ELSEVIER

Chemical modification of biocatalysts

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Although several powerful methods exist for the redesign of enzyme structure and function these are typically limited to the 20 most abundant proteinogenic amino acids. The use of chemical modification overcomes this limitation to allow virtually unlimited alteration of amino acid sidechain structures. If heterogeneous mixtures of enzyme products are to be avoided, however, the required chemistry should be efficient, selective and compatible with aqueous conditions. Recent advances have been made in the modification of proteinases, aminotransferases and redox enzymes.

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Abbreviations

CD	cyclodextrin
CRL	*Candida rugosa* lipase
EDCI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
GOx	glucose oxidase
GST	glutathione transferase
PT-PEO	phenothiazine-labelled polyethylene oxide
SBL	subtilisin *Bacillus lentus*

Introduction

The redesign of enzyme activity through the alteration of sidechains of functionally important amino acids is a tried and tested technique. Most 'protein engineering' modifications utilize mutagenesis to introduce a limited number of alterations. The choice of residue to be altered may be based on protein design, sequence similarity, structural modeling or determined through screening or selection-based forced evolution (random mutagenesis) approaches — or a combination of these techniques. However, these methods are typically limited to just the 20 primary proteinogenic amino acids. Ingenious molecular biological techniques have been developed for the introduction of non-natural amino acids into proteins [1,2]. The incorporation of non-coded residues is most successful for those that closely resemble their coded counterparts, and the use of more complex amino acids in such techniques can result in poor levels of incorporation.

Chemical modification of amino acid sidechains allows a greater, almost unlimited, variety of groups to be introduced, but the reactions used for their introduction are typically non-specific in nature. Thus, despite the many potential advantages, many classical methods used for protein modification create mixtures of proteins as a result of poorly discriminating or insufficiently efficient chemistry. This is also exacerbated by the limited variety and multiple copy numbers of chemical functional groups in proteins. Thus, there is still a strong requirement for novel strategies that exploit selective and efficient protein chemistry.

This review covers the period from 2001 until early 2003 and is intended as an update to the excellent reviews of Distefano [3,4] and Jones [5,6] on this topic, although several broader reviews have highlighted aspects of chemical modification during this period [7–11].

Non-specific chemical modification

Modification of proteases

Some surprising results, perhaps as a result of product heterogeneity, were obtained from the introduction of hydrophobic and hydrophilic groups into α-chymotrypsin. The groups were introduced using non-specific modification of lysine through reductive alkylation with aldehydes or reaction with anhydrides [12]. Unusually, both led to a broadening of the range of organic cosolvent concentration in water–organic mixtures at which enzyme activity was at least the same as that of the unmodified enzyme in water. Differential scanning calorimetry revealed a multicomponent system and the use of pyromelitic and succinic anhydrides might, in fact, lead to imide formation and hydrophobicity increase, rather than a decrease as assumed.

Couplings that use 1-ethyl-3-(3-diethylaminopropyl) carbodiimide (EDCI) have been used to introduce approximately two (as judged by mass of product increase) monoamino (i.e. one of the C-6 hydroxyl groups is a primary amine rather than a hydroxyl) α, β or γ cyclodextrins (CDs) into trypsin [13]. The modified trypsins were five- to eightfold more resistant to autolysis and showed some increase in esterase activity. The latter, it is suggested, may be due to an increase in local concentration by inclusion of substrate in the hydrophobic binding site of the CD. A similar study evaluated the effect of β-CD coupled using EDCI to trypsin with different spacer-arm lengths on C-6 (C-6 = CH_2NH where R = H or $(CH_2)_nNH_2$ where n = 2,3,4). Tryptic digests largely revealed modification at Asp153 and Glu186. Modified enzyme typically showed upto twofold enhanced esterase activity (k_{cat}/K_M),

with the exception of the propyl-spaced CD modifications, and enhanced thermolytic and autolytic stability [14]. The same group also used reductive alkylation of trypsin [15] and α-chymotrypsin [16] with a C-6 monoaldehyde of β-CD, obtained through Kornblüm oxidation, to enhance thermostability. Other examples of non-specific chemical modifications to increase thermostability have also been reported [17,18].

