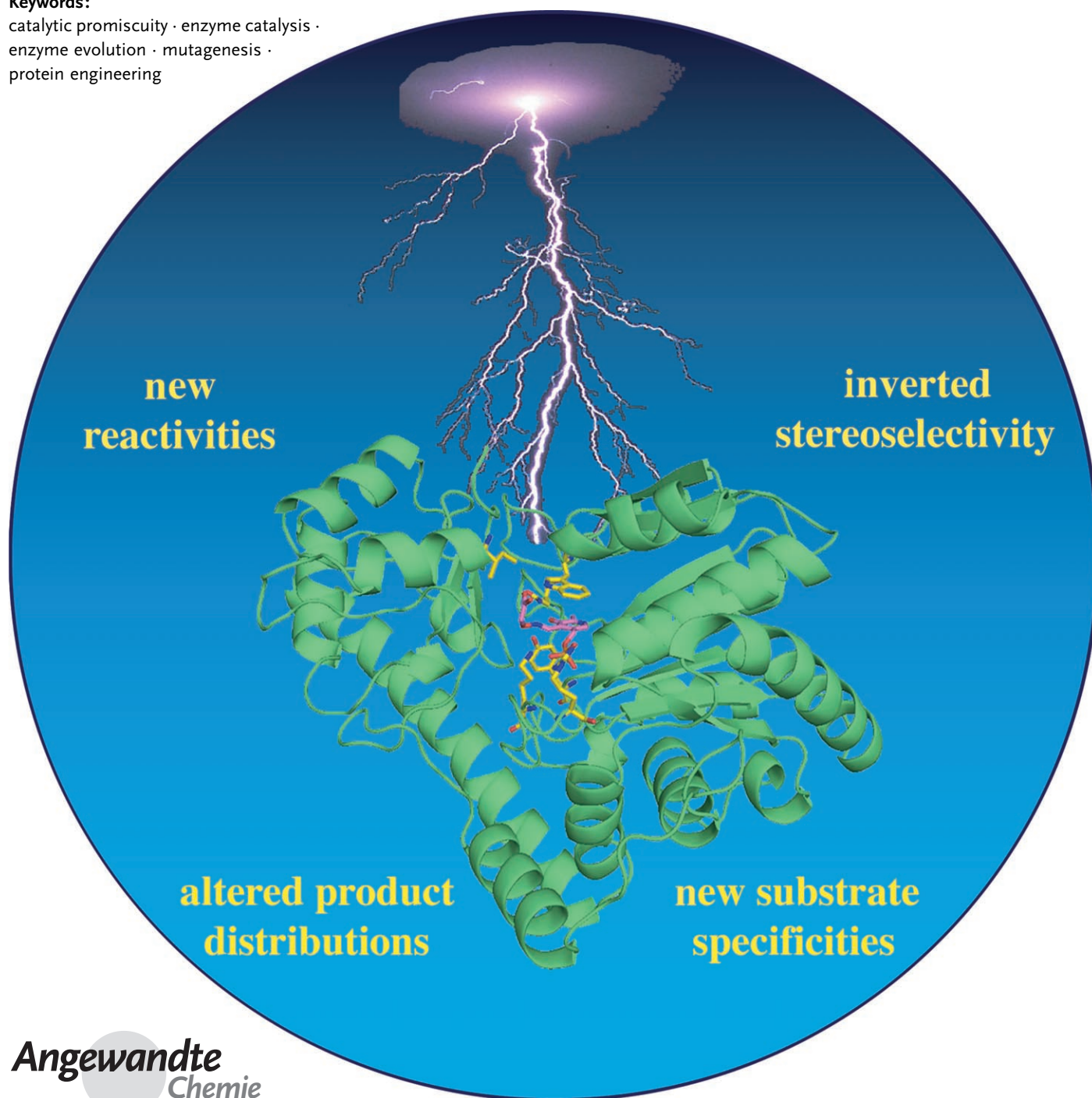# Minimalist Active-Site Redesign: Teaching Old Enzymes New Tricks

Miguel D. Toscano, Kenneth J. Woycechowsky, and Donald Hilvert*

Although nature evolves its catalysts over millions of years, enzyme engineers try to do it a bit faster. Enzyme active sites provide highly optimized microenvironments for the catalysis of biologically useful chemical transformations. Consequently, changes at these centers can have large effects on enzyme activity. The prediction and control of these effects provides a promising way to access new functions. The development of methods and strategies to explore the untapped catalytic potential of natural enzyme scaffolds has been pushed by the increasing demand for industrial biocatalysts. This Review describes the use of minimal modifications at enzyme active sites to expand their catalytic repertoires, including targeted mutagenesis and the addition of new reactive functionalities. Often, a novel activity can be obtained with only a single point mutation. The many successful examples of active-site engineering through minimal mutations give useful insights into enzyme evolution and open new avenues in biocatalyst research.

From the Contents

1. Introduction

Natural evolution has provided us with a remarkable set of enzymes that catalyze a great variety of chemical transformations.[1] These catalysts display enormous rate enhancements in water at neutral pH values and mild temperatures.[2] Increasingly, the chemical and pharmaceutical industries are taking advantage of these properties by utilizing enzymes to develop cheaper and environmentally friendlier synthetic processes.[3] However, the traditional view of enzymes is that their high catalytic efficiencies (often at or near diffusion control) are coupled with tight substrate specificities, limiting their usefulness in nonbiological applications. Protein engineering may provide a way to broaden the scope of enzyme-catalyzed transformations, enhancing their synthetic utility.

In attempting to engineer novel enzymes, the reactive centers of existing enzymes provide obvious starting points. An enzymatic reaction takes place at an active site in which a few amino acid residues compose a substrate-binding pocket. These precisely positioned residues promote catalysis by providing reactive groups, such as nucleophiles or acids/bases, and by setting the right microenvironment (hydrophobicity, charge complementarity, or hydrogen-bond donors and acceptors) to stabilize the transition state. Thus, the role of the active site is to bind the substrate(s), ease its conversion to the transition state, and then release the product(s).

It has long been recognized that the chemical space in enzyme active sites is not fully explored by nature. This observation reflects the fact that a limited number of folds and catalytic mechanisms are used by natural enzymes to produce an enormous diversity of biological compounds.[4] Despite being well-tuned catalysts for specific chemical transformations of biological relevance, some enzymes have been shown to exhibit promiscuous activities, accepting alternative substrates and catalyzing secondary reactions.[5–7] We classify promiscuity by using three categories: substrate (the enzyme accepts structurally distinct substrates but catalyzes the same chemical reaction), catalytic (the enzyme accepts different substrates and catalyzes different overall reactions), and product (the enzyme accepts a single substrate and uses similar chemical mechanisms to catalyze the formation of different products). These promiscuous activities may be involved in natural enzyme evolution.[7,8]

Consistent with this view, an increasing number of enzyme superfamilies have been identified whose members have homologous structures and use related mechanisms to catalyze different reactions.[9,10] In these families, small changes in the amino acid composition of the active sites can account for their differing activities, which supports the hypothesis that a wide variety of enzymatic activities divergently evolved from a limited set of primordial enzymes. Such activity switching demonstrates that the inherent structural plasticities and catalytic versatilities of enzyme active sites can give rise to new biocatalysts. Thus, productive searching of chemical space within enzyme active sites may introduce or enhance promiscuous activities and thereby increase their effectiveness in synthetic applications.

Indeed, mutation of active-site residues often dramatically changes the properties of an enzyme. Most changes of this type are simply inactivating;[11] sometimes, however, the altered enzymes have broadened substrate specificities or altered reaction profiles from which novel activities emerge.[12] How then can one efficiently identify interesting changes? The use of sequence, structural, and mechanistic information has proven to be a productive basis for designing active-site-directed mutations that expand the catalytic repertoire of enzymes. This strategy relies on targeted substitutions that do not disrupt the global protein fold, allowing (rational) exploitation of the unchanged residues in the

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context of the "new" active site. The recycling of functional features minimizes the number of mutations required to access a new function. Often, only a single amino acid substitution is sufficient to give a novel activity to an enzyme. So far, this type of active-site engineering has generated enzymes with broadened substrate specificities, altered control of product formation, improved trace activities, and new reactivities and reaction mechanisms.

How useful is the rational redesign of enzyme active sites? In this Review we aim to illustrate some remarkable successes in active-site engineering by using minimal sets of mutations, focusing on those that result in novel transformations rather than simple changes in substrate specificity (of which there are numerous examples in the literature[12,13]). Comparison of the different approaches used allows us to evaluate the potential of this method in the development of new biocatalysts. We conclude that making a small number of defined changes in the atomic structure of an enzyme is often a relatively easy way to access alternative catalytic activities. Although novel catalysts generated in this way are usually not optimally efficient, they are promising starting points for

further evolution. With the advent of tools for enzyme engineering, such as directed evolution and computation, active-site redesign is gaining importance as a strategy for meeting the ever-increasing demand for efficient, tailor-made enzymes.

2. Subtilisin: A Playground for Protein Engineers

Hydrolases have been popular objects for enzyme engineering, not only because they were among the earliest characterized enzymes but also because their catalytic activities often have direct industrial applications. Serine proteases catalyze the hydrolysis of peptide bonds through the formation of an acyl-enzyme intermediate, which is in turn hydrolyzed to the free acid.[14] The catalytic machinery of serine proteases includes a catalytic triad (Ser-His-Asp), which activates the serine side chain for nucleophilic attack, and an oxyanion hole, which stabilizes the tetrahedral intermediates formed during the enzyme acylation and hydrolysis steps. Among serine proteases, the wealth of structural and functional information available for subtilisin[15] has made this enzyme a particularly productive framework for rational active-site engineering.

In a sense, the history of active-site engineering starts with subtilisin itself. During the 1960s, the research groups of Bender[16] and Koshland[17] independently used subtilisin to provide the first example of site-directed mutagenesis in a protein: the post-translational conversion of the catalytic serine residue (Ser221) to cysteine. This conversion took advantage of the specific reaction between Ser221 and phenylmethanesulfonyl fluoride. Treatment of the resulting adduct with thioacetate leads to an enzymatic thioester that spontaneously deacylates, unmasking the newly introduced Cys221. In the era before recombinant DNA technology, the defined substitution of one genetically encoded amino acid for another in a protein was a monumental achievement. By applying chemical tricks similar to that used to mutate subtilisin, it became possible to generate directed amino acid changes at uniquely reactive positions in a few other proteins as well.[18]

The (relatively conservative) substitution of cysteine for serine in the subtilisin active site was intended to examine the relative reactivities of oxygen and sulfur within otherwise identical protein contexts. The Ser221Cys variant of subtilisin, also called thiolsubtilisin, was expected to be as good as (or even better than) wild-type subtilisin at catalyzing amide-bond hydrolysis because of the intrinsically higher nucleophilicity of thiols relative to alcohols (Scheme 1). The finding that the mutation caused an approximately 10^5-fold drop in protease activity was therefore quite surprising.[19] In retrospect, this dramatic decrease in activity is all the more striking because the active sites of cysteine proteases provide analogous residue constellations to those of serine proteases. Given the relatively similar sizes and chemical properties of oxygen and sulfur, this result highlights the exquisite sensitivity of active sites towards substitution.

Although thiolsubtilisin is an almost inactive protease, it did retain some esterase activity with activated esters such as

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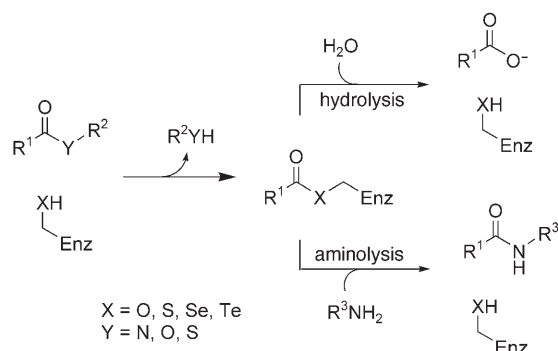
Scheme 1. Hydrolysis versus aminolysis: competing reactions catalyzed by subtilisin and variants (thiolsubtilisin, selenosubtilisin, tellurosubtilisin). Enz = enzyme.

p-nitrophenyl acetate.[19] The fact that thiolsubtilisin could be acylated but could not cleave amide bonds was exploited by Kaiser and co-workers, who demonstrated that this mutant protease can catalyze the chemoselective ligation of activated esters (*p*-chlorophenyl esters) with amines to form small peptides.[20] Indeed, the fate of the acyl intermediate was affected by the mutation, which caused a greater than 1000-fold increase in the ratio of aminolysis to hydrolysis compared to the wild type. Such differential partitioning is crucial for the observed ligase activity. The identification of this function in a crippled protease opened the eyes of protein engineers to the idea that a single, seemingly conservative, mutation can give rise to novel catalysts.

Still, there was room for improvement in the peptide ligase activity of thiolsubtilisin. Although the atomic radii of sulfur and oxygen differ by only 0.45 Å, the reactivity of the active-site nucleophile may be hindered by the introduction of a slightly bulkier thiol group into a pocket that was evolutionarily fine-tuned to activate a hydroxy group. Wells and co-workers made a second mutation in this active site[21] that was aimed at reducing the steric crowding provoked by the original Ser221Cys substitution. This double mutant (Ser221Cys/Pro225Ala), named subtiligase, lost a further 100-fold in amidase activity and gained 10-fold more peptide ligase activity (k_{cat}/K_m) relative to the parent single mutant. For example, glycolate (glc) esters, such as succinyl-Ala-Ala-Pro-Phe-glc-Phe-Gly-NH$_2$, gave good rates of ligation with the nucleophilic dipeptide Ala-Phe-NH$_2$. Another noteworthy feature of this catalyst is that it did not require the protection of any amino acid side chains or of the C-terminal carboxylate in the peptide that attacks the thioacyl-enzyme intermediate. The substrate specificity of subtiligase was found to be essentially equivalent to wild-type subtilisin for amide hydrolysis,[21] showing that, although the catalytic activity of the active site changed, the binding interactions with the substrates remained the same. Additional mutagenesis in the substrate binding pocket further showed that reactivity and substrate specificity determinants are uncoupled and could be evolved independently.[22]

The design of subtiligase was not only an exciting academic experiment, but it also has found practical applica-

tion in the semisynthesis of peptides and proteins. In one case, a biotinylated hexapeptide ester was ligated onto a folded protein (human growth hormone) in over 95% yield.[22] A more impressive example describes the use of subtiligase for the total synthesis of ribonuclease A, a 124 amino acid protein, through the stepwise ligation of six peptide fragments.[23] Three variants of ribonuclease A were produced in the same manner, with the unnatural amino acid 4-fluorohistidine (which has a lower pK_a value than histidine) incorporated in place of catalytically crucial histidine residues, to assess the importance of the proton-transfer steps in the rates of RNA cleavage and hydrolysis.

