Review

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Unnatural amino acid mutagenesis-based enzyme engineering

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Traditional enzyme engineering relies on substituting one amino acid by one of the other 19 natural amino acids to change the functional properties of an enzyme. However, incorporation of unnatural amino acids (UAAs) has been harnessed to engineer efficient enzymes for biocatalysis. Residue-specific and site-specific *in vivo* incorporation methods are becoming the preferred approach for producing enzymes with altered or improved functions. We describe the contribution of *in vivo* UAA incorporation methodologies to enzyme engineering as well as the future prospects for the field, including the integration of UAAs with other new advances in enzyme engineering.

Advent of novel enzyme engineering methods

Because of their eco-friendly nature, enzymes have found widespread applications as biocatalysts [1,2]. The production of biocatalysts has benefited from advances in protein science and the availability of genetic engineering techniques to develop new enzymes with improved or altered properties. Traditionally, enzyme engineering methods comprise three main strategies for improving enzyme stability and catalytic properties: rational design, directed evolution, and a combination of both methods ('semi-rational') [3–5]. Although these methods yield reliable results, being limited to using the side chains of natural amino acids in such engineered enzymes restricts the scope of possible applications.

In vivo UAA incorporation has become important in the protein engineering field as a means to confer novel functions upon proteins targeting a variety of desired applications [6–16]. In general, UAA incorporation can be achieved in two ways: in a residue-specific manner, which utilizes the misacylation of the endogenous tRNA, or in a site-specific manner, which utilizes an exogenously evolved orthogonal tRNA/synthetase pair (Box 1). Although either residue-specific or site-specific methods can be used to achieve the same goal, choosing the appropriate incorporation method depends on the nature of the target enzyme, the nature of the UAA, and the expected outcome. For example, the residue-specific approach allows UAA incorporation at multiple sites, and this can have synergistic effects in the enzyme. The site-specific method, by contrast, allows new chemical functionalities to be precisely introduced into enzymes very easily [17]. More recently, an increase in the diversity of UAAs and advances in incorporation methods have made it possible to overcome some existing challenges to engineering biocatalysts. We focus on discussing *in vivo* UAA mutagenesis-based enzyme engineering for functional applications and for improving or altering enzyme properties. We highlight the current advantages and limitations in the state of the art, and discuss the future prospects of UAA methodology.

Enzyme engineering via the residue-specific method

The residue-specific method has been a common approach for protein engineers and has led to many successful attempts at engineering enzymes for structural studies and property enhancement. In the following text molecular structures are referred to by number (bold font); structures **1–12** are depicted in Figure 1 and **13–32** in Figure 2.

Biophysical probes

Early implementation of the residue-specific method was successful in substituting methionine residues by selenomethionine, **1**, through the use of methionine auxotrophic strains [18]. Since then **1** has become valuable in the structural investigation of enzymes such as galactosidase, RNase H, and others [18–21]. Unlike other heavy atom substitution techniques for X-ray crystallography, substitution with **1** does not cause structural disturbances, and is considered advantageous for phasing studies, hence its continued use to the present day.

^{19}F NMR spectroscopy has been an important tool for the biophysical characterization of proteins and enzymes since the time of its invention [22,23]. For NMR studies, the major advantages of using fluorinated amino acids derive from the small atomic size of fluorine and its chemical properties, as well as from the fact that natural amino acids do not contain fluorine. The size of fluorine is similar

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Box 1. General methodology describing the *in vivo* incorporation of UAAs

In general, during protein synthesis, a cognate amino acid is added to its tRNA by a specific aminoacyl-tRNA synthetase enzyme. Once the tRNA is charged, the ribosome transfers the amino acid from the tRNA onto the growing polypeptide guided by an mRNA sense codon (Figure I).

Residue-specific method

In the absence of a specific cognate amino acid, endogenous tRNA synthetases can misacylate tRNA with isostructural analogs of the corresponding amino acid. The misacylated tRNA with UAA is carried to the ribosome and the UAA is then incorporated into the growing polypeptide guided by an mRNA sense codon. To completely remove the endogenous cognate amino acid from the host cells, auxotrophic strains are utilized.

Site-specific method

Evolved exogenous tRNA synthetases acylate suppressor tRNAs with UAAs, and acylated tRNAs with UAA are then carried to the ribosome and incorporated into the growing polypeptide chain in response to a nonsense codon (a stop codon and/or a quadruplet codon). This method involves an orthogonal exogenous tRNA/synthetase pair to minimize crosstalk with the host translational machinery, and these are typically derived from other species such as *Methanococcus jannaschii* or *Pyrococcus horikoshii*.

Combination of residue-specific and site-specific methods

In this combination method, within auxotrophic cells, the expression of an orthogonal tRNA/synthetase pair enables site-specific incorporation of UAA guided by a nonsense codon (stop codon). In addition, the presence of an endogenous tRNA synthetase facilitates the concurrent global incorporation of other UAA guided by sense codons in the mRNA.