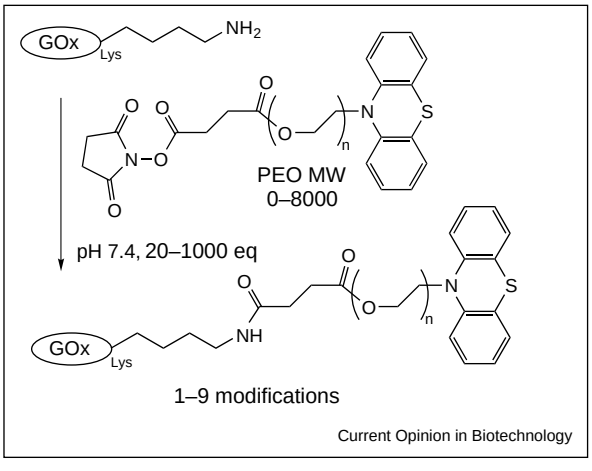
Modification of ribonuclease

EDCI was also used to couple aminoethanol, taurine and 1,2-diaminoethane to approximately six to eight of the 11 available carboxylates, as estimated by amino acid analysis, (to create $-CONHCH_2CH_2OH$, $-CONHCH_2CH_2SO_3^-$ and $-CONHCH_2CH_2NH_3^+$ sidechains) and hence to modify and/or alter the overall charge (+4, +10, +20) of several ribonucleases. The hydrolytic activity decreased steadily with increasing charge to 1.6% that of unmodified enzyme. Despite this loss of activity, the cytotoxicity of the enzymes increased from undetectable levels to a growth inhibition GI_{50} value of 0.17 μM towards 3T3-SV40 cells. These surprising results may be due to reduced intracellular inhibition of the RNase by RNase inhibitor protein or enhanced cellular uptake [19•].

Modification of glucose oxidase

A series of glucose oxidase (GOx) hybrids (GOx-phenothiazine-labelled poly(ethylene oxide) [PT-PEO] conjugates) that are capable of direct electrical communication with electrodes have been synthesized by randomly covalently attaching PT-PEO to lysine residues on the enzyme surface. The modification was achieved using activated *N*-hydroxy succinimide esters (Figure 1), which at molar ratios of 20–1200 allowed on average one to eight modifications (as judged by an increase in UV absorption) per GOx [20]. The length of the PEO chain (molecular weight 0–8000) and the number of PT groups were systematically altered. The catalytic current (i_{cat}) value increased with the number of PT groups introduced and indicated that most of the modified PT groups act as mediators. The maximum rate constant ($130\ s^{-1}$) for the electron transfer from $FADH_2$/FADH to PT^+ is obtained for a GOx hybrid modified with five PT-PEO groups and with a molecular weight of 3000. In a similar study, EDCI was used to form amide linkages between surface Glu/Asp groups on GOx with amine-terminated PEO-PT modifiers; this work also highlighted PEO3000 as optimal [21].

Figure 1



Lysine modification was used to create GOx-PT-PEO hybrids that showed activities dependent on the PEO length [20]. GOx, glucose oxidase; PT-PEO, phenothiazine-labelled polyethylene oxide.

Modification of lipases

The non-specific modification, as judged by 2,4,6-trinitrobenzenesulfonic acid (TNBS) titration, of amines in *Candida rugosa* lipase (CRL) with benzyloxycarbonyl (Z), Z-NO_2, lauroyl and acetyl enhanced enantioselectivity in lipase-catalyzed esterification of 2-(4-substituted phenoxy)propanoic acids with *n*-BuOH in isopropyl ether [22]. It also reversed the trend of increasing the logP (octanol/water partition coefficient) of various organic solvents upon enantioselectivity (as judged by E). The authors speculate, based on the evidence of circular dichroism and electron spin resonance studies, that upon acylation of charged lysine sidechains, CRL adopts a more compact, less flexible conformation. This change decreases the rates of reaction for both enantiomers, but the effect is more pronounced for the unfavoured *S* enantiomer. In the context of this model, it is interesting that the site-selective use of 'polar patches' in proteases to potentially increase the flexibility of binding loops led to a relaxation of enantioselectivity (*vide infra*). A similar study of amine modification in semi-purified CRL, *Pseudomonas cepacia* lipase (PCL) and lipase from *Alcaligenes* species revealed that CRL in which 84% of accessible amine groups had been modified with the Z group, as judged by TNBS titration, exhibited a 15-fold increase in enantioselectivity (E value) in the hydrolysis of butyl 2-(4-ethylphenoxy)-propionate, although the overall activity was decreased at least 10-fold [23]. Modulation of the enantioselectivity of CRL by non-specific modifications has been observed previously [24]. Indeed, modification of 59% of the accessible amine groups in *Candida arctica* lipase B (CAL-B) with polyethylene glycol (PEG) chains using tresyl chemistry increased the enantioselectivity (E = 214→277) in a CAL-B-catalyzed esterification of 3-methylbutan-2-ol in hexane with a concomitant decrease in rate [25]. Again, in this system a decrease in E was observed for other organic solvents (see also Update).

