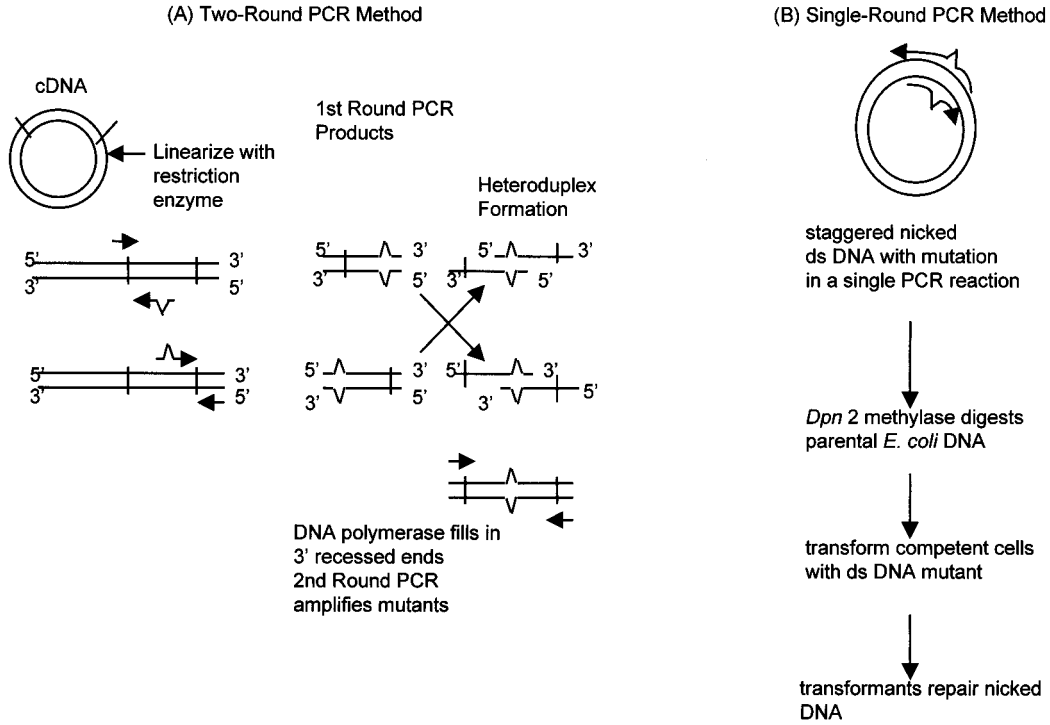



**Figure 1.** Common methods for introducing point mutations. (A) The two-round PCR method produces two products with the desired mutation at either the 5′- or 3′-end. Only the resulting heteroduplex with the mutation at the 3′-recessed end is amplified in the second round of PCR. (B) The single-round PCR method, which is increasingly popular, produces the desired mutation directly from a plasmid template.

two proteins from the same family.[5] Creation of enzyme chimeras uses two approaches. In the first, the two enzymes may share common restriction sites in their cDNAs that will permit swapping regions of sequence. However, naturally occurring restriction sites often do not allow for swapping of a specific functional domain (i.e., helix A may be the targeted region, but the readily available restriction sites cover loop 2, helix A, and loop 3). To solve this problem, the use of degenerate codons can introduce new compatible restriction sites that leave the amino acid codons unchanged. This allows for a more precise domain swap between the donor and acceptor proteins.

Peptide ligation is the second approach.[6,7] In this method, a target protein is assembled from a series of synthetic or biosynthetic peptide fragments. The key to peptide ligation involves a reversible *trans*-thioesterification reaction based on the mechanism of protein splicing mediated by naturally occurring inteins (Figure 2). The first step in protein splicing involves an N → S or N → O acyl shift involving transfer of the *N*-extein unit to the side-chain sulfhydryl or hydroxyl group of a conserved Cys/Ser/Thr residue located at the N-terminus of the intein. Then, transfer of the entire *N*-extein unit to a second conserved Cys/Ser/Thr residue at the intein-*C*-extein boundary occurs in a *trans*-thiolesterification reaction. Subsequent cyclization by an intein-*C* asparagine results in formation of a succinimide and loss of the intein. This leaves behind an *N*-extein-Cys-*C*-extein structure that joins the two starting pieces. Importantly, techniques for creating N-terminal cysteine proteins exist and allow for *trans*-thioesterification reactions with C-terminal thioester proteins using either expressed protein ligation or intein-mediated protein ligation methods. Synthesis of HIV-1 aspartyl protease from two 50 amino acid peptides followed this approach and permitted manipulation of its structure−function relationship through unnatural amino acid substitutions that would be inconceivable by standard mutagenesis techniques.[7]

## D. Directed Evolution

The use of random mutagenesis to achieve a desired change in function may seem like irrational design or no design altogether; however, this is a powerful protein-engineering tool when directed at short regions of sequence. In the strictest sense, a "Darwinian" approach is used which involves random mutagenesis followed by biological selection for the desired activity. The usefulness of random mutagenesis is that it permits a search through all of sequence space in a discrete region of a protein to achieve the desired outcome. This is clearly valuable when a lack of structural or mechanistic information results in no clear prediction of how to engineer a target. Since the evolution is directed by selection, a library can be enriched rapidly for the targeted change in function. If necessary, the progeny can be taken through multiple rounds of random mutagenesis to reach the desired goal.

The most common methods for generating random mutations that can be adopted for short segments are either phage display[8,9] or error-prone PCR[10,11] (Figure 3). In phage display, degenerate reverse primers in the PCR reaction randomly mutate the starting cDNA through a target region. The mixture of PCR

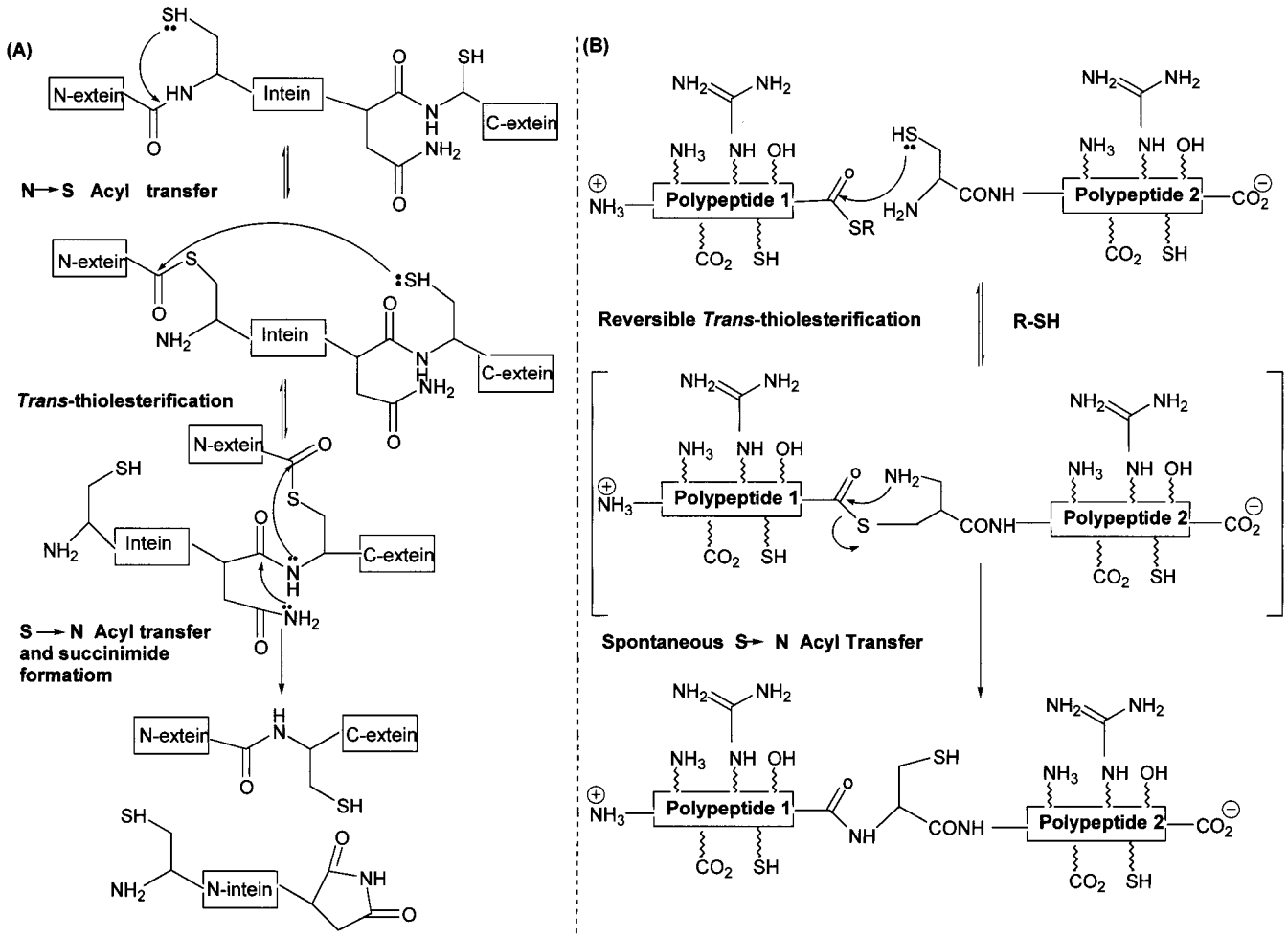



**Figure 2.** Peptide ligation. (A) Self-splicing catalyzed by extein-inteins sequentially involves N → S transfer, *trans*-thiolesterification, and S → N transfer with resultant succinimide formation. (B) Peptide ligation involving *trans*-thiolesterification involving a C-terminal thiol−ester and an N-terminal cysteine. In both instances a larger polypeptide is produced from two smaller peptide fragments.

products is then subcloned into an M13 bacteriophage vector encoding coat protein III of the filamentous phage f1. Each phage in the library expresses and displays a mutated protein fused to the coat protein on the phage surface. This allows screening or "biopanning" of the phage library for the desired function. Phage-DNA is plaque-purified from the positive clones and sequenced to obtain the sequence of the protein with the desired activity.

Two methods exist to promote error-prone PCR. The first involves a modified PCR protocol that uses varied $MgCl_2$ or $MnCl_2$ concentrations to achieve an average mutation frequency of 2−5 base substitutions per gene copy which corresponds to an error of one amino acid per mutated protein.[10] The second method uses mutation-inducible nucleosides in the PCR reaction. For example, 8-oxo-dGuo can pair with either A or C, whereas the amino tautomer of 3,4-dihydro-8*H*-pyrimido-[4,5-*c*]oxazine-7-one can pair with G and the imino tautomer with either A or G. Using these nucleosides, mutation frequencies ranging from 9.7 to 32.2 substitutions per 1000 nucleotides occur. These mutation frequencies far exceed those achieved by the first method.[11]

To vary the sequence over an entire gene, perhaps the most powerful method involves recombination of parent genes in a process akin to natural sexual recombination. This is accomplished by gene shuffling as first described by Stemmer.[12,13] In sexual recombination the progeny can have no more than two parents. The advantage of DNA shuffling is that many mutations can be introduced because pool-wise recombination of multiple parent genes is encouraged[14] (Figure 4). In one method, chimeric libraries are created by random fragmentation of the DNA sequences using DNase I followed by a reassembly of the fragments in a self-splicing chain-extension reaction catalyzed by DNA polymerase. DNA shuffling can also be achieved within the same protein family by replacing DNase I with restriction endonucleases. Digestion of each of the parental genes with several enzymes followed by digest-reassembly results in chimeras with diverse properties.[15] Other methods of varying sequence over an entire gene include incremental truncation[16] and a staggered extension process.[17] Each method uses a different strategy to increase the sequence diversity of a given set of genes.