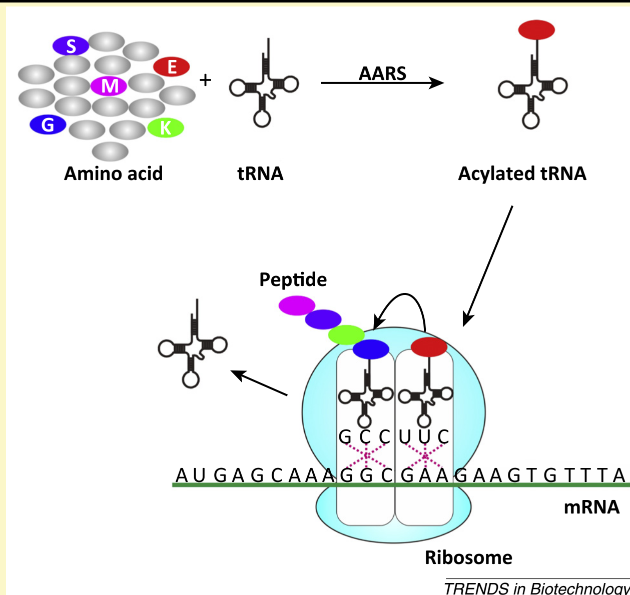

Figure I. Each amino acid is acylated to its cognate tRNA by a specific aminoacyl-tRNA synthetase (AARS) and then delivered to the ribosome. Based on the codon–anticodon interaction between the mRNA and aminoacylated tRNA, the tRNA-bound amino acid is linked to the amino acid of the adjacent aminoacyl-tRNA, extending the growing polypeptide and releasing the free tRNA.

to that of hydrogen with respect to its covalent radii (1.35 Å and 1.2 Å), and most likely does not cause any structural perturbations, thus allowing ^{19}F to offer sensitivity comparable to that of ^{1}H [24]. With such advantages, fluorinated amino acids have emerged as useful structural probes to investigate the chemical microenvironments of residues in enzymes. For example, 6-fluorotryptophan, **2**, was incorporated into lactate dehydrogenase from *Escherichia coli* to investigate interactions with detergent micelles and assess enzyme stability [25]. To demonstrate the utility of fluorinated aliphatic amino acids as ^{19}F NMR probes, the stereoisomer (2*S*,4*S*)-5-fluoroleucine, **3**, was incorporated into dihydrofolate reductase (DHFR) from *Lactobacillus casei*. This is the first example of a fluorinated aliphatic amino acid being used as a ^{19}F NMR probe with overall range of chemical shift 15.2 ppm almost as large as that found with aromatic fluorine-containing amino acids in proteins [26]. Likewise, when incorporated into bacteriophage λ lysozyme, difluoromethionine, **4**, which is diastereotopic in nature, exhibited a significant difference in the chemical shifts observed between the surface-exposed methionine residues and those found in the tightly packed core of the enzyme [27]. Similarly, trifluoromethionine, **5**, was incorporated into phage lysozyme to probe the functions of methionine residues in the protein, and has also been used as a unique probe to study protein–ligand interactions [28]. Incorporation of **4** into the crucial methionine-turn region of alkaline protease was used to investigate the role of methionine in the structural and catalytic properties of the protein [29]. Taken together, the use of fluorinated amino acids as NMR probes opens new perspectives in understanding the importance of substituted amino acids for analyzing enzyme structure and function.

Enhancing stability and activity

A major challenge in the use of natural enzymes as biocatalysts lies in the difficulty of maintaining enzyme stability and optimum activity under harsh conditions, such as during exposure to heat and organic solvents [30]. The residue-specific incorporation method, and the use of fluorinated amino acids in particular, have been important in enhancing stability. This is because fluorination can provide a unique tool for stabilizing proteins by increasing hydrophobicity while closely protecting the shape of the side chain [31]. Engineering enzymes for stability and activity enhancement includes residue-specific fluorination of aromatic residues of lipase B from *Candida antarctica*. Global replacement of fluorinated analogs such as 4-fluorophenylalanine, **6**, 5-fluorotryptophan, **7**, and 3-fluorotyrosine, **8**, into this enzyme gave reduced catalytic activity, but nevertheless prolonged the shelf-life of the lipase activity [32]. Likewise, global incorporation of **6** into phosphotriesterase (PTE) led to enhanced protein refolding after heating to over 70°C. Around 30% of the native structure of the enzyme was maintained in the variant incorporating **6**, whereas the wild type enzyme completely lost its structural conformation. The calculated melting temperature of the fluorinated variant was 1.3–2.5°C higher than that of the native enzyme. Surprisingly, the variant incorporating **6** exhibited a 3.7-fold loss in K_{cat}/K_m compared to the parent enzyme [33]. Fluorination can thus yield more thermostable PTE enzymes with enhanced refoldability, most likely owing to stabilization of the