Modification of glutathione peroxidase

A synthetic glutathione peroxidase that reduces hydroperoxides using glutathione as a cofactor has been created using chemical modification [26•]. Such peroxidases typically use selenocysteine as a catalytic group, which makes them difficult to modify using traditional recombinant DNA technology. Modification of a glutathione-binding

enzyme, glutathione transferase (GST), used Hilvert's methodology [27] of sequential reaction with (phenylmethyl)sulfonylfluoride (PMSF) and then NaSeH to alter a serine close to the substrate-binding site. A modified Se-GST was created that efficiently catalyzed the reduction of hydrogen peroxide, with an activity that was greater than the activity of some natural counterparts. The chemical modification in this case did not allow specific targeting of sidechains in the active site alone and other hydroxyl sidechains were modified to selenols. In a range of proteins approximately three to six hydroxyl groups were modified, as judged by Ellman's titration. The modification of the key active-site Ser11 in the Se-GST was implicated by the comparative modification of a Ser11Ala mutant, which created a protein that showed little activity and one less incorporated selenium.

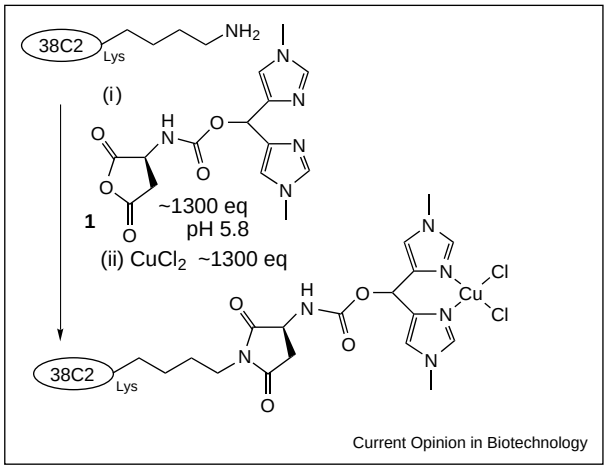
Isotope coding modifications

Chemical modification rescue experiments, in which the deleterious effects of a mutation are reversed through chemical modification, often suffer from difficulties in separating the interpretation of effects resulting from non-specific unwanted modifications from those that are desired. In experiments on fructose 1,6-bisphosphate aldolase [28], electrospray-ionization Fourier-transform mass spectrometry was used to detect the amount of aminoethylation caused by modification of an active-site cysteine with bromoethylamine through isotope coding. Under nondenaturing conditions, Cys107 was aminoethylated, whereas the four buried thiols remained unlabelled. The level of modification was measured at each site after double-labelling the protein with a 1:1 mixture of d_0-:d_4-bromoethylamine followed by labelling under denaturing conditions with only d_0-bromoethylamine. By normalizing the peak of the d_4-labelled protein or peptide the level of reacted and unreacted sites could be calculated. This showed that the 80% restoration of activity upon modification of the Lys107Cys mutant correlated with 80% modification of Cys107 with $-CH_2CH_2NH_2$. Aminoethylation of other residues was observed and correlated with regions of the protein containing histidine or methionine and the N terminus.