Whether random mutagenesis is achieved through a short region of sequence or through an entire gene, the power of directed evolution is its biological selection which can be used iteratively to accumulate

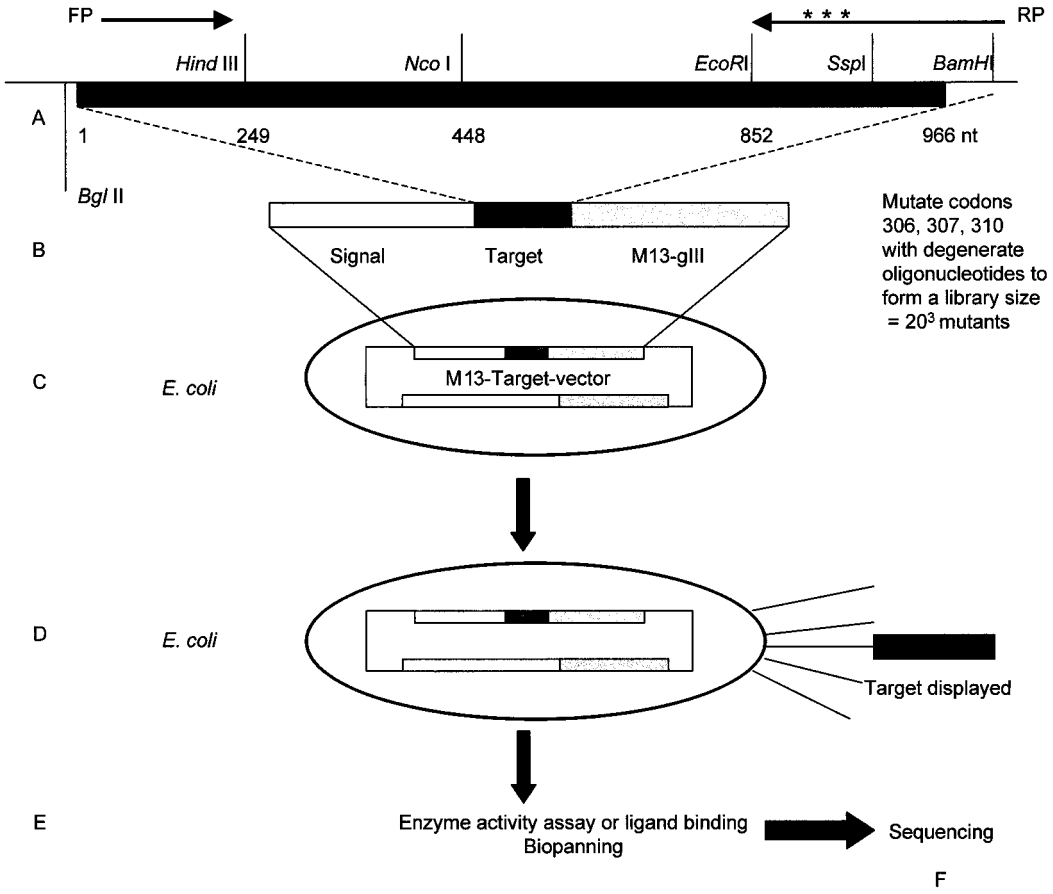



**Figure 3.** Generation of a phage display library of a C-terminal loop. (A) Forward and degenerate reverse primers are used in a PCR reaction to generate a library of products in which three amino acids (306, 307, and 310) are mutated to every other amino acid. (B,C) The PCR products are ligated into the nucleotide sequence of a M13-filamentous phage so that each mutant protein is fused to a coat protein. (D) Upon infection into the *E. coli* host, the coat protein is displayed as a fusion protein, and (E) the library is biopanned based on either ligand affinity or catalytic activity.

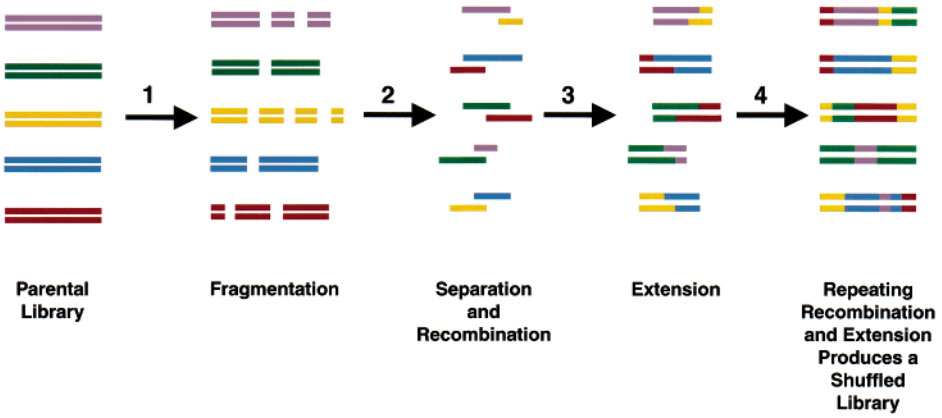



**Figure 4.** DNA shuffling. In Maxygen's Molecular Breeding, a library of parental genes is randomly fragmented by restriction digests (1). These fragments are heated to separate the DNA strands and then cooled to allow for recombination (2). This generates novel recombinations. These recombinants are extended (3). The process of re-annealing, recombination, and extension is repeated, resulting in a library of new full-length genes containing various recombinations (4). Screening of the new library can then identify a new enzymatic activity.

beneficial mutations and eliminate negative or neutral ones. Selection is best achieved through a tiered process in which the first round is based on a color change on an agar plate. Such methods can select mutants expressing enzymes that cause either a pH change (esterase) or a redox change (dehydrogenase). In both instances the agar plate can be impregnated with a pH- or redox-senstive dye so that desired mutations can be selected based on a halo effect. In the second round of selection positive mutants can be selected on microtiter plates based on their ability to produce a chromogenic or fluorescence change under optimized assay conditions. This rapid selection can lead to the identification of new enzymes that would be impossible to design by site-directed mutagenesis. Herein lies the rationale for this irrational process. Using this approach enzymes with inverted enantioselectivity and evolution of enzymes

with unusual stability or novel activities have been obtained.[17,18]

## *IV. Examples*

### A. Changing Substrate Specificity

Site-directed mutagenesis has been successful in redesigning the substrate specificity of a large number of common classes of enzymes, including oxidoreductases (dehydrogenases[19−24] and trypanothione/glutathione reductases[25−28]), hydrolases (acetylcholinesterases,[29−32] $\beta$-lactamases,[33−35] and proteases[36−59]), transferases (aminotransferases[60−64] and glutathione-*S*-transferase[65,66]), and restriction enzymes.[67−72] These experiments demonstrate that rational approaches based on a three-dimensional structure or amino acid sequence alignments can succeed at changing ligand recognition. In each of the examples that follow the chemical mechanism of the reaction remains unaltered.

#### *1. Oxidoreductases: Point Mutations and Loop Chimeras*

One of the early examples of changing substrate specificity was the conversion of lactate dehydrogenase (which converts lactate to pyruvate) into malate dehydrogenase (which converts malate to oxaloacetic acid).[19] A solid understanding of the structure and function of each enzyme aided in this feat. Three-dimensional structures of both enzymes provided clear templates that delineated the structural differences between each enzyme. Lactate dehydrogenase poorly catalyzes the reduction of oxaloacetic acid (compare $k_{cat}/K_m$ pyruvate $= 4.2 \times 10^6$ $M^{-1}$ $s^{-1}$ and $k_{cat}/K_m$ oxaloacetic acid $= 4.0 \times 10^3$ $M^{-1}$ $s^{-1}$). Oxaloacetic acid contains a carboxymethyl group which pyruvate lacks. Mutations of three residues that stabilize the substrate pocket of lactate dehydrogenase (D197N, T246G, and Q102R) individually increased the $k_{cat}/K_m$ for oxaloacetic acid. However, the shift in $k_{cat}/K_m$ for oxaloacetic acid with the Q102R mutant resulted in a dramatic $10^7$-fold increase for the desired reaction. Gln 102 is on a loop, and its replacement with an arginine residue provides a counterion for the additional carboxylic acid in oxaloacetic acid (Figure 5). Importantly, the lactate dehydrogenase Q102R mutant abolished the primary deuterium isotope effect associated with oxaloacetic acid reduction, indicating that hydride transfer is no longer rate limiting. In malate dehydrogenase, there is no primary isotope effect during the reduction of oxaloacetic acid suggesting that $k_{chem}$ in the Q120R mimics that of the target enzyme. Although these point mutants succeeded in optimizing an existing activity, there are examples where substrate specificity has been altered in oxidoreductases when there was no existing activity to optimize.

A good example is the conversion of rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD) into 20α-HSD.[24] 3α-HSD catalyzes the inactivation of androgens and reduces 5α-dihydrotestosterone into 3α-androstanediol (the reduction reaction occurs at the 3-keto position in the steroid A-ring). The target enzyme catalyzes the inactivation of progestins and

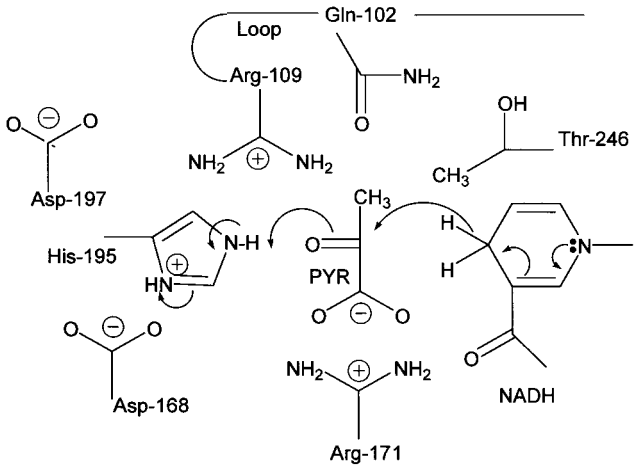



**Figure 5.** Changing lactate to malate dehydrogenase. The active site environment and catalytic mechanism of native lactate dehydrogenase is shown. The residues responsible for catalytic efficiency are indicated: Arg 109 polarizes the carbonyl of pyruvate, His 195 donates a proton, Arg 171 binds and orients the substrate, and Asp 168 stabilizes the protonated His 195. Mutation of Gln 102 to Arg effectively increases malate dehydrogenase activity by providing a counterion for the carboxymethyl group of oxaloacetic acid.

reduces progesterone to 20α-hydroxyprogesterone (the reduction reaction occurs at the 20-keto position of the steroid D ring) (Figure 6). Both enzymes are members of the aldo−keto reductase (AKR) superfamily and share 67% amino acid sequence identity. The crystal structure of 3α-HSD·$NADP^+$·testosterone complex identified 10 residues recruited from five loops that comprise the mature steroid-binding site. In 3α-HSD, the steroid A ring binds at the active site. For 20α-HSD activity, the steroid D ring must bind at the active site. Each enzyme is devoid of the other activity. Sequence alignments showed that six residues differ between the steroid-binding pockets of 3α- and 20α-HSD. Mutation of each of these amino acids individually and collectively failed to convert 3α-HSD into 20α-HSD. Using a chimeric approach, replacement of three of the five loops in 3α-HSD with the corresponding loops of 20α-HSD shifted the $k_{cat}/K_m$ for the reduction of progestins $10^{10}$-fold over that for androgens. There are few examples where changes in substrate specificity have been so discriminatory.

