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Chemical modification in the creation of novel biocatalysts

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Enzymes are able to perform a multitude of chemical and biochemical transformations with efficiencies that are typically unrivalled by chemical catalysts. However, these evolved systems may lack breadth or utility in other non-natural applications. Altering enzyme and protein scaffolds through covalent modification can expand the usefulness of native biocatalysts not only for synthetic application but also for therapeutic use. This review summarizes recent developments in the field of chemical modification of enzymes and how they can be applied to synthesis and biological research.

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Introduction

The design of novel biocatalysts is of major importance for biotechnological and pharmaceutical applications such as the investigation of more effective or new catalysts, the detection of biological analytes or the study of complex biomolecular interactions. Covalent alteration of these biomolecules can modulate their native functions and properties [1]. For instance, changes at some positions of an enzyme may alter substrate specificities, product distribution, stereoselectivity or even switch the enzyme's fundamental catalytic action.

The introduction of complex chemical reporters or modulators into a protein solely through expression methods is a complicated task. The main difficulties of such approaches are that the direct incorporation depends on the tolerance for the non-natural amino acid substrate and the variable efficiency of incorporation. For this reason, innovative combination of orthogonal reaction of amino acid sidechains and molecular biological techniques are gaining ground in the field of protein modification. Moreover, efforts in protein engineering and microbiology have been combined to both probe and make use of enzymes in living systems through the alteration of specific residues.

Herein, we provide a select overview of new chemical techniques to create more stable, more useful and more potent biocatalysts. First, we focus on nonspecific protein bioconjugation reactions. Then, we move to the development of novel selective bioorthogonal transformations on enzymes including methods which combine the incorporation of unnatural amino acid or artificial groups.

This review is intended as an update to our previous review on the chemical modification of biocatalysts [2] and will cover the period from late 2003 onwards.

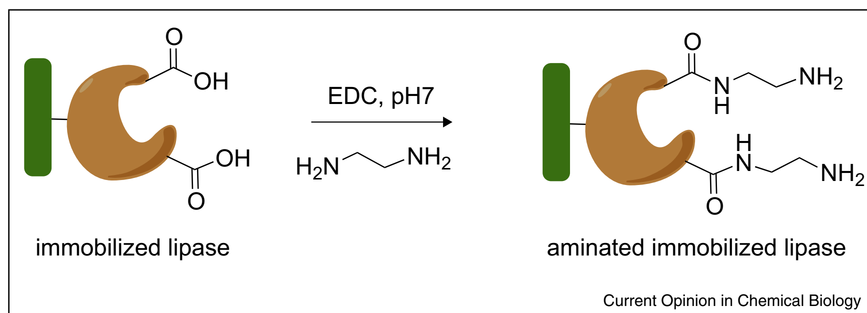
Nonspecific chemical modification

The exploitation of biocatalysts in synthetic organic chemistry, both in academia [3] and in industry [4], has increased dramatically during the past 20 years. Although some enzymes can be used in organic solvents, the main drawback of using biocatalysts is that enzymes may have evolved to work with a narrow substrate class in aqueous media. Therefore, many of these nonspecific approaches to enzyme modification are focused on understanding the effects of modifications on protein stability as well as on designing novel strategies to enhance selectivity, activity, and lifetime [5].

The modification of carboxylate groups in Asp and Glu residues in several immobilized lipases by chemical amidation using ethylenediamine has been described as one such method to modulate lipase activity (Scheme 1). This modification causes significant increase in enzyme activity in the case of *Candida antarctica* B (CAL-B) and *Thermomyces lanuginose* (TLL) lipases. However, the effect of this indiscriminate modification on the activity of immobilized *Pseudomonas fluorescens* (PFL) was the opposite. Surprisingly, enantioselectivity of modified CAL-B in the hydrolysis of (±)-2-hydroxyphenyl acetic acid methyl ester was increased from an $E = 2$ to $E > 100$ (ee > 99%) [6]. The unpredictable and difficult-to-explain nature of these results highlights that the undoubted utility of some nonspecific alterations can also on occasion be associated with little or no mechanistic insight.