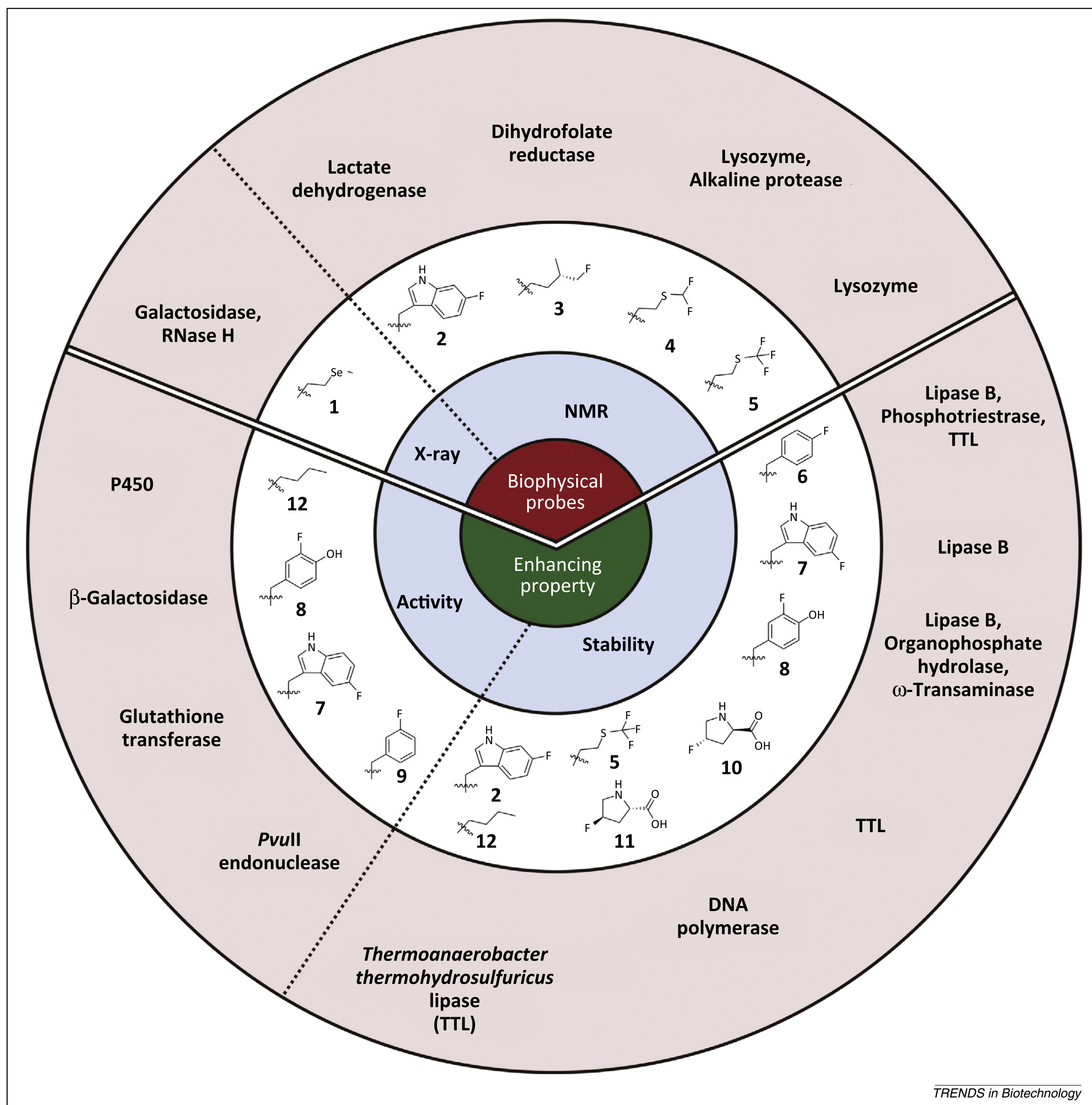

Figure 1. Enzyme engineering by the residue-specific method. General diagram illustrating the targeted incorporation of unnatural amino acids (UAA) into enzymes for structural studies and for improving enzyme properties such as increasing stability and catalytic activity.