#### *2. Hydrolases: Classic Templates*

Serine proteases share a conserved catalytic mechanism.[73] Peptide bond cleavage occurs through a tetrahedral oxyanion intermediate with an acyl−enzyme intermediate aiding group transfer to water. Proteases accommodate a wide variety of substrates by having ancillary binding sites referred to as S1, S2 or P1 and P2. Redesign of these sites leads to altered substrate specificity, as demonstrated by the engineering of trypsin into a "chymotrypsin-like" enzyme. Although trypsin and chymotrypsin share similar three-dimensional structures and a common reaction mechanism, they differ in substrate preference. Trypsin cleaves between basic residues (Arg/Lys) in a peptide, while chymotrypsin cleaves between bulky hydrophobic residues (Phe/Tyr) in a peptide. Chymotrypsin ($k_{cat}/K_m = 1.6 \times 10^6$ $M^{-1}$ $s^{-1}$)

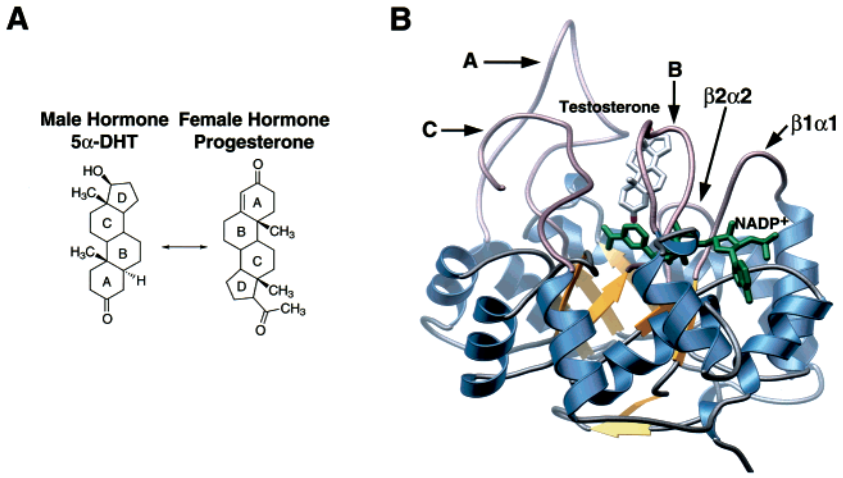



**Figure 6.** Changing 3α-HSD to 20α-HSD. (A, left) The structural relationship between the male sex hormone 5α-dihydrotestosterone and the female sex hormone progesterone. To convert 3α-HSD to 20α-HSD, the D-ring of progesterone must bind in the position of the A-ring of 5α-dihydrotestosterone. (B, right) The $(\alpha/\beta)_8$-barrel motif of the starting protein and the perpendicular relationship that exists between the cofactor $NADP^+$ and a steroid ligand in 3α-HSD validates the need to rotate the steroid at the active site.

exhibits trace trypsin activity ($k_{cat}/K_m = 4.5\ M^{-1}\ s^{-1}$). Point mutations in the S1 site of trypsin aimed at converting it to chymotrypsin produced only a modest increase in chymotrypsin activity.[36] Surprisingly, swapping of two loops that are not part of the S1 site of trypsin with the corresponding loops from chymotrypsin yielded a mutant trypsin with a $k_{cat}/K_m = 2.8 \times 10^3\ M^{-1}\ s^{-1}$ for chymotrypsin activity.[38] These loops are not responsible for substrate recognition, but they are responsible for bringing the S1 site into the correct conformation. These last three examples emphasize that specificity can reside beyond the amino acids directly contacting the substrate, requiring loop swaps to convert one enzyme into another.

### *3. Transferases: Changing Group Transfer*

Aminotransferases catalyze the interconversion of amino acids with their corresponding α-keto acids using pyridoxal phosphate as an amino group transfer reagent. Using the three-dimensional structure of *E. coli* aspartate aminotransferase as a template, Onuffer and Kirsch[61] generated a homology model of the *E. coli* tyrosine aminotransferase and identified six positions that differed between the two enzymes. Two positions (Thr 109 and Asn 297) are invariant in all aspartate aminotransferases but differed in tyrosine aminotransferase (Ser 109 and Ser 297). Four apolar residues in tyrosine aminotransferase (Leu 39, Tyr 41, Ile 47, and Leu 69) replaced the corresponding residues (Val 39, Lys 41, Thr 47, and Asn 69) in the active site pocket of aspartate aminotransferase. Mutagenesis of all six residues in aspartate aminotransferase to those found in tyrosine aminotransferase successfully altered substrate specificity. In another case, directed evolution changed aspartate aminotransferase into a valine aminotransferase.[60] A mutant enzyme with 17 substitutions showed a $2.1 \times 10^6$-fold increase in $k_{cat}/K_m$ for valine, a non-native substrate. Surprisingly, only one of the mutations directly contacts the substrate. The cumulative effect of the remaining mutations alters the overall structure of the enzyme that encloses the substrate-binding site and results in the shift in specificity. This study shows that it is unlikely that this change in specificity could have been achieved by a rational site-directed mutagenesis approach.

Glutathione S-transferases (GSTs) catalyze the conjugation of glutathione (GSH) to a diverse set of electrophilic substrates. Structurally, the major classes of GSTs (α, $\mu$, and $\pi$) have a site that binds hydrophobic electrophiles (the H subsite) and a functionally conserved site that binds GSH in an orientation that permits activation of the thiolate for catalysis (the G subsite). Variability in the H site accounts for substrate specificity and consists of 10 amino acid residues in GST A1−1. Random mutagenesis of these 10 residues generated a phage display library that was screened based on the ability to bind immobilized ligands, e.g., *p*-carboxy-*S*-benzyl-GSH conjugates. A set of mutants bound this ligand with a 5-fold higher affinity than wild-type GST A1−1. Several of the mutants also catalyzed rates of GSH conjugation to 1-chloro-2,4-dinitrobenzene (DCNB) at rates $10^3$-fold higher than that observed for the noncatalyzed reaction, suggesting that these randomly generated GST mutants may catalyze novel GSH transfer reactions.[65] Likewise, by targeting the H subsite site, Gulick and Fahl[66] demonstrated that random mutagenesis of three regions gave a more efficient drug detoxification GST. Selection of *E. coli* expressing the library yielded enzymes that confer nearly 10-fold greater drug resistance than wild-type GST to a nitrogen mustard.

## B. Altering Cofactor Requirements

Enzymes as biological catalysts use cofactors to act either as redox partners [NAD(P)(H), FAD(H), or FMN(H)] or for group transfer reactions (thiamine pyrophosphate, coenzyme A, or pyridoxal phosphate). Since cofactor requirements for enzyme-catalyzed reactions are functionally constrained, the engineering of cofactor requirements has been limited to changes in selectivity (i.e., preference for NAD(H) versus NADP(H) or substitution of unnatural cofactors).

### 1. NAD(H) versus NADP(H)

Nicotinamide adenine dinucleotides bind to target proteins using either a Rossmann-fold or non-Rossmann-fold motifs. Protein engineering has succeeded in redesigning the cofactor specificity in both types of binding fold. Structurally, NAD(H) and NADP(H) differ by a phosphate group at the 2′-position of the AMP moiety. This simple chemical difference affects the biological use of each cofactor. Oxidative degradation reactions primarily use $NAD^+$, whereas reductive biosynthetic reactions mainly use NADPH. As a consequence, the enzymes that use nicotinamide cofactors are often specific for either NAD(H) or NADP(H) and offer an attractive target for switching cofactor requirements. The conversion of a NADP(H) to an NAD(H) site is desirable if the target enzyme serves as a chiral catalyst in a synthetic reaction, since the diphosphate nucleotide is less expensive than its triphosphate counterpart.

The first example of altered coenzyme specificity changed glutathione reductase from an $NADP^+$-dependent enzyme into an $NAD^+$-dependent form.[74] Glutathione reductase catalyzes the reduction of oxidized glutathione (GSSG) to GSH with NADPH as a coenzyme. In the three-dimensional structure of human glutathione reductase, NADPH binds within a canonical $\beta\alpha\beta\alpha\beta$ dinucleotide-binding motif or Rossmann-fold.[75] This structure revealed a set of basic amino acid residues, including two arginines, interacting with the 2′-phosphate group of AMP. Modeling the structure of *E. coli* glutathione reductase on the structure of the human enzyme suggested a similar set of interactions. Mutation of the arginines in the *E. coli* enzyme reduced catalytic efficiency with NADPH 500-fold but only improved the catalytic efficiency for NADH 2.3-fold, indicating that other structural features impart NADH specificity. Ultimately, seven mutations (A179G, A183G, V197E, R198M, K199F, H200D, and R204P) converted glutathione reductase into an enzyme with a 18 000-fold preference for NADH over NADPH. Subsequent analysis of the crystal structure of the NADH-dependent mutant enzyme showed that the additional mutations caused rotation of a peptide bond close to the cofactor and changed the pucker of the ribose on adenosine.[76] This change in conformation allowed the formation of two additional hydrogen bonds between the 2′- and 3′-hydroxyl groups of ribose and the carboxylate of the newly introduced Glu 197 and accounts for NADH specificity. Similarly, introduction of a set of basic residues into the NAD(H)-binding Rossmann-fold motif of dihydrolipoamide dehydrogenase converts the cofactor specificity of the enzyme from NAD(H) to NADP(H).[77]

Redesign experiments also demonstrate how to switch nicotinamide cofactor specificity in enzymes lacking the Rossmann-fold motif. Enzymes of the aldo−keto reductase (AKR) superfamily bind pyridine nucleotide cofactors in an unusual extended conformation in which a "salt-linked" safety belt locks itself across the cofactor's pyrophosphate bridge. Importantly, the 2′-phosphate group of the AMP moiety forms an electrostatic linkage with a conserved arginine or lysine residue. Mutation of the conserved basic residue abolishes high-affinity binding for $NADP^+$, so that AKRs now bind $NAD^+$ with equal affinity.[78,79] Other examples of changing cofactor preference in enzymes that lack a Rossmann-fold are conversion of isocitrate dehydrogenase into an NAD-preferring enzyme[80] and changing isopropylmalate dehydrogenase into an enzyme with a 1000-fold preference for NADPH.[81] In nearly all cases elimination or introduction of basic residues that interact with the 2′-phosphate of AMP of NADP(H) often in combination with additional mutations leads to successful conversion of cofactor preference.

### 2. ATP versus Unnatural ATP Analogs

Recent engineering of protein kinases to accept unnatural nucleotides expands the goals of enzyme design beyond the understanding of cofactor recognition to probing signal transduction pathways.[82−86] Shokat and co-workers[82,83] modified the nucleotide specificity of the prototypical tyrosine kinase Src to nucleotides not used by wild-type Src. A single highly conserved amino acid in the ATP-binding site of Src, Ile 338, controls recognition of substituted ATP analogues. When mutated to an alanine or a glycine, the mutant kinase accepts ATP analogues with large substituents at the $N^6$ position of the adenine ring. By providing an unnatural ATP analogue as a phosphate donor, the modified kinase provides a tool to identify the direct protein substrates of Src. Extending this idea to inhibition of kinase targets, the redesign of kinase active sites to accept unique nucleotide inhibitors allows for direct investigation of the cellular function of a target kinase in a signal transduction pathway.[85,86]

## C. Inverting Reaction Stereochemistry

As asymmetric or chiral catalysts, enzymes distinguish between optical or geometric isomers to attain stereospecificity in their reactions. This property has allowed for incorporation of enzymes into a range of organic synthesis.[87] Altering the stereochemical outcome of an enzyme reaction for synthetic advantage represents a difficult problem for the enzyme designer whether using rational[88−96] or directed evolution[97,98] methods. However, mutagenesis experiments have successfully modified the enantioselectivity of target enzymes for substrates or products, and attempts have tried to alter the stereochemistry of hydride transfer.