The versatility of the subtilisin active site was further extended with the chemical conversion of the catalytic Ser221 to selenocysteine (Sec), creating selenosubtilisin[24] (Figure 1). Although the new active site is a poor catalyst of amide hydrolysis, it does promote acyl transfer reactions. Like thiolsubtilisin, selenosubtilisin also shows a high selectivity for aminolysis over hydrolysis of the acyl-enzyme intermediate (a 14 000-fold increase over the wild type and 20-fold over thiolsubtilisin). A more striking observation was that the incorporation of selenocysteine converted this protease into a peroxidase.[25]
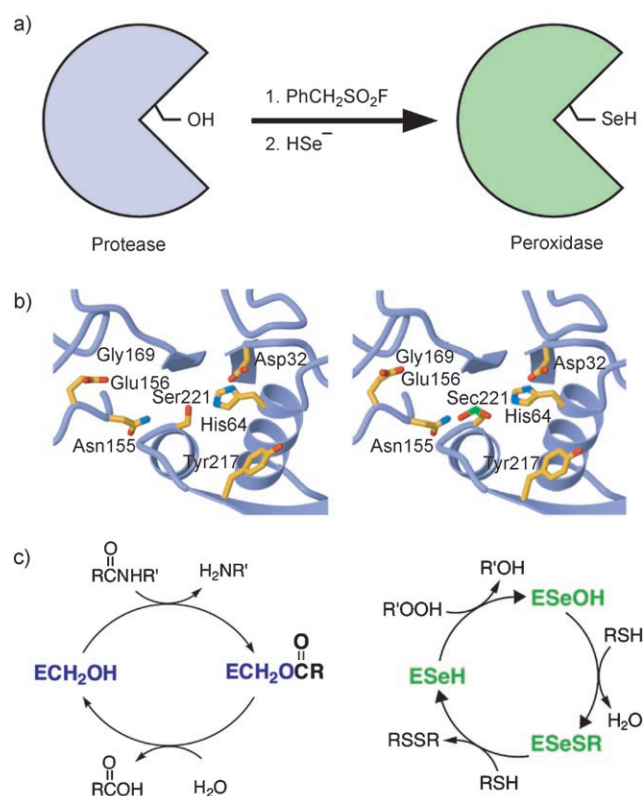
Figure 1. Selenosubtilisin: a) Chemical conversion of the catalytic serine in subtilisin into selenocysteine. b) Comparative views of the subtilisin (left) and selenosubtilisin (right) active sites (Sec221 in selenosubtilisin is oxidized to the seleninic acid (ESeO$_2$H); yellow C, red O, blue N, green Se). c) Catalytic cycles for amide hydrolysis (left) and thiol-dependent peroxidase (right) activities of subtilisin and selenosubtilisin, respectively (E = enzyme). The reactive ESeOH is obtained directly by reduction of the oxidized enzyme with thiols.

The ability of selenosubtilisin to catalyze the reduction of hydroperoxides by thiols was unprecedented in the subtilisin scaffold but could be rationalized by analogy with the natural selenoenzyme glutathione peroxidase (GPx).[26] A detailed picture of the catalytic cycle for the novel peroxidise activity (Figure 1) has been developed based on extensive characterization of selenosubtilisin by X-ray crystallography,[27] NMR spectroscopy,[28] kinetic analysis,[29, 30] and mutagenesis.[31] The rate-determining step appears to be the formation of the selenol (ESeH) group.[29] The pK_a value of the enzyme-bound selenol is unusually low, which can be explained by interactions with the subtilisin framework, particularly the formation of an ion pair with the histidine residue of the catalytic triad (which itself has an elevated pK_a value compared with wild-type subtilisin) and a hydrogen bond with an asparagine residue of the oxyanion hole.[27] Otherwise, the topology of the active site remains largely unchanged by the mutation. In contrast to GPx, glutathione is a poor substrate for selenosubtilisin, which exhibits a marked preference for aromatic thiol substrates (3-carboxy-4-nitrobenzenethiol being the best), mirroring the specificity of wild-type subtilisin.

Selenosubtilisin has been shown to accept a variety of secondary and tertiary hydroperoxide substrates and to form the corresponding alcohols stereospecifically. The substrate selectivity of selenosubtilisin is similar to that of wild-type subtilisin, suggesting that the hydroperoxides bind in a similar orientation to the peptide substrates and inhibitors in the active site of the wild-type enzyme.[32] The extensive binding information available for subtilisin could then be used to predict the substrate specificity of the seleno variant. For some substrates, selenosubtilisin catalyzed peroxide reductions with an *ee* value of 99%.[33] This characteristic was exploited for the resolution of racemic mixtures of hydroperoxides. The fact that selenosubtilisin has a similar catalytic efficiency but the opposite stereoselectivity to that of natural chloroperoxidase and horseradish peroxidase makes this designer enzyme a useful synthetic tool to obtain enantiomerically pure hydroperoxides.

Moving further down Group 16 of the periodic table, tellurium was also introduced at position 221 of subtilisin.[34] The resulting enzyme, tellurosubtilisin, exhibited similar redox activity to selenosubtilisin (and similar efficiency), catalyzing the reduction of hydrogen peroxide in the presence of aromatic thiols.

The chalcogenidic set of subtilisins described in these last examples demonstrates the plasticity of the protease active site in which differences at a single atom result in dramatic changes. These mutations obliterate the original catalytic activity, even though the newly introduced residues possess intrinsically similar functional capabilities. Yet, the altered active sites are not unreactive, they simply catalyze *different* chemical transformations than the wild type. These novel enzymes display predictable substrate specificities because the amino acid substitutions introduce only minor structural perturbations. Moreover, the synthetic applications of the designed variants imply that exploring new reactivities in other scaffolds will reap many rewards. Existing active sites may provide a vast, mostly untapped, well of great catalytic potential.

3. Strategies for Active-Site Redesign

Subtilisin is far from being the only example of catalytic versatility. The advent of recombinant DNA technology has brought with it facile methods for replacing one genetically encoded amino acid for another. Recent developments in altering the genetic code even allow the incorporation of nonstandard amino acids in enzymes,[35] vastly increasing the catalytic possibilities. Semisynthetic methods for the chemical modification of proteins have advanced as well. Consequently, site-specific changes of single amino acids and introduction of prosthetic groups can be exploited to effect alterations in substrate recognition and reactivity for many different enzyme scaffolds.

This section describes some examples of active-site redesign, organized according to the effects of the active-site changes and the strategies used. First, we look at the engineering of new activities in old active sites by the introduction or removal of catalytic residues based on structural and mechanistic information (Section 3.1). Many enzymes catalyze the formation of a reactive intermediate, which is then steered through a specific reaction path. In Section 3.2, we discuss active-site modifications that create new reaction paths to form alternative products. In some cases, natural enzymes possess promiscuous activities. Minor promiscuous activities can be enhanced and may even become the primary reaction through small numbers of active-site modifications (Section 3.3). Alternatively, the abilities of enzyme scaffolds to control the inherently promiscuous reactivities of enzyme cofactors can be harnessed to introduce new activities through active-site engineering (Section 3.4). These examples present interesting variations on the themes described above for the active-site engineering of subtilisin.
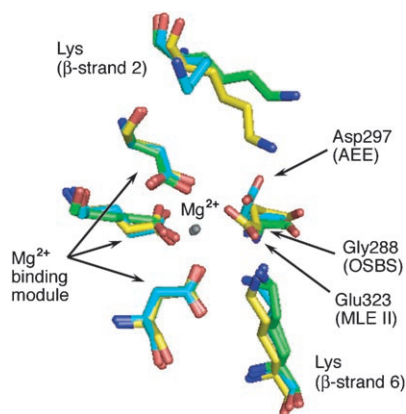
3.1. Introduction of New Reactivity

Completely new activities can often be conferred upon suitable scaffolds through the introduction or removal of essential catalytic machinery. As for the conversion of the protease subtilisin into a peroxidase, such efforts can take advantage of structural and mechanistic knowledge of the target reaction as well as the starting enzyme to obtain a new activity. This strategy depends on recycling features of the original active site, such as substrate binding pockets or interaction partners for the newly introduced residue. The reliance on some pre-existing functional features minimizes the number of required changes, and often, a single point mutation is sufficient to effect a switch in enzymatic function.

3.1.1. Interconversion of Homologous Enzymes

Homologous enzymes that catalyze different overall reactions use the same fundamental scaffold and mechanisms that make use of the same elementary chemical step.[9, 10] These similarities are thought to be indicators of descent from a common ancestor, a process known as divergent evolution. The increasing number of available enzyme
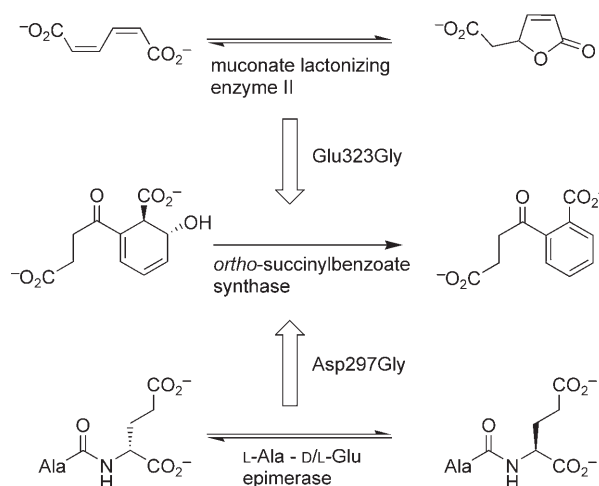
structures has resulted in the grouping of such homologous enzymes into superfamilies. The swapping of activities between superfamily members presents favorable test cases for active-site engineering because of the structural and mechanistic properties shared by such enzymes.

The enolase superfamily is one of the best-characterized enzyme superfamilies. These enzymes use a common triose-phosphate isomerase (TIM)-barrel fold to catalyze reactions involving α-proton abstraction from a carboxylate substrate, forming enediolate intermediates that are stabilized by coordination to an essential Mg^{2+} ion.[36] L-Ala-D/L-Glu epimerase (AEE), muconate lactonizing enzyme II (MLE II), and *ortho*-succinylbenzoate synthase (OSBS) are members of this superfamily and thus contain structurally similar active sites that exhibit these features (Figure 2). To illustrate plausible divergent evolutionary relationships in this superfamily, the active sites of AEE and MLE II were engineered to catalyze the OSBS reaction (Scheme 2),[37] an activity that is not detectable for the wild-type enzymes. Two different methods were used: site-directed mutagenesis in AEE and random mutagenesis in MLE II.

Scheme 2. Generation of OSBS activity in MLE II and AEE through a single active-site mutation.

Figure 2. Active-site overlay of the enolase superfamily enzymes (AEE light blue, MLE II yellow, and OSBS green); red O, blue N, gray Mg. The metal-binding motif, the lysine bases responsible for proton abstraction, and the residues targeted for mutagenesis (Asp297 AEE; Glu323 MLE II) are shown.

Comparison of the structures of AEE and wild-type OSBS showed that although the catalytic residues and the Mg^{2+} cofactor were in the same position in both active sites, the side chain of Asp297 in AEE overlapped the succinyl moiety of the *ortho*-succinylbenzoate (OSB) product in the active site of OSBS. To relieve the steric clash with the substrate, the Asp297Gly variant of AEE was designed based on homology to OSBS, produced, and shown to exhibit OSBS activity ($k_{cat} = 2.5 \times 10^{-3}$ s^{-1}, $k_{cat}/K_m = 13$ M^{-1} s^{-1}).[37] In parallel, random mutagenesis of MLE II also identified a single mutation, Glu323Gly, which conferred a greater than 10^6-fold increase in OSBS activity ($k_{cat} = 1.5$ s^{-1}, $k_{cat}/K_m = 1.9 \times 10^3$ M^{-1} s^{-1}) on this scaffold.[37] This mutant is analogous to the Asp297Gly AEE variant, demonstrating convergence of the catalytic solutions found by rational design and random approaches.

Mutagenesis of the active-site lysine residues in Glu323Gly MLE II and Asp297Gly AEE killed their new OSBS activities; as the homologous residues in natural OSBS are known to be essential for activity, these data support the notion that the engineered and natural enzymes act through similar mechanisms. Interestingly, Glu323Gly MLE II and Asp297Gly AEE also still retained their original activities, albeit with diminished catalytic efficiencies.

Although the OSBS efficiency of Asp297Gly AEE is still five orders of magnitude lower than that of wild-type OSBS, a follow-up study showed that, with a single additional round of random mutagenesis and selection, a new double variant (Ile19Phe/Asp297Gly) could be obtained with further increased catalytic efficiency (> 10-fold).[38] Saturation mutagenesis at position 19 identified a different mutation (Ile19Trp) that resulted in a 27-fold increase in catalytic efficiency relative to the parent Asp297Gly variant, which is mostly due to an increase in k_{cat}. These results illustrate how a newly introduced activity can be improved by further optimizing the active-site environment.

The interconversion of Δ^4-3-ketosteroid-5β-reductase (5β-reductase) and 3α-hydroxysteroid dehydrogenase (3α-HSD) shows that activity swapping through point mutation can be achieved in other superfamilies as well. 5β-Reductase and 3α-HSD are members of the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent aldo-keto reductase (AKR) superfamily[39] and catalyze consecutive steps in steroid hormone metabolism and bile acid biosynthesis: the product of 5β-reductase is the substrate of 3α-HSD. Members of this superfamily utilize a catalytic tetrad (Tyr-Lys-Asp-Xaa) in a "push–pull" mechanism for hydride delivery. The catalytic tetrads of both enzymes differ at the "Xaa" position, which is a histidine in 3α-HSD and a glutamate in 5β-reductase.

The His117Glu variant of 3α-HSD was produced and shown to have 5β-reductase activity ($k_{cat} = 4.2 \times 10^{-3}$ s^{-1} and $k_{cat}/K_m = 220$ M^{-1} s^{-1} for testosterone; Scheme 3).[40] As with the interconversion of activities in the enolase superfamily mentioned above, the engineered variant of 3α-HSD still displayed its original activity, albeit diminished by more than
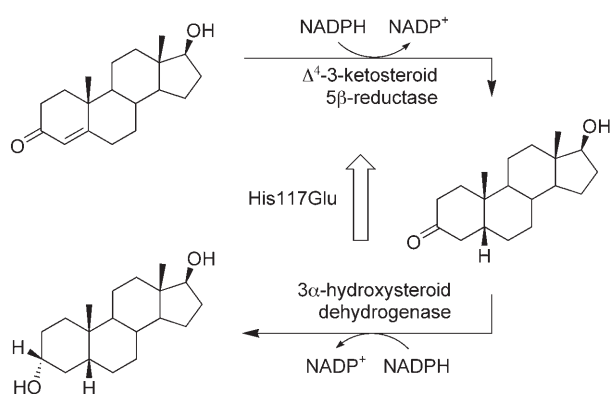
Scheme 3. Conversion of 3α-HSD into a 5β-reductase, homologous enzymes that catalyze consecutive steps in steroid metabolism.