interactions along the dimer interface. In terms of catalytic activity, introduction of **7** into M1-glutathione transferase enhanced its catalytic performance through an increased rate of product release. Structural analysis revealed that several subtle changes in structure arise as a result of steric interactions when **7** is incorporated at Trp146 and Trp214 in domain II, which in turn give rise to enhanced catalytic activity [34]. Likewise, global incorporation of **8** into β-galactosidase yields an enzyme with better catalytic activity. The fluorinated enzyme showed a 4.5-fold increased V_{max} towards phenyl-β-D-galactopyranoside and a twofold increase for ortho-nitrophenyl-β-D-galactopyranoside at pH 7.0. However, in terms of stability, 50% more activity was lost in the fluorinated enzyme within 2.5 min at pH 6.0 compared to the wild type enzyme [35]. Further, organophosphate hydrolase engineered by substituting tyrosine with **8** had an extended pH optimum of activity at acidic pH and improved thermal stability at alkaline pH. In addition, 40% residual activity was maintained in the fluorinated enzyme after heating at 55°C, whereas the wild type enzyme retained only 13% activity [36]. More recently, engineering ω-transaminase (ω-TA) by global substitution

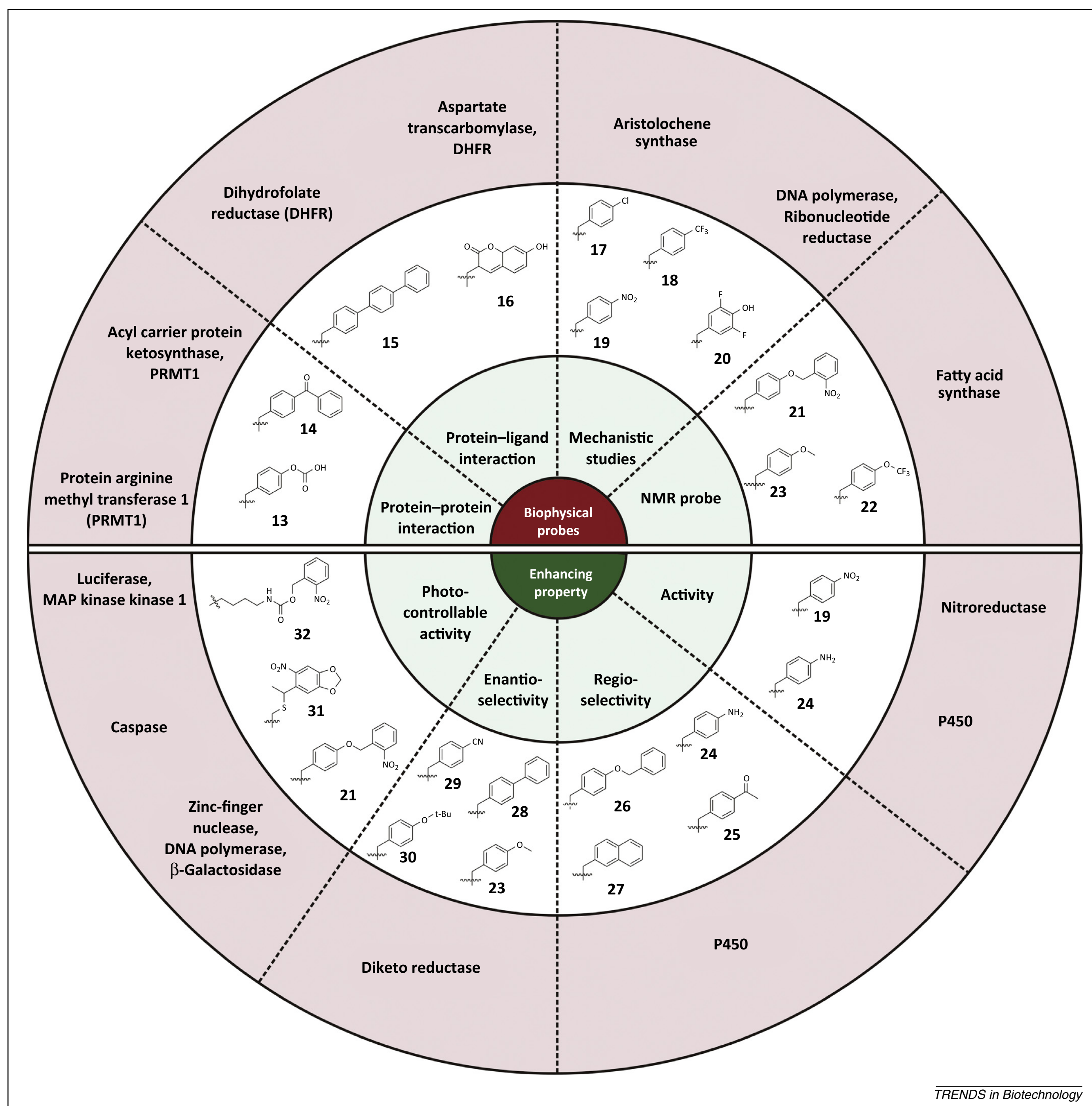

Figure 2. Enzyme engineering by the site-specific method. General diagram illustrating the incorporation of unnatural amino acids (UAA) into enzymes for functional studies and for improving or altering enzyme catalytic properties.