### 1. Substrates and Products

Changes in enzyme stereospecificity by mutagenesis benefits from examining structural templates. For example, Tsai and co-workers used the structure of yeast adenylate kinase to alter the chirality of the terminal phosphate transferred to AMP by chicken adenylate kinase.[88] By mutating an arginine that interacts with a phosphorothioate group of diastereomers of ADPαS at the active site, the adenylate kinase R44M mutant produces ($R_p$)-adenosine-5′-(1-thiodiphosphate) [($R_p$)-ADPαS] instead of ($S_p$)-ADPαS (Figure 7).

An approach that has worked is to mutate the active site of the target enzyme so that the substrate

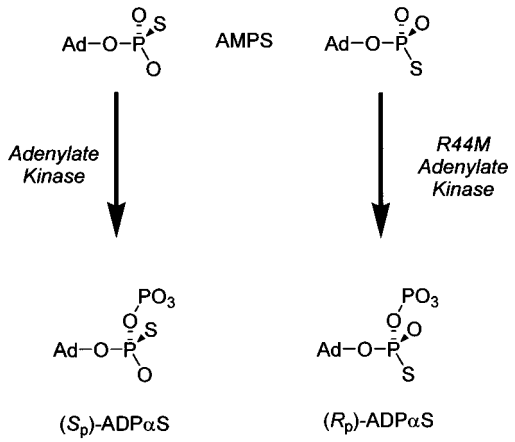



**Figure 7.** Inverting the stereochemical course of adenylate kinase. Adenylate kinase catalyzes the phosphorylation of adenosine 5′-monothiophosphate (AMPS) at the *pro-R* oxygen to yield ($S_p$)-adenosine-5′-(1-thiodiphosphate) [($S_p$)-ADPαS]. The R44M mutant generates ($R_p$)-ADPαS by phosphorylating the *pro-S* oxygen of AMPS.

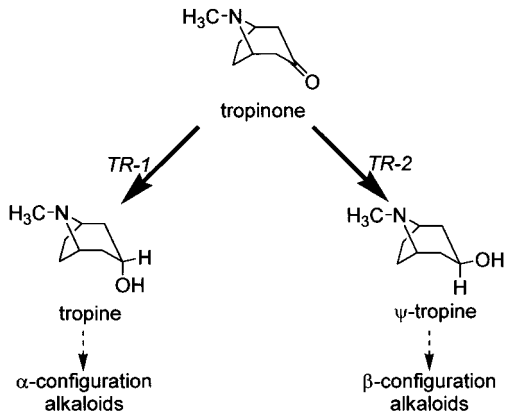



**Figure 8.** Reaction stereochemistry of tropinone reductases (TR) 1 and 2. Using tropinone as a common substrate, TR-1 produces tropine (3α-hydroxytropane) and TR-2 generates *ψ*-tropine (3*β*-hydroxytropane).

rotates 180° to invert the stereochemistry of the reaction. Recent work on tropinone reductase (TR) represents a thorough account of altering stereospecificity via this route.[94,99] In tropane alkaloid biosynthesis, two TRs serve as a metabolic branch point. Both enzymes catalyze the NADPH-dependent reduction of the 3-carbonyl group of their common substrate; however, TR-1 produces tropine (3α-hydroxytropane) and TR-2 produces *ψ*-tropine (3*β*-hydroxytropane) (Figure 8). TR-1 and TR-2 share 64% amino acid sequence identity and have nearly identical three-dimensional structures.[99] As expected, the substrate-binding sites of TR-1 and TR-2 were structurally different, and modeling studies suggested that the tropinone molecule binds in an inverted orientation in one active site leading to either the α- or *β*-enantiomer. Subsequent mutagenesis identified five positions within the active site responsible for the stereospecificity of TR-1 and TR-2.[94] Mutations at these positions switched the stereospecificity of TR-1 into that of TR-2 and vice versa, indicating that the substrate flips its orientation in the binding pocket to invert the reaction stereochemistry.

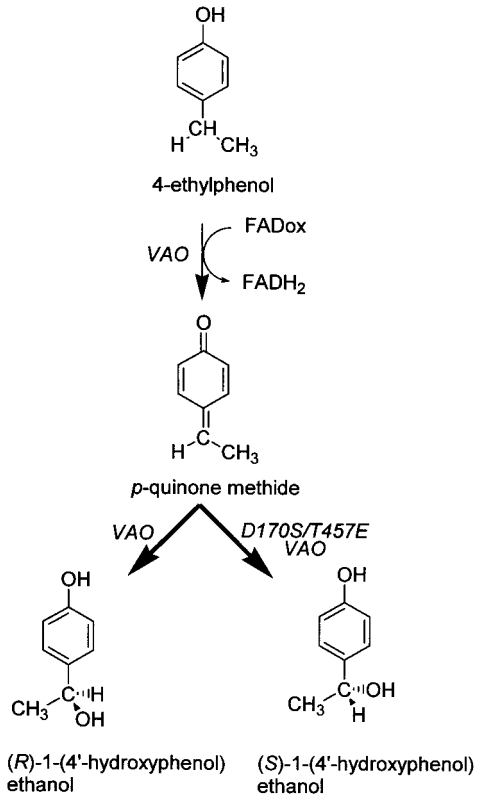



**Figure 9.** Inverting the reaction stereochemistry of vanillyl−alcohol oxidase (VAO). Wild-type VAO catalyzes the stereospecific hydroxylation of 4-ethylphenol into (*R*)-1-(4′-hydroxyphenol)ethanol. The VAO D170S/T457E double mutant produces greater than 80% of the (*S*)-enantiomer.

A similar tack is to relocate an essential catalytic residue within the active site, effectively inverting the active site relative to the reaction center on the substrate. van Den Heuvel et al.[95] demonstrated the success of this approach by inverting the stereospecificity of vanillyl−alcohol oxidase (VAO). VAO is a flavoenzyme that catalyzes the stereospecific hydroxylation of 4-ethylphenol (Figure 9). The three-dimensional structure of the enzyme suggested that Asp 170 serves as a general base during hydration of an intermediate *p*-quinone methide. Moving the base to the opposite side of the active site cavity resulted in a mutant (D170S/T457E) that was strongly *S*- rather than *R*-selective, yielding an enantiomeric excess of the *S*-isomer of >80%.

As an alternative to a structure-guided strategy, directed evolution approaches can alter enzyme stereochemical preference. Liebeton and co-workers[97] describe the evolution of a lipase from *Pseudomonas aeruginosa* with high selectivity for the hydrolysis of the chiral model substrate 2-methyldecanoic acid *p*-nitrophenyl ester. The native lipase displays no enantioselectivity for the hydrolysis of this substrate, yielding both product enantiomers. Through successive rounds of error-prone PCR and selection it was possible to increase enantiomeric excess from 2% to 51% in favor of one enantiomer. Mapping the mutations on to the three-dimensional structure of the lipase suggests that the increased flexibility of distinct loops determines the stereochemical preference of this enzyme.

Directed evolution can also broaden the stereospecificity of a target enzyme. *E. coli* 2-keto-3-deoxy-D-phosphogluconate (KPDG) aldolase requires phosphate for its reaction and is specific for D-glyceraldehyde-3-phosphate as a substrate. Using directed evolution methods, Fong and co-workers[98] generated a KPDG aldolase that is phosphate independent and accepts either D- or L-glyceraldehyde-3-phosphate to make D- and L-sugars. Interestingly, none of the six mutations that increase the stereochemical promiscuity of KPDG aldolase occur at the active site.

### 2. Cofactors: 4-pro-S versus 4-pro-R Hydride Transfer

Nicotinamide adenine dinucleotides are ubiquitous as hydride donors and acceptors in enzyme-catalyzed reactions. These cofactors transfer either a 4-pro-*R* or a 4-pro-*S* hydride ion from the A or B face of the nicotinamide ring to an acceptor group of the substrate. Dehydrogenases exhibit strict stereochemical preference for the hydride ion transferred, leading to a classification of dehydrogenases as being either A-face or B-face specific. Shielding of one side of the 1,4-dihydronicotinamide ring often determines whether A- or B-side hydride transfer occurs. Attempts to change an A-face dehydrogenase into a B-face dehydrogenase have met with limited success. To determine how successful these attempts are, it is important to access "how stereospecific are enzymes[100]"? For example, the frequency of nonstereospecific hydride transfers occurs less than 1 in $10^7$ events for lactate dehydrogenase,[101] suggesting that a fraction of an increase in enantiomeric inversion may represent a pronounced effect.

Inverting the stereochemistry of hydride transfer in dehydrogenases has proven difficult. Examination of the 3α-HSD·$NADP^+$ binary complex structure indicates that hydrogen bonds between Ser 166, Asn 167, and Gln 190 and the C3-carboxamide hold the nicotinamide ring in place and that 4-pro-*R* hydride transfer occurs because $\pi$-stacking with Tyr 216 shields the B-face.[102] The structural model also suggested that mutation of residues donating hydrogen bonds to the C3-carboxamide would allow the nicotinamide ring to rotate 180° around the *N*-glycosidic bond. The 3α-HSD S166A, N167A, and Q190A mutants resulted in decreased affinity for NADP(H) but not NAD(H). Using 4-pro-*R*-[$^3$H]-NADH as a hydride donor to the acceptor substrate, [$^{14}$C]-5α-dihydrotestosterone, the $^3$H:$^{14}$C ratio decreased by 50% in the mutant, suggesting that racemization of hydride transfer was successful. However, the primary kinetic isotope effect for tritide transfer fully accounts for the decrease in the observed $^3$H:$^{14}$C ratio. Since stereochemistry of hydride transfer is often invariant in a protein superfamily, DNA-family shuffling is also unlikely to yield the desired outcome.

## D. Engineering Catalysis: Looking for the Grail

So far all the examples of enzyme redesign have focused on changing substrate or cofactor specificity or altering the stereochemistry of a reaction, but none demonstrate alteration of reaction chemistry. The "Holy Grail" of enzyme redesign is the engineering of new catalytic activities. Strategies aimed at changing enzyme reaction mechanisms reach different levels of sophistication and include (1) the diversion of covalent reaction intermediates down alternative pathways, (2) the unmasking and optimization of side reactions, (3) modifying an existing active site to catalyze a new reaction mechanism, and (4) engineering catalytic activity into ligand-binding sites. We now survey examples of various approaches used for catalytic redesign.

