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Perspective

# A New Age of Biocatalysis Enabled by Generic Activation Modes

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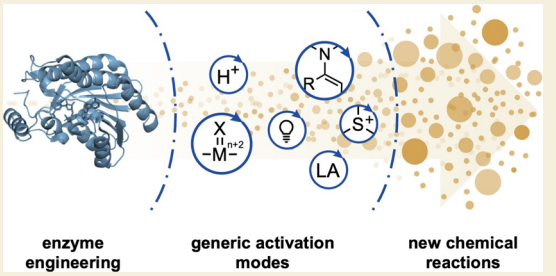
**ABSTRACT:** Biocatalysis is currently undergoing a profound transformation. The field moves from relying on nature's chemical logic to a discipline that exploits generic activation modes, allowing for novel biocatalytic reactions and, in many instances, entirely new chemistry. Generic activation modes enable a wide range of reaction types and played a pivotal role in advancing the fields of organo- and photocatalysis. This perspective aims to summarize the principal activation modes harnessed in enzymes to develop new biocatalysts. Although extensively researched in the past, the highlighted activation modes, when applied within enzyme active sites, facilitate chemical transformations that have largely eluded efficient and selective catalysis. This advance is attributed to multiple tunable interactions in the substrate binding pocket that precisely control competing reaction pathways and transition states. We will highlight cases of new synthetic methodologies achieved by engineered enzymes and will provide insights into potential future developments in this rapidly evolving field.




**KEYWORDS:** *Activation modes, Biocatalysis, Enzymes, Selective catalysis, Brønsted acids, Photoenzymatic reaction, Nitrene transfer*

## INTRODUCTION

Biocatalysis is an enabling technology that provides only limited solutions to chemical synthesis due to the relatively small range of reactions routinely catalyzed by enzymes.[1] Common uses of biocatalysts involve modifying functional groups and late-stage modifications of bioactive compounds.[2,3] In terms of synthetic organic chemistry, enzymes are frequently used to produce chiral molecules such as amines, alcohols, and different carbonyl compounds.[2] Applied biocatalysts typically include hydrolases and redox enzymes such as amine transaminases (ATAs), ketoreductases (KREDs), ene-reductases (EREDs), and lately imine reductases (IREDs). Beyond these success stories, which are driven by advances in enzyme engineering,[4] broader use in synthetic organic chemistry is hampered by the limited knowledge of enzymes' overall reactivities. Enzymes are historically seen as catalysts, that utilize specific mechanisms to catalyze a unique reaction on a small set of substrates.[5−8] Lately, cheaper access to synthetic DNA for genes of interest and simpler methods for efficient enzyme production and engineering has substantially lowered the barriers for chemists to investigate enzymes for diverse synthetic and catalytic purposes.[10] Many established and early career scientists have developed research programs to systematically explore reactivity patterns of natural and engineered enzymes. This research is significant because it shows that the underlying reactive centers in proteins have a much broader reactivity and applicability than originally thought. In this review, we argue that the field is currently entering a new age, as several generic strategies to activate molecules are now widely available in enzymes, allowing a more rational exploration of the reaction promiscuity of enzymes.[9]

A generic strategy to activate substrates ideally leads to reactive species that can participate in many different types of reactions (Figure 1a).[11] These reactive intermediates are generated by primary interactions of the catalyst with simple functional groups in a predictable fashion and consequently enable the development of multiple chemical transformations. The identification of generic activation modes has been extremely important for the rapid growth of the field of organocatalysis as well as photoredox catalysis.[11,12] This suggests that the field of biocatalysis, equipped with a panel of generic activation strategies, may experience similarly rapid growth in the near future. Not only by generating chemomimetic enzymes[13] that "copy" established chemocatalysts to catalyze the same bond-forming reactions, but by inventing new catalytic transformations based on precise control in enzyme active sites.

Next to primary catalytic interactions, enzymes offer a multitude of secondary interactions in their substrate binding pocket (Figure 1b).[14] These multiple secondary interactions create a confined[15] catalytic center and facilitate substrate preorganization and binding in reactive conformations.

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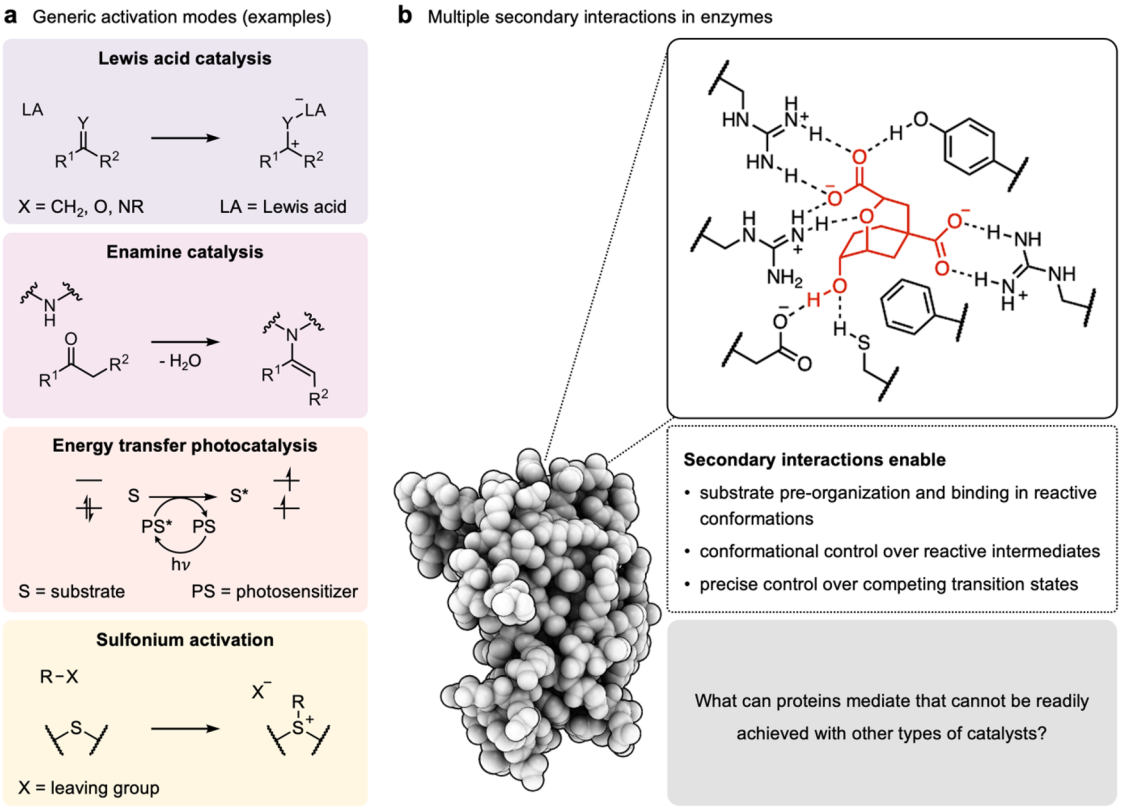


**Figure 1.** (**a**) Selected generic activation modes and (**b**) the potential of natural and engineered enzymes to access new chemical transformations.