of tyrosine by **8** yielded enhanced activity up to 20% higher than that of the wild type enzyme. In addition to enhanced catalytic activity, ~36% of the residual activity was maintained at 70°C, with a 2.3-fold longer half-life, and 90% residual activity was retained in presence of 50% (v/v) dimethylsulfoxide (DMSO), whereas the wild type enzyme exhibited only 51% of residual activity [37]. Interestingly, global fluorination of ω-TA does not change its original substrate specificity or enantioselectivity. A similar approach involving the substitution of fluorinated phenylalanine analogs into *Pvu*II endonuclease showed changes in enzyme catalytic behavior. Interestingly, 3-fluorophenylalanine, **9**, gave a twofold increase in average specific activity, with a similar conformational stability to the wild type enzyme. Structural analysis of *Pvu*II endonuclease revealed that the substituted phenylalanine residue was not located near the catalytic region or in a DNA-binding site [38]. Hence, this work showed that the incorporation of **9** at locations distant from the active site of *Pvu*II endonuclease can alter its catalytic behavior

through subtle changes in enzyme conformation. In addition, the multi-UAA acid incorporation strategy was used to incorporate (4*S*)-fluoroproline **10**, **6**, and **2** into *Thermoanaerobacter thermohydrosulfuricus* lipase (TTL) [39]. Interestingly, global substitution of the three different monofluorinated amino acids at 24 positions did not have any detrimental structural effects or abrogate enzyme activity [39]. UAA substitution into the KlenTaq polymerase (C-terminal fragment of *Taq*I polymerase analogous to the Klenow fragment of *E. coli* DNA polymerase I) was exploited to examine the general applicability of incorporating UAA into such large and highly-dynamic enzymes at multiple sites. Multi-fluorinated KlenTaq DNA polymerases were generated by the global replacement of methionine and proline residues with **5** and (4*R*)-fluoroproline, **11**, respectively. The enzymes with fluorinated methionine and proline residues exhibited highest activity at temperatures in the range 50–60°C, maintaining a temperature optimum similar to that of the wild type enzyme. Incorporation of **11** was accompanied a loss of thermostability at 95°C; however, the enzyme showed similar dNTP conversion and specific activity compared to the wild type [40,41]. Thus, fluorination of KlenTaq without any major loss in enzyme activity or fidelity represents a promising starting point for directed DNA polymerase evolution with UAA which could eventually be further extended for engineering other DNA polymerases with novel functions suitable for biotechnological applications.

The use of non-fluorinated amino acids has also played an important role in enzyme tailoring. For example, the global substitution of methionine residues with an hydrophobic analog *nor*-leucine **12** in TH-4 (a variant of BM-3 cytochrome P450 fatty acid monooxygenase, CYP102A1, derived through directed evolution) resulted in a twofold increase in peroxygenase activity despite a significant loss in thermostability [42]. Thus, the global substitution with **12** can produce an enzyme resistant to chemical oxidation accompanied by interesting catalytic behavior. The incorporation of non-fluorinated analogs such as **12** into TTL generated an enzyme that was highly stable at 75 °C for 180 min and that exhibited increased activity in aqueous phase without the need for thermal activation [43]. This change arises most likely due to alterations in the hydrophilicity/hydrophobicity balance of the lid domain when globally replaced with **12**, resulting in an open conformation state, and thereby promoting enzyme activity.

Enzyme engineering via the site-specific method

Rapid progress with the site-specific incorporation method has allowed protein engineers to successfully incorporate more than 100 diverse UAAs into enzymes and proteins for various applications. A wide range of studies enabled by the site-specific incorporation of UAAs as crosslinkers and 'handles' for bioconjugation, protein localization studies, and post-translational modifications have been extensively reviewed [44–48]. Similarly to the residue-specific method, the site-specific method has also been exploited for engineering enzymes for structural studies and activity enhancement. In addition, the site-specific method has been used successfully to alter enzyme properties such as enantioselectivity and regioselectivity as well as to develop photo-controllable enzymes which can potentially be used to precisely control their biological function (Figure 2).

Biophysical probes

The site-specific introduction of UAA as biophysical probes aids the analysis of local structure and dynamics in proteins and enzymes, and is an important technique for the study of protein folding and stability, conformational changes, and protein–protein interactions, allowing scientists to explore complex biological problems [10]. For instance, the introduction of 4-carboxymethyl-L-phenylalanine, **13**, protein arginine methyl transferase 1 (PRMT1) clearly demonstrated that tyrosine phosphorylation can alter protein–protein interaction and substrate specificity. Substitution of 4-benzoyl-L-phenylalanine, **14**, at Tyr291 revealed the involvement of phosphorylation changes in protein interactions with the histones [49]. Similarly, the introduction of **14** site-specifically into the acyl carrier protein provided a simple photocrosslinking method for rapid and sensitive detection of the acyl carrier protein–ketosynthase interaction [50]. The ability of UAAs to label enzymes at a specific site for use as a probe will certainly serve as a basis for new approaches to regulate and examine enzyme biological function. Incorporating fluorescent UAAs into DHFR was used to monitor enzyme conformational changes by studying the intermolecular interactions through the energy-transfer changes exhibited by biphenyl-L-phenylalanine, **15**, and L-(7-hydroxycoumarin-4-yl) ethylglycine, **16** [51]. Likewise, substituting **16** site-specifically into the regulatory site of aspartate transcarbamoylase was helpful in deciphering the molecular mechanism behind the allosteric regulation of this enzyme [52].