600-fold ($k_{cat} = 0.95 \text{ s}^{-1}$ and $k_{cat}/K_m = 160 \text{ M}^{-1} \text{ s}^{-1}$ for 5β-androstan-17β-ol-3-one). The ease of interconversion for these enzymes supports the suggestion that the evolution of some metabolic pathways could have followed a similar mutational course, developing backwards.[41]

Activity swapping between superfamily members can also go in the other direction, generating an enzyme that accepts the product of its original activity as a substrate for the next step of a biochemical pathway. Base-excision repair (BER) enzymes are involved in the recognition and repair of oxidative damage to DNA.[42] Members of this superfamily include monofunctional glycosylases with base-excision activity and bifunctional enzymes that also catalyze strand scission (bifunctional glycosylase/apurinic-apyrimidinic (AP) lyases). Bifunctional BER enzymes have a conserved lysine residue in the active site, which is crucial for catalysis of the strand scission reaction. Sequence alignments suggest that monofunctional glycosylases in the BER superfamily lack this conserved lysine. For example, MutY, a monofunctional BER enzyme that removes mispaired adenine bases, was converted into a bifunctional glycosylase/AP lyase by grafting an appropriately placed lysine into the active site through site-directed mutagenesis (Scheme 4).[43] In this variant, Ser120
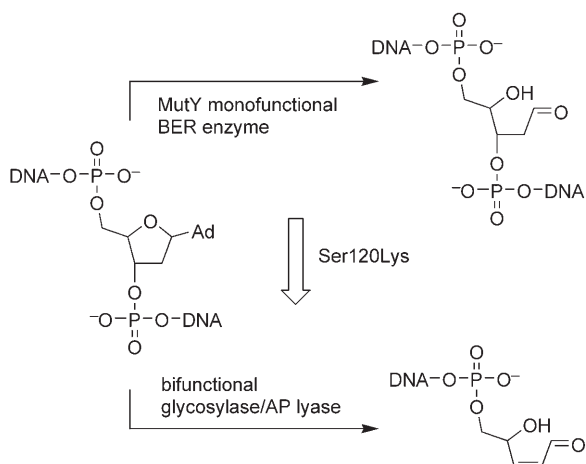
Lys MutY, the rates of both reactions were similar, although the glycosylase activity was reduced by 20-fold relative to the wild-type enzyme. This MutY variant thus catalyzes two consecutive steps in DNA repair (its original reaction as well as the next one), an outcome reminiscent of that seen for activity swapping within the AKR superfamily (His117Glu 3α-HSD catalyzed its original reaction as well as the previous step in its metabolic pathway). Such hybrid bifunctional enzymes represent plausible intermediates in pathway development.

The interconversion of catalytic activities among homologous enzymes can also lead to "cross-talk" between different metabolic routes. For instance, the enzymes HisA and HisF catalyze consecutive steps during histidine biosynthesis, and both of these enzymes are also homologous to the tryptophan biosynthetic enzyme TrpF (Scheme 5). Mutation of a single, analogous position in HisA and HisF endows each with TrpF activity.[44] The conferral of TrpF activity on these proteins required the replacement of a negatively charged aspartate residue with any one of several other residues (such as valine, threonine, or proline).

HisA and TrpF both catalyze Amadori rearrangements, but with distinct substrates. Thus, this conversion is formally just a change in substrate specificity. However, HisF catalyzes
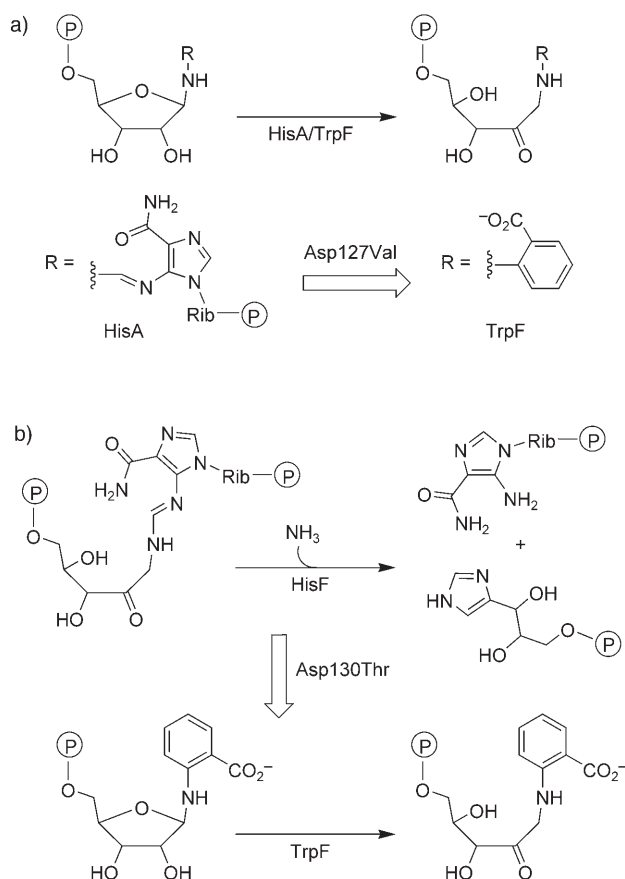
Scheme 5. Conferral of TrpF activity on HisA and HisF. a) Conversion of HisA into TrpF involves a change in substrate specificity. b) Conversion of HisF into a TrpF requires changes in both substrate specificity and reaction specificity.

Scheme 4. Conversion of a monofunctional BER enzyme into a bifunctional enzyme by homology-based active-site mutation.

a distinctly different type of reaction (an ammonolysis/cyclization), albeit with a low level of promiscuous HisA activity. Although the HisA variants have 10- to 20-fold higher activities than the HisF variants, switching the specificity of the minor promiscuous activity in HisF required essentially the same change in active-site structure as for HisA, which was already optimized for carrying out the chemistry of the target reaction. Thus, HisA, HisF, and TrpF might be reminiscent of an ancient ancestor enzyme that had multiple functions and operated in independent metabolic pathways.

3.1.2. Introduction of Catalytic Machinery

When redesigning active sites to promote novel transformations, the recycling of catalytic mechanisms helps to minimize the number of required mutations, as was seen for the activity swapping within the superfamilies described above. However, in some cases, it is also possible to "design in" new mechanistic features through one or a few point mutations. In these cases, the original active site is already predisposed to bind the substrate, and the introduced residues provide new reactive capabilities within the context of the enzyme–substrate complex.

One example of introducing catalytic machinery to promote a different reaction is provided by the generation of oxaloacetate decarboxylase activity in 4-oxalocrotonate tautomerase (4-OT). In small-molecule[45] or peptide model systems,[46] catalysis of oxaloacetate decarboxylation simply requires formation of a Schiff base between the substrate and a primary amine (Scheme 6). The enzyme 4-OT does not normally form a covalent intermediate with its substrate, but rather uses the secondary amine of Pro1 as an acid/base to effect a proton shift.[47] To explore the possibility of catalyzing oxaloacetate decarboxylation by using imine formation, a single mutation (Pro1 Ala) was made in the active site of 4-OT to introduce a primary amine without disrupting the ability to bind α-ketocarboxylates.[48] This variant did indeed show oxaloacetate decarboxylase activity ($k_{cat} = 0.08\ \text{s}^{-1}$, $k_{cat}/K_m = 114\ \text{M}^{-1}\text{s}^{-1}$) in contrast to the wild-type enzyme, which had no detectable activity for this reaction. The activity of Pro1Ala-4-OT is 4700-fold lower than natural oxaloacetate decarboxylase. However, the 4-OT variant is over 30 times more active than model peptide catalysts of this reaction,[46] providing evidence that a significant catalytic contribution is made by the protein scaffold.

As (at least some of) the catalytic machinery is incorporated into the active site by this engineering strategy, prior enzymatic activity is not required on the part of the starting scaffold. For instance, the introduction of a single reactive residue conferred phosphatase activity on a catalytically inert phospho(serine/threonine/tyrosine) binding protein (STYX).[49] The residues involved in ligand binding by STYX are mostly conserved in a class of enzymes known as dual-specificity phosphatases (dsPTPases), which hydrolyze phosphorylated serine, threonine, or tyrosine residues. One difference between dsPTPases and STYX is that an active-site cysteine in the former is replaced by a glycine in the latter. This position was hypothesized to act as an activity switch in this scaffold. Indeed, the Gly120Cys mutant of STYX was
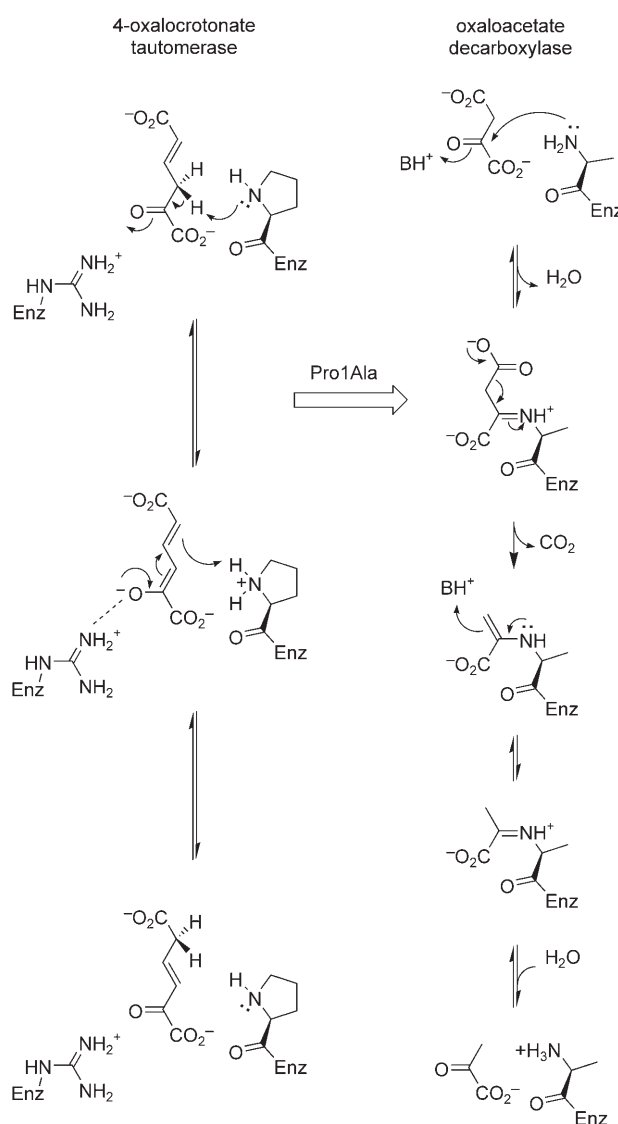
Scheme 6. Mechanisms of the wild-type 4-oxalocrotonate tautomerase and the Pro1Ala oxaloacetate decarboxylase.

shown to catalyze the hydrolysis of *p*-nitrophenyl phosphate ($k_{cat} = 4.6\ \text{s}^{-1}$; $k_{cat}/K_m = 490\ \text{M}^{-1}\text{s}^{-1}$) as well as the dephosphorylation of both phosphotyrosine and phosphothreonine in a diphosphorylated mitogen-activated protein (MAP) kinase peptide.

As noted above for selenosubtilisin, the substitution of an active-site residue by selenocysteine can provide an enzyme with novel redox properties. This method of generating peroxidases is not limited to the subtilisin fold. It has also been used to confer thiol-dependent peroxidase activity on phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[50] and *Lucilia cuprina* glutathione-*S*-transferase (GST).[51] By introducing a selenocysteine into the active site of the latter scaffold, the stabilization of the new covalent intermediate could benefit from the specific binding site for a substrate common to both the new and the old activities. As with the other examples of artificial selenoenzymes, this seleno-GST variant was shown to efficiently catalyze the

reduction of H_2O_2 by glutathione ($k_{cat} = 39.2\,s^{-1}$; $k_{cat}/K_m =$ $1.7 \times 10^5\,M^{-1}\,s^{-1}$, at $[GSH] = 2.00\,mM$). In fact, the catalytic efficiency of seleno-GST is only two orders of magnitude lower than that of natural GPx ($k_{cat}/K_m = 1.4 \times 10^7\,M^{-1}\,s^{-1}$),[52] which is striking because this enzyme is believed to have evolved almost to perfection for the reduction of hydroperoxides.

The versatility of the GST scaffold was further demonstrated by converting GST A1–1 into a thioesterase.[53] In this case, addition of *S*-glutathionyl benzoate to the wild-type enzyme leads to nucleophilic attack by an active-site tyrosine on the substrate and the formation of a dead-end enzyme–substrate conjugate. Using structural information as a guide, a variant was designed in which a newly introduced histidine residue was strategically placed to promote hydrolysis of the previously inert adduct. The catalytic versatility of histidine was further exploited by introducing this residue into the active site of a glycoside hydrolase.[54] This histidine performs nucleophilic attack on the substrate, thus promoting a new transgylcosidase activity and abolishing the original activity.

The hydroxy group of serine is not normally nucleophilic under physiological conditions. Natural enzymes that use serine as a nucleophile have evolved precise networks of electrostatic interactions to activate this residue. Nonetheless, placement of a serine in the active site of cyclophilin (ECyP) by rational design was sufficient to confer protease activity onto this scaffold (Scheme 7).[55] Cyclophilin catalyzes the *cis*–
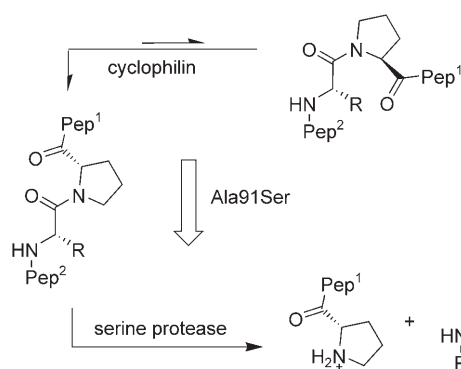
Scheme 7. Conversion of cyclophilin, a peptide bond isomerase, into a serine protease.

trans isomerization of Xaa-Pro bonds in peptides (where Xaa is any amino acid except Pro) and therefore already has an active site that specifically recognizes these substrates. Visual inspection of the structure of ECyP in complex with prolyl-containing peptides guided the design of the Ala91Ser variant, which was capable of catalyzing amide-bond hydrolysis ($k_{cat} = 0.044\,s^{-1}$; $k_{cat}/K_m = 73\,M^{-1}\,s^{-1}$). To further improve this initial activity, histidine and aspartate residues were placed in positions adjacent to the introduced Ser91 to mimic the Ser-His-Asp catalytic triad characteristic of naturally evolved serine proteases. The triple mutant, Ala91Ser/ Phe104His/Asn106Asp ECyP (cyproase), did indeed show greater hydrolase activity towards a chromogenic substrate containing an Ala-Pro amide bond ($k_{cat} = 4.0\,s^{-1}$; $k_{cat}/K_m =$

$1.7 \times 10^3\,M^{-1}\,s^{-1}$). Cyproase was specific for Xaa-Pro peptide bonds, indicating that the substrate-specificity determinants of the active site were left intact. The success of this design strategy also relied on the fact that the mutants preserved the native ability to polarize the C=O of the Xaa-Pro amide bond.