### 1. Diverting Covalent Enzyme Reaction Intermediates

Enzymes that conduct catalysis through covalent reaction intermediates offer the opportunity to alter the chemistry at the level of these intermediates to yield new reaction products. For example, $\beta$-lactamases, which confer $\beta$-lactam antibiotic resistance to bacteria, catalyze hydrolysis through a serine-linked acyl−enzyme intermediate (Figure 10). Mutations of an active site glutamate (Glu 166), believed to act as a general base in the deacylation reaction, leads to accumulation of a normally transient acyl−enzyme intermediate.[103−104] Mutation of an asparagine residue (Asn 170) at the bottom of the substrate-binding cavity within hydrogen-bonding distance of Glu 166 leads to a chemical mechanism that yields a substantially altered product profile.[105] The N170L $\beta$-lactamase mutant undergoes deacylation by a postulated intramolecular rearrangement (Figure 10), instead of hydrolysis, to yield a thiazolidine−oxazoline that degrades into *N*-phenylacetylglycine and *N*-formylpenicillamine.[105]

Pyridoxal phosphate-dependent enzymes share a common central intermediate, a carbanion stabilized by an electron sink via a Schiff base with pyridoxal phosphate. Depending upon whether the carbanion results from abstraction of an α-proton or decarboxylation, the intermediate aldimine can initiate transamination, deamination, or decarboxylation of L-amino acids. L-Aspartate aminotransferase has served as a template for altering the partitioning of the aldimine down one particular pathway. Graber et al.[106] described a double mutation in this enzyme (Y225R/R386A) that catalyzed the $\beta$-decarboxylation of L-aspartate to L-alanine 1330-fold faster than wild-type enzyme, although the transaminase activity of this mutant still exceeded the $\beta$-decarboxylase activity by 2.5-fold (Figure 11). With the addition of a third active site mutation (Y225R/R292K/R286A), the ratio of $\beta$-decarboxylase activity to transaminase activity increased greater than 25 million-fold.[107] Importantly, no pyruvate was produced by the triple mutant, indicating that deamination did not occur as a side reaction. These experiments show that although aspartate aminotransferase accelerates a specific reaction and suppresses potential side reactions, it is possible to alter the ratios between two reactions at the same active site with a handful of mutations. Diversion of enzyme reaction intermediates by altering active site residues also leads to new products with 5-enolpyruvylshikimate-3-phosphate synthase,[108] ornithine decarboxylase,[109] and ketoacyl synthase-related enzymes.[110−112]

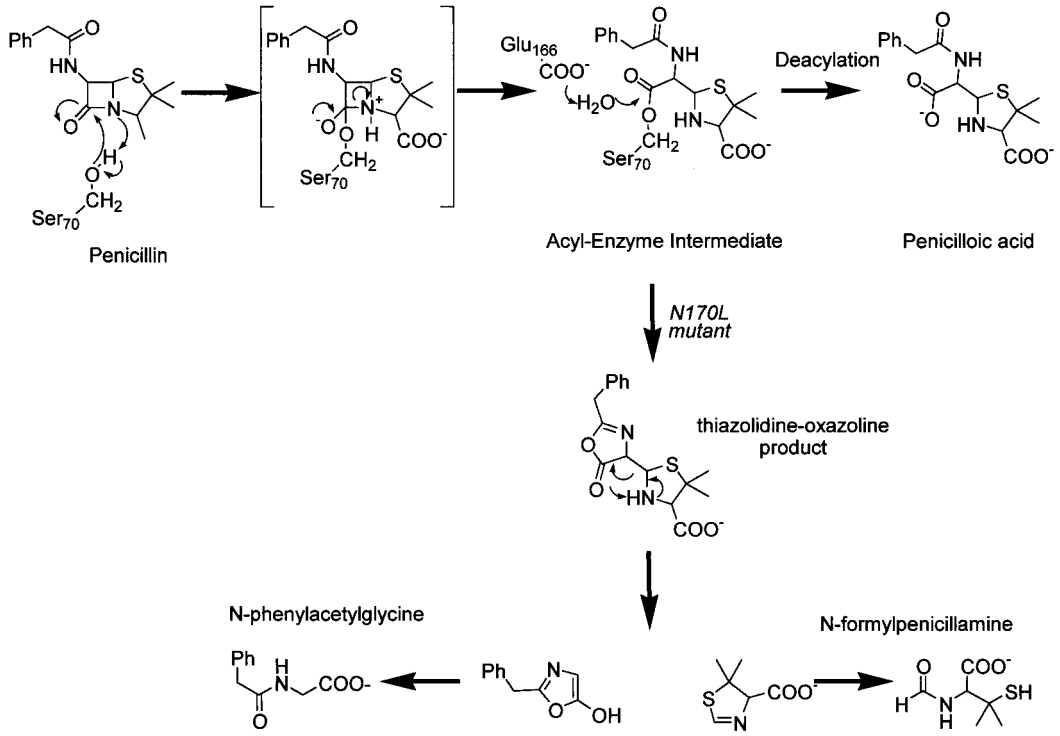



**Figure 10.** Formation of the acyl−enzyme intermediate in $\beta$-lactamase and alternate product formation in the N170L mutant. The catalytic reaction of $\beta$-lactamase occurs via an enzyme−acyl intermediate that is deacylated by Glu 166 acting as a general base. Mutation of the glutamate results in accumulation of the intermediate. The N170L mutant undergoes deacylation by an intramolecular rearrangement to yield a thiazolidine−oxazoline which breaks down to form *N*-phenylacetylglycine and *N*-formylpenicllamine.

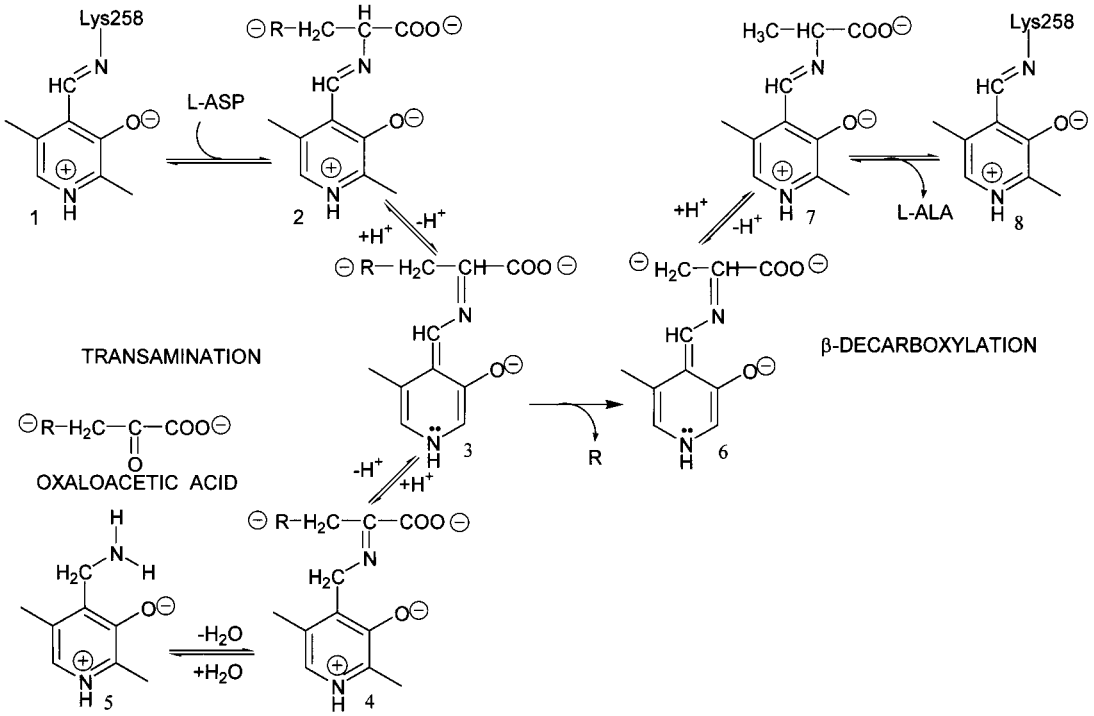



**Figure 11.** Conversion of a transaminase to a $\beta$-decarboxylase. In the transamination reaction an aldimine (2) is formed; subsequent removal of the α-proton and stabilization of the carbanion leads to imine (3). Reprotonation converts the amino acid to an α-keto acid and leaves behind an aminated pyridoxal phosphate for subsequent transamination (5). In the $\beta$-decarboxylation reaction, the imine (3) is again formed; however, $\beta$-decarboxylation occurs instead of reprotonation to give (6) and the aldimine is reformed (7) and hydrolyzed to give L-alanine, where R = $CO_2$.

### *2. Unmasking of Alternate Reactions*

Many enzymes catalyze alternate reactions involving different chemistry with catalytic efficiencies that are significantly lower than the physiologic reaction.[113] While these secondary activities are often kinetically below the threshold of physiologic useful-

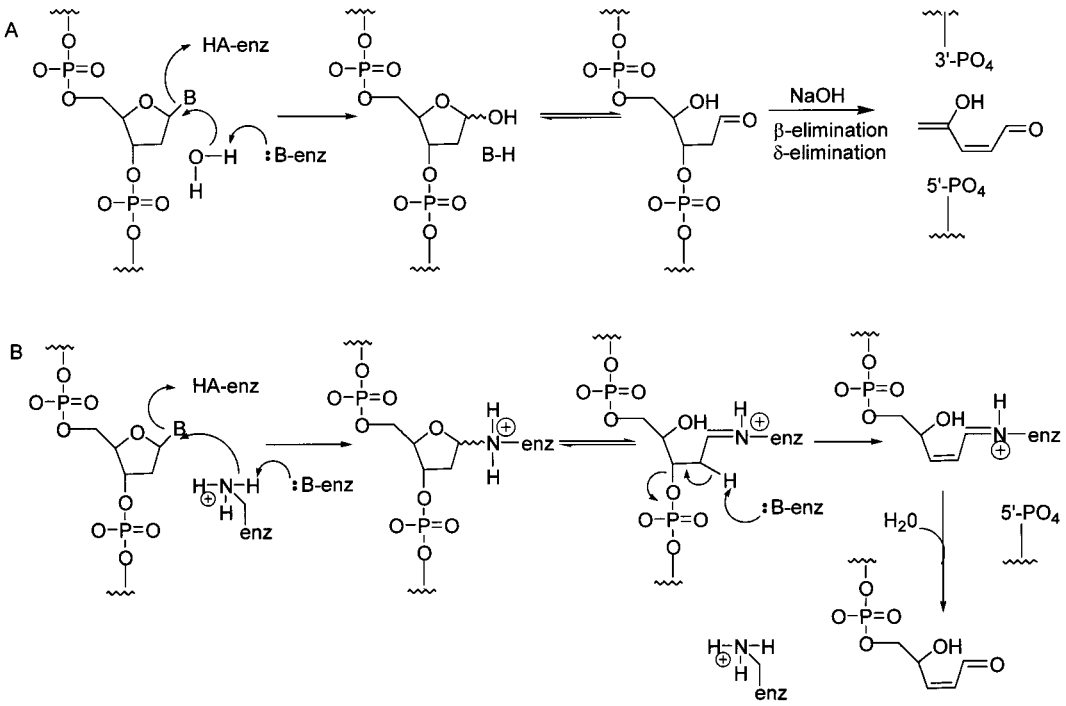



**Figure 12.** Changing monofunctional glycosylases into bifunctional glycosylase/apurinic lyases. (A) Monofunctional glycosylases catalyze the cleavage of the *N*-glycosidic bond by nucleophilc attack of a base-activated water molecule. (B) Bifunctional glycosylases catalyze the cleavage of the *N*-glycosidic bond and cleavage of the 5′-phosphoester bond via imine formation with an active site amine.

ness, mutations that shift the balance between the wild-type and side reactions can exploit this catalytic promiscuity.[114] Numerous examples highlight the usefulness of this unmasking approach for engineering new enzymatic activities and for providing an understanding of enzyme evolution.[115−121]

Myoglobin, known as a carrier of molecular oxygen, catalyzes the hydrogen peroxide supported peroxygenation of a variety of substrates, leading to olefin epoxidation and thioether sulfoxidation, with low turnover numbers.[119] Comparison of the three-dimensional structures of myoglobin and cytochrome *c* peroxidase (CCP) suggested that repositioning the histidine proximal to the heme in myoglobin would improve the efficiency of ferryl−oxygen transfer reactions. Relocation of this histidine (L29H/H64L) by site-directed mutagenesis converted myoglobin into an active and stereospecific peroxygenase of styrene and methyl phenyl sulfide with an enantiomeric excess of greater than 80% for the formation of the corresponding *R*-isomers.[119]

*E. coli* adenine glycosylase MutY prevents mutations caused by 7,8-dihydro-8-oxo-2′-deoxyguanosine (OG) by removing adenine from OG:A base pairs.[120] Mechanistically, MutY performs a C1′ nucleophilic attack by a base-activated water molecule to displace the purine/pyrimidine. This contrasts with the reaction catalyzed by bifunctional glycosylase/apurinic-apyrimidinic lyases, which use a conserved lysine as the initial nucleophile to displace the purine/pyrimidine and form a Schiff's base. Subsequent removal of the C2−H′ proton by an enzyme base leads to hydrolysis of the 5′-phosphodiester bond. A single-point mutation of an active site serine into a lysine (S120K) changes the monofunctional MutY protein into a bifunctional glycosylase/lyase (Figure 12).