Furthermore, site-specific incorporation of UAAs has been used as an effective tool to provide structural insights into the structures of enzymes and their roles in governing enzyme action. The role of cation–Π interaction in catalysis by aristolochene synthase was investigated by replacing Trp334 by a strong electron-withdrawing substituent containing phenylalanine analogs such as 4-chlorophenylalanine, **17**, 4-trifluoromethylphenylalanine, **18**, or 4-nitrophenylalanine, **19** [53]. Likewise, site-specific incorporation of 2,3,5-trifluorotyrosine, **20**, into ribonucleotide reductase was performed to investigate the significance of a stable tyrosine radical pathway for maintaining enzyme conformation and activity during catalysis [54]. Incorporating **20** site-specifically into the KlenTaq DNA polymerase active site allowed the investigators to probe the role and importance of specific hydrogen bond interactions in the abasic bypass site, based on the modulation of pKa values [55]. Further, site-specific incorporation of isotopically labeled UAA such as 2-nitrobenzyltyrosine, **21**, 2-amino-3-(4-(trifluoromethoxy)phenyl)-propanoic acid, **22**, and 4-methoxyphenylalanine, **23**, at 11 different positions around the proposed binding site in the thioesterase domain of human fatty acid synthase demonstrated the utility of NMR-active UAAs as a tool to probe the structure, dynamics, and ligand binding of enzymes [56].

Enhancing activity

The nitroreductase enzyme has recently raised enormous interest in view of its potential use in enzyme prodrug

therapy applications. To improve the catalytic activity of the enzyme, engineering the Phe124 site with natural amino acids such as Tyr, Lys, and Asn, and incorporating diverse phenylalanine analogs into the enzyme, changed the catalytic properties of the enzyme. Among the various analogs incorporated, enzyme containing **19** showed a substantial increase in catalytic efficiency and a >2.3-fold improvement over the best possible natural amino acid, lysine. Results from this study clearly demonstrate the feasibility of the site-specific approach in improving enzyme catalytic properties in a way that cannot be achieved using natural amino acids [57].

Altering regioselectivity and enantioselectivity

Engineering the properties of enzymes by modifying their enantioselectivity and regioselectivity is a fruitful biotechnological application [1–3]. The site-specific incorporation method has recently been employed successfully for modifying enzymes to improve their functional activity. For instance, a P450 enzyme was engineered by the site-specific incorporation of the tyrosine analogs 4-amino-phenylalanine, **24**, 4-acetyl-phenylalanine, **25**, 2-benzyl-tyrosine, **26**, and 3-(2-napthyl) alanine, **27**, into an engineered variant termed CYP102A1-139-3. The parent enzyme converts (*S*)-ibuprofen methylester into benzylic alcohol (62%) and allylic alcohol (38%) derivatives. However, the incorporation of **26** at Leu181 enabled the conversion of the (*S*)-ibuprofen methylester into benzylic alcohol (15%) and allylic alcohol (85%) derivatives. Another variant, in which **27** was incorporated at Ala32, also converted the (*S*)-ibuprofen methylester into benzylic alcohol (5%) and allylic alcohol (95%) derivatives. These results clearly demonstrate that the site-specific incorporation of UAAs at the active-site positions can change enzyme regioselectivity in a promising way. Further, the introduction of **24** into the Leu75 variant resulted in an increased total turnover number (34650) for (+)-nootkatone, and this is the highest value reported to date [58].

Over the past few decades, controlling the enantioselectivity of a biocatalyst has been a major challenge, and various attempts to address this issue have been reported [59]. More recently, mutagenesis studies with diketoreductase showed that Trp222 is crucial for substrate binding and catalysis. The wild type enzyme showed an (*R*)-preference, with 9.1% enantiomeric excess (*e.e.*) value towards the substrate 2-chloro-1-phenylethanone. The incorporation of tyrosine analogs such as 4-methoxy-L-phenylalanine, **23**, 4-phenyl-L-phenylalanine, **28**, 4-cyano-L-phenylalanine, **29**, or 2-*tert*-butyl-L-tyrosine, **30**, into the enzyme at Trp222 altered the enantioselectivity of the enzyme. For example, the variant incorporating **30** gave a 33.7% *e.e.* value. More interestingly, the enzyme containing **29** showed an inversion of enantiopreference from (*R*) to (*S*), with 33.3(*S*)% *e.e.* [59]. Taken together, these data clearly demonstrate the potential of UAA in increasing the enantioselectivity and also in changing the enantiopreference of enzymes.

