Review

CellPress

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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0167-7799/
 http://dx.doi.org/10.1016/j.tibtech.2015.05.002

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.

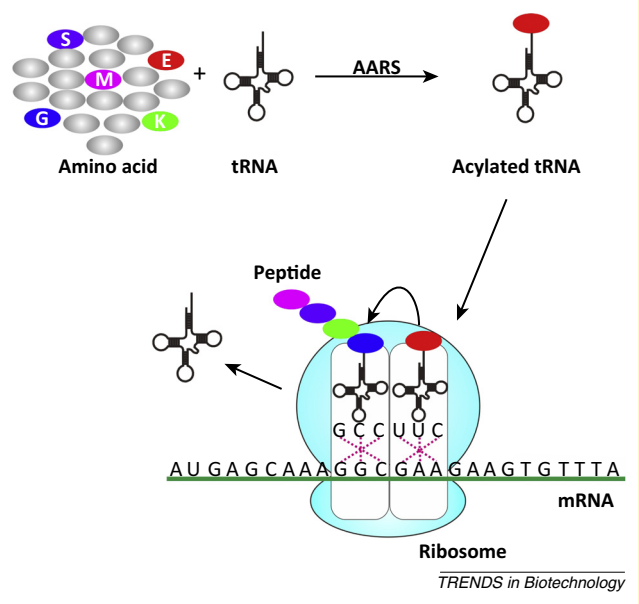

Figure I. Each amino acid is acylated to its cognate tRNA by a specific aminoacyl-tRNA synthetase (AARS) and then delivered to the ribosome. Based on the codon–anticodon interaction between the mRNA and aminoacylated tRNA, the tRNA-bound amino acid is linked to the amino acid of the adjacent aminoacyl-tRNA,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