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REVIEW PAPER

Recent Advances in Enzyme Engineering through Incorporation of Unnatural Amino Acids

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Abstract The development of new enzyme engineering technologies has been actively pursued as the industrial use of biocatalysts is rapidly increasing. Traditional enzyme engineering has been limited to changing the functional properties of enzymes by replacing one amino acid with the other 19 natural amino acids. However, the incorporation of unnatural amino acids (UAAs) has been exploited to manipulate efficient enzymes for biocatalysis. This has been an effective enzyme engineering technique by complementing and extending the limits of traditional enzymatic functional changes. This review paper describes the basic functions of the new functional groups of UAAs used in enzyme engineering and the utilization of UAAs in the formation of chemical bonds in the proteins. The recent developments of UAA-mediated enzymology and its applicability in industry, pharmaceutical and other research areas to overcome the limitations of existing enzymes is also emphasized.

Keywords: unnatural amino acids, biocatalysis, covalent and noncovalent bonds, enzyme engineering

1. Introduction

Enzymes, due to their excellent catalytic properties, have become the center of attraction in the field of applied research. Owing to their eco-friendly nature, enzymatic syntheses are widely preferred in various industries such as fine chemical, agrochemical and pharmaceutical. Continuous research is going on to discover new functional enzymes to utilize them for such industrial applications. In addition, recent years have evidenced an increasing effort to find new methodologies to alter or enhance the properties of existing enzymes [1]. Chemical modification is an attractive tool for enzyme engineering because, it allows to improve properties of conventional enzymes. Many researchers have introduced a variety of chemical modifications into enzymes to alter their functions such as increased stability, expanded range of acceptable substrates, rapid catalytic reactions and development of artificial metalloenzymes [2-5].

Over the last few decades, engineering of enzymes using the incorporation of unnatural amino acids has emerged as a new trend of chemical modification of enzymes. It has proved to be promising strategy to develop enzymes with enhanced properties and functionalities, thereby increasing medical and industrial applicability thereof. Previously published reviews have mainly focused the UAA-mediated engineering of proteins for functional applications and altered properties of the target enzymes [1,6,7]. This review endeavors to provide the role of newly formed chemical bonds by the incorporation of UAAs into the target protein, especially an enzyme, in the improvements in the certain properties of them (Fig. 1). Furthermore, recent advances in the UAA-mediated enzyme engineering and its applicability in the industrial, pharmaceutical and other research fields to overcome the limitations of existing enzymes are also discussed. This review also provides a perspective on the progressive challenges for the advancement of enzyme engineering utilizing UAAs and industrial applicability of such engineered enzymes.

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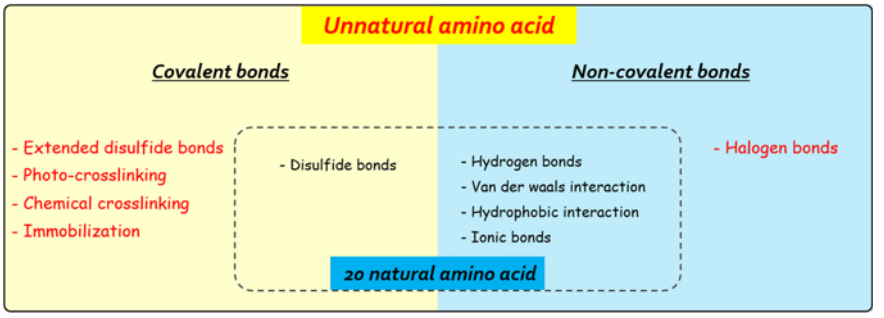


Fig. 1. Extended covalent and non-covalent bonds formation of enzymes through the incorporation of UAAs in UAAs-fusion enzyme engineering.

2. *In vivo* Incorporation of UAAs into Enzymes through Residue-specific and Site-specific Methods

Generally, *in vivo* incorporation of UAAs into enzymes is accomplished by two complementary approaches- either at defined site (termed as site-specific manner) or throughout the proteome via protein translation (called as residue-specific approach).

2.1. Residue-specific incorporation of UAAs

Residue-specific approach involves the incorporation of UAAs into growing polypeptide chain in complete absence of its isostructural analog (cognate amino acid) among 20 natural amino acids (Fig. 2) [8]. In this method, UAA gets incorporated at multiple sites throughout the protein without changing the genetic code of a target protein. To achieve the incorporation of particular UAA by residue-specific