The nonspecific modification of amino groups of papain with dicarboxylic anhydrides of citraconin, maleic, phthalic, and succinic acids shifted the optimum operating pH of this enzyme from 7 to a more alkaline pH of 9. This pH shift is due to the change in the surface charge distribution. This modification also resulted in a slight

Scheme 1



Chemical amidation of Asp and Glu residues [6].

decrease in activity [7]. Such changes of optimum pH may be useful for performing reactions only possible in alkaline media or in helping to solubilise particular substrates. This same strategy has been used to modify lysine residues of *Bacillus licheniformis* α-amylase (BLA). Such a modification causes fundamental changes in its specificity (increasing maltosidase activity). Furthermore, this chemical modification alters the pattern of cleavage of enzyme substrates to an *exo*-type instead of the native *endo*-type [8]. Again, both are striking results, the mechanistic cause of which remains unclear.

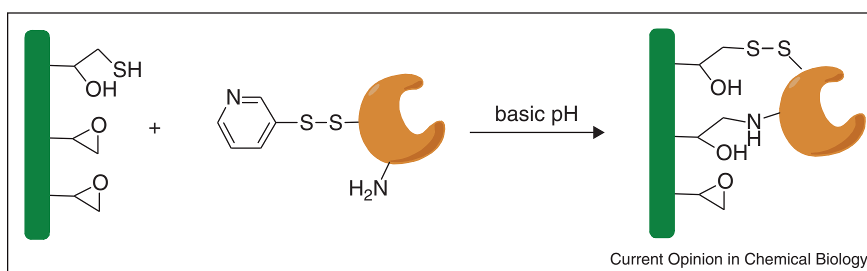
Another example of nonspecific modification has been reported by Villalonga and co-workers. Glycoconjugation of *Bacillus badius* phenylalanine dehydrogenase (PheDH) with different monoactivated cyclodextrins [containing seven α-1,4-linked D-glucopyranose units (β-CD)] was achieved using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as coupling agent. Conjugates increased in operating temperature by around 10°C and displayed enhanced affinity for L-phenylalanine by 1.4-fold [9]. However, the enzyme activity fell to 60% of wild type (WT).

An important covalent attachment often used in therapeutics is PEGylation and some recent stability studies have been focused on the application of these methods to biocatalysts. For instance, chymotrypsin was modified with poly(ethylene glycol) (PEG) creating conjugates in different molar ratios (from 0.4 to 96). The stability of these aggregates was investigated during encapsulation in poly(lactic-co-glycolic) microspheres by determining the specific enzyme activity. An increase in the number of PEG chains destabilized the system and residual enzyme activity in the hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide decreased. Thermal stability appears to be maximal in this system when an average of 6.3 molecules of PEG are bound to the enzyme [10].

Immobilization strategies

Covalent chemical immobilization of enzymes is a simple method to positively alter activity, specificity or stability. Grazú *et al.*, for instance, have developed a two-step method for the preparation of multipoint-thiolated immobilized Penicillin G acylase from *Escherichia coli* (PGA) and lipase from *Rhizomucor miehei* (RML) [11•]. The first step in this procedure is the chemical introduction of thiol group through treatment with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and subsequent reaction of the protein with previously thiolated Eupergit®C or EP-Sepabeads support (Scheme 2). Those supports consist of polymethacrylate-based resin functionalized with oxirane groups. These multipoint

Scheme 2



Two-step strategy for multipoint covalent attachment onto epoxy support [11•].

enzyme support attachments increase the enzyme thermal stability between 12-fold and 15-fold.