The examples provided by cyproase and the other engineered enzymes discussed in this section illustrate that by taking advantage of the substrate binding properties inherent to an existing active site, a single mutation can alter the reactivity of an enzyme in a defined way by using a different mechanism to catalyze a new reaction. Further, as shown by the engineering of a catalytic triad into cyclophilin, a small number of additional mutations can predictably alter the microenvironment around the introduced residue (the second shell of the active site) to optimize its chemical properties.

3.1.3. Removal of Nucleophilic Residues: Addition by Subtraction

Rather than adding reactive groups to an active site, their removal can sometimes provide an alternative way to access new catalytic activities. Efficient enzymatic catalysis usually results from the cooperative action of multiple reactive groups clustered in the active site. Often, mutation of a nucleophilic active-site residue has catastrophic consequences for activity by precluding an essential step in the catalytic mechanism (such as the formation of a covalent intermediate). Nevertheless, the remaining active-site residues can usually still interact (and react) with the substrate. Thus, an enzyme variant lacking a nucleophile necessary for its original activity may catalyze a different chemical transformation by using a mechanism that had previously been superceded by the nucleophilic attack on the substrate.
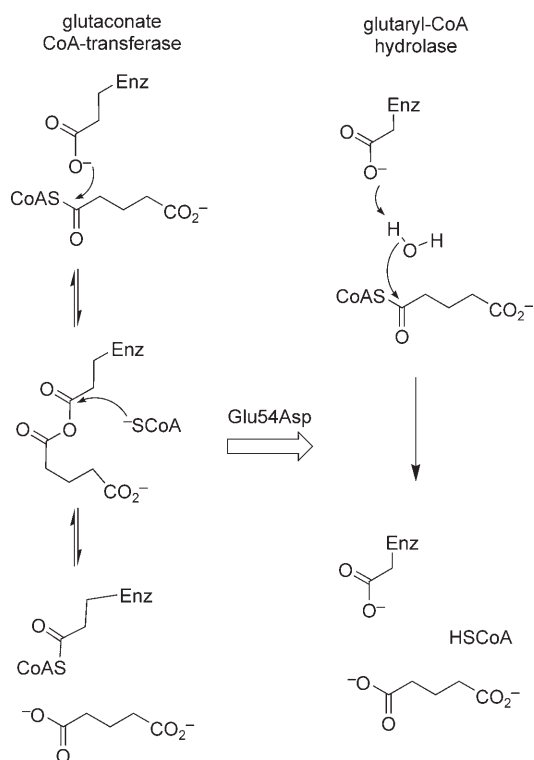
One example is provided by the Glu54Asp mutation in *Acidaminococcus fermentans* glutaconate-CoA-transferase (CoA = coenzyme A).[56] The wild-type enzyme catalyzes the reversible transfer of CoA from glutaryl-CoA to acetate by using a mechanism that involves transient thioester formation between Glu54 and CoA. Mutation of this residue to aspartate resulted in an inactive transferase. However, the new variant hydrolyzed glutaryl-CoA with impressive efficiency ($k_{cat} = 16\,s^{-1}$; $k_{cat}/K_m = 2.3 \times 10^7\,M^{-1}\,s^{-1}$; Scheme 8). Deleting a methylene group from the side chain of residue 54 presumably suppresses the original nucleophilic mechanism because the carboxylate can no longer reach the carbonyl group of the CoA ester. Instead, the newly introduced aspartate residue may activate a water molecule for addition to the substrate. This mutant mimics the action of *Saccharomyces cerevisiae* acetyl-CoA hydrolase, a related enzyme that has an aspartate residue positioned similarly to Glu54 in glutaconate-CoA-transferase.

Deletion, as opposed to shortening, of an essential nucleophilic group from an enzyme active site can also give rise to a new catalytic activity. As a case in point, a cysteine-to-alanine mutation (Cys149Ala) in the active site of phosphorylating GAPDH resulted in non-phosphorylating dehydrogenase activity.[57] The mechanism of oxidative phosphorylation by GAPDH begins with the formation of a covalent thiohemiacetal intermediate. Oxidation of this intermediate
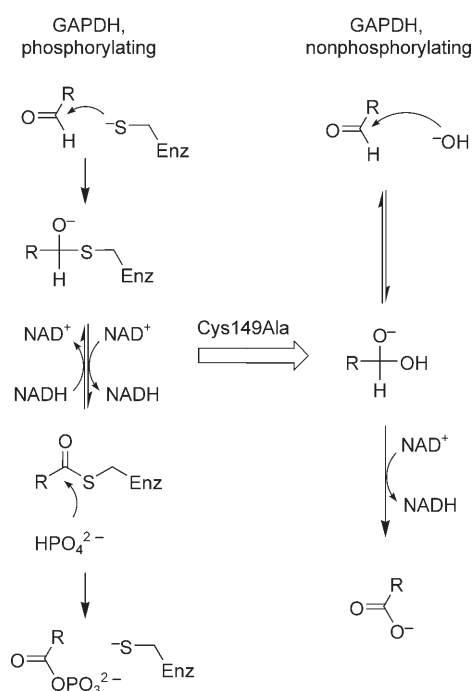
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by nicotinamide adenine dinucleotide (NAD⁺) results in a thioester. Acyl transfer to inorganic phosphate then generates the product and regenerates the enzyme for another round of catalysis (Scheme 9). Deletion of the thiol group at position 149 prevents nucleophilic attack on the substrate and thus abolishes catalysis of oxidative phosphorylation. However, removal of the active-site thiol does not disrupt substrate binding and leads to the formation of an acetal group in the active site of Cys149Ala GAPDH through the addition of a water molecule to the substrate. Oxidation of the acetal group by NAD⁺ results in the formation of the new product, a carboxylate.

Although this variant loses its ability to form a covalent intermediate with the substrate, it retains its ability to act as an oxidase, with the altered mechanism giving rise to (efficient) catalysis of a different overall chemical reaction ($k_{cat} = 5.2\ s^{-1}$; $k_{cat}/K_m = 1.4 \times 10^3\ M^{-1}\,s^{-1}$). Removal of a reactive group was also used to promote the formation of carbon–carbon bonds by a lipase, although the mechanism remains unclear.[58]

The emergence of new catalytic activities upon the removal of active-site nucleophiles is not uncommon. Another example is the Cys161Gln mutation in the β-ketoacyl synthase domain from rat fatty acid synthase.[59] The wild-type active site catalyzes the decarboxylative transfer of a malonyl group (or methylmalonyl group) from an acyl carrier protein (ACP) to the enzyme-linked acyl chain precursors of fatty acids. In the absence of the acceptor, the decarboxylation of the malonyl group occurs only slowly because it is no longer coupled to the acyl transfer step.
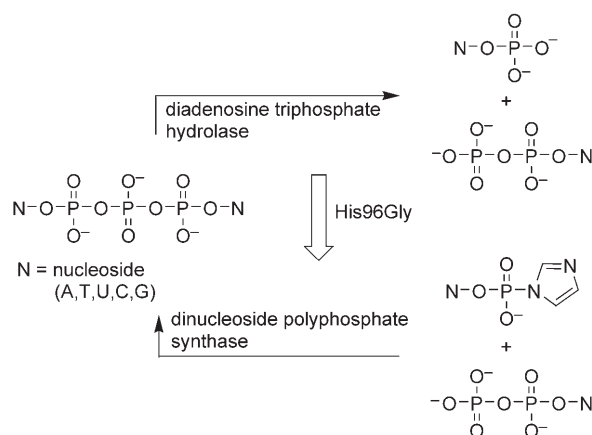

Scheme 9. Phosphorylating and nonphosphorylating mechanisms of wild-type GAPDH and Cys149Ala GAPDH.

However, the Cys161Gln variant, which cannot be loaded with the acceptor, enhances the decarboxylation reaction by more than two orders of magnitude. Importantly, mutation of Cys161 to serine or asparagine (or other amino acids) did not increase malonate decarboxylase activity relative to the wild type. In this instance, successful redesign depended not merely on the removal of a nucleophile, but also required particular structural or functional aspects of the replacement residue.

In the case of diadenosinetriphosphate hydrolase, mutation of the nucleophilic His96 to glycine led to the catalysis of phosphoanhydride-bond formation through the transfer of a nucleoside phosphate from imidazole to a nucleoside di- or triphosphate (Scheme 10).[60] The choice of glycine as a

Scheme 8. Mechanisms of wild-type glutaconate CoA-transferase and the Glu54Asp variant with glutaryl-CoA hydrolase activity.

Scheme 10. Conversion of a triphosphate hydrolase into a polyphosphate synthase by a single mutation.

replacement for histidine was likely crucial to create a binding pocket for the imidazole moiety of the nucleoside-5′-phosphimidazolide substrate. This example is reminiscent both of the conversion of the protease subtilisin into a peptide-bond ligase[20] and of "chemical rescue" experiments,[61] whereby an essential active-site group is removed from an enzyme by mutagenesis and the addition of a small molecule containing the missing functional group (partially) reactivates the mutant enzyme.

The removal of an active-site nucleophile was also able to convert glycosidases into glycoside ligases (glycosynthases), which have potential applications in oligosaccharide synthesis. Retaining glycosidases utilize bifunctional active sites containing a nucleophilic group and an acid/base catalyst. The covalent intermediate partitions between glycosyl transfer and hydrolysis, which can be problematic for synthetic applications. Replacement of the nucleophile, a glutamate in β-glucosidase/galactosidase or β-mannosidase, by alanine or serine prevents the formation of the glycosyl-enzyme intermediate and therefore hydrolysis.[62] However, the engineered variants did catalyze the transglycosylation of activated α-glucosyl fluorides with various sugars through direct S_N2 displacement.[63] Substitution of the acid/base by alanine similarly obliterates the glycosidase activity but still allows formation of the covalent intermediate. Addition of more-nucleophilic thiosugars to this adduct results in the synthesis of thioglycosides.[64] A variant that combines both mutations catalyzes thioglycoside formation from α-fluoro- and thiosugars. Apparently, the binding apparatus remains intact in these redesigned active sites, and the catalytic effect of the variants relies upon bringing the substrates closer together (substrate approximation).

3.2. Partitioning Reactive Intermediates

Many reactions proceed through the formation of intermediates and, if uncontrolled, these species can partition into complex mixtures of products. However, enzyme active sites frequently provide an environment that directs intermediates down particular reaction channels. The control of product distribution by kinetic partitioning can involve both promoting one particular reaction and foreclosing alternative chemical outcomes. In this manner, enzymes achieve reaction specificity. Further, intrinsically unfavorable reactions can even be enhanced by channeling unstable intermediates down appropriate reaction channels, which is of particular relevance for synthetic applications of biocatalysts.[65]

Alteration of active sites to disrupt the reaction channels available to an enzyme-bound reaction intermediate is a common tactic in mechanistic enzymology. However, in those cases, the mutations are usually intended to block any further reaction of the intermediates to facilitate their isolation for structural characterization. There are numerous examples of such modifications,[11] usually involving the substitution of a catalytically essential residue by an inert residue such as alanine. Sometimes, though, active-site redesign can go one step further and not only block one of the steps in the original mechanism, but also open up a new reaction path for the intermediate, leading to the formation of a different product.

3.2.1. Channeling Covalent Enzyme–Substrate Intermediates in New Directions

The reactivities of acylated enzymes have been the focus of numerous active-site redesign efforts. Many hydrolases and amide- or ester-bond ligases undergo acyl-transfer reactions with their substrates (Scheme 11). These transformations often involve formation of acyl-enzyme intermediates. Simple active-site mutations can have a dramatic effect on such species, as we saw in the case of thiolsubtilisin.

The conversion of a hydrolase into a ligase boils down to a competition between acyl transfer from the enzyme to a designated acceptor (ligation) or to water (hydrolysis). A pertinent example is provided by the Ser101Cys/His237Arg variant of rat mammary gland thioesterase II, which gained acyltransferase activity.[66] In this case, acylation of the introduced cysteine by an activated ester substrate, p-nitrophenyl decanoate, resulted in the formation of a slowly hydrolyzed thioester intermediate. In the presence of thiols, such as β-mercaptoethanol or CoA, however, the acylated variant performed efficient transthioesterification to form the thioester product. The k_{cat} value of $0.91\ s^{-1}$ for the ligase activity of the doubly mutated enzyme (with β-mercaptoethanol as the nucleophile) compares favorably to that of the thioesterase activity of the wild type ($k_{cat} = 0.11\ s^{-1}$). The
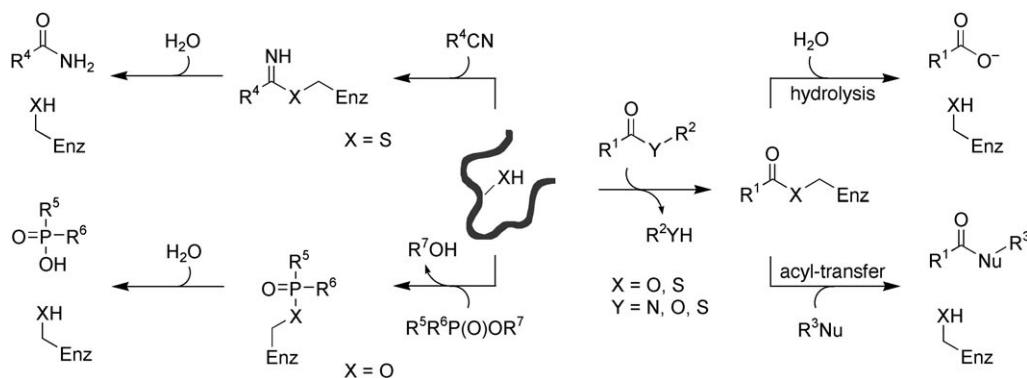
Scheme 11. Different possibilities for the partitioning of acyl-enzyme intermediates.

Ser101Cys substitution was sufficient by itself to confer acyltransferase activity, and the His237Arg mutation increases the acyltransferase activity by further suppressing hydrolysis of the intermediate.

Some hydrolase inhibitors form covalent, dead-end adducts with active-site nucleophiles. Minimal active-site redesign can often turn such inhibitors into substrates for new catalytic activities by enabling the further processing of normally unreactive species. For example, nitriles are known reversible inhibitors of cysteine proteases, such as papain, that form covalent thioimidates with the catalytic cysteine (Scheme 11). In the active site, hydrolysis of thioimidates is slow compared to reversion of the unprotonated thioimidate back to the nitrile, probably because thioimidate hydrolysis (in solution) is acid catalyzed, whereas thioester hydrolysis in papain is base catalyzed.