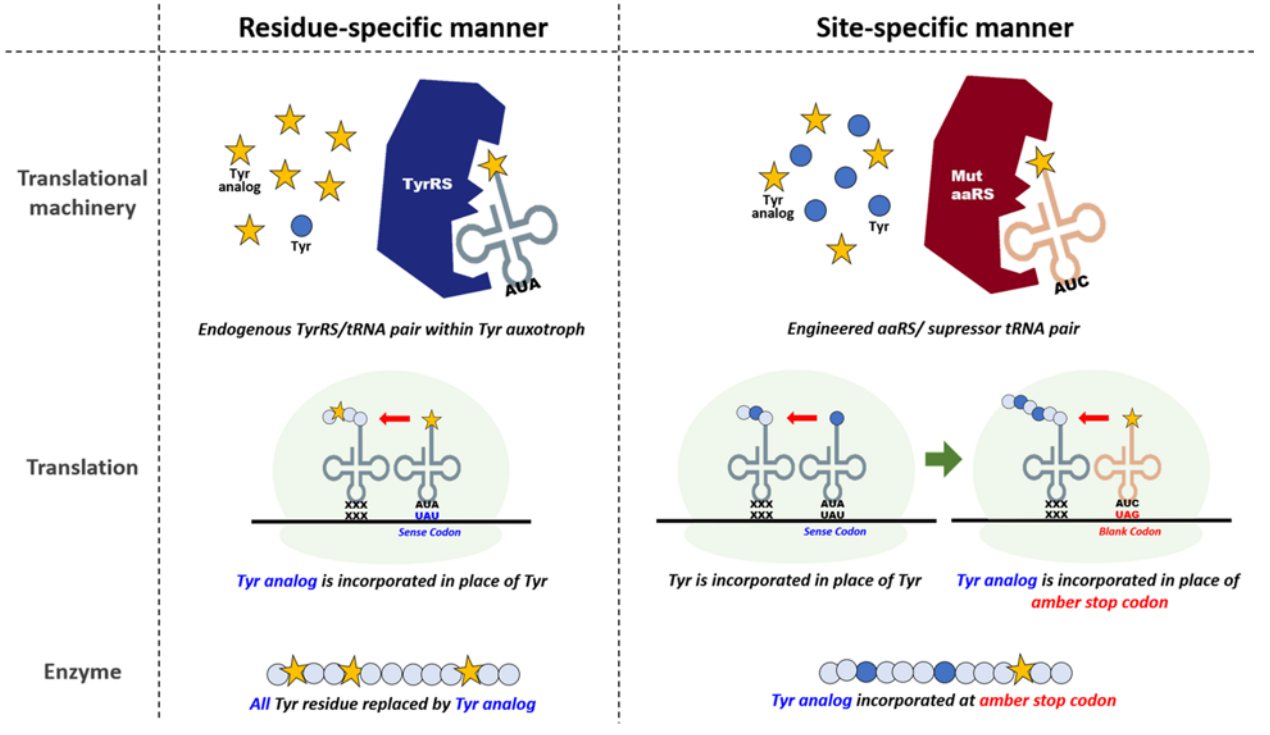


Fig. 2. Strategies for genetic incorporation of UAAs into the enzyme in *E. coli*. Residue-specific methods utilize a host's translational machinery to incorporate one type of natural amino acid globally into structurally similar UAAs. The enzymes synthesized as a result of this manner shows improved properties by synergy effects through the introduction of UAAs. Genetic encodings of UAAs through site specific methods are achieved by co-transformed an exogenous aaRS/suppressor tRNA pair into *E. coli*, which enables the utilization of UAAs with various functional groups by mutating a single site in the enzyme.

manner, three conditions should be satisfied; a) natural amino acid to be replaced with UAA must be encoded in the genetic sequence of a protein; b) UAA must be recognized by aminoacyl tRNA synthetase (aaRS) and tRNA in the absence of natural amino acid; and c) auxotrophic host cell lacking the ability to synthesize natural amino acid, which replaced with UAA, and thereby facilitating the incorporation of UAA [6]. In a particular experiment, the host cells are grown to a mid-log phase (OD_{600} = 0.8-1.0) in the presence of all 20 natural amino acids with limiting concentration of cognate amino acid. The concentration of cognate amino acid is optimized in such a way that it should be depleted completely from the media after achieving the mid-log phase growth. Then UAA is supplied to that media and protein translation is induced to express the protein containing UAA. Presence of very small amount of cognate amino acid can crucially affect the efficiency UAA incorporation. Thus, adding "washing step" to remove cognate amino acid using 0.9% NaCl after achieving mid-log phase growth and then shifting the host cells to another media, completely deprived from cognate amino acid can highly increase the efficiency of UAA incorporation [9,10]. Residue-specific manner incorporates the UAA at multiple sites which leads to synergistic effects in altering the properties of enzymes. In addition, higher protein yields are obtained in comparison to the site-specific manner [11]. However, the residue-specific manner has limitations in improving the properties of enzymes due to the potential risk of interfering with the folding structure of the enzyme by replacing unacceptable residues with UAA. In addition, it is possible to inadvertently remove residues essential for the activity of the enzyme, resulting in a substantial loss of activity of the target enzyme [7]. Several strategies have been studied to overcome this problem. Selection of UAA having similar size of that cognate amino acid is important to minimize the interference with structure and function of target proteins. For example, substitution of all phenylalanine, proline and tryptophan residues constituting 10% of the amino acid sequence of lipase B with mono-fluorinated amino acid did not induce deleterious structural problems or change in activity of the enzyme based on the fact that size of the fluorine atom is similar to hydrogen but considerably increases the hydrophobicity of the enzyme [12]. Substitution of an amino acid such as tryptophan or methionine, which constitute relatively small amount in the protein's primary structure, with its structural analog minimally disturbs the enzyme's structure than that by replacement of moderately abundant residue such as histidine [13,14]. Residue-specific manner, with this high fidelity and good protein yield (20-93%) similar with that of wild-type ones, can be effective in producing artificial biocatalysts for a variety of bioprocess applications [11].