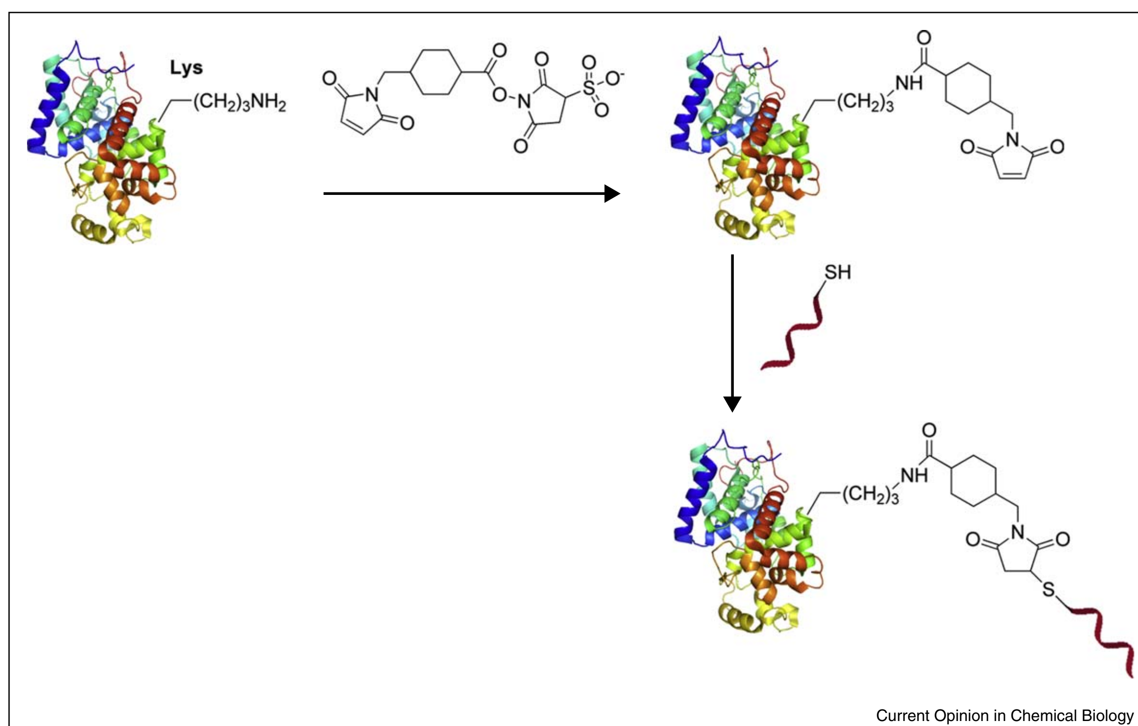
An alternative approach, which also addresses stability and activity, has been explored by Sheldon and co-workers. This relies on the preparation of cross-linked enzyme aggregates (CLEA) and expands related work on cross-linked crystals and precipitates developed over several years. The cross-linking reaction consists of the coupling of free amino groups present on the surface of the enzyme with a dialdehyde, commonly glutaraldehyde; such methods have also been used by several groups previously. For instance, chloroperoxidase (CPO)–CLEA [12] were synthesized by precipitation with 1,2-dimethoxyethane, albumin, and pentaethylenehexamine. CLEA showed higher temperature and pH stability compared to the free enzyme. Although the activity was lower (68%), these results proved to be important because, by using this methodology, the enzyme's tolerance to H_2O_2 was greatly increased. In a previous report, *Linum usitatissimum* hydroxynitrile lyases were immobilized as 'LuCLEA' [13] in a similar fashion and these aggregates showed high enantioselectivity in the addition of cyanide to the prochiral butanone substrate in microaqueous media: 87% ee was obtained, although activity was quite low. The possibility of recycling these supported-enzymes makes them potentially suitable for industrial applications.

Villalonga and co-workers [14] took advantage of the Ugi-four component reaction to prepare trypsin–polysaccharide neoglycoenzymes. Lysine amino groups located on the trypsin surface react in the presence of formaldehyde and *tert*-butyl isocyanide with carboxylate groups of *O*-carboxymethylcellulose (CMC) or sodium alginate (ALG). Catalytic properties of these polyglycoconjugated enzymes decreased to 61–69% compared to the native counterpart. Despite this decrease in activity, the conjugates showed a noticeable increase in thermal stability. Autolysis studies revealed that modified enzymes are around 16 times more stable. The authors hypothesized that this stabilization is caused by the formation of isopeptide structures.