Site-specific chemical modification

While the introduction of a unique functional group into a protein provides a robust method for ensuring site-selective chemical modification (*vide infra*), an alternative approach is to exploit the existing differences in reactivities between functional groups of the same type in different environments within a given protein (i.e. regioselective modification). In particular, active-site residues that act as nucleophilic catalysts are *defacto* prone to more rapid reaction with suitable electrophiles. This strategy was first used by Polgar and Bender [29] and Neet and Koshland [30] to modify the nucleophilic $-CH_2OH$ sidechain of serine proteases, but has only recently been applied to catalytic antibodies [31] and in an elegant

Figure 2



Novel anhydride **1** allowed chemical modification of the active-site lysine of aldolase antibody 38C2 to create a metalloprotease-like antibody [32•].

method has been used for the introduction of metals by Janda and co-workers [32•]. The construction of bisimidazole anhydride (**1**; Figure 2) allowed the formation of an imide at the nucleophilic Lys^{H93} of catalytic antibody aldolase 38C2. Cu^{2+} was introduced either before or after modification. Modification was assessed by inhibition of inherent 38C2 aldolase activity and Cu analysis; potentially competing hydroxyl acylation was discounted by treatment with hydroxylamine. The resulting Cu-containing metalloproteins were unable to catalyze oxidations, but did display metalloprotease-like hydrolytic activity of a pyridine-based carboxylate at k_{cat} levels $\sim 10^4$ higher then a comparable bis-imidazolyl-Cu(II) complex alone (see also Update).

A single thiol group, as determined by Ellman's titration, was introduced at the N terminus of subtilisin Carlsberg by exploiting differences in the pK_a of α and sidechain amines. Lack of Edman degradation following reaction with 2-iminothiolane hydrochloride (Traut's reagent) at pH 8 supported the N-terminal location of the modification site. The single thiol allowed coupling to maleimide-modified poly(allylamine) to create enzyme systems that were more autolytically and thermolytically stable [33].

Site-selective chemical modification through combined site-directed mutagenesis and chemical modification

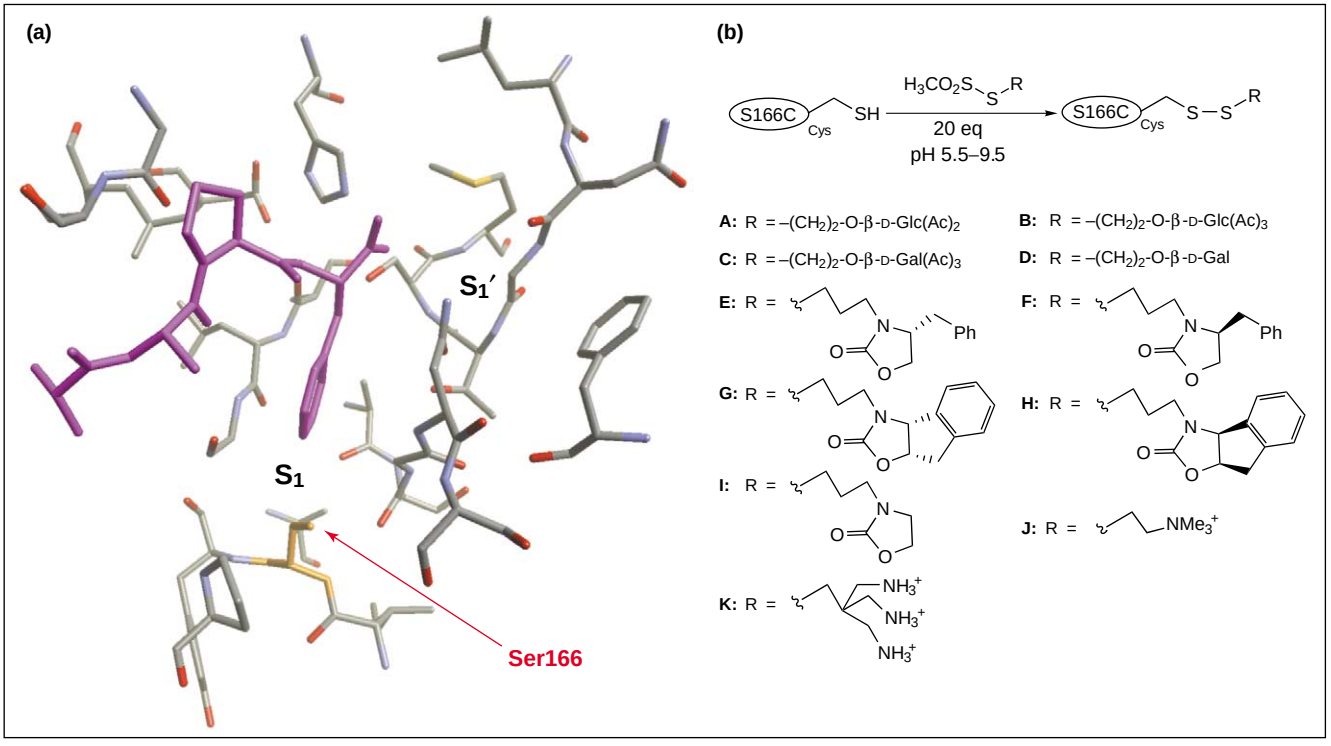
A major factor in the synthetic use of enzymes is their lack of generality and this has often hampered their widespread acceptance as synthetic catalysts. In this sense, their often exquisite substrate specificity is a limitation. Site-selective chemical modification using combined site-directed mutagenesis and chemical modification [6] has recently been used to address this problem and to expand