2.2. Site-specific incorporation of UAAs

In site-specific method the orthogonal aaRS/tRNA pair engineered for specific UAA is used to incorporate that UAA on growing polypeptide chain in response to a blank codon on mRNA (Fig. 2). In the method of site-specific incorporation, codon of residue to be substituted with UAA is mutated to amber stop codon (TAG), which is recognized by the pair of orthogonal aaRS and suppressor tRNA derived from other species [15]. The orthogonal aaRS/tRNA pair used to minimize confusion with host's protein translation machinery is mainly derived from *Methanocaldococcus jannaschii* or *Pyrococcus horikoshii.* These orthogonal aaRSs can specifically aminoacylate its cognate orthogonal tRNA as highly specific substrate but cannot aminoacylate endogenous tRNA of the host. In contrast to residue-specific method, site-specific method incorporates the UAA at genetically defined position with the less structural perturbation of protein structure. This method is more useful to study the structural and functional aspects of the proteins [16-19]. One of the biggest obstacles to incorporate UAAs by this method is to achieve higher specificity of aaRS to aminoacylate tRNA with the specific UAA. Many researchers have been trying to diversify the substrate specificity of aaRS for UAAs with various functional groups through direct evolution based on the structural analysis of exogenous aaRS enzymes [7]. For example, error-prone PCR can be used to generate mutant libraries based on wild-type orthogonal aaRS [20,21]. Furthermore, desired functional mutations can be deduced using protein docking programs such as Rosetta [22]. Among the many efforts, Schultz's group have developed powerful library screening tool for the development of mutant aaRSs needed for the incorporation of various UAAs [23]. As a result, more than fifty types of UAAs containing special functional groups have been successfully incorporated using a site-specific method in *E. coli* and yeast [7]. Owing to its dependence on the expression level of the exogenous tRNA/aaRS pair or the presence of release factor terminating the translation, the protein yield obtained using the site-specific manner is relatively low compared with that of residue-specific method. Nevertheless, the efficiency of UAA incorporation in a site-specific manner is comparatively high (> 98%) because of its dependence on response to the specific blank codons [8,24-26]. An important advantage of this site-specific approach is that it allows the incorporation of various UAAs (more than 100) with specific functional groups at desired positions of the proteins, thereby expanding the functional properties corresponding to the number of UAAs incorporated into the proteins.