Couplings mediated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) have been used to attach Fe_2O_3-chitosan nanoparticles to papain [15]. This conjugated system was analyzed by transmission electron microscopy and X-ray diffraction after conjugation and revealed the presence of spherical and monodisperse nanoparticles with a mean diameter of 9.6 nm. This superparamagnetic system exhibited better tolerance to reaction conditions (pH and temperature) and an enhanced enzyme activity.

Cross-linking strategies have been used to covalently tether DNA to enzymes for the fabrication of potential biosensors. For instance, horseradish peroxidase (HRP)–DNA conjugates were synthesized by chemical

Scheme 3



Synthesis of HRP–DNA conjugates by cross-linking [16].

cross-linking using thiolated oligonucleotides and bifunctional sulfo-succinimidyl-4-(*N*-maleimido-methyl)-cyclohexan-1-carboxylate (sSMCC, Scheme 3) [16]. This reaction was not specific and several conjugates were obtained with different ratios of DNA per enzyme since at least four lysine residues are relatively accessible at the HRP surface. The HRP–DNA conjugates were then immobilized on gold electrodes through DNA hybridization methodologies. As amperometric currents were low, a redox mediator such as ortho-phenylendiamine was necessary. However, the K_M of covalent conjugates was higher than that of reconstituted-noncovalent HRP conjugates, presumably due to changes in the relative orientation of the active site at the electrode surface. The authors demonstrated that a weak catalyst in oxidation reactions such as myoglobin can have its activity tuned by 30-fold to 280-fold simply by changing the attached DNA sequence, although they did not find a general trend for this behavior [17]. Other examples of electrochemical biosensors based on enzyme immobilization have been reported [18,19].

An elegant bioluminescence system that can be applied for molecular imaging *in vivo* has been reported by Rao and co-workers [20•]. EDC was also used to couple CdSe/ZnS quantum dots and a mutant of *R. reniformis* luciferase generating carboxylate-quantum dot conjugates. This bioconjugate can be imaged in cells, small animals and is even detectable in deep tissues. Such semiconductor quantum dot conjugates provide potential for enhanced bioluminescence imaging, therapeutics, and other applications. These techniques are based on energy transfer that takes place between a luminescent or fluorescent donor and a fluorescent acceptor. The convenience of these systems is their sensitivity and stability. Moreover, the ability to tune the optical properties of quantum dots can make them appealing for monitoring biological processes. Other examples in this field have been recently reported [21].

When considering such nonselective approaches, it is undeniable that these strategies have proven to be practically useful since simple groups or even complex biomolecules can be easily introduced for a variety of applications. However, limitations remain. Since in most cases there is not a well-defined structure after modification, reproducibility of these reactions can be questionable. Thus, these examples may not be as applicable to cases where the modification is intended to occur in a particular region of the enzyme. Moreover, when striking changes are observed their causes and origins may remain opaque.

Site-specific chemical modification

Targeting proteinogenic amino acids

To better control the fidelity of structure and function of modified biocatalysts, a selective approach may be employed. Consequently, specific bioconjugation reactions in enzymes have provided valuable information to elucidate their roles and modulate activities in biological processes.

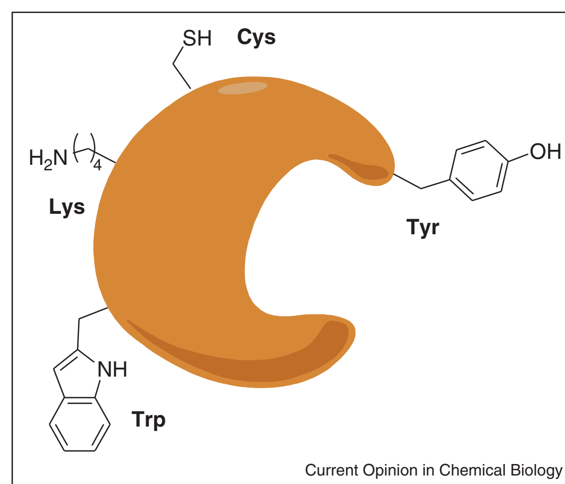
Lysine and cysteine have been broadly used for the modification of proteins since they can easily react with a variety of electrophiles. Of the canonical proteogenic amino acids, cysteine is the most versatile residue for selective modification due to its relatively low abundance and high nucleophilicity [22].