Secondary interactions provide precise conformational control over transient reactive intermediates in each step of a catalytic cycle and control competing transition states. The precision offered by the macromolecular structure of enzymes is highly appreciated in the catalysis community, and one can foresee that broad access to generic activation modes in enzymes holds an enormous potential to develop potentially useful synthetic transformations. Consequently, an exciting current question is ***what can proteins mediate that cannot be readily achieved with other types of catalysts***? In the following chapters, we will highlight the potential of identifying and exploring generic activation modes in engineered and even natural enzymes. This is an extremely fruitful approach, not only to generate enzyme functions that are "new" to biology, but to accomplish sought-after bond-forming reactions that were previously not accessible in general.[16−18]

## BRØNSTED ACID CATALYSIS

Among others, Brønsted acids activate alkenes, alkynes, carbonyls, imines, and hydroxy groups to form carbocation, oxonium, and iminium species (Figure 2a), all of which promote nucleophilic addition and other types of transformations such as pericyclic reactions.[19] This strategy has been used for example with BINOL phosphate-derived Brønsted acids to catalyze a huge variety of reactions in an asymmetric manner.[20] In a broad definition, hydrogen-bond catalysis as for example utilized by thiourea catalysts may fall under the classification of Brønsted acid catalysis, yet, this activation mode is not the focus of this chapter. Brønsted acid and hydrogen-bond catalysis are two different modes of activation (with a fluid boundary).[21] In hydrogen-bond activation, the catalyst acts by stabilizing a transition state or an intermediate through hydrogen bonds, without undergoing any net chemical change. In contrast, Brønsted acids transfer a proton to generate an intermediate which subsequently undergoes further reaction steps to yield the products. Although enzymes such as lipases have been broadly investigated as hydrogen-bond catalysts,[22] it is only in recent times that researchers have identified "stronger" Brønsted acids in other enzyme classes and begun to explore their potential in general Brønsted acid catalysis.[23]

Numerous enzyme classes demonstrate exceptional acidity within their active sites, extending beyond typical acid−base chemistry prevalent in enzyme catalysis. This includes enzymes such as squalene hopene cyclase (SHC),[24,25] limonene epoxide hydrolase (LEH),[26,27] and fatty acid hydratase (FAH),[28] among others,[29,30] that in part even activate simple olefins for asymmetric reactions. The origin of the high acidity in these enzymes remains incompletely understood.[31] A common scheme in many enzymes is that the Brønsted acidic center is part of a bigger network of amino acids connected by hydrogen bonds. In addition, the carboxylic acids that act as Brønsted acids are often placed to transfer the proton in the *anti* conformation (Figure 2b). It has been estimated that carboxylic acid protons in the *anti* conformation are $10^4$ times more acidic than those in the *syn* conformation, which is more consistent with the capability for olefin protonation.[32]

In recent years various promiscuous enzymes with Brønsted acid activation have been engineered to catalyze challenging transformations. An early example is the engineering of squalene hopene cyclases (SHCs) to catalyze the Prins/carbonyl-ene reaction of citronellal to isopulegol (Figure 2c) as part of menthol synthesis.[23,33] While the Prins/carbonyl-ene reaction of citronellal is catalyzed by various Lewis and Brønsted acids, it can be difficult to control the selectivity as


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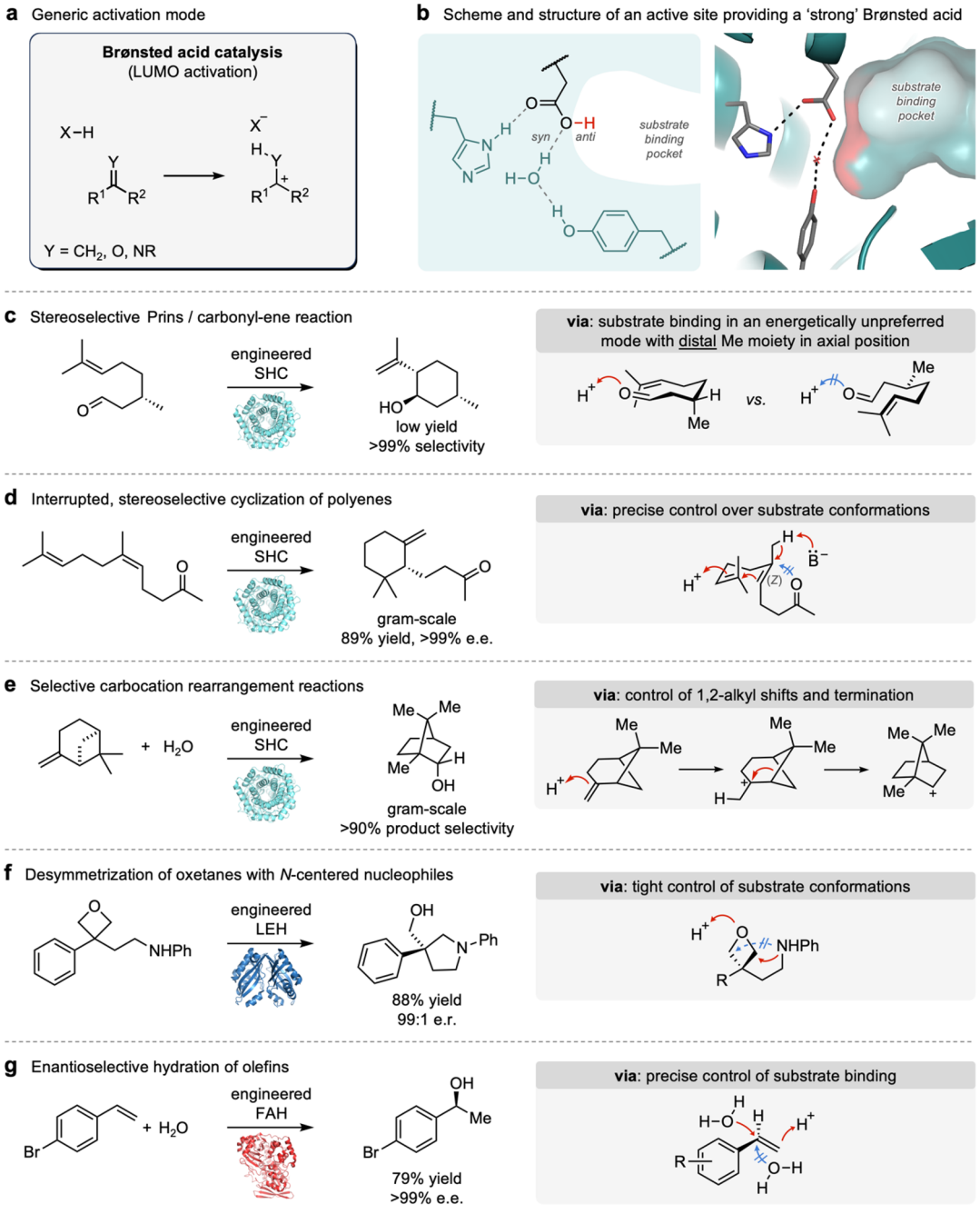