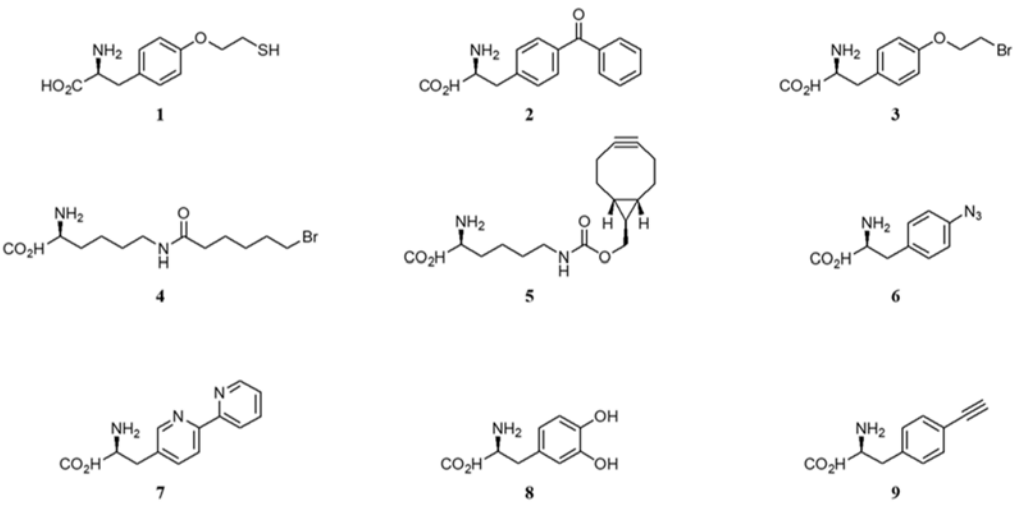


Fig. 3. List of extended UAAs utilized to form additional chemical bonds within proteins. The name of the structures corresponding to UAA are as follows: *O*-(2-mercaptoethyl)-L-tyrosine, **1**; *p*-benzoyl-L-phenylalanine, **2**; *O*-(3-bromoethyl)-L-tyrosine, **3**; (*S*)-2-amino-6-(6-bromohexanamido)-hexanoic acid, **4**; N6-[(1*R*,8*S*,9*R*)-bicyclo[6.1.0]non-4-yn-9-ylmethoxy]carbonyl-L-lysine, **5**; *p*-azido-L-phenylalanine, **6**; (2,2'-bipyridine-5yl)alanine, **7**; L-3,4-dihydroxyphenylalanine, **8**; *p*-ethynylphenylalanine, **9**.

3. Expansion of Enzyme Engineering through Chemical Reactions Using UAAs

Unnatural amino acids with functional groups, capable of forming photocrosslinking or chemical crosslinking, incorporated into protein through site-specific manner are powerful tools to identify many metabolic processes such as protein signal transduction systems (Fig. 3) [27,28]. Generally, it is difficult to observe the binding between proteins formed in metabolic processes because it is weak and transient. However, the most basic information to understand biological processes can be obtained by forming covalent bonds using UAAs [29]. In recent years, the formation of covalent bonds using UAAs has also been used to improve the biocatalytic properties of the enzymes. Furthermore, formation of long covalent bonds between the monomers constituting the multimer of enzyme improves the thermal stability [22]. Also, artificial metalloenzymes with desired activity can be developed by covalent bonding of metal ions essential for enzyme activity to UAAs. In this section, utilization of UAAs in covalent bond formation for enzyme engineering will be discussed in detail. In addition, present prospect of the applications of UAAs for the identification of intracellular metabolic processes is also discussed.

3.1. Utilization of UAAs for the formation of disulfide bonds

Genetic engineering by modifying one of the 20 natural amino acids enabled to produce enzymes with enhanced stability and catalytic properties but has a limited scope for research. Chemical modification depends on reactivity of the natural amino acid to form covalent bonds which particularly depends on the distances and orientations of the specific residues. To overcome these limitations, protein engineering through UAAs has become an attractive alternative in recent decades. Special functional groups of UAAs lead to form additional bonds for various reactions, enabling enhanced properties and functionalities of enzyme [11,28,29]. As a typical example, chemical modification utilizing natural amino acid is performed using thiol group of cysteine, which forms disulfide bond between two cysteines or reacts with other molecules to form covalent bonds, thereby causing protein folding, stability and protein ubiquitination [30-33]. Also, thiol group can function as metal chelator that allows the formation of entirely novel metalloenzymes [34,35]. However, disulfide bonds derived from cysteine in protein are typically constrained to short bond length of ~5.5Å between two β-carbons and dihedral angle of approximately 90° [36,37]. This restriction to irreversible cross-link formation can be overcome by genetic encoding of UAAs with long thiol-containing side chains. For instance, incorporation of *O*-(2-mercaptoethyl)-L-tyrosine (SetY), **1**, *O*-(3-mercaptopropyl)-L-tyrosine (SprY) and *O*-(4-mercaptobutyl)-L-tyrosine (SbuY) resulted in the formation of an extended length disulfide bond (more than ~11Å) over the surface of β-lactamase (Fig. 4) [30]. This led to the development of enzyme mutants that had improved melting temperatures (Tm) without losing their catalytic activity at nonpermissive temperatures. Thus, the

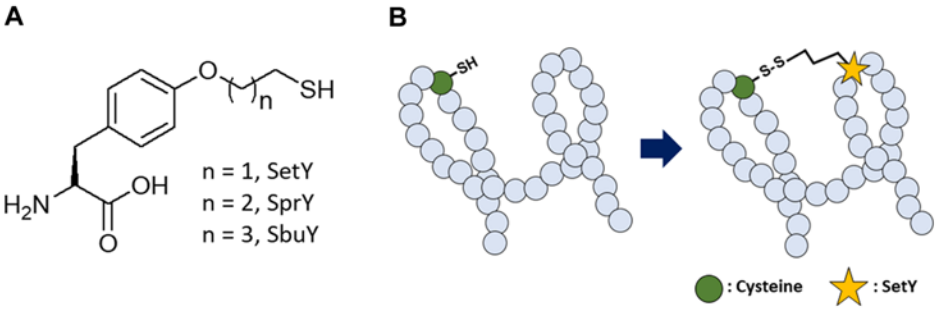


Fig. 4. (A) Genetic incorporation of UAAs containing long-side-chain thiols. (B) Formation of extended disulfide bonds using *O*-(2-mercaptoethyl)-L-tyrosine (SetY) containing long-side chain thiol group.

geometric constraints of a natural disulfide bond could be overcome by genetically encoding UAAs that can form long-distance disulfide bonds.