The key to these approaches is to selectively modify a single residue in the presence of a plethora of other functional groups within a protein. Therefore, an equally important factor is the rate (relative and absolute) of the chemical reaction to be used. Several interesting studies have examined methods for determining the kinetics of some chemoselective reactions of amino acids in proteins, especially with electrophiles [23–25].

As a result, new strategies have emerged not only for the modification of thiols and amines but also for tyrosine and tryptophan residues in enzymes, and other proteins, in a chemoselective fashion (Scheme 4). For a detailed discussion of chemoselective protein modifications we direct the reader to recent and interesting reviews [26•,27].

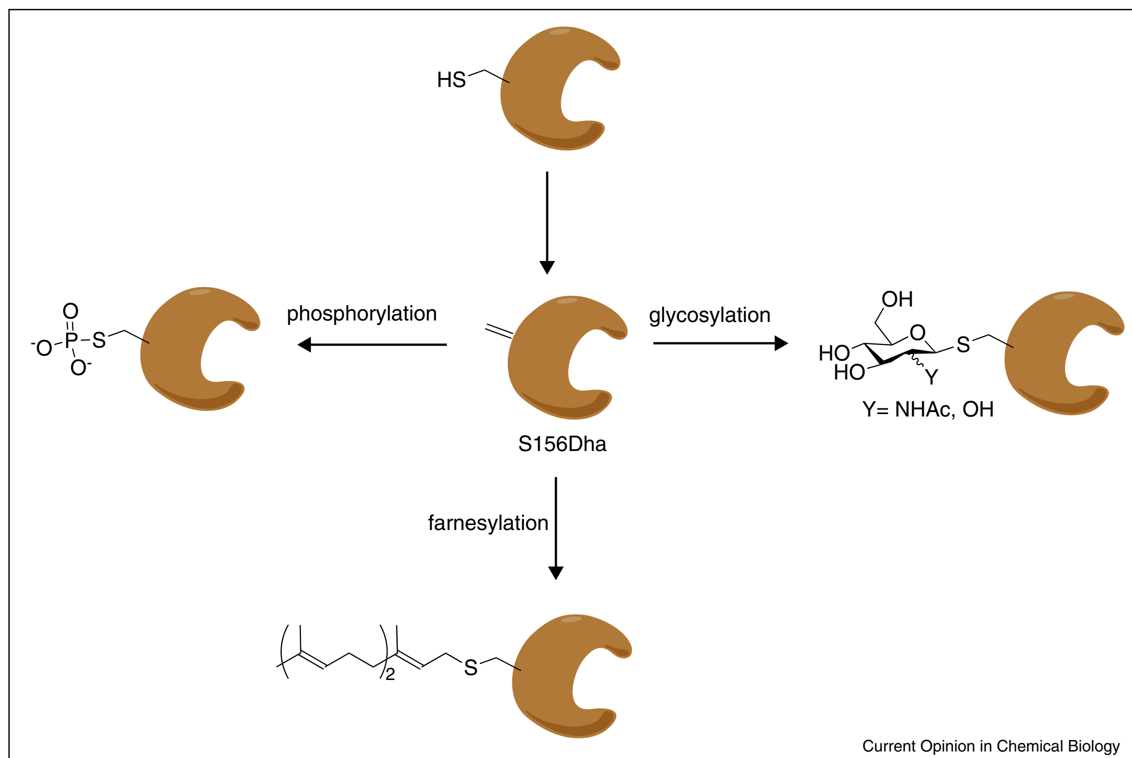
The serine protease mutant subtilisin *Bacillus lentus* (SBL) with a cysteine residue at position 156 has been modified through a two-step methodology. In a first step, the cysteine is transformed into dehydroalanine by using *O*-mesitylenesulfonylhydroxylamine (MSH) and then treatment of SBL156-Dha with different thiol nucleophiles yields a variety of thioether modified proteins.