Consistent with this hypothesis, the thioimidate hydrolysis activity of papain was promoted by introducing a residue capable of donating a proton to the nitrogen atom of the thioimidate.[67] Using structural information, Gln19, which comprises part of the oxyanion hole, was mutated to glutamic acid, resulting in an increase in both k_{cat} (4×10^5-fold) and k_{cat}/K_m (4×10^4-fold) for nitrile hydratase activity, relative to the wild-type enzyme. The amide product can also act as a substrate for the variant, although the amidase activity of the papain mutant becomes rate limiting and the amide accumulates. This (Gln19Glu) papain variant has found application in the synthesis of an amidrazone.[68]

The potential generality of this approach is indicated by the conferral of nitrile hydratase activity on asparagine synthetase B,[69] an amidotransferase whose catalytic mechanism utilizes a nucleophilic cysteine residue but whose overall structure and sequence show no significant relationship to the cysteine proteases. A single mutation (asparagine to aspartic acid) increased nitrile hydratase activity by more than 2500-fold, while decreasing the native glutaminase activity by more than 5000-fold, which suggests a similar nitrile hydratase mechanism to Gln19Glu papain.

Other catalytic activities can also be gained by opening up new reaction channels for otherwise inert enzyme conjugates. For example, a single mutation (Gly117His) endowed human butyrylcholinesterase with the ability to hydrolyze organophosphorous compounds.[70] Addition of such compounds to cholinesterases normally results in the formation of a hydrolysis-resistant phosphoester bond with the nucleophilic serine in the active site (Scheme 11), causing irreversible inhibition of the esterase. Consequently, this class of inhibitors has been applied as pesticides, drugs, and nerve gases (such as sarin and VX). However, the esterase activity of the Gly117His variant exhibits time-dependent reactivation following treatment with GB (sarin) or VX, which suggests that it might be able to hydrolyze its phosphoester linkage with the inhibitor. Indeed, this mutant was shown to catalyze the hydrolysis of numerous organophosphorous compounds (such as the phosphotriester paraoxon, $k_{cat} = 0.013$ s^{-1}) with rate enhancements of up to 10^6-fold over the uncatalyzed reaction. Although natural phosphotriesterases are 10^3–10^5-fold more active than this variant, they use a fundamentally different, metal-dependent mechanism.[71] It is not completely

clear how Gly117His butyrylcholinesterase catalyzes organophosphate ester hydrolysis. In the first step, the active-site serine reacts with an organophosphorous substrate, which is similar to the inactivation of the wild-type enzyme. The introduced histidine, which is positioned in close proximity to the oxyanion hole, probably promotes the breakdown of the phosphoester intermediate either by correctly orienting a water molecule for nucleophilic attack on the phosphorus and/or by distorting the orientation of the intermediate in the active site, thereby exposing it to such a nucleophilic attack.

Together, these examples show how unrelated enzymes with a common mechanistic feature—the formation of a covalent intermediate—can gain new activities by changing the ways they process these adducts. Alternative reactivities can emerge by either enhancing disfavored reaction channels and/or curbing more favorable ones. Useful mutations tend to target either the active-site nucleophile or the residues surrounding the intermediate and can generally be guided by chemical intuition.

3.2.2. *Control of Product Formation*

Many enzyme active sites navigate their substrates through a complex reaction manifold. New catalytic activities can therefore be obtained by changing the reactivity of an enzyme with its substrate or intermediate(s), as described above. However, enzymes can be engineered to catalyze the formation of new products while still employing their natural substrates and mechanisms. Changes in product specificities have been achieved by modifying the size and shape of the active site, or by changing the position of catalytic residues relative to the substrate.

3.2.2.1. *Changing the Extent of Polymerization*

The product sizes of some enzyme-catalyzed polymerizations are determined by the dimensions of the reaction (binding) pocket. In these cases, the polymer lengths might be changed in a (qualitatively) predictable manner by varying the size of this pocket: a bigger pocket should give a higher chain-length product and a smaller pocket should give a lower chain-length product. Isoprenyl diphosphate synthases were chosen to test this hypothesis because they catalyze the addition of multiple isopentenyl diphosphate (IPP) units to dimethylallyl diphosphate (DMAPP) with narrow distributions in product size. To design alterations in product distribution, the structure and sequence of farnesyl (C_{15}) diphosphate synthase (geranyltransferase) was compared with isoprenyl diphosphate synthases involved in the synthesis of isoprenoid diphosphates of various chain lengths.[72] This analysis suggested that residues Phe112 and Phe113 in the geranyltransferase could be involved in setting the size of the binding pocket for the growing isoprenoid chain (Figure 3). Mutations that decreased the size of these residues gave products with increased chain lengths, such as geranylgeranyl (C_{20}) diphosphate (Phe112Ala), geranylfarnesyl (C_{25}) diphosphate (Phe113Ser), and even longer isoprenoid diphosphates (Phe112Ala/Phe113Ser). Conversely, two other variants (Ala116Trp and Asn144Trp) were designed to decrease
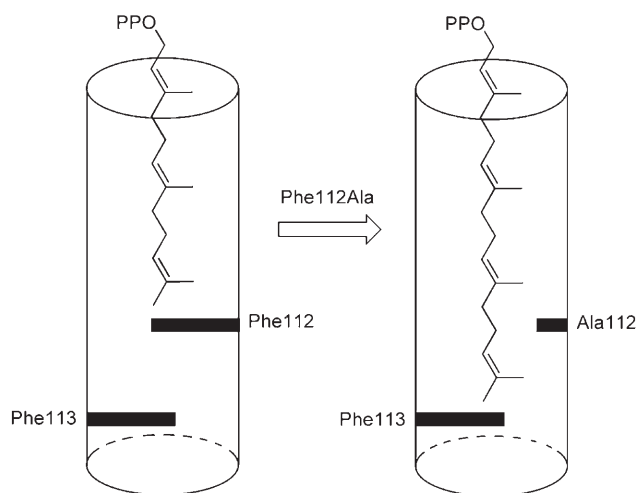
Figure 3. Farnesyl diphosphate polymerase. Schematic view of the active-site determinants of product length. PP = diphosphate.

the room available in the product binding pocket, and these variants yielded the smaller geranyl (C_{10}) diphosphate as the major product.[73] Single amino acid substitutions have also modified the product distributions of other medium-chain isoprenyl diphosphate synthases,[74] suggesting that this approach to set product size may be fairly general for this class of enzymes.

Control of product chain length is also crucial in the biosynthesis of polyketides, natural compounds with a wide variety of biological activities. In type III polyketide synthases, the geometry and volume of the active site influences both the length of the polyketide formed and the choice of the starting molecule. For example, 2-pyrone synthase (2-PS) and chalcone synthase (CHS) catalyze the addition of malonyl-CoA units to acetyl-CoA (2-PS) and *p*-coumaroyl-CoA (CHS) to give the products 6-methyl-4-hydroxy-2-pyrone and chalcone, respectively. Combining three active-site mutations in CHS (Thr197Leu/Gly256Leu/Ser228Ile) changed the geometry of the initiation/elongation cavity and resulted in a variant functionally identical to 2-PS (Scheme 12).[75] Thus, the activity switch in the CHS variant involved not only a change in substrate (from *p*-coumaroyl-CoA to acetyl-CoA),
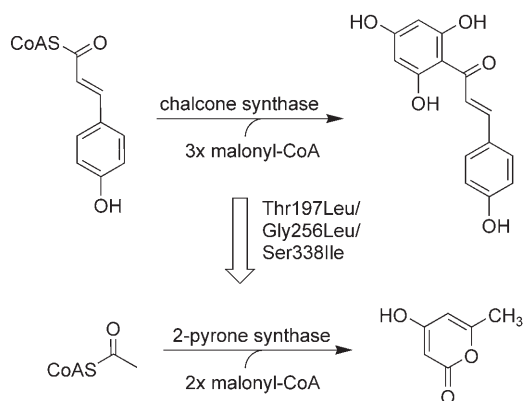
but also in product specificity (from three catalyzed malonyl-CoA additions to two).

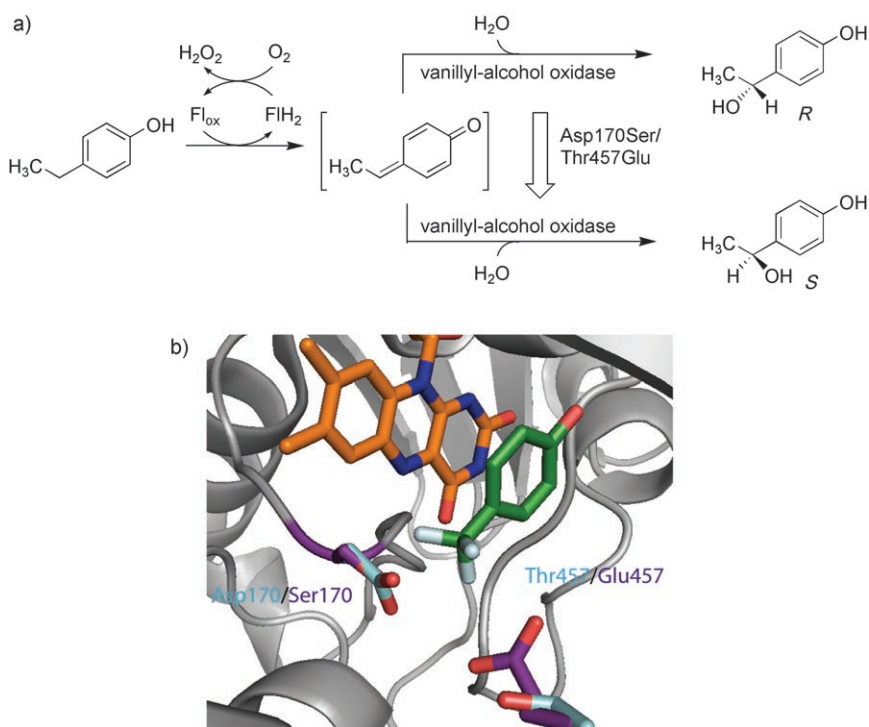#### 3.2.2.2. Controlling Stereo- and Regiochemistry

Enzyme-catalyzed processes often exhibit exquisite stereo- and regiospecificities, which provides a major motivation for the use of biocatalysts in asymmetric synthesis. Sometimes, however, enzymes with the proper selectivity for a desired application may not exist. Therefore, considerable effort has been directed toward controlling stereo- or regiospecificity in enzymatic reactions. Although directed-evolution methods have achieved some success, such laboratory-evolved enzymes usually accumulate multiple mutations outside of the active site.[76] Sometimes, changing residues that line the active site can yield greater effects.[77] Indeed, the switching of stereo- and regiospecificities has also been achieved by rational design targeting these positions.

One approach to designing an enzyme with inverted specificity is to move an essential reactive residue to the opposite face of the substrate. This strategy was employed in vanillyl-alcohol oxidase, a flavin-dependent oxidoreductase.[78] This enzyme is highly promiscuous, catalyzing a variety of reactions with phenolic substrates among which is the stereospecific hydroxylation of 4-ethylphenol to give (*R*)-1-(4′-hydroxyphenyl)ethanol (Figure 4a). The hydroxy group is thought to come from a water molecule activated by the carboxylate group of Asp170. The double variant Asp170Ser/Thr457Glu was designed to shift the activating carboxylate to the other side of the bound substrate (Figure 4b) and did indeed catalyze the formation of the *S* isomer with an 80% *ee* value. Likewise, repositioning a proton donor (cysteine) by double mutation inverted the enantioselectivity of arylmalonate decarboxylase.[79]
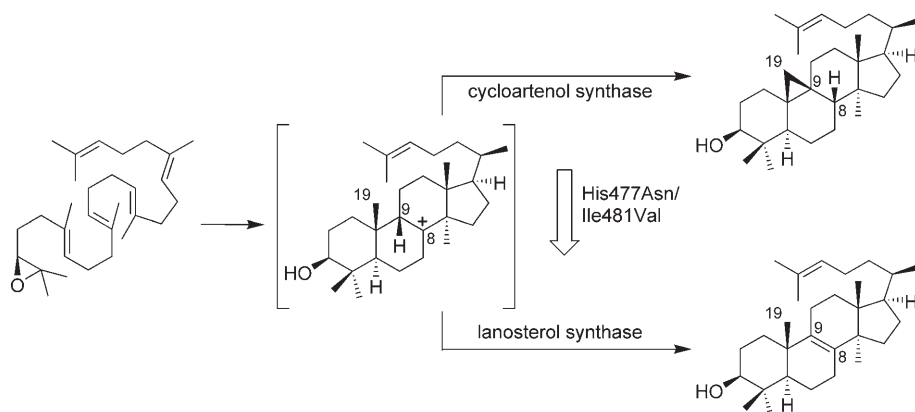
An alternative solution to the problem of inverting stereospecificity was found with adenylate kinase. Rather than rearranging the reactive parts of this enzyme, a single mutation (Arg44Met) was found to change the orientation of the substrate adenosine-5′-phosphorothioate (AMPαS) within the active site.[80] Although the wild-type enzyme specifically phosphorylates the *pro-R* oxygen of the phosphate group to give (S_p)-ADPαS (ADP = adenosine diphosphate), the engineered variant has reversed stereospecificity, forming mainly (R_p)-ADPαS.

Active-site architecture can also dramatically influence regioselectivity. The cyclases involved in steroid and terpene biosynthesis are exemplary. The versatility of these enzymes, which use carbocation chemistry to construct a wide variety of products from a small set of isoprenoid building blocks, stems from their ability to precisely steer the reactivity of identical (or closely related) intermediates in different directions.

An investigation into the product specificity determinants of cycloartenol synthase (CAS) resulted in a variant with highly specific lanosterol synthase activity (Scheme 13).[81] Three highly conserved residues (Tyr410, His477, and Ile481), which may contribute to correct product formation in CAS, were identified from sequence alignment and homology modeling by using the structure of squalene-hopene cyclase (which is closely related to CAS). Mutation

Scheme 12. Conversion of a chalcone synthase into a 2-pyrone synthase by mutations that change the dimensions of the active site.

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Figure 4. Engineered inversion of vanillyl-alcohol oxidase stereospecificity: a) The wild-type vanillyl-alcohol oxidase catalyzes the formation of (*R*)-1-(4′-hydroxyphenyl)ethanol, whereas the engineered Asp170Ser/Thr457Glu variant produces mainly the *S* isomer (80% *ee*). Fl = flavin. b) Superimposed active-site structures of the wild-type vanillyl-alcohol oxidase (light blue) and the engineered Asp170Ser/Thr457Glu variant (magenta). The flavin cofactor is shown in orange, whereas 4-trifluoromethylphenol, a substrate analogue, is green.

cyclization, hydride and methyl shifts, and finally deprotonation. The double variant and the wild type carry out carbon deprotonation at different positions, which then dictates product identity. Although CAS activity involves a hydride shift from C9 to the C8 cation followed by deprotonation at C19, synthesis of lanosterol requires direct deprotonation at C9. Examination of the homology model suggested that the switch in product specificity resulted both from the increased space created by the Ile481Val mutation, which allowed for the rotation of the intermediate to expose the C9 position, and from the His477Asn modification, which helped to position an active-site base for deprotonation of C9.