3.2. Utilization of photo-crosslinking UAAs

Incorporation of unnatural amino acids that form cross-linking in response to light at wavelengths that do not damage biomolecules is used as attractive tool to characterize protein-protein interactions by forming photo-crosslinking with nearby residues. UAAs that form these light-induced crosslinking are used to map, identify, stabilize and modulate the structures of proteins by forming crosslinking of varying lengths from rigid and short side chains (6 to 8Å) to long and flexible linkers (12 to 15Å) [38-43]. *p*-benzoyl-L-phenylalanine (pBzF), **2**, a photo-reactive phenylalanine-derivative that is commercially available at relatively low cost and easy to handle was successfully incorporated into glutathione-S-transferase in *E. coli* by *Methanococcus jannaschii* tyrosyl-tRNA synthetase (MjTyrRS) mutants [38]. pBzF forms long-life ketyl diradicals upon exposure to light, which can form covalent bonds with nearby C-H bonds with a high efficiency [44]. Photo-crosslinking formed through site-specific incorporation in *E. coli*, yeast and mammalian cell has been applied to protein interactions such as receptor-ligand interaction mapping in signal transduction pathways, chromatin biology decoding [45-49]. The covalent bonds not only enable to study transient protein-protein interactions but also can be applied to enzyme engineering. The formation of irreversible covalent bonds not only studies the mapping of signal transduction by immobilizing transient protein-protein interactions but also applies to enzyme engineering to improve the stability. Shultz group constructed a mutant library that randomly introduced various UAAs including pBzF into homoserine-O-succinyltransferase (metA), which is essential for the growth of *E. coli*, to identify mutant enzymes with improved thermal properties [50]. F21pBzF mutant in which Phe21 residue of metA was replaced with pBzF generally allowed *E. coli* to grow at 44ºC, which is not a favorable temperature

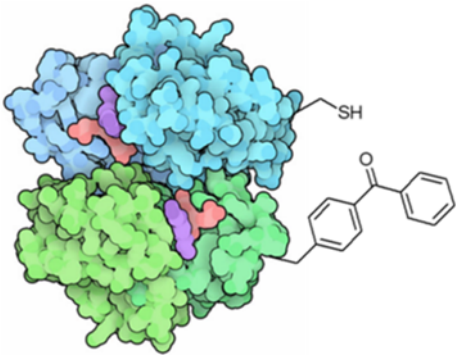


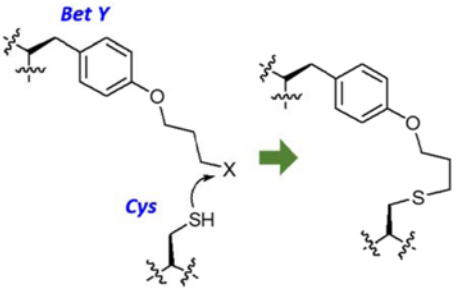
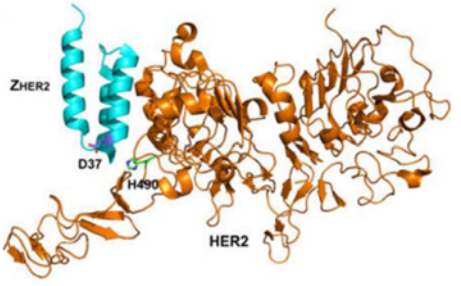
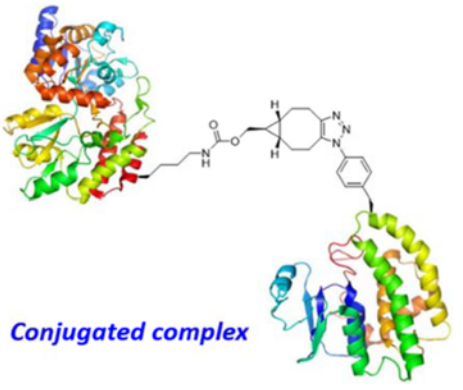
Fig. 5. A schematic diagram of homoserine-O-succinyltransferase (metA) capable of forming photo-induced crosslinking by incorporation of pBzF.

for growth of *E. coli*. It is worth emphasizing that the melting temperature (Tm) of F21pBzF mutant was found 74ºC, which was approximately 21ºC higher than that of wild-type. This extreme change in the enzyme's thermal stability was due to the stabilization of the dimer form via a covalent bond between the thiol group of Cys90 and the keto group of pBzF (Fig. 5). These studies have shown that extended building blocks of proteins can lead to the evolution of enzymes with improved properties by forming photo-induced cross-linking.