Scheme 4



Common proteinogenic amino acid targets for site-selective enzyme modification.

Scheme 5



Modification of SBL by addition of thiol reagents to a Dha-protein intermediate [28].

Phosphorylated, farnesylated, and glycosylated proteases can all be obtained by using this methodology (Scheme 5) [28]. In all cases these reactions proved mild enough to maintain and even modulate the proteolytic activity of SBL. Moreover, use of excess MSH allowed removal of modifications and the subsequent reinstallation of other groups in an iterative process.

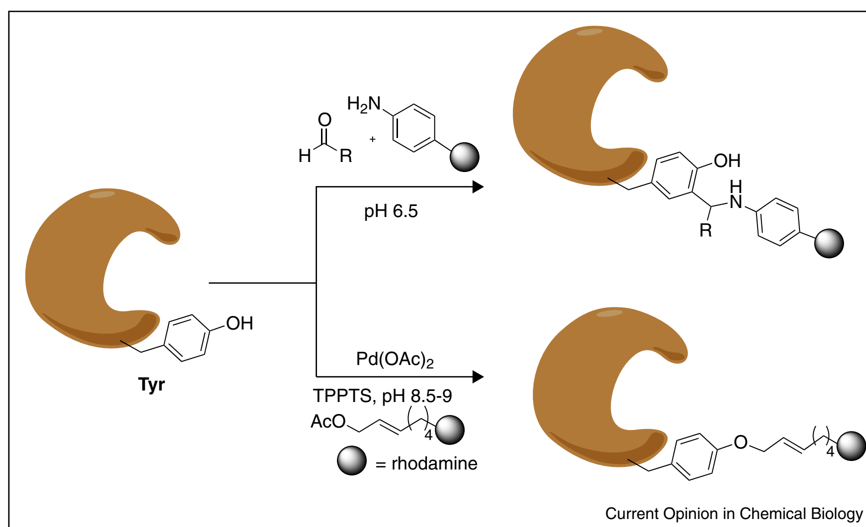
A selective reaction on a tyrosine residue has been used to introduce novel functionalities into several glutathione transferases such as fluoro-derivatives, aldehydes, and fluorescent probes. A wide range of ligands react through a site-directed acylation on a single tyrosine residue Y9 using a set of thioesters of glutathione (GS-thioesters). More than 70% of tested GS-thioesters were accepted [29]. While this method is applicable only to modify Y9 at the active site of this class of GSH transferases, it is one of only few reported examples of Tyr modification.

Another Tyr-selective modification has been reported by Francis and co-workers. A three-component Mannich reaction was used for the installation of several functional groups in tyrosine residues in chymotrypsinogen A and other enzymes bearing accessible tyrosine residues such as lysozyme and RNase A (Scheme 6). Yields were dependent on the nature of the coupling partners. No reaction was observed when a given protein substrate has less accessible tyrosine residues such as in horse heart myoglobin [30].

Recent studies have begun to explore the possibility of using transition metal complexes as promising reagents to carry out protein modification in aqueous media. Some of these methodologies have been applied to the introduction of new functionalities in biocatalysts. In this context, Francis and co-workers have reported that chymotrypsinogen can rapidly react via a Tyr residue with rhodamine allyl acetate to yield the monoalkylated product in 50% yield using a water-soluble phosphine ligand in the presence of $Pd(OAc)_2$ [31•]. The selectivity of this protein Tsuji–Trost reaction was determined via tryptic digestion. Allylation of Y171 was the major pathway and a minor second modification site was also observed at Y146. This study highlighted the great potential of palladium, especially if a method could be found to exclusively direct this modification to a single residue on more complex protein targets.