**Figure 2.** Brønsted acid catalysis. (**a**) The figure shows a general scheme for the activation mode as well as an example of an enzyme active site from a squalene hopene cyclase (SHC, pdb: 1UMP) highlighting the Brønsted acid source (**b** and **c**). Various catalytic transformations are emphasized to showcase the variety of challenging transformations that are accessible in engineered enzymes using Brønsted acid catalysis for substrate activation. This includes the stereoselective Prins/carbonyl-ene reaction (**c**),[23] interrupted, stereoselective cyclization of polyenes (**d**),[36,37] selective carbocation rearrangement reactions (**e**),[38] desymmetrization of oxetanes (**f**),[42] and enantioselective water addition to olefins (**g**).[46] Please note that many of these transformations do not have other catalytic solutions.

four different stereoisomers are accessible through different reactive conformations. Methods that selectively generate energetically less favored isopulegol isomers by preferably binding and activating citronellal in an energetically less favored conformation are scarce. In this context, SHCs have been engineered to access single products with very high selectivity (>99%), including a variant that selectively binds and activates (*S*)-citronellal with the distal methyl moiety in an energetically less favored axial orientation (Figure 2c). The energetically preferred all-equatorial conformation and product was not generated.

SHCs catalyze the cationic polyene cyclization of squalene in nature, which inspired a whole field of biomimetic cascade polyene cyclization.[34,35] Polyene cyclizations are used to assemble complex carbon scaffolds in an efficient manner. However, interrupting these cyclization reactions at specific positions remains underdeveloped and is highly challenging, as it relies on precise control over the conformations of flexible alkyl chains over a long distance. Engineered SHCs enabled


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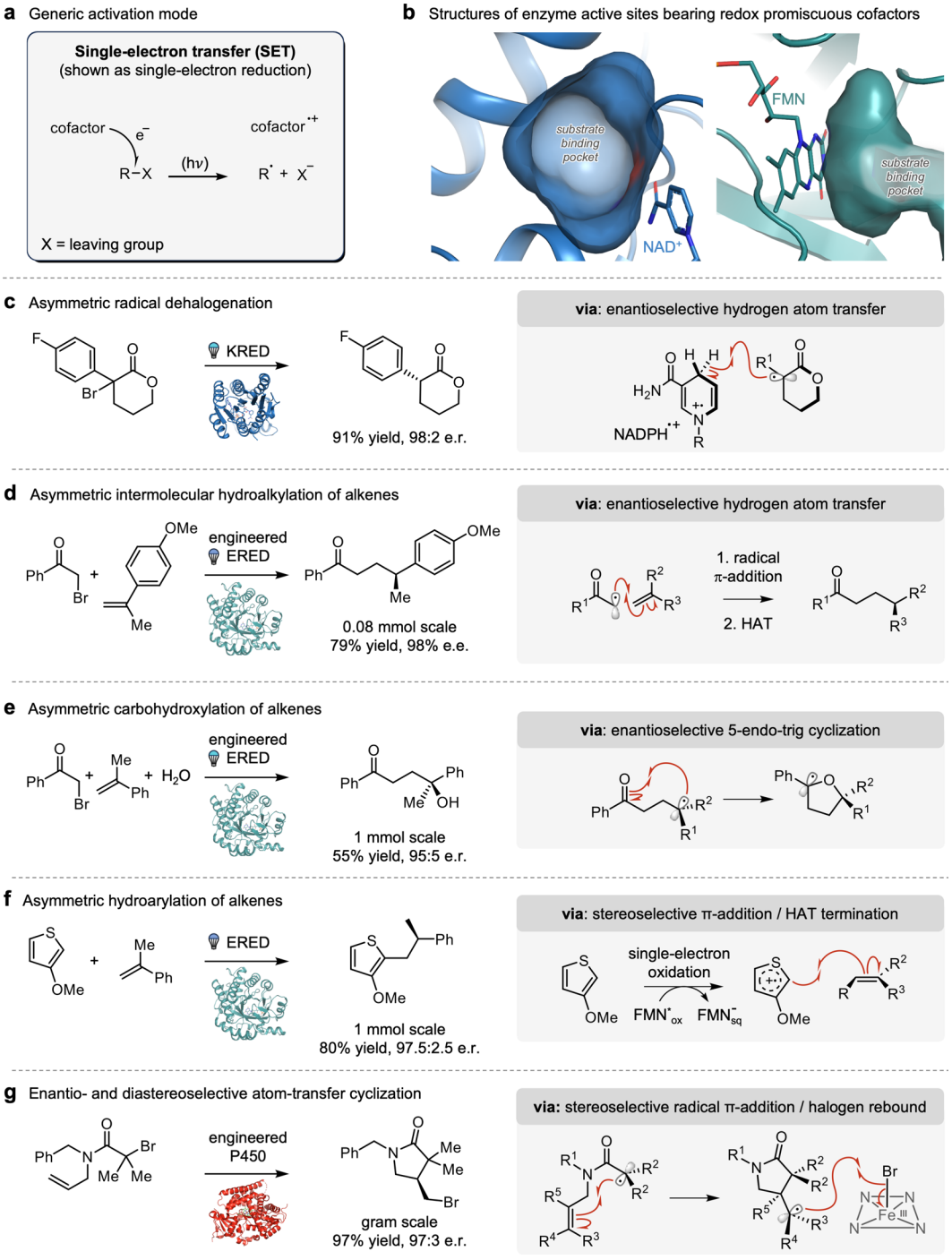