3.3. Utilization of UAAs for click chemistry

One of the other strategies to form covalent bonds is to utilize UAAs that can react with specific chemical moieties to form a conjugation (Table 1) [51-53]. The formation of covalent bonds with reactive chemical moieties has higher efficiency and selectivity than that by photo-induced crosslinking interactions. UAAs with electrophilic side chains that can react with natural nucleophilic amino acids such as His, Lys and Cys residues of the protein in a proximity-dependent manner to form chemical crosslinks. Haloalkane UAAs, **3** can be incorporated into proteins using orthogonal tRNA/synthetase to form irreversible

Table 1. Applications of UAAs for click chemistry

UAAs	Enzyme (Protein)	Related interaction	Comments	Ref.
3	Z protein ZSPA affibody		Increased the melting temperature	[54]
4	Z protein (Z_{HER2})		Covalently cross-link native membrane receptors with affibody in live cells	[55]
5, 6	GST MBP		Formation of protein-protein conjugation	[56]

covalent thioether bonds as result of S_N2 reactions with adjacent cysteine residues [54]. Such proximity-enabled protein crosslinking (PEPC) strategy can be applied both intramolecular and intermolecular reactions. For example, the formation of intramolecular thioether linkages within the affibody has been observed to improve the denaturation temperature of the affibody compared to the wild type (Tm values of wild-type and mutant-type were 46 and 60°C, respectively) [55]. Similarly, another haloalkane UAA, (*S*)-2-amino-6-(6-bromohexanamido)-hexanoic acid (BrC6K), **4** was able to react with adjacent Cys, His, and Lys residues of the protein and formed long and flexible inter- and intramolecular protein crosslinking [56]. These irreversible covalent bonds can be expected to be used not only in the field of biological diagnostics, bioimaging tools and therapeutics but also in the formation of enzymes to improve stability and other properties [57-59].

Furthermore, formation of long and flexible conjugation through chemical moieties of UAAs can also be utilized as linkers for two or more proteins. The strain-promoted azide-alkyne cycloaddition (SPAAC) used to design artificial metalloenzymes has also been used for the direct formation of fusion-protein [60]. Previously, by adding a long amino acid sequence as a linker between the two proteins to be linked, the two proteins were translated into a single peptide and formed one complex. However, the addition of a long amino acid sequence between two proteins resulted in unintended protein folding or low yield [61]. Lee *et al*. genetically introduced UAAs to form protein-protein linkers through strain-promoted alkyne-azide cycloadditions (SPAAC). Lee *et al.* genetically incorporated *p*-azido-L-phenylalanine (AzF) with an azide group, **6,** and N6-[(1*R*,8*S*,9*R*)-bicyclo[6.1.0]non-4-yn-9-ylmethoxy]carbonyl-L-lysine (BCNK) with an alkyne group, **5,** into two other proteins, glutathione S-transferase (GST) and maltose-binding protein (MBP), respectively

and expressed separately. As a result of the introduction of UAAs, two mutants with azide and alkyne groups have been shown to enable the formation of binding complexes through SPAAC. The formation of protein-protein conjugation through the above method can be formed between two or more enzymes involved in a multistage reaction and used to form a multiple enzyme complex. The multi-step reactions using multi-enzyme complexes formed through UAAs can be expected to have higher efficiency by enabling rapid substrate transfer in the enzyme-relay reaction [62-64].