An unusual example of capitalizing on the catalytic promiscuity of an enzyme occurs upon elimination of the essential general acid−base in 3α-HSD.[121] Mutation of the catalytic tyrosine, Tyr 55, into a phenylalanine demolishes the ability of the enzyme to catalyze oxidation and reduction reactions on steroid and xenobiotic metabolites. Surprisingly, the Y55F mutant retains a quinone reductase activity that nearly matches that of the wild-type enzyme. Subsequent characterization of the mutant suggests that quinone reduction occurs via a mechanism distinct from that of steroid or xenobiotic reduction. This represents a rare example where one enzyme can catalyze the same chemical reaction (in this case carbonyl reduction) by two different reaction mechanisms.

### *3. Engineering the Same Active Site to Catalyze New Reactions*

Site-directed mutagenesis plays an essential role in dissecting enzyme reaction mechanisms and identifying the active site residues responsible for catalysis. Since enzymatic reactions are analogous to those performed in organic chemistry (e.g., enolate and enol chemistry, nucleophilic addition or substitution, proton and hydride transfer, acyl- or phosphoryl-group exchange, dehydration−hydration reactions, and elimination reactions, etc.), the designer can select the best residues from the repertoire of amino acids available to alter existing active sites to catalyze new reactions.

**Enolate and Enol Chemistry.** All members of the enolase superfamily of proteins, including mandelate racemase, muconate lactonizing enzyme, and enolase, are $(\alpha/\beta)_8$-barrel proteins that catalyze the abstraction of the α-proton from a substrate carboxylic acid.[122] Partitioning of the resulting enolic inter-

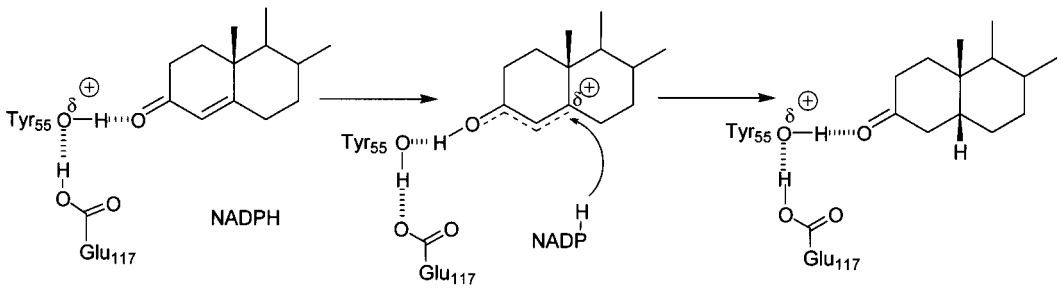



**Figure 13.** Engineering 3α-HSD to 5β-reductase. Substitution of Glu 117 into the active site of 3α-HSD increases the acidity of the general acid Tyr 55 permitting acid-catalyzed enolization of a $\Delta^4$-3-ketosteroid. The resultant carbocation ion facilitates hydride transfer and double-bond reduction.

mediates into different chemical reactions generates novel products. Mandelate racemase and related enzymes catalyze a 1,1-proton transfer, muconate lactonizing enzyme catalyzes a cyclo-isomerization reaction, and enolase catalyzes the β-elimination of water. Comparison of enzymes across this enzyme superfamily revealed that naturally occurring mutants have altered the reaction trajectory. This suggests that similar success could be achieved with mutations introduced by the designer.

**Nucleophilic Substitution versus Nucleophilic Addition.** GST A1−1 catalyzes the GSH conjugation of DCNB, a nucleophilic substitution reaction, but engineering the H site results in preferential 1,4-Michael addition of GSH to alkenals, a nucleophilic addition reaction.[123] Four-point mutations coupled with the substitution of a helical segment from GST A4−4 into GST A1−1 yielded a mutant with a $k_{cat}/K_m = 1.5 \times 10^6$ $M^{-1}$ $s^{-1}$ for addition of GST to nonenal. This gave a 10-fold decrease in the $k_{cat}/K_m$ for DCNB but a 300-fold increase in the $k_{cat}/K_m$ for nonenal. Thus, repositioning of the substrate at the H site changes reaction chemistry in the engineered GST. Alterations in the H site also decrease the p$K_a$ of Tyr 19, which may affect the ionization of the thiolate on GSH.

**Proton Transfer: Acid−Base Catalysis.** The AKR enzyme superfamily contains two classes of steroid-transforming enzymes: the HSDs and the $\Delta^4$-3-ketosteroid-5β-reductases (5β-reductases).[124] The HSDs interconvert steroid alcohols and ketones; while the 5β-reductases reduce the carbon−carbon double bond in $\Delta^4$-3-ketosteroids, a functionality present in nearly all steroid hormones (Figure 13). Chemically, the reduction of a double bond is more difficult than reduction of a carbonyl moiety. The HSDs of the AKR superfamily catalyze a common reaction mechanism using a strictly conserved amino acid tetrad (Tyr 55, Lys 84, Asp 50, and His 117) with the tyrosine serving as a general acid/base. Comparison of the catalytic tetrads in HSDs and 5β-reductases of the AKR superfamily revealed that the histidine is a glutamic acid in the reductases. The 3α-HSD H117E mutant significantly impaired the $k_{cat}/K_m$ for 3α-HSD activity but introduced 5β-reductase activity with a $k_{cat}/K_m$ similar to that of native rat 5β-reductase.[125] In this mutant, the p$K_b$ of the catalytic tyrosine was decreased, suggesting enhanced $TyrOH_2^+$ character. The H117E/Y55F mutant eliminated both 3α-HSD and 5β-reductase activities. These data indicate that Tyr 55 mediates the acid-catalyzed enolization of the $\Delta^4$-3-ketosteroid by facilitating hydride transfer to a carbocation at C5 of the steroid. Since 5β-reductase precedes 3α-HSD in steroid hormone metabolism and both are homologous AKRs, this suggests that this major pathway of steroid hormone metabolism evolved by gene duplication with divergence of sequence and enzymatic activity.

**Acyl Transfer: Enzyme−Acyl Intermediates.** Members of the 2-enoyl-CoA hydratase/isomerase superfamily share a common active site that includes a CoA-binding site, an acyl-group-binding pocket, an oxyanion hole for polarizing the thioester carbonyl, and acidic and basic residues involved in proton transfer. Given this architecture, repositioning of the acid/base residues within the active site could tailor the overall reaction mechanism.[126] To test this hypothesis, the goal was to convert 4-chlorobenzoyl-CoA dehalogenase into crotonase (Figure 14). Here, the starting enzyme catalyzes nucleophilic displacement of a halogen by an enzyme−acyl intermediate while the target enzyme catalyzes double-bond hydration mediated by an enzyme general base. Introduction of eight mutations (G117E, A136P, W137E, N144P, D145G, T146A, A147G, and T148G) into 4-chlorobenzoyl-CoA dehalogenase abolished the native activity and yielded a mutant enzyme that catalyzed the crotonase reaction with a $k_{cat}$ 15 000-fold lower than native crotonase. Optimization of catalytic behavior will require further modifications of the target protein.

Two groups engineered nitrile hydratase activity into the cysteine protease papain[127] and asparagine synthetase.[128] Dufour and co-workers[127] exploited structure−function relationships of papain to choose a single active site mutation that introduced the desired activity (Figure 15). They rationalized that a mutation in the oxyanion hole would alter the normal reaction course. Peptide nitriles react with papain to form thioimidates. Mutation of Gln 19 to Glu 19 in the oxyanion hole introduces a carboxylate at the active site. The glutamic acid provides a proton to form the reactive protonated thioimidate that undergoes acid-catalyzed hydrolysis, where $k_{cat}/K_m$ is $1.15 \times 10^3$ $M^{-1}$ $s^{-1}$ and $<0.00003$ $M^{-1}$ $s^{-1}$ for nitrile hydrolysis catalyzed by the Q19E mutant and the wild-type papain, respectively. Subsequent work demonstrates the use of the modified papain as a biocatalyst for formation of a peptide amidrazone.[129] A related experiment that introduces an aspartic acid in place of an asparagine in the active site of

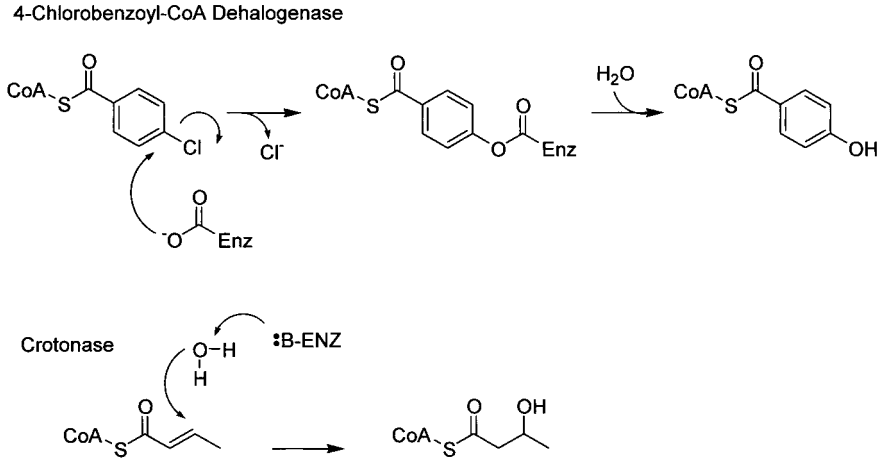



**Figure 14.** Reactions catalyzed by 4-chlorobenzoyl-CoA dehalogenase and crotonase. The dehalogenase catalyzes a nucleophilic displacement via an enzyme−acyl intermediate, while crotonase catalyzes hydration of a double bond where water is activated by an enzyme general base.

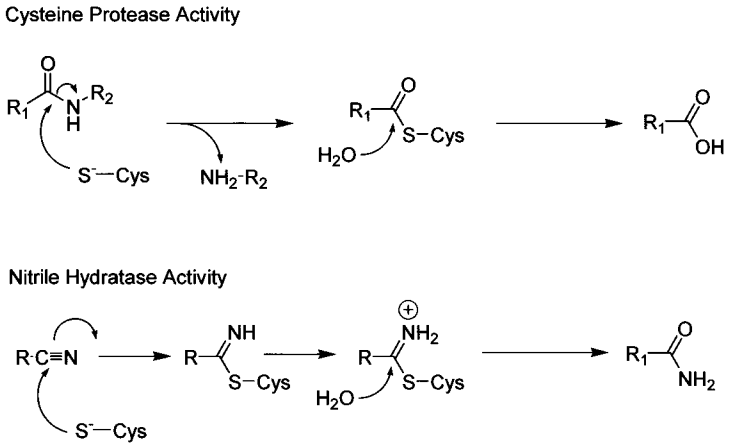



**Figure 15.** Protease versus nitrile hydratase activity. The cysteine protease papain hydrolyzes a peptide bond via the formation of a thiol acyl−enzyme intermediate. It is converted into a nitrile hydratase activity in the Q19E mutant which enhances protonation of the intermediate thioimidate and hydrolysis to produce the amide.

asparagine synthetase also yields nitrile hydratase activity with a concomitant decrease of native enzymatic activity.[128] Thus, in papain and asparagine, synthetase formation of protonated thioimidates leads to nitrile hydrolysis.