Since Trp residues are quite rare on protein surfaces, their single-site modification is plausible. Francis and co-workers have modified Trp residues to obtain Subtilisin Carlsberg and myoglobin conjugates by using rhodium

Scheme 6



Two strategies to modify tyrosine residues [30,31•].

carbenoids. However, the insertion reaction proceeds in acidic conditions which may be incompatible for many enzymes. Crude reaction mixtures at pH <4 revealed that the major product of this reaction comes from the O–H insertion of the carbenoid with H_2O, a competing side reaction that erodes reagent. However, this limitation can be resolved by adding *t*BuNHOH as additive instead of H_2NOH. With this additive, they were able to selectivity modify hen egg white lysozyme under thermal denaturing condition (>75°C) at pH 6. Additionally, for this modification to occur, tryptophan should be placed at the surface [32].

Unnatural amino acids come into play

An alternative methodology to biologically incorporate novel functionalities into proteins has been pioneered by Tirrell and co-workers [33]. Through this method cells are able to metabolize unnatural amino acids in depleted media by using aminoacyl-tRNA synthetase machinery. Following this methodology, surrogates for Met, Phe, and Trp can be replaced by analogues within an enzyme, allowing incorporation of amino acids containing, for example, alkenyl-groups, alkynyl-groups or azido groups that can then undergo further reactions.

In this way using a 'tag-and-modify' approach [34], it is then possible to chemically modify an enzyme containing such amino acids. Such a strategy has been used through the introduction of azidohomoalanine (Aha) as a methionine surrogate into a relevant industrial enzyme lipase. In this way, a *C. antarctica* lipase B variant was engineered in a methionine auxotrophic *E. coli* strain. Aha-containing Cal-B was treated with alkyne-functionalized ligands in order to obtain potential fluorescent conjugates; dansyl derivatives only reacted when the Aha residue was placed at the *N*-terminus [35]. After the entire modification procedure, some catalytic hydrolysis activity was lost. This was attributed to both the incorporation of the nonproteinogenic amino acid and the reaction conditions used ($CuSO_4$, ascorbate, and ligand). Such examples also highlight the need for reaction optimization.

Although an important number of examples of site-selective modification for enzymes have been described in the literature, most of them are limited to a single reaction/modification. Davis and co-workers have reported examples in which two chemical reporters are introduced into a beta-glycosidase from the *archaeon Sulfolobus solfataricus* (SSβG) in an efficient and compatible manner. This multi-site modification strategy combined the incorporation of Cys and unnatural amino acids such as Aha and homopropargylglycine (Hpg) as sites for subsequent modification. In a first step, they used methanethiosulfonates to target the Cys residue and then Huisgen cycloaddition was achieved at the noncanonical amino acid. This second reaction not only was compatible with the first one but also proceeded in good conversion (>95%). These modified SSβG enzymes were shown to be active, allowing the innate glycosidase activity to be exploited as chemical reporter for the tracking of the protein. Additionally, suitably modified enzymes were able to bind lectins, which led to the conclusion that a plausible application will be to track natural modification of these substrates *in vivo* during pathological diseases [36••]. Indeed, the authors tested this system in the detection of inflammation in Plasmodium-induced brain disease.

Budisa and co-workers have recently published the incorporation of different fluorinated amino acids into a lipase from *Thermoanaerobacter thermohydrosulfuricus* [37•]. The resulting so-called 'teflon proteins' could be an important step forward for designing novel catalysts with interesting properties.