3.4. Utilization of UAAs for designing novel metalloenzymes

Metal ions bound to specific sites of protein through interaction with residues play a crucial role as cofactor in bio-catalysis or in maintaining the structure of the enzyme [65-69]. Based on the importance of the metal ions, many researchers have developed new metalloenzymes based on the structure and function of native metalloenzyme [70-73]. Among such efforts, research is underway on enzyme engineering to expand the function of proteins by introducing UAAs, which can function as metal binding ligands into non-catalytic scaffolds (Table 2) [74]. For instance, a new approach of enzyme engineering for the production of artificial metalloenzymes has been developed based on the formation of novel active site that can catalyze enantioselective reaction. Incorporation of (2,2'-bipyridine-5yl)alanine (Bpy-ala), **7**, one of the metal-binding UAA, into Lactoccocal multidrug resistance Regulator (LmrR) to bind Cu^{2+} ions as transition metal ion, that resulted in 83% of product yield with catalytic asymmetric vinylic Friedel-Cras alkylation reaction [75]. Also, substitution of Bpy-ala has successfully developed artificial nuclease [76]. Replacement of Lys26 residue present at the protein-DNA interface with Bpy-ala converted the catabolite activator protein (CAP) into a site-specific DNA cleavage protein. K26CAPBpy-ala, DNA binding protein containing UAA was able to cleave a specific sequence of DNA with high affinity for double-stranded DNA in the presence of Cu (II) and 3-mercaptopropionic acid. Furthermore, artificial metalloenzymes based on strain-promoted azide-alkyne cycloaddition (SPAAC) containing *p*-azido-L-phenylalanine (Az), **6** was developed that could be used for the covalent anchoring of bicyclo[6,1,0]nonyne (BCN)-substituted metal complex into scaffold of enzymes. Bioconjugation of metal ions via SPAAC does not require additional reagents for formation of artificial metalloenzymes since it allows the biosynthetic linkage in mild condition between azide-containing amino acid present in protein scaffold and alkyne containing metal cofactors [77]. Therefore, by introducing UAAs, which are capable of forming covalent bonds with indispensable metal ions in bio-catalysis, into scaffolds proteins, artificial metalloenzymes can be developed that can selectively catalyze the desired reactions.

3.5. Utilization of UAAs for protein immobilization

Developing tools for the site specific immobilization of proteins on solid supports is crucial in terms of maintaining proper orientation for effective surface-based analysis of proteins (Table 3) [78,79]. In particular, L-3,4-dihydroxy-phenylalanine (DOPA), **8**, which is tyrosine analog, is used as selective chemical crosslinking agents because they play an important role in adhesion of mussel adhesive proteins (MAP) and protein-protein conjugation [80,81]. In the case of green fluorescent protein (GFP) with DOPA introduced, efficient and simple methods of forming protein-chitosan complexes have been studied [82]. Furthermore, same research group has reported the incorporation of multiple UAAs into ω-TA by both residue specific as well as site specific manner. Residue specific incorporation of 4-fluoroproline into ω-TA improved the thermostability and site-specific incorporation of L-3,4-dihydroxyphenylalanine (DOPA), **8** lead to immobilization of ω-TA onto chitosan or polystyrene beads. Incorporation of these two UAAs showed excellent reusability and stability of the enzyme

Table 2. Applications of UAAs for metalloenzyme design

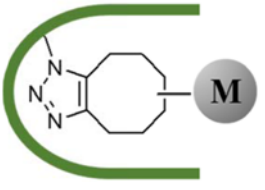
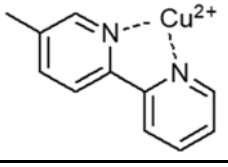
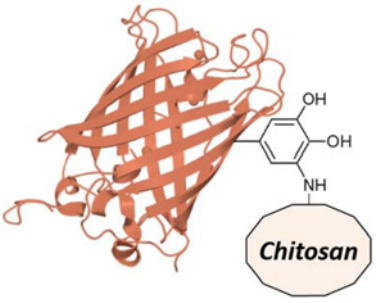
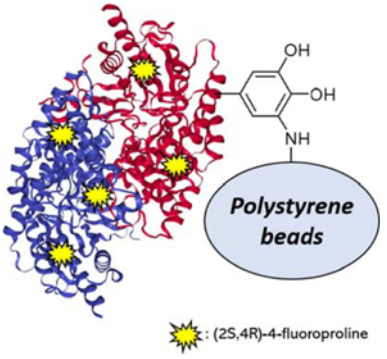
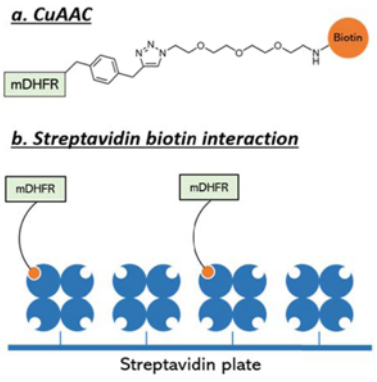
UAAs	Enzyme (Protein)	Related interaction	Comments	Ref.
6	tHisF (scaffold protein)		Catalyzes the SiH and olefin insertion reactions and decomposition of diazo compounds	[77]
7	LmrR (scaffold protein)		Catalyzes Friedel-Cras alkylation reaction	[75]
7	CAP		developed artificial nuclease	[76]