**Figure 3.** Enzymatic SET initiation. Scheme showing a general reductive SET that is often exploited in biocatalysis. Initiation can occur in the ground state or be triggered by light excitation, usually by forming a charge-transfer complex (**a**). Examples of enzyme active sites of a KRED (left, pdb: 4BMS) and an ERED (right, pdb: 6O08) bearing common redox promiscuous cofactors next to the substrate binding pocket (**b**). Highlighted radical reactions catalyzed by enzymes after SET initiation events. Different catalytic radical transformations are shown to illustrate the high control exerted by enzymes including asymmetric radical dehalogenation (**c**),[65] intermolecular hydroalkylation (**d**),[67,74] carbohydroxylation (**e**),[80] and hydroarylation (**f**)[70] of alkenes as well as stereoselective atom-transfer cyclization (**g**).[68] Except for one case (**c**), the yields correspond to isolated yields.

the selective monocyclization of various polyenes with high yield, enantioselectivity (>99% e.e.) and on gram scale (Figure 2d).[36,37] The generated products are relevant, among other things, as flavor ingredients or fragrances.

Next to controlling substrate conformations, SHCs have been engineered to control the stereochemistry of carbocation rearrangements, including Wagner-Meerwein shifts. In an outstanding example, these biocatalysts have been optimized for the acidic isomerization of pinene.[38] Pinenes are renewable building blocks and acidic pinene isomerization can generate >15 important monoterpenes.[39] However, developing catalytic selective isomerization for this transformation proves exceedingly challenging, particularly in generating the thermodynamically less favored products. Controlling acidic isomerization of


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pinenes depends on controlling the chemistry of several aliphatic carbocation intermediates that are similar in structure and energy and can react through different competing reaction pathways. Directed evolution optimized a promiscuous SHC for selective Brønsted acid-catalyzed isomerization of (+)-(β)-pinene to (+)-borneol (Figure 2e).[38] This reaction generated (+)-borneol with >90% selectivity, on gram-scale and sets the stage for catalyst-controlled, acidic isomerization to upgrade broadly abundant terpenes.

In addition, SHCs have been developed for various reactions that are not further discussed here, which includes highly stereoselective polyene cyclization reactions and semipinacol rearrangement reactions.[40,41] Beyond SHCs, other enzyme classes offer Brønsted acids with remarkable acidity in their active sites. This includes limonene epoxide hydrolases (LEH), which catalyze enantioselective opening of epoxides with water.[26,27] LEHs have been engineered for desymmetrization of oxetanes with different *N*-centered nucleophiles to access chiral pyrrolidines with high yield and enantioselectivity (98% e.e., Figure 2f).[42] Desymmetrization of such oxetanes is not unique to engineered enzymes and has been achieved with small molecule catalysts such as chiral Lewis and Brønsted acids.[43] Nevertheless, this example highlights that biocatalytic, asymmetric Brønsted acid catalysis is not limited to engineered SHCs.

Recently, fatty acid hydratases (FAHs) attracted attention, as they use Brønsted acid catalysis to activate aliphatic olefins for hydration of unsaturated fatty acids.[28,44] While the exact Brønsted acidic site is under debate, these enzymes have recently been engineered for asymmetric hydration of aliphatic and aryl alkenes.[45,46] What stands out is that these engineered enzymes enable highly enantioselective synthesis of chiral alcohols (>99% e.e.) from simple alkenes and water in an atom economic process (Figure 2g). Apart from these engineered FAHs, there are no documented reports of catalytic asymmetric hydration reactions of unactivated alkenes.[47,48]

These examples illustrate that asymmetric Brønsted acid activation in engineered enzymes is an enabling approach to develop unique, useful, and sought-after catalytic processes. In our opinion, enzymatic Brønsted acid catalysis shines particularly when precise preorganization of flexible substrates and conformational control of reactive intermediates enable transformations that are difficult to achieve with chemocatalysts.

## RADICAL CHEMISTRY THROUGH SINGLE-ELECTRON TRANSFER (SET) INITIATION

Radical reactions have a broad chemical space, often enabling transformations that are complementary to polar reactions and otherwise highly challenging.[49,50] The ability of radicals to tolerate reactive functionalities and to be generally unaffected by steric effects facilitates orthogonal transformations under mild conditions.[49−51] Due to this unique combination of innate properties, the utilization of radicals has played an essential role in synthesis of complex molecules, and is now becoming an essential piece of retrosynthetic logic.[52] The field of radical chemistry is continuously growing, spurred by numerous strategies available for the mild generation of radical species through single-electron transfer (SET) using organic photoredox and transition-metal catalysis.[51,53,54] However, despite significant recent advances, catalytic methods for selective transformations utilizing radical intermediates remain underdeveloped.[50,55−57]