The product distributions of enzymes involved in sesquiterpene biosynthesis can also be affected by point mutations that alter regiospecificity. δ-Selinene synthase and γ-humulene synthase are product-promiscuous enzymes that catalyze the cyclization of farnesyl diphosphate to produce a large number of natural products. As with steroid synthases, such as CAS, the product distribution depends heavily on the active-site architecture, which influences the deprotonation site as well as the orientation of the substrate and the intermediate(s). This dependence was explored in a study in which single point mutations at a number of active-site residues in each enzyme showed significant (and in some cases dramatic) changes in product specificity.[82]

Homology-based residue swapping between two sesquiterpene synthases, 5-epiaristolochene synthase (TEAS) and premnaspiro-

Scheme 13. Engineering product specificity in cycloartenol synthase with two active-site mutations.

of these residues individually resulted in the formation of multiple products, indicating decreased regiocontrol. However, a double variant (His477Asn/Ile481Val) exhibited high (99%) selectivity for the formation of lanosterol and also complemented a yeast strain deficient in lanosterol synthase. The CAS and lanosterol synthase activities both share the same substrate, oxidosqualene, but catalyze its cyclization to different products.

The mechanism is complex in that the initial protonation of the epoxide leads to carbocation formation, followed by

diene synthase (HPS), was able to achieve a nearly complete switch in product distribution in both directions.[83] As the substrate-contacting residues of the active sites were identical, nine second-shell residues were therefore targeted for mutagenesis. The results indicate that the activity determinants lie almost completely in the second shell. Consecutive addition of mutations in TEAS showed an increase in product promiscuity up to the variant with six mutations. This was followed by a sharp increase in preference for the HPS product over the remaining three mutations. This example

supports the notion that the evolution of new enzyme activities might proceed through nonspecific intermediates.[7,8,84]

In a parallel study, Keasling and co-workers performed a thorough examination of how product distribution is controlled by γ-humulene synthase.[85] In this work, 19 active-site amino acids were subjected to saturation mutagenesis, which led to the identification of several positions that make major contributions to product specificity. An analysis of the relative amounts of each product formed by each single mutant led to the development of a predictive mathematical algorithm (assuming that each single modification acts independently) to find the combination of mutations that should maximize any given terpene synthase activity. Seven novel enzymes were designed by using this method and all showed good selectivity for the desired terpene product with catalytic efficiencies similar to the wild type (30-fold lower to 3-fold higher; Table 1). The knowledge gained from extensive mutagenesis can thus be used to predict and control catalytic activity.
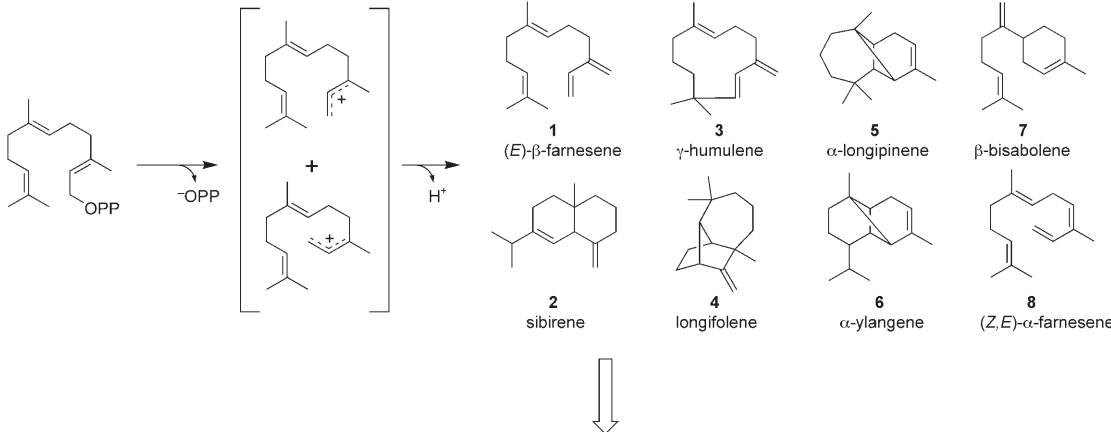
3.3. Improving Promiscuous Enzyme Activities

A traditional view of enzymes holds that their catalytic activities, while optimized by evolution, also represent highly specialized dead ends (one gene, one function). However, this notion is belied by the use of enzymes with non-natural substrates in organic synthesis.[86] Increasingly, many natural enzymes are also found to display substrate, catalytic, or product promiscuity. The high frequency with which enzymes are found to be promiscuous and the ready evolvability of these minor activities to evolve in the laboratory suggest that the set of natural promiscuous activities might provide common starting points for the evolution of efficient and specialized enzymes in nature (for recent reviews, see references [6,7]). Further, the examples described above show that redesigned enzymes frequently display catalytic promiscuity while retaining a fraction of their original activities. Active-site redesign has also found some success in enhancing these minor activities.

In one case, the determinants of "cross-activity" were explored for the homologous $(\beta/\alpha)_8$-barrel enzymes, 3-keto-L-gulonate-6-phosphate decarboxylase (KGPDC) and D-arabino-hex-3-ulose-6-phosphate synthase (HPS), which are members of the orotidine-5′-monophosphate decarboxylase suprafamily.[87] Both enzymes are promiscuous; KGPDC possesses weak HPS activity and HPS possesses weak KGPDC activity (Scheme 14). The two activities are believed to utilize a common intermediate, a Mg^{2+}-stabilized cis-enediolate, although both differ in the mechanisms by which it is formed (decarboxylation in KGPDC versus deprotonation in HPS) and decomposed (protonation in KGPDC versus aldol condensation with formaldehyde in HPS). In an attempt to illuminate the basis for the different catalytic preferences of these two enzymes, three active-site residues were identified that are conserved in KGPDC but not in HPS. These positions were mutated in E. coli-KGPDC to the corresponding residues of HPS.[88] The resulting variant, Glu112Asp/Arg139Val/Thr169Ala-KGPDC, showed a 260-fold increase in catalytic efficiency for HPS activity ($k_{cat}/K_m = 21 M^{-1} s^{-1}$) relative to the wild type.

***Table 1:*** Product distribution [%] for γ-humulene synthase (wild type) and its variants.[85]

Products	Wild type	W315P	F312Q/M339A/M447F	M339N/S484C/M565I	A317N/A336S/S484C/I562V	A336C/T445C/S484C/I562L/M565L	S484A/Y566F	A336V/M447H/I562T
1	3.0	54.1[a]	6.4	2.7	1.7	7.7	2.6	6.4
2	23.1	2.9	**78.1**	0.4	1.4	n.d.[b]	0.4	0.4
3	45.1	2.2	11.4	**85.7**	12.6	11.7	54.6	3.8
4	13.4	n.d.	2.6	3.4	**63.0**	13.3	3.5	4.7
5	4.7	n.d.	0.1	3.5	12.6	**61.5**	15.8	1.1
6	3.8	n.d.	1.0	4.3	3.2	2.2	**14.7**	0.1
7	6.9	6.7	0.4	n.d.	5.5	3.6	8.4	**83.6**
8	n.d.	**34.0**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Values in bold indicate the highest yield obtained for a given product. [b] Not detected.
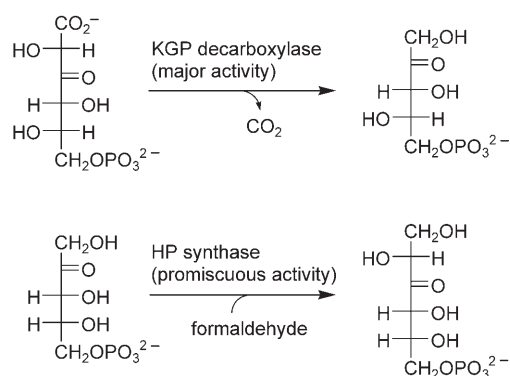
Scheme 14. Reactions catalyzed by KGPDC.

By using a similar approach, the structure of dihydrodi-picolinate synthase (DHDPS) was used to guide mutation of *N*-acetylneuraminate lyase (NAL) to increase its promiscuous DHDPS activity (Scheme 15).[89] Comparison of their homol-
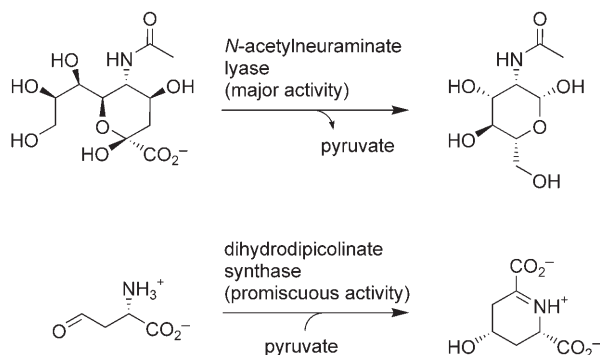

Scheme 15. Reactions catalyzed by NAL.

ogous $(\beta/\alpha)_8$-barrel structures led to the identification of a single active-site mutation (Leu142Arg) that enhanced the DHDPS activity 19-fold. Interestingly, catalytic promiscuity was maintained in both redesign efforts, which suffered only threefold and 30-fold drops in their original NAL and KGPDC activities, respectively.

The already-mentioned GST scaffold (Section 3.1.2) is also naturally promiscuous, both in terms of substrates and catalytic activity.[90] This versatility was exploited in GST A2-2, a glutathione peroxidase with weak steroid isomerase activity. Based on homology with GST A3-3, an efficient steroid isomerase, the active site of GST A2-2 was redesigned by targeting mutations to a set of five sequence positions.[91] Both single and triple mutants exhibited significant improvements in steroid isomerase function, but the largest gain was seen for the quintuple mutant, which was 5 000-fold more active than wild-type GST A2-2. Indeed, the quintuple mutant achieved a catalytic efficiency for steroid isomerization that is similar to that of GST A3-3, whereas its original peroxidase activity decreased by only threefold.

In contrast with the introduction of new functions, the gains in catalytic efficiencies of pre-existing promiscuous

activities seen in these last three examples came at a low cost in terms of the original primary functions. This phenomenon has also been observed in other laboratory-evolved variants with improved promiscuous activities.[92] The changes required for catalysis of a new reaction might cause extreme disruptions in the established catalytic machinery, whereas promiscuous active sites are by definition compatible with the different activities. Therefore, the "native" activity may be relatively insensitive to mutations that enhance a minor one.

However, this is not always the case, as evidenced by the improvement of perhydrolase activity in a serine hydrolase[93] and oxygenase activity in an aldolase,[94] both of which lose more than two orders of magnitude in their respective original hydrolase and aldolase activities. A more dramatic example is provided by the engineering of lactate dehydrogenase (LDH) to improve a weak malate dehydrogenase (MDH) activity (Scheme 16).[95] The design process used structural informa-
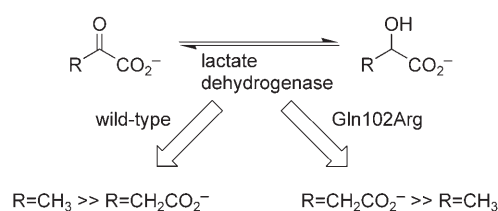
Scheme 16. Swapping the substrate preference in lactate dehydrogenase with a single active-site mutation.

tion to augment the binding of the unnatural substrate without disturbing the residues that are important for turnover. Computer modeling suggested that the introduction of a single mutation (Gln102Arg) would create favorable interactions with the new oxaloacetate substrate while destabilizing the enzyme–substrate complex with pyruvate. Indeed, this variant displayed a 10^3-fold increase in catalytic efficiency for oxaloacetate reduction, with k_{cat} and k_{cat}/K_m values (250 s^{-1} and $4.2 \times 10^6 M^{-1} s^{-1}$, respectively) identical to those of the wild-type LDH with pyruvate as the substrate. Remarkably, this mutation also reduced the LDH activity by four orders of magnitude; the substrate selectivity of the variant was thus fully inverted relative to the wild type. This early example of active-site engineering demonstrates the potential power of a single point mutation, in this case causing a more than millionfold swing in specificity.

3.4. Cofactor Promiscuity

The 20 standard genetically encoded amino acids give rise to an enormous variety of protein scaffolds that promote an incredibly diverse set of chemical transformations, but they cannot do everything. To expand the range of accessible chemistries, many enzymes use nonstandard amino acids, either through ribosomal incorporation (such as selenocysteine[96] or pyrrolysine[97]) or post-translational modification (formation of pyrroloquinoline quinone, oxidation of thiols to

disulfides, etc.).[98] Alternatively, reactive ligands can serve as cofactors. Examples include flavins, nicotinamides, metal ions, and pyridoxal phosphate. These species tend to be inherently promiscuous when free in solution, catalyzing many different reactions with many different substrates. In cofactor-dependent enzymes, the protein scaffold provides a binding pocket, lined with reactive residues, that fixes the substrate in a particular orientation relative to the cofactor. Together, these features promote a reaction along a particular manifold and minimize the occurrence of side reactions. Thus, an active-site environment that specifically favors one reaction path might easily be altered to enable a different chemical outcome, reminiscent of how enzymes can be manipulated to control the fate of reactive intermediates (see Section 3.2).

3.4.1. Flavin-Dependent Enzymes

Flavin-dependent enzymes are widely used in nature for the catalysis of oxidation/reduction reactions and electron transport.[99] Cofactors, such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are particularly versatile because they can carry out both one- and two-electron-transfer reactions (as a consequence of their three possible oxidation states). This attribute allows flavins to mediate the reaction of diverse substrates with molecular oxygen, the most powerful biological oxidant.

Changes in the protein environment around the flavin can have dramatic effects on reactivity. For example, a single active-site mutation of a noncatalytic cysteine (Cys106Val) in bacterial luciferase was enough to convert this mono-oxygenase into an oxidase.[100] Luciferase catalyzes the FMN-dependent oxidation of aldehydes to carboxylic acids, an activity that is lost in the Cys106Val variant. Instead, this variant catalyzes the direct oxidation, by O_2, of the cofactor $FMNH_2$ to FMN, with concomitant formation of hydrogen peroxide. Similarly, a single mutation in the active site of L-lactate mono-oxygenase (Gly99Ala) changed this enzyme into an oxidase.[101] Apparently, the greater steric crowding in the mutant active site causes the release of pyruvate rather than further enzymatic processing to acetic acid and water.

A specialized flavin binding site is not even necessary to steer the activity of this cofactor. In the late 1970s, Kaiser and Lawrence tethered flavin analogues to papain, a cysteine protease, through a covalent linkage with the sulfhydryl group of the active-site cysteine (Cys25; Figure 5).[102] The attachment of the prosthetic group converted this protease into an oxidase. With substrates such as dithiols (for example, dithiothreitol (DTT)), and dihydronicotinamides (for example, NADH), the rate enhancements were 17-fold and 600-fold, respectively, when compared with protein-free model reactions. Flavopapains showed selectivity among different N-substituted dihydronicotinamide substrates and were also stereoselective, favoring pro-R-hydride transfer. These results are all the more impressive considering that the papain active site evolved to recognize polypeptide substrates, which look nothing like the substrates for the novel oxidase activity of the semisynthetic variants. The inherently chiral environment of the protein can influence the specificity of the reactions even without an optimized active site.
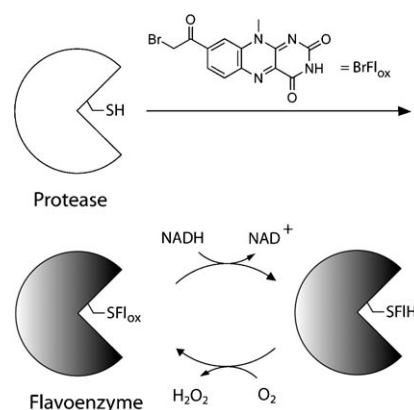

Figure 5. Semisynthetic flavoenzymes: covalent attachment of flavin cofactors to the catalytic cysteine of papain. The resulting semisynthetic enzyme catalyzes the oxidation of nicotinamides with the regeneration of the oxidized form of the cofactor by molecular oxygen.