Photo-controllable enzymes

Incorporating photocaged UAAs site-specifically into proteins and enzymes enables researchers to precisely control their biological activities via the photochemical reaction [60,61]. Optochemical control studies began with the incorporation of **21** into β-galactosidase and 2-nitrobenzylcysteine (**31**) into caspase-3, respectively, to monitor their enzymatic activities [62,63]. Likewise, incorporation of **21** into *Taq* DNA polymerase successfully enabled researchers to regulate the enzyme activity [64]. Later, 2-nitrobenzyllysine (**32**) was introduced at the active site of firefly luciferase to facilitate the measurement of intracellular ATP dynamics in HEK293T cells [65]. More interestingly, investigating signaling pathways through the optochemical control method was accomplished by incorporating **32** into a highly conserved lysine site of the mitogen-activated protein kinase kinase 1 (MAP kinase kinase 1, MEKK1) [66]. Recently, the principle of photocaging has also been extended to gene-editing studies. A light-activatable zinc-finger nuclease was developed by site-specifically labeling the enzyme with 2-nitrobenzyl tyrosine (**21**) which can be used as an alternative over the Cu(I)-catalyzed click cycloaddition because activation by light is non-toxic to cells and enables precise temporal control in gene-silencing applications [67].

Challenges and opportunities for enzyme engineering with UAAs

Residue-specific incorporation is an simple approach for preparing single UAA-containing proteins with novel functionalities. In particular, the synergistic effects of multisite UAA replacements can be exploited to improve the structural and biological features of the proteins. However, the major drawback of this approach is that there is no specific control of the site of introduction [68]. Further, enzyme engineering with residue-specific incorporation enables multisite incorporation of UAAs that often induces perturbations in the folded structure, especially for larger proteins [17,69,70]. This can be overcome by employing directed evolution (Box 2) [69,71,72] or by exclusion of the non-permissive site [70] with the help of structural analysis. In the residue-specific incorporation approach, the presence of trace levels of the corresponding natural amino acid in the cell can compete with the UAA, often leading to heterogeneous proteins [39,73]. For the efficient incorporation of UAA, complete depletion of the corresponding natural amino acids in the medium after reaching a specific optical density (in general, 1 OD_{600} in flask cultures) is a crucial step in UAA incorporation, and this complicates the process, notably for large-scale production of enzymes in fermenters. Taken together, it is important to develop standardized protocols and customized approaches for efficient incorporation of UAA during large-scale fermentation. In addition, the residue-specific incorporation method relies on the substrate specificities of endogenous tRNA synthetases that are restricted to isostructural analogs of the 20 common amino acids [74]. To achieve efficient incorporation of desired UAA, the substrate specificity of the endogenous tRNA synthetase needs to be engineered to permit an increased spectrum of UAAs [74].

In contrast to residue-specific incorporation, site-specific incorporation is a unique system that enables selective control of UAA incorporation. In addition, this approach is generally not restricted to isostructural UAAs because it

Box 2. Directed evolution with UAAs

Directed evolution is based upon the principle of natural evolution, whereby the introduction of random mutations into a protein allows the creation of high-level sequence diversity and screening/selection for favorable variants [92]. In the current decade, the directed evolution strategy has been enhanced by an expanded set of amino acids that enlarge the sequence space of proteins and in turn increase the chances of evolving a desired mutant [69,93–95]. Recent progress in protein evolution using UAAs is outlined below.

Directed evolution with the residue-specific incorporation method

Although the introduction of UAAs using residue-specific incorporation provides new functionalities to the proteins, it often leads to a substantial loss of activity or stability through perturbations of the correctly folded structure [17,69]. Tirell and colleagues described a new approach to engineer enzymes with novel compositions by combining residue-specific incorporation of UAAs with directed evolution [69]. The complete replacement of 13 leucine residues of chloramphenicol acetyltransferase (CAT) with the leucine surrogate 5′,5′,5′-trifluoroleucine resulted in a 20-fold reduction in the half-life of thermal inactivation of the parent enzyme at 60 °C. However, two rounds of random mutagenesis and screening yielded a variant of CAT containing three amino acid substitutions, which in fluorinated form demonstrated a 27-fold improvement in half-life, demonstrating full recovery from the loss in thermostability caused by 5′,5′,5′-trifluoroleucine incorporation [69]. Later, a similar strategy was demonstrated for other proteins such as green fluorescent protein and the single-chain variable fragment (scFv) of an antigenin antibody [71,72]. These studies showed that directed evolution provides an effective means for adapting protein sequences for the inclusion of novel side chains and intramolecular interactions.