the utility of the serine protease subtilisin *Bacillus lentus* (SBL) in peptide synthesis. Firstly, a cysteine residue was introduced at position 166 at the base of the primary specificity S_1 pocket of SBL through the creation of mutant Ser166Cys. This was then followed by chemical modification with a variety of simple methanethiosulfonates ($R\text{-}SSO_2CH_3$) to create enzymes with greater tolerance for a wider range of amino acid substrates [34].

Glycosylation of the S_1 pocket of the same enzyme, SBL, created a strikingly broad substrate tolerance, including the acceptance of D-amino acids, and it was speculated that stereochemical mismatching effects might be observed in such glycosylated enzymes [35]. A detailed study of these effects of glycosylation has recently been conducted using carbohydrate, homochiral oxazolidinone auxiliary and polar substituents, such as alkylammonium groups, again introduced using methanthiosulfonate reagents (Figure 3) [36•]. This work revealed that the key to broad substrate tolerance was high polarity in the modifying groups, at levels not normally found in single amino acid sidechains. It is speculated that perhaps the presence of high polarity within the normally hydrophobic S_1 pocket leads to an 'inside-out' folding of the pocket to allow solvation of the polar group, which in turn greatly loosens the pocket and hence substrate tolerance. Typical increases in isolated yields of dipeptides of 60–80% for modified over unmodified SBL (e.g. 0% yield of Z-D-Glu-$GlyNH_2$ using wild-type SBL increased to 74% using $Ser166Cys\text{-}S\text{-}(CH_2)_2NMe_3^+$) demonstrated the synthetic utility of this 'polar patch' strategy. The balanced yields of products with both L-and D-amino acids (e.g. 91% yield of Z-L-Ala-$GlyNH_2$ and 86% yield of Z-D-Ala-$GlyNH_2$ using the same catalyst Ser166Cys-S-(3*R*,4*S*)-indeno-oxazolidinone) was exploited in a small parallel library synthesis with a view to demonstrating their use as potential 'combinatorial biocatalysts' (see also Update).