3.4.2. Metalloenzymes

Metals may be the most useful of all cofactors. Indeed, 30% of all natural enzymes are metalloenzymes.[103] Transition metals have a wide variety of properties and reactivities that are utilized by organometallic catalysts. The high regio- and stereocontrol of these systems has led to their widespread application in organic synthesis. Both the organic shell (in organometallic catalysts) and the polypeptide scaffolds (in metalloenzymes) harness the chemistry of metals by providing substrate specificity, stereocontrol, and an appropriate local electronic environment.[104] To obtain new metal-dependent enzymatic activites, an enzyme engineer can introduce a new metal center and/or change the active-site topography.

An early success in turning a protein into an organometallic catalyst was reported by Wilson and Whitesides, and involved the introduction of a rhodium(I) catalytic center at the biotin binding site of avidin,[105] an experiment analogous to Kaiser's flavopapain (Section 3.4.1). Avidin is a well-characterized protein that binds biotin with an affinity ($K_d = 10^{-15}$ M) that is among the strongest known noncovalent interactions. The metal center, rhodium(I) complexed by diphosphine ligands, was introduced into the protein's binding site through a biotin analogue. This artificial metalloenzyme showed enantioselective hydrogenation activity towards the substrate α-acetamidoacrylic acid, with full conversion and approximately 40% ee for (S)-N-acetylalanine. The free ligand, however, lacked detectable enantioselectivity (Scheme 17).

This system has been recently optimized through changes in the biotin ligand, metal, and the enzyme scaffold.[106] Modifications in the ligands gave rise to improved enantioselectivities and were even able to induce a swap in the preferred product enantiomer. Better ee values (92% for the hydrogenation of α-acetamidoacrylic acid) were obtained with streptavidin, a related protein with similar affinity for biotin, possibly because of its deeper binding pocket. In contrast with avidin, this scaffold favored the formation of the R product. The contribution of the protein was underlined by a single amino acid substitution, Ser112Gly in streptavidin,
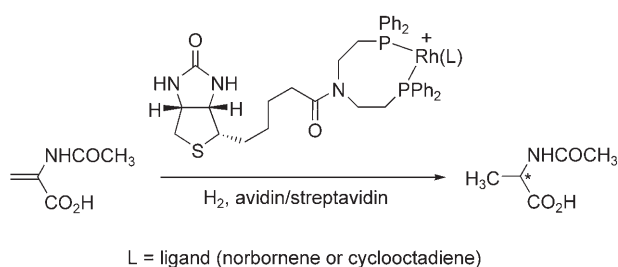
Scheme 17. Noncovalent attachment of a biotin–Rh complex in biotin-binding proteins (avidin and streptavidin) introduces new stereoselective hydrogenation activity.

which resulted in an improved *ee* value (96%). This strategy has also been applied and optimized for the hydrogenation of ketones by using Ru^{II} complexes. Based on structural information and molecular docking, the residue closest to the metal complex, Ser112, was targeted for saturation mutagenesis.[107] The resulting variants showed remarkably different stereoselectivities. Although aromatic residues increased the selectivity for the (*R*)-alcohol products (up to 97% *ee* for Ser112Tyr), positively charged residues shifted the selectivity towards formation of the (*S*)-alcohols (up to 70% *ee* for Ser112Arg).

The importance of active-site topography for the activities and stereoselectivities of metalloenzymes has been further illustrated with myoglobin. The main function of this heme-containing protein is the transport of molecular oxygen, but it also exhibits low peroxidase activity. Structural comparison with a natural heme-containing enzyme, cytochrome *c* peroxidase, suggested that the position of a histidine residue above the heme iron could be important for activity. The Leu29His/His64 Leu myoglobin variant was produced, shifting the position of the targeted histidine residue (Figure 6).[108] For the hydrogen peroxide-dependent oxidation of methyl phenyl sulfide, this variant indeed showed a 200-fold increase in catalytic rate ($k_{cat} = 0.092\ s^{-1}$) as well as increased enantioselectivities (97% versus 25% *ee* for the wild type). A similar
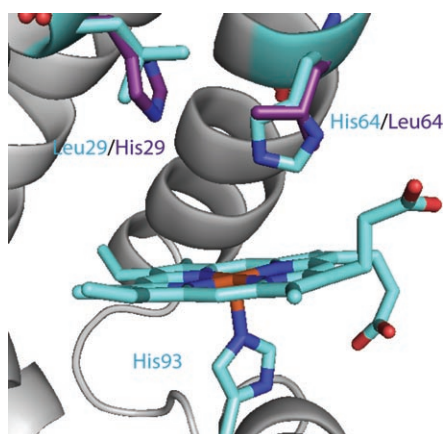
effect was observed for the epoxidation of styrene. Two other approaches, involving the replacement of the heme with Cr^{III}-[109] and Mn^{II}-containing[110] complexes, also resulted in myoglobin variants with peroxidase activity. Other examples of engineering novel peroxidases include the introduction of Mn^{II} in carbonic anhydrase by metal exchange[111] and the incorporation of vanadate in phytase.[112]

Changes in oxidation/reduction activities have also been achieved through the redesign of natural metalloenzymes. *Arabidopsis thaliana* oleate desaturase (FAD2) and *Lesquerella fendleri* oleate hydroxylase (LFAH) are two structurally and mechanistically related membrane-bound enzymes involved in the modification of fatty acids (Scheme 18). Their active sites include a non-heme di-iron cluster and their mechanisms involve the formation of a high-energy radical intermediate through hydrogen abstraction by an iron-oxo species.[113]
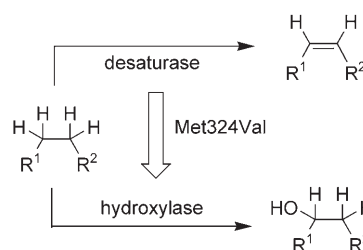
Scheme 18. Desaturase (FAD2) versus hydroxylase (LFAH) activities in iron-dependent fatty acid modifying enzymes.

Residue swapping at seven nonconserved active-site positions resulted in increased hydroxylase activity in FAD2 and an increased desaturase activity in LFAH.[114] In a further study, a single mutant (Met324Ile) of FAD2 was obtained that showed a 54-fold increase in the hydroxylation/desaturation ratio relative to the wild type.[115] A similar strategy was employed to convert *p*-hydroxyphenylpyruvate dioxygenase, a Fe^{II}-dependent enzyme, into a *p*-hydroxymandelate synthase by using either one or two active-site mutations.[116] As both of these examples change the partitioning of a common intermediate, the observed switches in activity can also be viewed as manipulations of the reaction pathway (see Section 3.2.2).

The engineering of novel metalloenzymes has not been limited to redox chemistry. The introduction of a Cu^{II} complex into albumins resulted in highly enantioselective catalysts (up to 98% *ee*) of Diels–Alder reactions.[117] Indeed, the scope of reactivities that can be explored is only limited by the choice of metal; the protein scaffold provides stereoselectivity. However, it is worth noting that the presence of a protein scaffold alone does not necessarily lead to rate enhancements or stereoselectivity.[118] Better control of metal properties might be achieved by engineering metal binding sites directly into the protein scaffold.[119] As with the development of organometallic catalysts, progress will be driven by optimizing the shell surrounding both the metal and the substrate.

Figure 6. Comparison of the active sites of wild-type myoglobin (light blue) and the Leu29His/His64Leu variant (magenta). Changing the position of the histidine relative to the heme greatly increased the rate and stereoselectivity of the secondary peroxidase activity.

3.4.3. PLP-Dependent Enzymes

Pyridoxal phosphate (PLP)-dependent enzymes catalyze a wide range of reactions with amino acid substrates (racemizations, decarboxylations, aldol condensations, and transaminations, among others).[120, 121] The reactivity of this cofactor in solution has been extensively studied and shows little substrate or reaction specificity, although some measure of control has been obtained in the presence of metal ions[122] or with cofactor analogues.[123] PLP-catalyzed reactions share a common mechanistic feature: the formation of an imine bond between the substrate and the cofactor that acts as an electron sink to stabilize carbanion formation at the Cα position. The active-site architecture is responsible for recognition of the substrate and orients the reaction path towards the desired product. Dunathan's hypothesis, first communicated in 1966,[124] states that the labile bond (which breaks to generate the Cα carbanion) must be perpendicular to the plane of the cofactor pyridinium ring, aligning the σ bond with the conjugated π system of the cofactor (Figure 7). Enzyme active sites can exploit noncovalent interactions to orient the substrate, influencing which bond is perpendicular to the cofactor ring, and can therefore strongly favor a specific reactivity. Structural information has shown that much of the cofactor-binding and catalytic machinery is conserved between different enzymes, even those possessing completely different folds.[120]

Following this line of thought, a single active-site mutation was sufficient to convert alanine racemase from *Geobacillus stearothermophilus* (AR) into a D-amino acid aldolase, catalyzing the retro-aldol reaction of D-β-phenylserine (Scheme 19).[125] Natural D-amino acid aldolases have been purified and characterized,[126] but no structure has been reported. Although both the D-amino acid aldolases and AR have been classified as type-III fold PLP-dependent enzymes, there is not enough sequence similarity between these enzymes to allow comparison of the active sites. Therefore, the structure of L-threonine aldolase (TA) from *Thermotoga maritima* was used to guide the redesign of AR. TA and AR are evolutionarily unrelated enzymes that have different
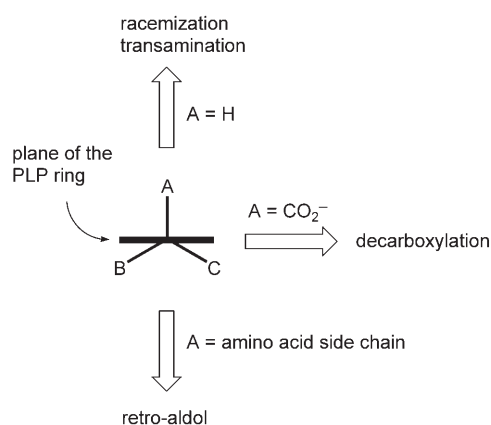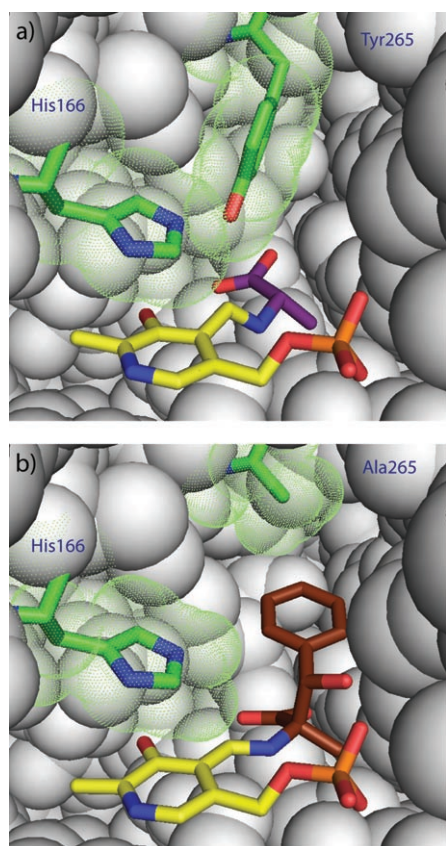

Figure 7. Dunathan's hypothesis concerning the reaction specificity of PLP-catalyzed reactions. The nature of the chemical transformation is determined by the identity of the bond oriented perpendicular to the plane of the pyridinium ring of PLP.

tertiary folds (type I and type III, respectively) and quaternary assemblies; however, their mechanisms involve a common aldimine intermediate. Analysis of the two active sites and superimposition of the respective PLP ligands has shown that the essential catalytic residues common to both enzymes are near mirror images of each other, relative to the cofactor. This example illustrates how nature converged upon similar solutions to two distinct catalytic problems using two different folds that evolved independently.

D-β-Phenylserine aldolase activity was introduced in AR by mutating a catalytic base, Tyr265, to alanine ($k_{cat} = 0.095\ s^{-1}$; $k_{cat}/K_m = 11\,M^{-1}s^{-1}$), which resulted in a more than 3000-fold rate enhancement.[125] This mutation creates a cavity that can accommodate the phenyl ring of D-β-phenylserine (Figure 8). The D-amino acid is the preferred substrate of the Tyr265Ala variant. This stereoselectivity is consistent with the design in that it places the Cα–Cβ bond of D-β-phenylserine orthogonal to the plane of the cofactor. The residual methyl-binding pocket was recycled to gain improved
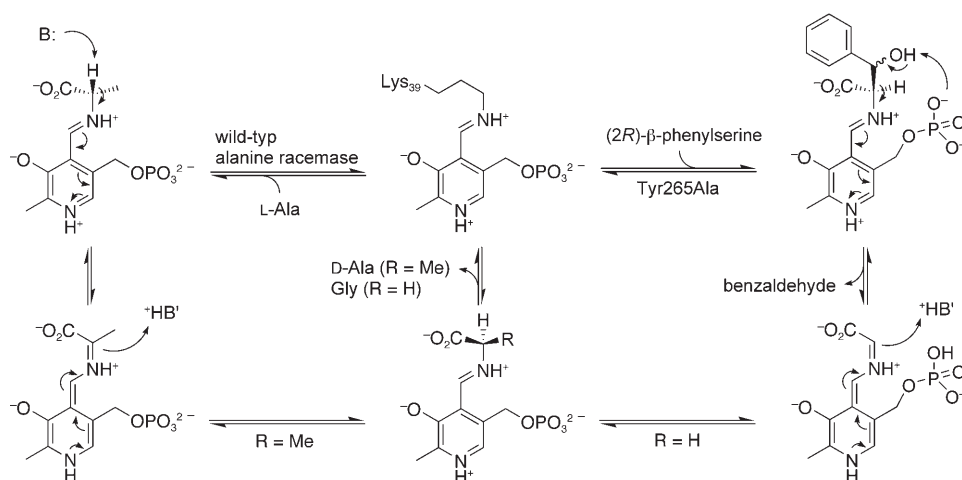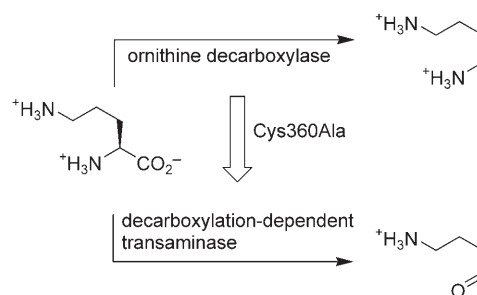

Scheme 19. The reaction mechanisms of wild-type alanine racemase and the aldolase activity of the Tyr265Ala variant (B = Tyr265, B′ = Lys39).