Generation of subtiligase, a modified version of subtilisin BPN′, represents a classic example of using protein engineering to meet synthetic requirements by introducing a new catalytic activity into a serine protease.[130−131] Serine proteases strongly favor hydrolysis of the acyl−enzyme intermediate over aminolysis. This presents a problem for the synthesis of new peptide bonds. Nakatsuka et al.[132] originally generated a thiolsubtilisin variant with a cysteine replacing the active site serine (S221C) that shifted the preference for aminolysis over hydrolysis but with a catalytic efficiency below the esterase activity of wild-type enzyme. The addition of a second mutation (P225A) in the thiolsubtilisin relieves steric crowding and increases the catalytic efficiency of the double mutant.[130] The complete synthesis of ribonuclease A containing unnatural amino acids by subtiligase demonstrates the power of engineering approaches directed at peptide-bond formation.[131]

**Phosphoryl Transfer: Enzyme−Phosphoryl Intermediates.** Modification of the reactions catalyzed by butyrylcholinesterase and acetylcholinesterase leads to organophosphorous acid anhydride hydrolase activity in both enzymes.[133−135] Organophosphorus compounds, commonly found in insecticides and nerve gas agents, irreversibly inhibit butyrylcholinesterase and acetylcholinesterase. These compounds undergo hydrolysis leading to a phosphoryl−enzyme intermediate in place of the acyl−enzyme intermediate. The phosphoryl−enzyme intermediates are very stable and lead to enzyme inactivation. Introduction of a histidine residue into the oxyanion hole of the active site of either butyrylcholinesterase or acetylcholinesterase allows these enzymes to catalyze the hydrolysis of organophosphate esters when it was not possible before.

### *4. Changing Binding Sites into Active Sites*

Contrary to the “chemistry first” view of enzyme evolution is the “specificity first” view of enzyme evolution. Conceptually this approach to enzyme redesign capitalizes on introducing catalytic residues into a ligand-binding site to create an active site capable of catalyzing a chemical reaction. This strategy requires detailed structural information of the template protein and has worked with artificially created and naturally occurring binding sites.

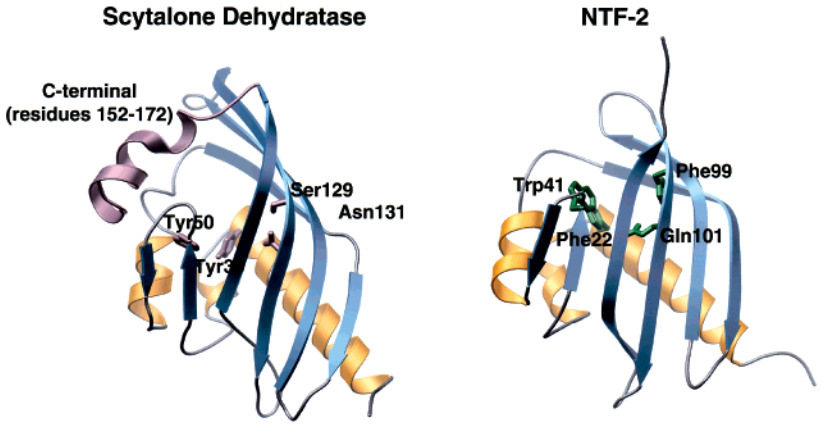



**Figure 16.** Grafting the active site of scytalone dehydratase (left) onto nuclear transport factor (NTF) 2 (right). The ribbon traces (α-helices in gold and *β*-strands in blue) of scytalone dehydratase and NTF2 emphasize the overall structural similarity of both proteins, despite less than 20% amino acid sequence identity. The portions of scytalone dehydratase introduced into NTF2 are highlighted in rose. Addition of the C-terminal of scytalone dehydratase (residues 152−172) to the C-terminal of NTF2 and mutation of Phe 22, Trp 41, Phe 99, and Gln 101 of NTF2 (green residues) into Tyr, Tyr, Ser, and Asn, respectively, achieved the desired goal.

Engineering experiments on CCP show how building a cavity near the heme center that binds small molecule ligands tailors the oxidative power of the enzyme.[136−138] In CCP, mutation of Trp 191 to a glycine opens a well-defined cavity at the heme center that binds substituted imidazoles.[136] Screening of small heterocyclic compounds identified 2-aminothiazole as an unnatural substrate for oxidation by the W191G mutant.[137] Using this complementation approach, mutation of Arg 48 into an alanine at the CCP heme site expands a water-filled cavity above the distal heme face and allows oxidation of *N*-hydroxyguanidine.[138]

Quemeneur et al.[139] used a grafting technique to introduce a serine protease active site into a peptide-binding cleft. Cyclophilins bind specific amino acid sequences and catalyze the *cis*−*trans* isomerization of proline residues but do not hydrolyze peptide bonds. Using structural information on how an Ala-Pro dipeptide binds to an *E. coli* cyclophilin, mutation of three amino acids proximal to the peptide-binding cleft introduced a catalytic triad similar to that of the serine proteases. The resulting A91S/F104H/N106D mutant catalyzed hydrolysis of an Ala-Pro dipeptide and displayed a proline-specific endoproteolytic activity with a 61 amino acid snake toxin as a substrate.

Expanding on the approach of grafting active sites, Nixon et al.[140] engineered the catalytic activity of one enzyme into a structurally related protein. In melanin biosynthesis, scytalone dehydratase catalyzes the elimination of water through an α-proton abstraction to form 1,3,8-trihydroxynaphthalene. Nuclear transport factor 2 (NTF2) possesses no catalytic activity and participates in protein translocation across the nuclear membrane. Although related by less than 20% amino acid sequence identity, scytalone dehydratase and nuclear transport factor 2 (NTF2) share similar three-dimensional structures and belong to the α,*β*-fold group of proteins. Comparison of the two structures revealed conservation of two active site residues from the active site of scytalone dehydratase in NTF2 (Figure 16). This provided a starting point for redesigning NTF2. Scytalone dehydratase also has an additional C-terminal α-helix and an extended *β*-sheet compared to NTF2. By adding the C-terminal α-helix (residues 152−172) of scytalone dehydratase and introducing four active site residues (F22Y/W41Y/F99S/Q101N), NTF2 catalyzed scytalone dehydratase activity with a 150-fold increase in $k_{cat}/k_{noncat}$.

In these active site grafting experiments, the ability to map the active site of one enzyme onto the structure of another protein resulted in the introduction of new catalytic activities when none existed before. Surprisingly, the examples required a relatively small amount of computer modeling. Construction of an iron superoxide dismutase activity into thioredoxin, a polypeptide that does not contain a transition-metal-binding site,[141] demonstrates the value of incorporating computer algorithms in the enzyme redesign process.[1,142]

### 5. Combining Rational Design and Directed Evolution

Evolution remains an excellent teacher of how to modify biological catalysts, especially when an initial round of rational engineering of a protein target stacks the evolutionary odds. In a technical tour de force, Altamirano et al.[143] demonstrate the benefit of combining both rational and irrational schools of enzyme redesign. Phosphoribosyl-anthranilate isomerase (PRAI) and indole-3-glycerol phosphate synthase (IGPS) catalyze sequential steps in tryptophan biosynthesis (Figure 17A). Related by 22% amino acid sequence identity, PRAI and IGPS share common $(\alpha/\beta)_8$-barrel scaffolds and bind a common biosynthetic intermediate. Structural comparison of PRAI and IGPS suggested the most likely modifications required to convert IGPS into PRAI (Figure 17B). The first change was the removal of 48 amino acids from the N-terminus of IGPS. Second, a mixed library of loops containing 4−7 residues replaced the *β*1α1 loop which was 15 amino acids long. Finally, loop *β*6α6 of IGPS was swapped with the corresponding loop of PRAI and a catalytic aspartate introduced at position 184 near the active site. The resulting IGPS template physically resembles PRAI, and the mutant IGPS binds an inhibitor of both enzymes. In

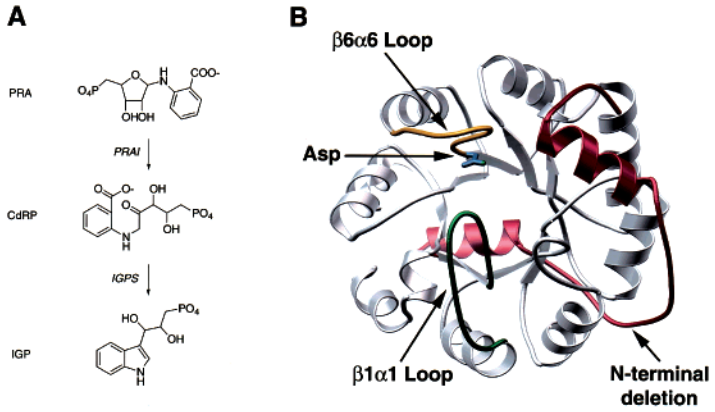



**Figure 17.** Converting indole-3-glycerol phosphate synthase (IGPS) into phosphoribosyl-anthranilate isomerase (PRAI). (A, left) PRAI converts *N*-(5′-phosphoribosyl)-anthranilate (PRA) into 1′-(2′-carboxyphenylamino)-1′-deoxyribulose-5′-phosphate (CdRP) by an intramolecular Amadori rearrangement. IGPS uses CdRP as a substrate to form indole-3-glycerol phosphate (IGP) by an irreversible ring closure. (B, right) Structural conversion of IGPS into PRAI focused on four modifications: deletion of 48 N-terminal residues (red), generation of a 4−7-residue amino acid library to replace the $\beta$1$\alpha$1 loop (green), swapping the $\beta$6$\alpha$6 loop of IGPS with the same loop from PRAI (gold), and introduction of a catalytic aspartate (blue).

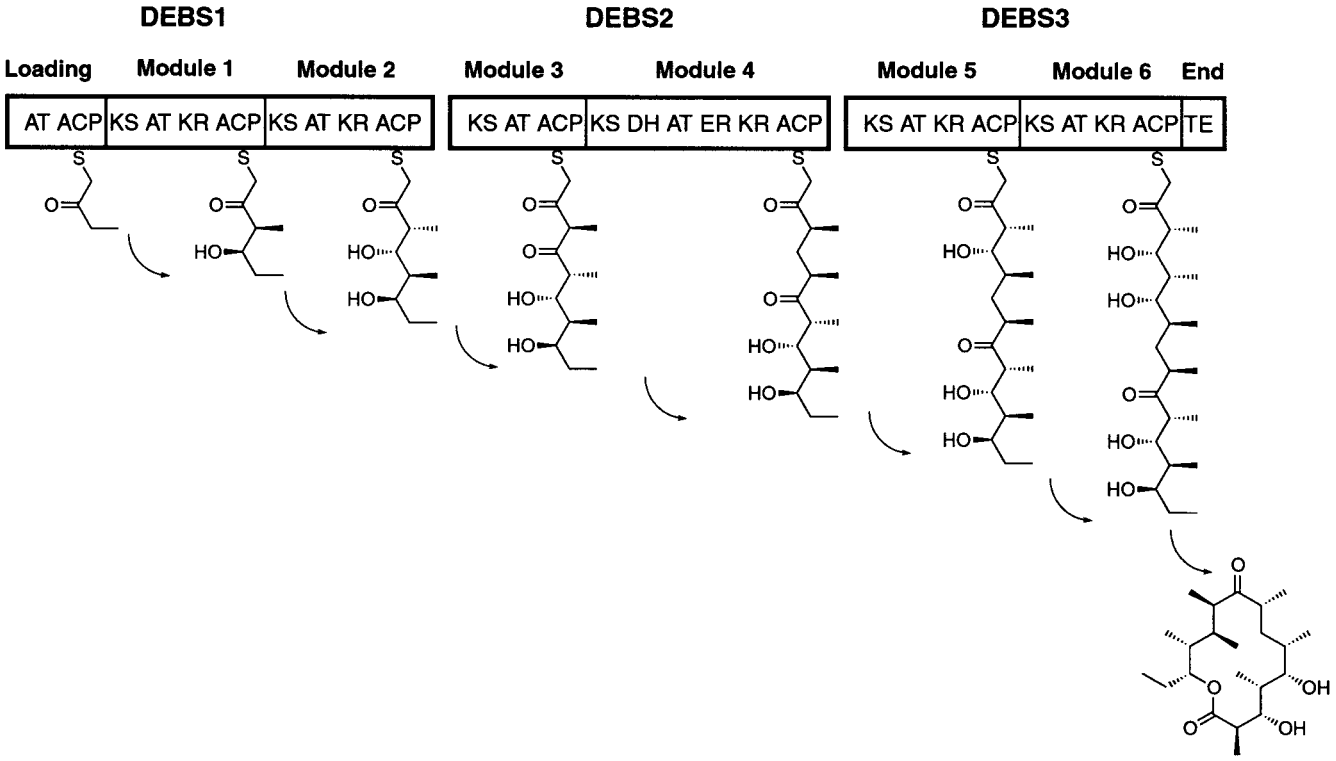



**Figure 18.** Modular design of polyketide synthases. 6-Deoxyerythronolide B synthase (DEBS) is a multiprotein complex of three proteins (DEBS1−3). Each DEBS protein is comprised of modules that catalyze the extension and modification of the growing polyketide through a set of distinct activities, including acyltransferase (AT), acyl-carrier protein (ACP), ketosynthase (KS), ketoreductase (KR), dehydratase (DH), enoylreductase (ER), and thioesterase (TE) functions.