De novo biocatalysts through chemical modification

Enzymes are capable of performing a wide range of chemical reactions yet high substrate specificity often limits synthetic applications. For these reasons chemists have also focused their attention on the construction of completely artificial enzymes, especially metalloenzymes. This has often involved searching for unusual functions not found in nature. Inspired by this idea, several groups have recently explored methods to covalently modify proteins by anchoring metal complexes for achieving enantioselective catalysis [38]. Among these, Ward and co-workers have reported the creation of an artificial metalloenzyme based on biotin–avidin technology to perform enantioselective hydrogenation of acetamidoacrylic acids. Although essentially based on a noncovalent modification, these systems nonetheless allow the alteration of catalytic properties by modifying the attached metal complex, the protein, or both. Thus, enantioselectivities of these reduction processes are increased up to 96% ee in a modified-variant of streptavidin mutant S112G [39••]. During these studies, it became apparent that the efficiency of this system depends highly on the affinity of the substrate for the active site. To overcome this problem, Reetz has proposed an approach that unifies this concept of synthetic achiral metal–ligand centers and the concept of directed evolution for tuning the enantioselectivity of the so-called 'hybrid catalyst system' [40]. In this last case, they also employed a biotinylated diphosphine–Rh complex but used the esterified substrate to facilitate work-up and subsequent analysis by gas chromatography. They applied CASTing [41] methodology where amino acid sites around the complete binding site are randomly mutated in an iterative manner. The best variant obtained after a single round of saturation mutagenesis shows only a 35% (*R*) ee. However, iterative processes led to a single mutant (Asn49Val) with an ee of 65% (*R*). In subsequent work, they applied the same selection process to generate a library of mutants of a thermophilic synthase tHisF from *Thermotoga maritima*. In this work, the mutant Cys9Ala/Asp11Cys was treated with maleimides or α-halo carbonyl derivatives to form bioconjugates for potential metal ligation. This protein appears to be a robust system [42•].

An intriguing example in this field has been reported by Lu and co-workers [43]. In this paper, a two-point covalent attachment approach has been performed to introduce a Mn(salen) complex into sperm whale myoglobin. This transformation involves the use of two methanethiosulfonate groups and a mutant with cysteine at two positions (L72Y/Y103C). Comparison of this dual modified myoglobin with WT or single modification proteins demonstrates that this metalloprotein shows a relevant increase in ee (51%) and rate (0.390 min^{-1}) in the sulfoxidation of thioanisole.

Conclusions

The classical view of biocatalysts is changing. Enzyme capacities are not fully explored by nature and engineering native enzyme or protein scaffolds to create new functions is opening a myriad of possibilities. Throughout this review we have highlighted a number of advantages that exist for modified biocatalysts that are limited or lacking in native systems: first, chemical alteration of enzymes can change affinity, specificity or stability. Second, selective modifications enable the tagging of enzymes which can allow insight into complex biological processes. Significant improvement has been made in the field of chemical modification/trapping strategies for proteome analysis. These methodologies have emerged to enable the profiling of enzyme activities *in vivo* and the chemistries used are often active site-directed variants of those chemistries used more generally to modify proteins and enzymes. In this way, activity-based protein profiling (ABPP) can help to unpick the role that these proteins are playing in pathological processes [44,45] and provides a rich example of conceptual cross-fertilization of strategies in this field. Finally, enzyme scaffolds can be tuned through expression techniques and by introducing non-natural groups to create enzymes that can act as valuable starting points for further modification. In this way it is starting to become possible to obtain more potent biocatalysts or to generate entirely novel protein catalysts.

It is clear that the combination of novel chemical modification and sophisticated biochemical technologies will continue to be in the spotlight. It is likely that additional orthogonal reactions will emerge to enhance selectivity and kinetics of transformations. For instance, metal-catalyzed reactions have proven to be feasible in water and will likely become a widespread tool for enzyme engineering. The introduction of more complicated chemical reporters to track small metabolites or recognition processes is another focus of research. Undoubtedly, future efforts on the design of modified biocatalysts should be channelled to create enzyme-based constructions with unique properties and functions not found in nature and/or chemistry not easily achieved by traditional methods. It should not be forgotten that these principle could be applied equally to other biocatalysts such as modified ribozymes.

This attractive field is also open to the arrival of specifically designed chemical modification to study the largely unknown *in situ* behavior of enzymes in a cellular environment.

Acknowledgements

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