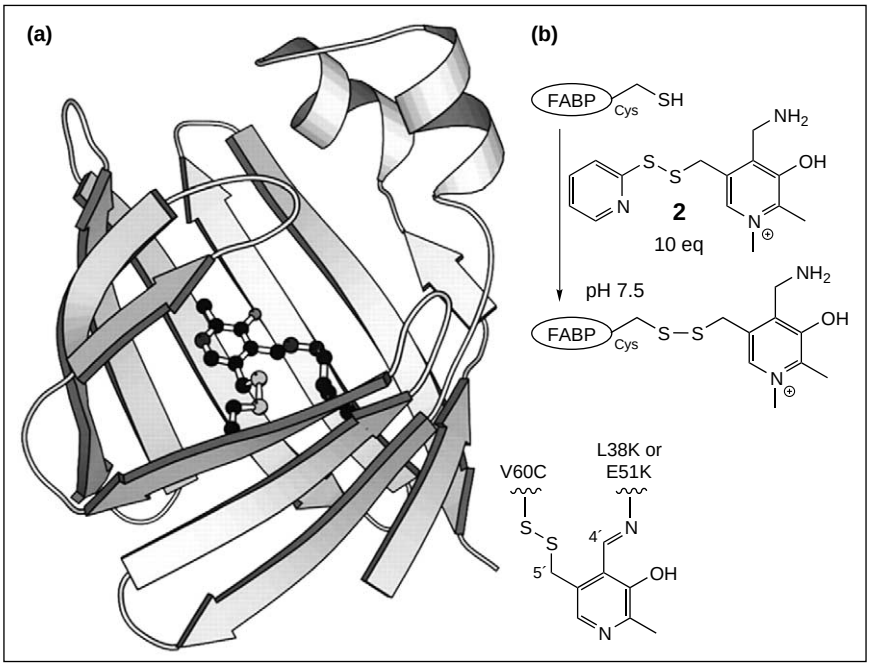
Using thiopyridyl modification reagents Distefano and co-workers [37] continue their elegant work on the site-selective modification of intestinal fatty acid binding protein (IFABP) (Figure 4). Through the N-methylation of the pyridoxamine in reagent **2**, a cationic cofactor was created and tethered, as judged by increase in UV absorbance of the protein, to Cys60 of the IFABP mutants Val60Cys/Leu38Lys and Val60Cys/Glu51Lys. It was reasoned that the use of a synthetic N-methylated pyridoxamine derivative with a permanent positively charged ring would result in a similar electron sink effect to that suggested for natural transaminase enzyme mechanisms.

Figure 3



Modifications to broaden substrate tolerance. The introduction of 'polar patches' to position 166 at the base of the primary specificity determining pocket of a serine protease greatly broadened the substrate tolerance to allow its use in the coupling of D-amino acids. **(a)** Structure of the specificity determining pocket with bound substrate mimic shown in purple; Ser166 is in yellow and other atoms are in standard colours. **(b)** The various carbohydrate, oxazolidinone and trimethylammonium 'polar patches' A–J were attached to position 166 using methanethiosulfonate chemistry. (Figure reproduced from [36•] with permission of Wiley-VCH.)

Figure 4



Modification of intestinal fatty acid binding protein (FABP). **(a)** Structure of FABP with modification shown in ball-and-stick representation. **(b)** A synthetically methylated pyridoxamine derivative **2** was used to modify FABP and increased both the k_{cat} and K_M in transamination reactions as compared with non-methylated. (Figure reproduced from [37] with permission of Oxford University Press.)

The role of this electron sink is thought to be critical to the deprotonation of the α-amino acid proton during transamination to form ketimine, and hence quinoid intermediates. These newly created transaminases catalyzed the transamination between α-ketoglutarate and various amino acids with enantiomeric excesses of up to 96% and were also selective for the amino acid source used for transamination (e.g. 17 turnovers for L-Tyr as compared with ~4 for D-Tyr). Control reactions of the unmodified FABP mutants Leu38Lys or Glu51Lys effectively failed. The k_{cat} values for these synthetic enzymes were 1.2–2.5-fold higher than those using non-methylated cofactor and, in fact, the highest that Distefano's artificially generated catalysts have reached. These k_{cat} enhancements were counterbalanced by large increases in K_M (17–36-fold), which severely modified overall activity. The limited space within the cofactor cavity may explain the reasons for the much higher K_M values upon addition of only one methyl group.