vivo selection experiments using the engineered IGPS to complement a PRAI-deficient *E. coli* strain identified a set of clones with a low level of activity. Next, DNA shuffling and staggered extension methods ultimately improved the mutant's PRAI catalytic efficiency 6-fold over that of native PRAI and eliminated IGPS activity. A related study showed that directed evolution approaches can also convert PRAI into *N*′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide isomerase, an enzyme of histidine biosynthesis.[144]

## E. Engineering Modular Enzyme Systems

Modular proteins frequently occur in signaling systems but are comparatively rare as biological catalysts.[145] The two best-studied modular enzyme systems are the polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) involved in the biosynthesis of structurally complex and pharmacologically important natural products, including many antibiotics.[146−147] Large, multifunctional proteins organized in an "assembly line" synthesize the polyketides and nonribosomal peptides (Figure 18). The

order and composition of the modules, each catalyzing a distinct set of reactions, that form a PKS or NRPS dictate the final chemical structure of the polyketide or non-ribosomal peptide product. The organization of the PKS and NRPS suggested that combinatorial biosynthesis involving the mixing and matching of different modules could lead to the production of hybrid or novel antibiotics.[148−150] Repositioning modules within a given system[151−153] or exchanging modules between different systems[154−156] increases the diversity of reaction products formed by PKS and NRPS systems.

## V. Future Directions

More than 15 years ago the ability to remodel the amino acids of an enzyme active site introduced the power of protein engineering to the field of enzymology.[157−159] From the earliest experiments which probed how enzymes function, the challenge of designing new biocatalysts continually loomed on the horizon. Since its inception, rational design methods have successfully altered substrate specificity, cofactor preference, stereospecificity, and reaction mechanisms of numerous enzymes through a variety of approaches. Later, the development of combinatorial or directed evolution opened alternative paths for redesigning biocatalysts.[10−18] These philosophies of enzyme redesign, tailoring by rational design versus directed evolution via natural selection, are now converging and permitting greater flexibility for altering catalytic properties of enzymes. Examples exist using both approaches where difficult feats have been accomplished, i.e., altering stereospecificity and reaction chemistry.

When the two approaches are compared, rational design is information intensive and computer assisted and is likely to become automated and predictive (see below). In contrast, directed evolution does not require structural or mechanistic information. Also, because evolutionary drift occurs, the directed approach avoids using the closest parents to obtain the desired progeny, which is often the guiding principle in rational design. Finally, directed evolution often results in solutions to attain desired enzymatic activity that may not be foreseen using rational engineering. However, the satisfying feature of a rational design approach is that when the proposed change has the predicted outcome the designer really does understand the principles of engineering the structural template.

Has the promise of either the de novo design of biocatalysts or directed evolution of truly novel enzymes been fulfilled? No. Protein engineers simply do not have the requisite understanding of the interplay between protein folding, stability, structure, and catalysis to build an enzyme entirely from scratch. Likewise, the inability to generate sufficient molecular diversity for selection and lack of universal

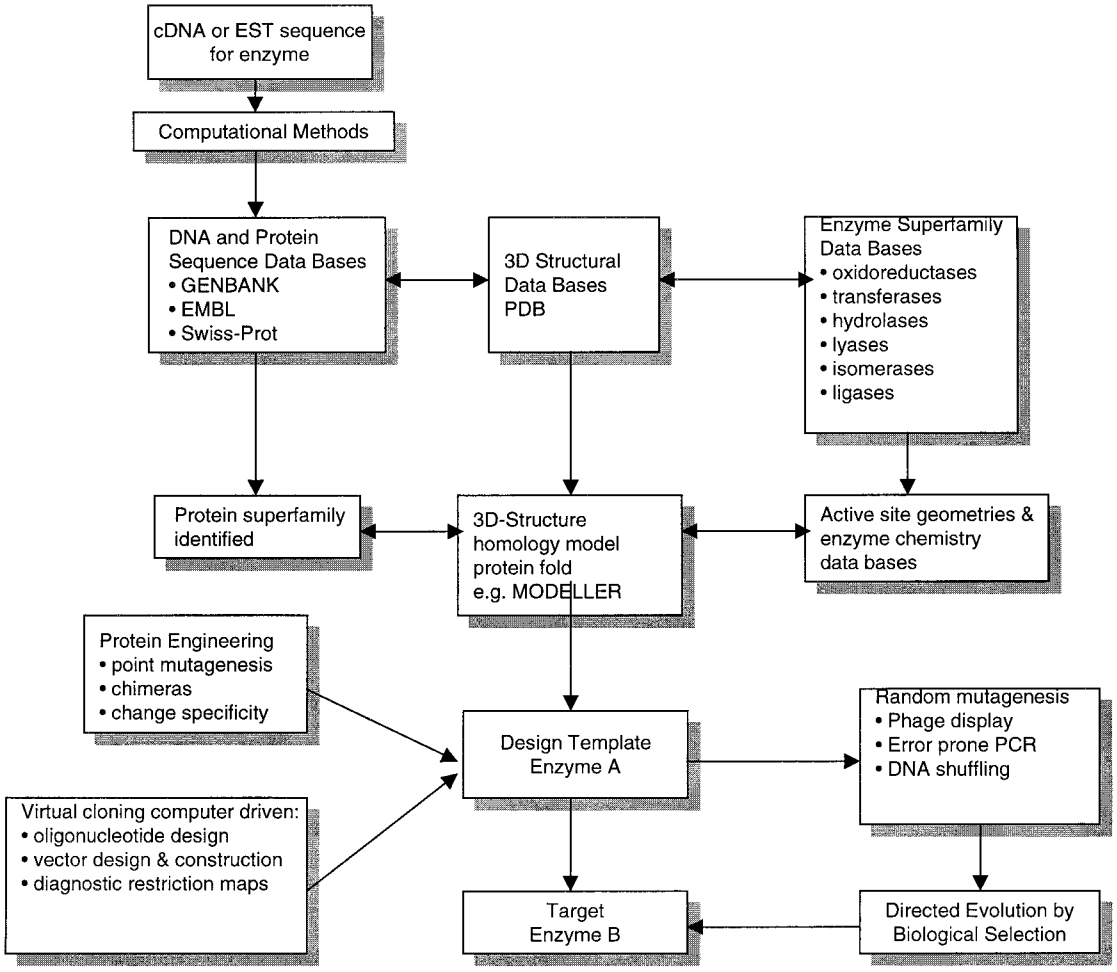



**Figure 19.** Future of enzyme redesign.

selection procedures hampers the directed evolution of an enzyme's activity into a completely novel reaction. Although this "Holy Grail" of enzyme design remains undiscovered, the successes in understanding how to modify enzyme specificity and catalysis achieved so far point toward an exciting future.

With the advent of structural and functional genomics, nature's gold-mine of engineering experience will reveal itself to the experimentalist. At present, the cDNA or expressed sequence tag (EST) for a target enzyme can be subjected to computational methods which permit the mining of DNA, protein sequence, and motif databases, with the goal of assigning the enzyme to a protein superfamily (Figure 19). Crystallographic and NMR methods continue to identify the major protein folds that exist, and bioinformatics will help assign these protein folds to the six major enzyme classes (oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases). As the major chemical reactions catalyzed by enzymes of various folds are classified (e.g., enolate chemistry, proton and hydride transfer, acyl- or phosphoryl- transfer, dehydration, and elimination, etc.), the appropriate geometries of active site residues required for different reactions will be deciphered and maintained in databases, e.g., PROCAT.

Armed with this increasing mass of structural and functional data, computational methods will play an important role in producing a design template for Enzyme A. Automation of the design process from selecting target residues within a template to optimizing the solution will ultimately evolve. Programs such as TESS already enable searches of structural databases for conserved active site motifs to guide the designer how best to modify catalytic residues or to graft entire active sites onto noncatalytic proteins (i.e., building a protease active site into cyclophilin[139]). Also, simple comparative modeling studies can suggest major structural alterations, like those used to graft the scytalone dehydratase active site onto the NTF-2 scaffold[140] or to convert IGPS into PRAI,[143] that alter enzymatic activity. As databases of protein sequences, three-dimensional folds, chemical reactions, and enzyme active sites expand, the increasing sophistication of computational methods will aid the protein designer. These methods will predict what changes are required to alter enzyme A into enzyme B or how to introduce a new enzymatic activity into a protein scaffold. Likewise, using the deposited cDNA sequence of a starting protein, virtual cloning programs will streamline the generation of point mutations or chimeras required at the nucleotide level. Thus, by combining the information in the different databases, an automated approach to redesign is envisioned.

Also, the ability to incorporate unnatural amino acids into proteins will expand the existing genetic code. The power of incorporating unnatural amino acids into designer proteins offers the chance to introduce novel reaction chemistry at an active site. Unnatural amino acids can be incorporated into a protein of interest by using acylated suppressor tRNAs that recognize stop codons at the target site.[160] The major drawback of this approach is that it is dependent on in vitro translation techniques and therefore is currently not amenable to producing large amounts of protein for detailed structure−function studies.[161] The creativity in methods developed for directed evolution, random mutagenesis, DNA shuffling (recombination) coupled with selection methods has led to increased automation and production of synthetic enzymes. Such enzymes will reshape the production of chiral pharmaceutials and fine chemicals. DNA shuffling is now commercially available through Maxygen's Molecular Breeding system.

Finally, the exploitation of blending rational and directed enzyme design approaches has only just begun.[143] With the improvement of rational design strategies and the development of new genetic tools and selection methods, the potential for generating altered or new biocatalysts via a combination of these methods remains fertile territory. In addition, the field of metabolic engineering[162] is just incorporating protein-engineering methods.[163,164] For example, the recently described heterologous biosynthesis of novel carotenoids in *E. coli* coupled traditional metabolic engineering approaches with the introduction of modified enzymes that resulted from directed evolution.[165]

The explosion of genomic information will expand the number of potential template enzymes for the designer, coupled with the diversity of available reaction chemistry, and the emergence of new computational methods, enzyme redesign will continue to open new avenues and redefine the field of protein engineering.

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