Scheme 20. Introduction of transaminase activity into PLP-dependent ornithine decarboxylase.

In the second example, a triple mutant of aspartate aminotransferase (AAT), Tyr225Arg/Arg292Lys/Arg386Ala, was rationally designed to catalyze the β-decarboxylation of L-aspartate, based on its structural similarity with aspartate decarboxylase (Scheme 21).[130] The engineered mutant showed a 1300-fold increase in decarboxylase activity (k_{cat}), whereas the transaminase activity decreased by 18000-fold. The Tyr225Arg/Arg386Ala mutations were shown to elicit β-decarboxylase activity, whereas the Arg292Lys substitution suppressed transaminase activity.
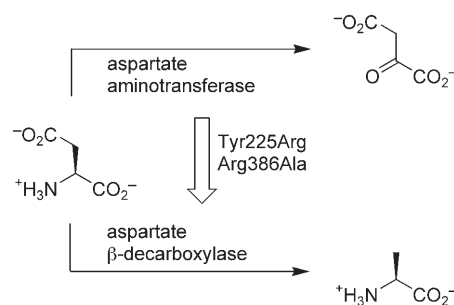
Scheme 21. Conversion of aspartate aminotransferase into a β-decarboxylase.

4. When are Minimal Active-Site Changes Insufficient?

Together, the many success stories summarized in the previous section indicate that new activities are readily accessible, often through a single mutation, from naturally occurring enzymes. However, the simple substitution or introduction of residues does not always result in a desired new enzymatic activity. This is sometimes just a case of not choosing the right scaffold. Even with a well-chosen starting point, it might be necessary in some cases to extensively remodel the active site to accommodate the new catalytic residues and/or create the right environment for the desired reaction to take place. Either the new substrate/reaction places more demands on the active site than can be met by the kinds of straightforward redesign strategies discussed above or the newly introduced residues required for function cause (unintended) catastrophic changes in the structure. Regard-

Figure 8. Engineering aldolase activity into a PLP-dependent alanine racemase: a) Active site of *G. stearothermophilus* alanine racemase complexed with the aldimine between PLP (yellow) and L-Ala (magenta); b) model of the Tyr265Ala variant complexed with the aldimine between PLP (yellow) and (2R,3S)-2-methyl-β-phenylserine (brown). The residues at position 265 and an active-site histidine (His166) are shown in green.

aldolase efficiency with α-methylated β-phenylserines, a non-natural activity.[127] This variant was not stereospecific at the β-position of these substrates, a property also seen with natural PLP-dependent β-hydroxy amino acid aldolases. A second mutation (Arg219Glu) causes an increase in the promiscuous transaminase activity of AR, although the aldolase activity in this double mutant was not measured.[128]

Two other examples have been reported that illustrate different ways in which the reactivity of PLP may be redirected by active-site engineering. Ornithine decarboxylase (ODC) is a type-III PLP-dependent enzyme that catalyzes the formation of putrescine. Two ODC variants, Cys360Ala and Cys360Ser, were produced and shown to catalyze a decarboxylation-dependent transamination to form pyridoxamine-5-phosphate (PMP) and γ-aminobutyralde-hyde, an activity that is not detected with the wild-type enzyme (Scheme 20).[129] In the wild-type active site, the cysteine residue seems to be involved in directing protonation at the Cα atom after the decarboxylation step, preventing the transamination reaction. For the mutants, protonation occurs at the C4′ position of the cofactor instead, resulting in the non-natural reaction.

less, single mutations are not the answer to every design problem.

A salient example of the need for more thorough active-site remodeling is provided by efforts to convert 4-CBA-CoA (4-CBA = 4-chlorobenzoyl) dehalogenase into a crotonase, both members of the 2-enoyl-CoA hydratase/isomerase superfamily.[131] Grafting the two essential catalytic glutamate residues of crotonase onto the 4-CBA-CoA dehalogenase scaffold based on simple homology considerations resulted in an unstable variant. This instability precluded verification of the desired switch in activity and was attributed to a steric clash with other residues in the active site. Therefore, six additional mutations, mainly in a single segment, were introduced into the unsuccessful Gly117Glu/Trp137Glu variant of 4-CBA-CoA dehalogenase. These new changes were designed both to open up space for and to help correctly align the glutamates introduced in the original design. The variant produced after the second round of engineering did indeed exhibit crotonase activity ($k_{cat} = 0.06$ s^{-1} and $k_{cat}/K_m = 1.3 \times 10^3$ M^{-1}s^{-1}; Scheme 22) and showed an increase of greater than 64 000-fold over the background activity. Other, more extreme cases include the introduction of scytalone dehydratase activity to a homologous, but catalytically inert, scaffold[132] and the conversion of glyoxalase II, a metallohydrolase, to a metallo-β-lactamase.[133] The extensive protein engineering required to obtain the desired activities in these latter two examples included full-chain segment substitutions/additions in addition to point mutations.
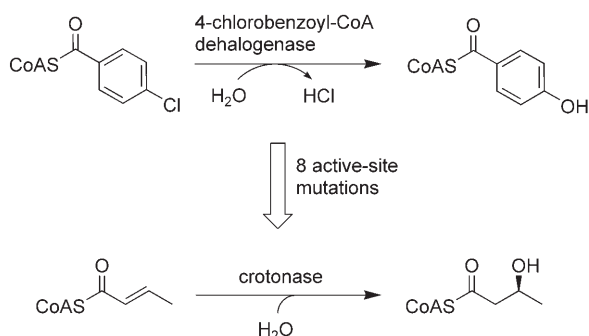
Scheme 22. Rational engineering of crotonase activity into the 4-CBA-CoA dehalogenase scaffold based on structural homology.

In principle, minimizing the changes to an active site increases the reliance on functional aspects of the conserved amino acids. Although smaller numbers of mutations simplify the design process, limiting the extent of mutagenesis places restrictions on the leaps of activity that are accessible to a given scaffold. Certainly, naturally evolved enzymes are biased in favor of the sizes, shapes, and electrostatic properties of their cognate substrates/transition states. For the applications described in the previous paragraph, the simple substitution of catalytic residues is apparently not enough to overcome this bias. Such situations require greater tenacity and insight to identify the necessary adjustments in the active site without upsetting the delicate balance between function

and overall structural stability. They consequently represent a new frontier in enzyme design.

Indeed, for more difficult redesign challenges it may be necessary to focus not only on those residues that directly interact with the substrate (the primary shell) but also those residues that help to align the active-site residues (the secondary shell). Although the primary shell is certainly more important for dictating activity, changes in the secondary shell can sometimes significantly influence the size and shape of the active site or the orientation/reactivity of catalytic residues.

To cite but one example, in human carbonic anhydrase, the carboxylate group of Glu117 is essential to set the polarity of His119 (which coordinates the reactive Zn^{2+} metal center), and a structurally conservative substitution with Gln leads to an almost complete loss of activity (because of a sharp increase in the pK_a value of the metal-bound water, resulting from stabilization of the histidinate anion).[134]

Thus, expanding the set of mutations should extend the scope of active-site engineering. Distant mutations, which are often identified in directed evolution experiments, can fine-tune activities and selectivities.[135] This observation suggests that the most general strategy for enzyme redesign might be to initially target the active site, where modifications have the potentially highest impact,[77] followed by sequential mutagenesis of the protein scaffold from the second shell outwards. Comprehensive knowledge of enzyme structure and function will be crucial for the success of these endeavors.

5. Evolutionary Lessons

Rational design efforts are normally based on information about natural enzymes. Analysis of the outcomes may, therefore, provide insights into how natural evolutionary processes take place. The observation that two different enzymes can lie only one (or a few) point mutation(s) away from each other in sequence space provides a feasible pathway for natural divergent evolution by gene duplication and random point mutation. Although the idea that many contemporary enzymes diverged from a common ancestor has gained wide acceptance based on their structural and mechanistic similarities,[9, 10] related enzymes with different activities usually possess low sequence similarity (less than 50%). Therefore, it is difficult to identify which mutations are truly important for their distinct activities. The examples discussed in the previous sections are unambiguous: the redesigned variants contain only those mutations absolutely necessary for changes in activity, showing that a small alteration in sequence can result in catalysis of a different chemical reaction.

Examples of recent natural evolution reinforce this lesson. For instance, an investigation on blowfly strains that are resistant to organophosphate (OP) pesticides (such as Diazinon) suggested that mutations in a gene encoding a carboxyesterase could be responsible for a new OP hydrolase activity.[136] It was observed that a single glycine to aspartate mutation at the active site (in the vicinity of the oxyanion hole) was present in all resistant strains, although other

scattered mutations were also found.[137] The Gly137Asp mutant of the (parent) carboxyesterase E3, from an OP-susceptible strain, was in fact shown to possess OP hydrolase activity, at the expense of the original one. This E3 mutant appears to use a similar OP hydrolase mechanism as the engineered butyrylcholinesterase discussed in Section 3.2.1. This is not an isolated case; others include atrazine chlorohydrolase[138] and melamine deaminase,[139] which are involved in the degradation of synthetic compounds introduced into the environment within the last century. Although these enzymes are specific for their reactions and have diverged only recently, their sequences differ in only nine amino acids, most of which are not essential for the new activity.[140] The significance of the nonessential mutations is unclear. They might represent activity optimization, structure stabilization, or just neutral genetic drift.

It has been proposed that the evolution of new functions proceeds through nonspecific intermediates.[7,8,84] The observation of catalytic promiscuity in both naturally evolved and engineered enzymes is consistent with this view. However, not all activity switches lead to promiscuity (e.g. subtilisin to a peroxidase and glutaconate-CoA transferase to glutaryl-CoA hydrolase). Single mutations can sometimes radically alter the environment in the active site, obliterating the original activity while introducing a new function. Although small sequence changes may generate promiscuous enzymes, some modifications result in new and specialized catalysts.

Minimal active-site redesign has illuminated various paths available for evolving new enzymes. It is also relevant to understanding the mechanisms by which metabolic pathways might evolve.[141] These include retrograde evolution, in which enzymes evolve sequentially in reverse order along the pathway[41] (e.g. conversion of 3α-HSD to 5β-reductase, Section 3.1.1), specialization of a promiscuous enzyme[7,8] (e.g. NAL to DHDPS, Section 3.3), and enzyme recruitment from a different pathway[8] (e.g. HisA to TrpF, Section 3.1.1). From an active-site engineering point of view, each of these proposals seems equally feasible. The choice of the parent scaffold may depend on an evolutionary race between substrate binding and reactivity.

The few mutations required to obtain new (or improved) catalytic activities should be accessible through the inherent error rate in DNA replication, and therefore it is not unreasonable to think that new enzyme activities from a given fold could be spontaneously produced in an organism. These activities either find use in metabolic pathways or are discarded. Selection pressure and the dynamics of genome duplication may "simply" accelerate enzyme evolution.

6. Outlook

Natural enzymes are clearly not evolutionary dead ends. Enzymatic active sites seemingly lie at or near chemical "tipping points" where well-chosen single mutations can have powerful effects, often suppressing "old" activities and conferring new ones. Although active-site redesign methods are chosen on a case-by-case basis, the collection of novel enzymes obtained by rational point mutagenesis demon-strates the generality of this strategy, both in terms of starting scaffolds and target activities. The engineering of a new activity generally boils down to a change in specificity, either binding (affinity for a new substrate), functional (using the same catalytic machinery in different ways), or mechanistic (adding new reactive centers).[10]

The development of biocatalysts for synthetic reactions that do not have a natural counterpart represents a particularly exciting opportunity for enzyme engineers. Although many of the engineered activities discussed in this review have been based on natural homology, the first steps have already been taken to access non-natural activities. In addition to minimal redesign of existing enzymes, two other strategies have been employed to meet this challenge: catalytic antibodies and computational design (in silico screening).

Although catalytic antibodies have provided one of the most general and reliable ways of generating novel protein catalysts,[142] their catalytic efficiencies still lag far behind those of natural enzymes. The affinity maturation of antibodies elicits residues that are useful for the tight binding of a ground-state ligand but are not necessarily useful for substrate turnover. In contrast with catalytic antibodies, rational redesign is not limited to the immunoglobulin scaffold, but rather can access the set of all known protein folds, many of which may prove more tractable for purposes of activity optimization. Furthermore, the catalytic versatility of enzymes simplifies the design process because many features of their active sites can be recycled.

Computational design offers many of the same advantages as minimal redesign and potentially greater generality as it is not restrained by homology with natural enzymes. One remarkable success has been reported to date, the introduction of triosephosphate isomerase activity into a ribose-binding protein.[143] However, computational methods are still in their infancy, and in order to realize their enormous potential a more complete understanding of enzyme energetics is needed. Current enzyme engineering software lacks some fundamental aspects of the minimalist approach, such as chemical intuition and the ability to match chemistry with scaffold. In the near future, more accurate and accessible versions of these programs will likely become a valuable part of the enzyme engineer's toolkit and will enable the routine analysis of large sets of possible mutations. The combination of chemical insight and computational methods will undoubtedly accelerate the development of catalysts for non-biological reactions. Some initial efforts in this direction have been undertaken to tailor the properties of enzymes.[144] Therefore, computational design should be viewed as a complementary technique to minimal redesign, rather than as an alternative.

Computational design methods could, in principle, also be applied to the second major challenge facing enzyme engineers, namely activity optimization. However, our understanding of structure–function relationships is not yet mature enough for this approach to be reliably successful. Directed evolution, however, provides a general route to improved catalysts that does not require foreknowledge of structure or mechanism. More and more sophisticated library designs and improved screening and selection methods are constantly

being developed.[76,145] These advances both expand the application of directed evolution to new activities and improve the chances of identifying interesting molecules by increasing the number of sampled sequences. To date, the application of directed evolution to catalyst discovery has been limited by the insufficient sensitivity of current detection techniques for spotting the very low levels of activity that are likely to be present in a large library of mutants. This limitation underscores the importance of methods able to obtain starting activities above background levels.

Based on the examples discussed in this review, it might be productive to view the active-site structure as providing a local code that can be translated into a particular function.[146] This code is often modular and residue substitutions can either render it nonsensical (i.e., inactivate the enzyme) or alter its output (i.e., switch the enzyme's catalytic activity). The residues that line the active site are of primary importance. The secondary shell of the active site (and even more distant parts of the protein structure) can also play significant roles, but these are generally more difficult to interpret and predict. A synergistic combination of rational design, computational methods, and directed evolution is likely to provide a general strategy for deciphering and meaningfully rewriting active-site codes, opening the door to custom-made, optimized enzymes.

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