Interestingly, nature constantly tames radical species with unparalleled precision in biosynthetic pathways of complex natural products.[58−60] In these cases, radical formation is frequently initiated by a selective hydrogen atom transfer (HAT) from the substrate involving enzymes from different major families, including iron-dependent oxygenases and radical *S*-adenosylmethionine (SAM)-dependent transferases. The activation of substrates through HAT has the potential to shortcut synthesis as it allows direct functionalization of C−H bonds without preactivation.[61] While radical biocatalysis through HAT by iron-dependent oxygenases is partly practical,[62,63] the application of radical SAM-dependent transferases is currently very limited.[64] In addition to HAT activation, radical formation via single-electron transfer (SET) has recently driven an emerging field of radical biocatalysis (Figure 3a). SET induces radical formation, for example through mesolytic cleavage of C−X bonds (X = halides, esters, and other leaving groups) and can be achieved by "redox promiscuity" of natural cofactors like flavins, nicotinamide adenine dinucleotide, and metal ions. Harnessed enzymatic SET mechanisms include single-electron reduction of substrates from (photoexcited) flavine hydroquinone ($FMN_{hq}$) and reduced nicotinamide adenine dinucleotide (NADPH) as well as from $Fe^{II}$ ground-state (Figure 3b).[65−69] Alternatively, cofactors can also initiate radical formation by single-electron oxidation of substrates, for example by a photoexcited flavine quinone, which is in analogy to the mechanism of natural fatty acid photodecarboxylases.[70,71] Based on the inherent redox promiscuity of natural cofactors, widely used enzymes such as flavin-dependent "ene" reductases (EREDs), which typically perform two-electron hydride transfer processes, have lately been exploited for SET-induced radical chemistry.[72] This yielded biocatalysts that catalyze unique chemical transformations and address long-standing selectivity challenges in radical chemistry.

The potential of using enzymes to overcome challenges that have largely eluded efficient and selective catalysis is highlighted by biocatalytic enantioselective HAT to terminate radical reactions. This was initially demonstrated with the enantioselective radical dehalogenation of racemic α-bromolactones.[65] In this context, ketoreductases (KREDs) were used, which upon photoexcitation of a charge-transfer complex, triggered a SET to induce reductive cleavage of the C−Br bond (Figure 3c). The prochiral radical intermediate is then converted to generate chiral lactones through a stereocontrolled HAT (96% e.e.). In follow-up studies, engineered EREDs were also successfully applied in radical dehalogenation to synthesize α-substituted esters with high enantioselectivities.[73] Please note that in the latter case, the SET-induced radical formation proceeds from ground state $FMN_{hq}$ (without photoactivation).

The combination of SET-induced radical formation and enantioselective HAT was key to the development of a unique set of enzymatic intermolecular radical C−C bond-forming reactions.[72] In these examples, SET is typically used for reductive cleavage of C−X bonds, followed by the addition of the radical intermediate to an alkene. The radical reaction is then terminated by an enantioselective HAT. In early studies, this strategy has been used for intermolecular radical alkene hydroalkylation.[67,74] This includes the elusive control of the remote γ-stereocenter (Figure 3d) through the stereoselective HAT termination (98% e.e.), potentially from an active site tyrosine. Various photoenzymatic methods for radical alkene


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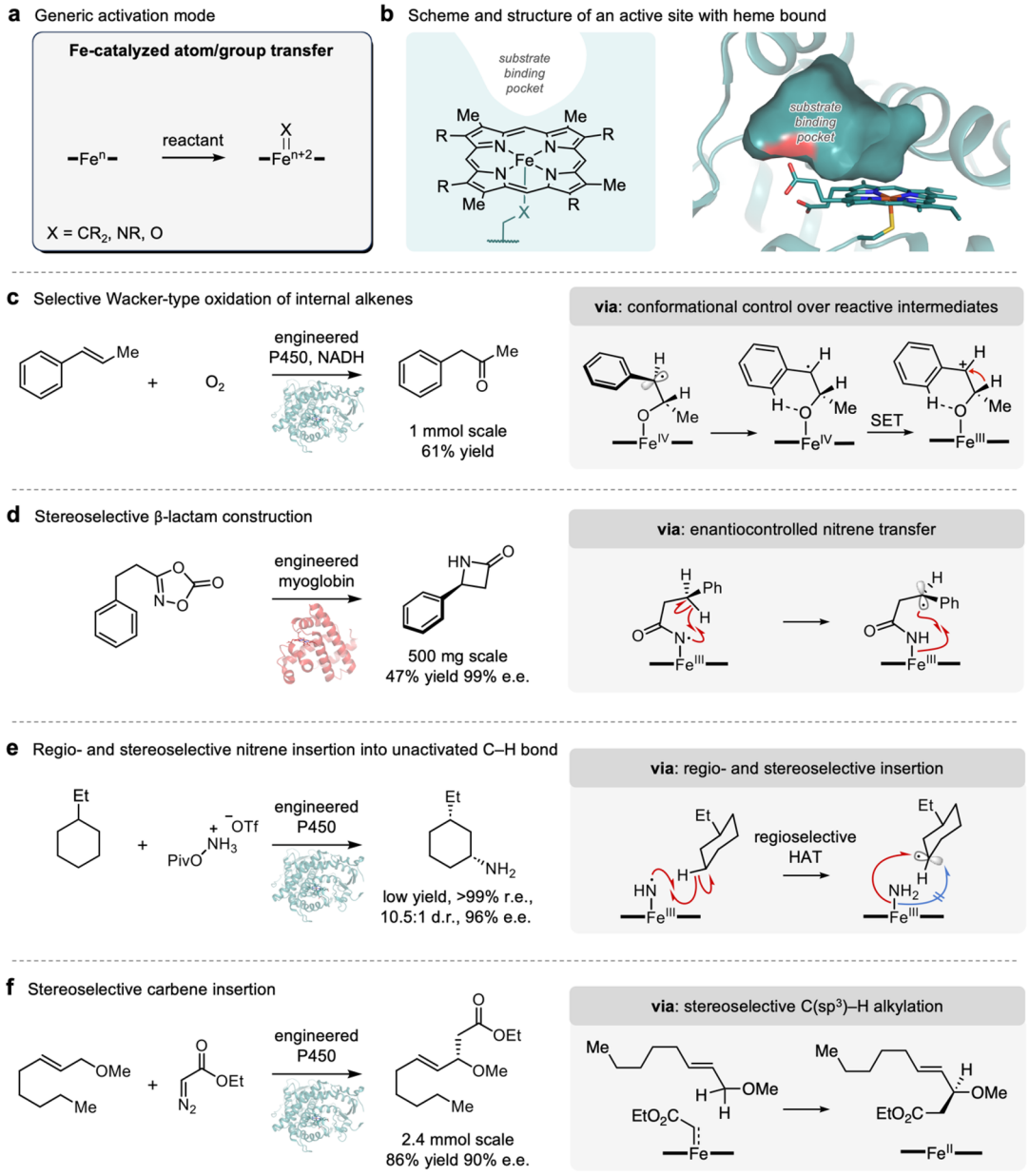