Directed evolution with site-specific incorporation method

In contrast to the residue-specific incorporation method, UAAs can be genetically encoded at a desired site by using a stop codon, thereby enabling site-specific incorporation [73]. Translation with UAA using site-specific incorporation utilizes the same fundamental paradigm as translation with natural amino acids, resulting in unrestricted 21 amino acid protein evolution [92,93]. The initial groundwork for the directed evolution of proteins with UAAs was carried out in the phage-display system targeting the immunoglobulin complementary-determining region 3 of the heavy chain (CDR3H) [93]. The phage-displayed antibody libraries that randomly incorporated UAAs into CDR3H were subjected to *in vitro* selection experiments. A phagemid expression system comprising bacteriophage M13 coat protein pIII and an orthogonal tRNA/synthetase pair was utilized for the incorporation of UAAs in response to the amber stop codon (TAG) [93]. Antibodies containing boronate or sulfotyrosine residues were found to outcompete natural antibodies in binding to acylic glucamine resins and the HIV coat protein gp120, respectively [96–98]. Although this strategy was demonstrated with an antibody, it can also be extended to enzymes where the incorporation of novel amino acid codons into the genetic code can be advantageous for the evolution of enzymes with novel or enhanced functions. In addition, directed evolution methods have been developed that allow random substitution of a contiguous trinucleotide stop codon sequence (TAG) throughout a target gene for use in conjunction with site-specific incorporation [94].

relies on the use of an orthogonal system developed by guided-evolution approaches [75]. Although great progress has been made in enzyme engineering via site-specific incorporation of UAAs, it also has limitations such as poor protein yield and single-site incorporation of a single type of UAA [76–78]. Recent breakthrough strategies enable increased protein expression levels and multisite incorporation of UAAs by engineering ribosomes or by the removal of a translation release factor [76,77,79–81]. Further, efforts have also been focused on multi-UAA incorporation, and this has been accomplished by evolving quadruplet codons and encoding at two different types of stop codon [79,82]. Exploiting these strategies in enzyme engineering will have an enormous influence on evolving novel enzymes with attractive multifunctional properties. Finally, structural insights into the target enzyme and understanding the effects of novel side-chain UAAs will be necessary for choosing the optimum site and the appropriate UAA. Although these emerging strategies have become now become firmly established, UAA diversity and breakthrough strategies can be further expanded and more widely applied in enzyme engineering.

One way to overcome the challenges inherent in each method is to combine them in a single *in vivo* expression experiment. To make this possible, a simple approach was developed by combining residue-specific and site-specific incorporation of UAA to generate a multifunctional protein with great promise in several applications (Box 1) [68,75, 83–85]. By means of this coupling approach, a bifunctional ω-TA enzyme was engineered that exhibited increased thermostability, owing to the synergistic effects of globally substituted (4*R*)-fluoroproline, and enhanced chitosan immobilization as a result of site-specifically added L-dihydroxyphenylalanine, thereby facilitating the reusability of the enzyme [84]. In terms of thermal stability, two different UAA-containing ω-TA variants showed a 2.4-fold increase in half-life at 70°C, compared to the wild type enzyme, and the immobilized enzymes exhibited excellent reusability for up to 10 cycles in the kinetic resolution of chiral amines [84].

Concluding remarks and future perspectives

In vivo incorporation of UAA has proved to be a remarkably effective strategy in engineering enzymes with improved and altered enzyme activity, stability, regioselectivity, or enantioselectivity. The technique is also helpful in investigating photo-controllable activation and for the biophysical characterization of enzymes using UAA as biophysical probes. Although tremendous efforts have been invested in advancing protein engineering using UAAs [75–85], the newly developed strategies and recent advances have not yet been fully exploited in enzyme engineering applications. The high cost of the unnatural building blocks and their commercial unavailability are major barriers to the industrial application of UAA-containing enzymes. To overcome these limitations, enzymatic synthesis of UAA amino acids such as fluorotyrosine and tryptophan analogs has been accomplished using tyrosine phenol lyase and tryptophan synthase, respectively [86–88]. Further development of chemical and enzymatic routes for the synthesis of UAAs in an easy and cheap way will be a milestone in enzyme engineering. Alternatively, host cells can be engineered for the biosynthesis of UAA such as phenylalanine and pyrrolysine analogs and their subsequent incorporation into target proteins [89–91]. Ultimately, evolving host cells for the metabolic production of UAAs from cheap sources and concurrently incorporating them into target enzymes will lead to a boom in the industrial applications of engineered enzymes.

In conclusion, rapid developments in UAA incorporation strategies are opening new doors in enzyme engineering and will serve as a toolbox to evolve and engineer enzymes with desired functions.

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