Reetz has proposed an approach that unifies the use of forced evolution and site-selective chemical modification to create so-called 'hybrid catalysts' [38•]. This would involve cycles of random mutagenesis, site-selective chemical modification at a pre-identified site in all the mutants generated, and *en masse* screening. In this way the protein fold around a prelocalized chemical modification could be optimised for a particular catalytic function. The primary target reactions of the Reetz group are transition-metal catalyzed processes that are not performed by enzymes, such as olefin metathesis or allylic substitution. As a first step they have described the chemistry that allows the introduction of ligand–metal systems into protein scaffolds [39]. In this scenario it would be expected that the usual steric and electronic effects of a given organometallic catalyst will still operate, but they will be coupled with the effect of the protein microenvironment in place of local and/or bulk solvent. The nucleophilic cysteine of papain was modified using maleimide-containing ligand–metal reagents. Low enantiomeric excess (<10%) levels were observed for epoxidation using manganese-salen modified papain and for reductions by rhodium-containing papain. The use of *para*-nitrophosphonate reagents to modify lipase serine residues with a diphosphine group proved to be reversible and, therefore, not of general utility in this approach. An alternative use of random mutagenesis simply to identify sites suitable for chemical modification has also been proposed [40], which is essentially an extension of methods that have previously been used to identify 'hot spots' for saturation mutagenesis [41–43] but with access to a greater range of sidechains. A crude thio-cyclen preparation has been used to introduce cyclen via a disulfide bond into a DNase enzyme using combined site-directed mutagenesis and chemical modification. The DNase still binds to an inhibitory protein, but the effect upon activity is not discussed [44].

Conclusions

Most of the currently employed methods for biocatalyst chemical modification are still based upon reactions of limited selectivity and efficiency. Non-specific modifications of multiple sites to create heterogeneous mixtures are still, unfortunately, commonplace. If we are to more precisely understand and control biocatalyst function, methods for introducing unusual amino acids in a site-selective manner should be the focus of future research.

The increasing ease of acylated-tRNA technology as a means of introducing chemical 'tags' for further chemoselective reactions in other protein systems [45] provides one potential avenue. Improved and novel protein chemistries will provide another. Many natural examples highlight interesting post-translational modifications that may be viewed as informative for the chemical modification of enzymes. For example, an interesting substrate self-modification of a haem highlights a potentially new approach to altering cytochrome P450 activities. In this example, aldehyde (3-phenylpropionaldehyde or 3-phenylbutyraldehyde) induced alkylation of a cytochrome P450 mutant with a widened substrate-binding pocket (the well-studied Phe87Gly mutant) gave rise to modified enzymes with altered oxidative activities. Intriguingly, the modification abolished the activity towards some substrates (e.g. lauric acid), but not towards others (e.g. *p*-nitrophenol) [46].

In all of this work precise structural characterization is becoming increasingly important, and one could argue essential. Advances in techniques such as mass spectrometry are proving invaluable in this respect. Given the inherent ease of chemical modification, the attractiveness of the modification of existing protein scaffolds, and the wide range of 'sidechain space' that can be explored, chemical protein modification has a rosy future.

Update

A similar result to that observed for lipases [21–24] has been observed for non-specifically decanoyl-modified chymotrypsin. The enantioselectivity of the hydrolysis for D- and L-*N*-dodecanoyl-Phe-*p*NP was markedly enhanced, although an overall reduction in activity was observed [47]. Non-specific palmitoylation of lysine sidechains using palmitoyl chloride has been used to create superoxide dismutase 'enzymosomes' (liposomes containing the 30%-modified enzyme) in which the modified enzyme is more exposed on the surface of the liposome than unmodified enzyme [48]. In myoglobin, His93 binds an iron-porphyrin with a distal His64 on the opposite face. Double point-mutation (His64Asp, Ala71Gly) of apomyoglobin allowed reconstitution with a Cr(III)salophen 'cofactor'. The cofactor is presumed to coordinate to His93 via Cr to allow the creation of a semisynthetic sulfoxidation 'metalloenzyme' catalyst, albeit with low activity and enantioselectivity (ee 13% or lower) [49•]. Combined site-directed mutagenesis and chemical modifications using methanethiosulfonates has been used to create chimeric proteinases that can selectively target-and-destroy preselected proteins (antibodies, binding proteins or enzymes), following a strategy of attaching an appropriate low molecular weight ligand (epitope, ligand or inhibitor) that binds selectively to a protein at its functionally relevant site. Hydrolytic degradation near the binding site then ensues. When the binding site is sufficiently degraded, the proteinase is released and becomes free to seek-and-destroy additional target molecules [50•].

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- •• of outstanding interest

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