**Figure 4.** Fe-catalyzed atom/group transfer. Illustrated in the figure is a general scheme depicting activation via iron species (**a**), alongside a scheme and a structure of a heme cofactor in the active site of cytochrome P450 (pdb: 2IJ2). Challenging transformations that have been achieved in engineered enzymes include the selective Wacker-type alkene oxidation of internal alkenes in an NAD(P)H-dependent reaction (**c**),[103] stereoselective $\beta$-lactam construction (**d**),[104] selective nitrene insertion into unactivated C−H bonds (**e**),[110] as well as stereoselective carbene insertion into C−H bonds (**f**).[115] Please note that accessing many of the intermediates solely through an iron porphyrin system is not feasible.

hydrofunctionalization yielding highly stereoselective intermolecular C−C bond formations have been reported in follow-up studies.[72,75−78] The same approach has been used for carbamate activation to generate *N*-centered radicals (instead of carbon radicals) and catalyze radical alkene hydroamination with high enantioselectivities.[79]

EREDs have also been engineered to facilitate the asymmetric synthesis of tertiary alcohols via radical carbohydroxylation of alkenes (Figure 3e).[80] This multicomponent reaction involves an $\alpha$-haloketone and an olefin, for which catalytic asymmetric solutions remain inaccessible. The evolved enzyme shares the same initiation step with the enzymatic alkene hydroalkylation reactions mentioned above. The single-electron reduction of the substrate generates a radical that adds to an olefin. What is striking is that the engineered enzyme prefers C−O bond formation over HAT termination. Mechanistic studies suggested that the enantio-determining step is an intramolecular 5-endo-trig cyclization with the keto carbonyl to generate an $\alpha$-oxy radical. The final chiral alcohol is obtained after oxidation of the $\alpha$-oxy radical via a SET to the flavin semiquinone ($FMN_{sq}$), followed by hydrolysis. Overall, the reaction results in a redox-neutral process that requires only catalytic amounts of reductant. Note that this transformation is performed with photoexcited $FMN_{hq}$, while ground state $FMN_{hq}$ can initiate the SET as well, although with reduced activity.

Next to single-electron reductions, oxidative single-electron processes have also been used in engineered enzymes to activate substrates and initiate radical chemistry. This is demonstrated by applying EREDs in the asymmetric radical


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hydroarylation of olefins with electron-rich arenes (Figure 3f).[70] In contrast to the reports mentioned above, the photoexcited flavine quinone acts as a single-electron oxidant for electron-rich arenes, leading to the formation of an aryl cation intermediate. This event subsequently triggers a radical-mediated $C(sp^2)-C(sp^3)$ bond formation. It has been proposed that an anionic $FMN_{sq}$ species generates the stereocenter through a protonation and enantioselective HAT reaction sequence in an overall redox-neutral manner. Moreover, the identified ERED enzymes served as an enantiodivergent platform for this challenging radical hydroalkylation reaction, addressing a recognized stereoselectivity problem.

Next to flavins and nicotinamide adenine dinucleotide, ferrous iron has also been used as a cofactor in engineered enzymes to initiate radical generation through single-electron reduction.[68,69,81] For example, P450s were developed to catalyze stereodivergent atom-transfer radical cyclization (ATRC), producing chiral bromolactams from $\alpha$-bromoamides bearing an alkene group (Figure 3g).[68] The reaction starts from the ferrous ($Fe^{II}$) state and a reductive SET to the $\alpha$-bromoamides to generate a transient radical intermediate and ferric ($Fe^{III}$) bromide. Intramolecular addition of the transient radical to the alkene moiety generates a product radical, which reacts with the ferric ($Fe^{III}$) bromide to produce the lactam product and regenerates the metalloenzyme catalyst. One difference between radical termination using halogen rebound instead of HAT is that the halogen remains in the molecule, allowing further functionalization. While enantio- and diastereocontrol in such ATRCs is a long-standing challenge in the field, the engineered enzymes control both C−C and C−Br bond formation with very high stereoselectivity. Enantio- and diastereodivergent enzymes have been reported and the reactions have been carried out on a gram scale.

Organic reactions that proceed via free-radical intermediates are difficult to control with chemocatalysts. Notably, cofactor-induced SET has become an excellent choice to initiate radical formation in enzymes under very mild conditions. The examples described above highlight that engineered enzymes provide access to unique and potentially very useful radical reactions.

## FE-CATALYZED ATOM/GROUP TRANSFER REACTIONS

Transition metals provide a simple platform for transferring a myriad of groups through specific substrate activation processes. A common reaction mode catalyzed by transition metals is atom/group transfer.[82,83] Transition metals such as iron have a wide range of oxidation states which enable the formation of reactive (high-valent) species such as metal-carbenes, metal-nitrenes, or metal-oxo compounds (Figure 4a). These intermediates are highly reactive and participate in many different types of reactions, including insertion into C=C and C−H bonds, enabling a diverse range of functionalizations.[84−86] Using metal-carbenes as an example, a general (simplified) catalytic cycle begins with the formation of the intermediate via transfer of electrons and removal of a leaving group (e.g., $N_2$ in the case of a diazo compound as a carbene precursor). The metal-carbene can then, for example, undergo HAT from a substrate followed by radical rebound or directly insert into a specific bond.[87] Similarly, (high valent) metal-nitrene and metal-oxo intermediates can be generated and transferred.[83,88]

Since seminal work published in 2013,[89] atom/group transfer reactions (beyond natural oxo-transfer) are also investigated in engineered enzymes. Typical enzyme classes include iron-dependent proteins, such as heme-dependent globins and cytochrome P450 monooxygenases (P450s, see Figure 4b), as well as $Fe^{II}/\alpha$-ketoglutarate-dependent hydroxylases.[90] In many intriguing studies, a plethora of *new-to-nature* enzyme functions have been developed, which refers to engineered enzymes catalyzing reactions that are known from chemocatalysis but new to biology.[91−94] These examples illustrate that efficient and selective enzymes can be engineered[95] and even designed[96] to utilize activation modes not typically found in nature.[97] In the context of this review, we highlight examples where engineered enzymes enabled unique reactivities that were previously out of range in chemocatalysis.

Oxo-transfer with high-valent metal-oxo species is well established in catalysis. Yet until recently, this reactive species could not be fully exploited for direct Wacker-type alkene oxidation.[98] High-valent metal-oxo species typically epoxidize alkenes, since this reaction pathway is strongly preferred.[99,100] Interestingly, P450s have lately been engineered to override the intrinsic epoxidation reaction and catalyze Wacker-type alkene oxidation with high activity and selectivity (Figure 4c).[101−103] This was achieved by controlling the accessible conformations of a covalent radical intermediate that is generated after radical attack of the high-valent Fe-oxo species to the alkene. Conformational control disfavors epoxidation and facilitates the formation of a transient carbocation intermediate that generates the carbonyl product through a fast 1,2-hydride migration. So far, enzymes have been generated that directly oxidize styrenes to phenylacetaldehydes and internal aryl alkenes to the corresponding ketones (Figure 4c). Remarkably, the evolved enzymes exhibit catalytic efficiencies of average natural enzymes and control stereoselectivity during 1,2-hydride migration, thus, providing the first instances of enantioselective Wacker-type alkene oxidations (not shown in Figure 4c).[101−103]

Apart from oxo-transfer reactions, engineering of a myoglobin yielded biocatalysts for the stereoselective construction of $\beta$-, $\gamma$- and $\delta$-lactam rings via enzymatic C−H amidation reactions (Figure 4d).[104] The reactions proceed through metal-nitrenoid intermediates generated from stable dioxazolone nitrene precursors, which are synthesized from simple carboxylic acids. Nitrene transfer has been applied in intramolecular C−H functionalizations to synthesize oxazolidinones, sulfamates, or pyrrolidines. Accessing the important class of lactams via this approach possesses a unique challenge, as the corresponding acyl nitrene intermediates readily decompose and form isocyanates via a Curtius-type rearrangement. Iridium and ruthenium catalysts have been developed to favor lactam formation over Curtius rearrangement reactivity and control enantioselectivity.[105−107] However, developing a catalytic process that controls regio- and enantioselectivity in C−H amidation to yield lactams of varying ring sizes remains a puzzling task. The recently reported Fe-catalyzed reactions by engineered myoglobins are exciting as they promoted predictable, stereoselective synthesis (99% e.e.) of lactams of different sizes, including gram-scale examples.[104]

In various studies, enzymatic nitrene transfer has also been expanded toward primary amination of benzylic, allylic, propargylic, and even unactivated C−H bonds (Figure 4e).[108−111] These reactions are catalyzed with engineered


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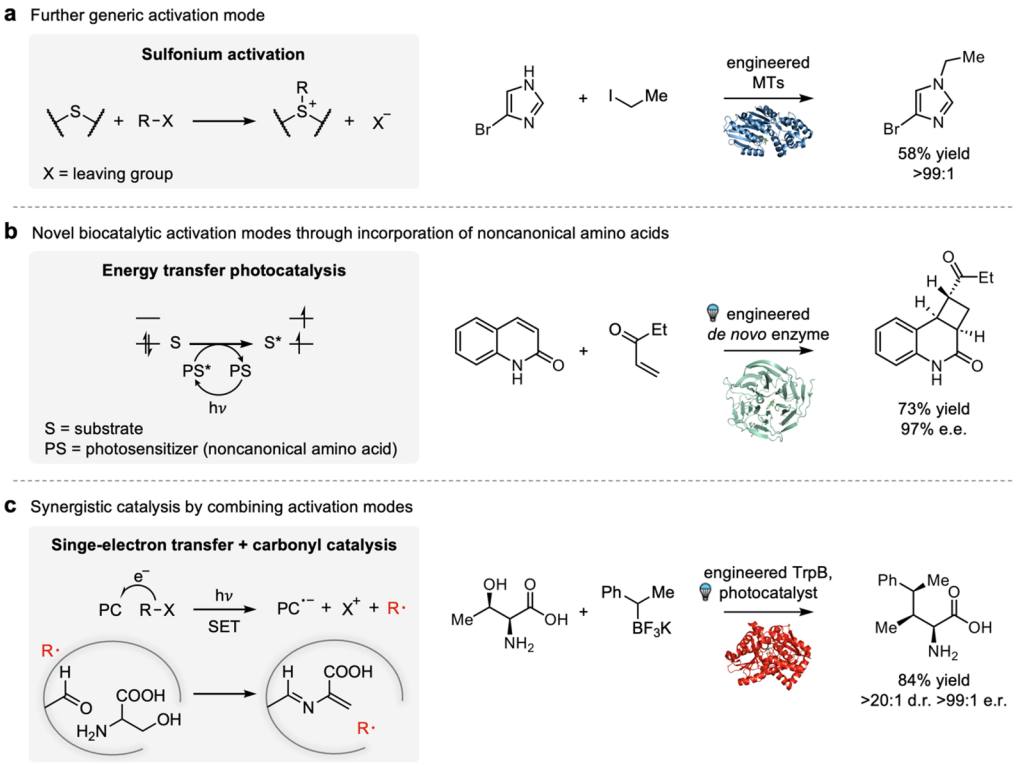


**Figure 5.** Examples of emerging activation modes in enzymes (**a** and **b**)[131,137,138] and synergistic catalysis through independent activation of two substrates in a chemo-biocatalytic process (**c**).[144]

P450s and most likely proceed through an unprotected iron nitrenoid intermediate. The selective primary amination of unactivated C−H bonds (as shown in Figure 4e) is outstanding due to the challenge of differentiating nearly identical C−H bonds in the substrate.[110] Although this method is currently not universal and produces in part very low yields, such transformations are exceptional and potentially very useful. Lately, this biocatalytic approach has been expanded to aminate tertiary C($sp^3$)−H bonds with high efficiency (up to 2300 TTNs) and selectivity (up to >99% e.e.) in an enantioconvergent manner.[111] Despite substantial efforts,[112−114] a chemocatalytic approach for direct primary amination of C($sp^3$)−H bonds remains elusive, further highlighting the potential of enzymes to accelerate desirable reactions and complement small-molecule catalysis.

Engineered P450s for biocatalytic carbene transfer chemistry are described, among others, for alkene cyclopropanation and C−H alkylation. An outstanding example is the stereoselective insertion of a metal carbenoid into benzylic, allylic, and propargylic C−H bonds (Figure 4f).[115] The enzyme facilitated specific binding of flexible linear alkyl substrates, enabling the carbene transfer with high stereoselectivity. Prior to this work, iron-catalyzed enantioselective C−H insertion was recognized as a formidable challenge.[115]

These examples exemplify the unparalleled atom/group transfer chemistry achievable through engineered heme-dependent enzymes. In many cases, novel catalytic selective reactions are achieved through controlling competing reaction pathways (Figure 4c and 4d) or accessing highly reactive species such as transient carbocations (Figure 4c) or unprotected iron nitrenoid intermediates (Figure 4d). This illustrates that enzymes provide numerous, tunable secondary interactions to modify catalytic reactions and precisely control the entire environment in which a reaction takes place.

## FUTURE PERSPECTIVES

In addition to the general activation modes discussed above, natural enzymes provide a variety of reactive centers whose chemical reactivity is currently being explored in the laboratory. This includes imine/enamine catalysis, which allows activation of ($\alpha,\beta$-unsaturated) carbonyl groups to either yield efficient electrophilic[116,117] or nucleophilic[118,119] intermediates. Similarly, amine groups can be activated by pyridoxal 5-phosphate (PLP)-dependent enzymes.[120−123] Further, radical processes through either selective HAT using high-valent iron oxo species, or cobalamin activation of C−Cl bonds have recently attracted attention.[124,125] Hydrogen bonding and Lewis acid activation are additional areas being investigated,[22,126] and there are many more reactive sites in enzymes just waiting to be explored. Much of this research had a strong chemomimetic[13] focus, producing enzymes that mimic chemical catalysts. Given the generality of these activation modes, we expect that many new and unique transformations will be enabled in engineered enzymes soon.

An activation mode that sparked our interest (and that of others) is the generation of sulfonium ions (Figure 5a). Sulfonium ions are represented in nature by the *S*-adenosyl methionine (SAM) cosubstrate. Simple access to a broad panel of different sulfonium ion cosubstrates from "off the shelf" haloalkanes will ease the exploration of these species' reactivity within engineered enzymes.[127−129] Currently, sulfonium ions are mainly utilized as electrophiles in biocatalytic reactions.[130] One potential application is the regioselective alkylation (>99% r.r.) of azoles with simple haloalkanes (Figure 5a),[129,131] a transformation that is on the wish list of many


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chemists.[132] Beyond serving as an electrophile, α-CH deprotonation of alkyl sulfonium ions produces sulfur ylides, oxidative addition of sulfonium salts to low oxidation state metals occurs at one of their S−R bonds, and single-electron reduction can lead to the formation of radical intermediates.[133] It will be interesting to see how these species and processes can be controlled and utilized in engineered enzymes.

Beyond natural activation modes, artificial cofactors and noncanonical amino acids are introduced into proteins to expand the accessible chemistry.[134−136] These strategies have been explored for introducing several key activation modes into enzymes. For example, through genetic code expansion, a noncanonical amino acid photosensitizer (PS) was incorporated in enzyme active sites[137,138] to activate substrates through triplet energy transfer photocatalysis.[139] Engineering such photoenzymes enabled intra- and intermolecular enantioselective [2 + 2] cycloadditions (Figure 5b) with high selectivity (97% e.e.).[137,138] Surprisingly, and in contrast to many small-molecule photocatalysts, the engineered enzymes can operate under aerobic conditions. It is very likely that triplet energy transfer photocatalysis can be exploited in engineered enzymes to enable very challenging and unique transformations.

Ultimately, catalysis with engineered enzymes is not limited to a single activation mode. Enzymes have been engineered to perform synergistic catalysis with two activation modes, for example by activating two different molecules in one active site. A recent example combines iminium with Lewis acid activation for asymmetric Michael addition within one enzyme.[140] In another study, a single active site proline has been utilized for both, enamine and iminium activation, to form various C−C bonds by one multifunctional enzyme.[141] In addition, synergistic catalysis by two activation modes can also be achieved in combined chemo-biocatalytic processes,[142] in particular through synergistic bio- and photocatalysis.[143−145] An exemplary instance involves the production of amino acids possessing several stereocenters (Figure 5c). In this case, threonine was activated through carbonyl catalysis using a PLP-dependent enzyme, coupled with chemocatalytic photoredox activation through SET-induced radical formation. Without any doubt, combining the reactivities of more than one activation mode with the selectivities of engineered enzymes is an exciting future area of research.

## CONCLUSIONS

The field of biocatalysis is experiencing a thriving and exciting development. The examples highlighted here clearly illustrate how the field is facing a reshaping phase. They demonstrate that enzymes are no longer seen under the historical paradigm as selective catalysts that use specific mechanisms over a small set of substrates. Instead, enzymes are catalytic platforms that can host general activation mechanisms to produce reactive species with predictable reactivity. This ability to identify and describe different general activation modes allows chemists to imagine and design new enzymatic platforms for previously unthinkable transformations. Therefore, it is reasonable to expect that biocatalysis can experience meaningful growth like the fields of organo- and photoredox catalysis through harnessing generic activation modes.

Nowadays, it is common to find enzymatic reactions that emulate transformations reported by organocatalysts or other chemocatalysts. However, enzymes have the potential to expand the known chemical space and lead chemists to unexplored transformations. Unlike small-molecule catalysts, engineered enzymes can exert a unique precise control over single steps in a catalytic cycle, including conformational control over substrates and reactive intermediates like carbocations and radicals. This is well exemplified by enantioselective hydrogen atom transfer (HAT) or asymmetric hydration of olefins. Therefore, exploring the reactivity of generic activation mechanisms in engineered enzymes comprises a priceless opportunity for chemists. It will be exciting to see what kind of transformations are enabled by engineered enzymes in the coming years. The discovery of new reactions might lead to new retrosynthetic disconnections and hopefully simplify the synthesis of important molecules.

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‡S.J. and F.O. contributed equally to this work and are listed in alphabetic order. All authors contributed to the conception and writing of this manuscript. CRediT: **Shubhanshu Jain** conceptualization, visualization, writing-original draft, writing-review & editing; **Felipe Ospina** conceptualization, visualization, writing-original draft, writing-review & editing; **Stephan Christian Hammer** conceptualization, funding acquisition, project administration, supervision, validation, visualization, writing-original draft, writing-review & editing.

### Notes

The authors declare no competing financial interest.

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