Table 3. Applications of UAAs for immobilization

UAAs	Enzyme (Protein)	Related interaction	Comments	Ref.
8	GFP		Forming protein-chitosan complexes	[82]
8	ω-Transaminase		Immobilization of enzymes with improved thermal stability	[83]
9	mDHFR		Immobilized enzyme maintaining the activity	[84]

for kinetic resolution of chiral amines [83]. Kwon *et al.* reassessed the effect of copper-catalyzed azide-alkyne cycloaddition (CuAAC) on enzyme catalytic activity using murine dihydrofolate reductase (mDHFR) as a model system [84]. After introducing *p*-ethynylphenylalanine (pEthF), **9** with alkane functional group at a specific site of mDHFR, it was conjugated via CuAAC to form site-specific bioconjugation in enzyme. Site-specific bioconjugation between fluorescence dye or biotin and mDHFR-43pEthF was effectively formed without losing substantial catalytic activity. In addition, biotin-conjugated enzyme, mDHFR-43biotin, was successfully immobilized on streptavidin-coated plates while maintaining the activity of enzyme through biotin-streptavidin bond formation. This is a useful tool for the bioconjugation with preserved catalytic activity and has demonstrated the potential to be used in the near future for the development of biosensors through the immobilization of useful enzymes.

4. UAA-mediated Enzyme Engineering through the Formation of Non-covalent Bonds

For an enzyme to act as biocatalyst, maintaining correct folding of tertiary and quaternary structures is a necessary. With few exceptions, it is difficult for conventional enzymes to maintain their structure at higher temperature or chemically harsh environments (Fig. 6) [85]. In order to overcome such limitations, research efforts have been devoted to produce enzymes that can withstand harsh conditions by formation of non-covalent bonds in enzymes through incorporation of UAAs. The advantage of UAA-

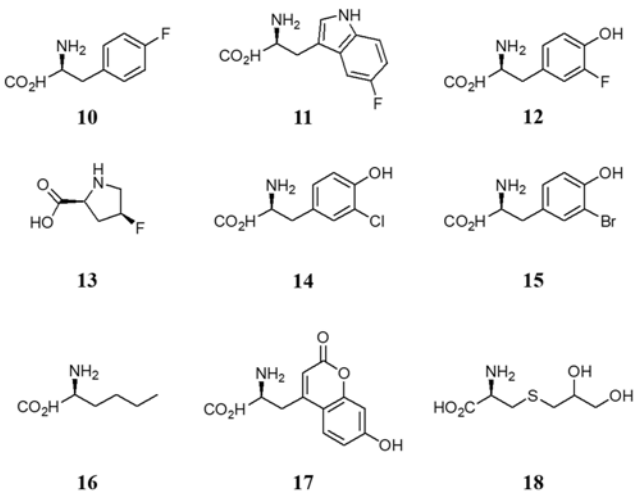


Fig. 6. List of extended UAAs utilized to form non-covalent bonds within proteins. The name of the structures corresponding to UAA are as follows: 4-fluoropheylalanine, **10**; 5-fluorotryptophan, **11**; 3-fluorotyrosine, **12**; 4-(*R*)-fluoroproline, **13**; 3-chloro-L-tyrosine, **14**; 3-bromo-L-tyrosine, **15**; norleucine, **16**; L-(7-hydroxycoumarin-4-yl)ethylglycine, **17**; 2,3-dihydroxypropyl cysteine, **18**.

mediated introduction of non-covalent bonds in the enzymes is reduction in the detrimental effect on enzyme's activity by preserving the reactivity of the surrounding residues.

4.1. Applications of halogenated UAAs

Halogen bonds perform important functions at the bio-molecular level such as substrate specificity, molecular recognition and binding [86-88]. Among them, 'Fluorous effect' is the best example of such enzyme engineering. Presence of fluorine leads to different bond energies, electron distribution, hydrogen bonds, and steric interactions. Because fluorine is only 0.15 A˚ larger than hydrogen and the C-F bond is 0.4 A˚ longer than the C-H bond, many fluorinated amino acids are recognized by the natural aminoacyl tRNA synthetase (aaRS) in *E. coli* and incorporated into proteins *in vivo*. Thus, incorporation of FAAs leads to minimal internal changes with large global changes in the properties. Residue-specific incorporation of FAAs into protein materials can impart stereoelectronic as well as steric effects that can influence stability [89]. Over the time, various fluorinated analogs of leucine, tyrosine, phenylalanine, proline etc. have been incorporated in enzymes to study the stability and functionality (Table 4) [90-93]. Global replacement of aromatic amino acids such as phenylalanine, tyrosine and tryptophan in lipase B from *Candida antarctica* with their respective monofluorinated analogs 4-fluoropheylalanine, **10**, 5-fluorotryptophan, **11**, and 3-fluorotyrosine, **12**, respectively resulted in prolongation of shelf life of all the three mutants compared to their wild type counterpart [93]. In a similar study, residue-specific incorporation of 3-fluorotyrosine, **12**, into industrially important enzyme ω-transaminase (ω-TA) from *Vibrio fluvialis* resulted in enhanced thermostability and organic solvent tolerance without altering the enzymes substrate specificity and enantioselectivity [83,94-96]. As examples of other halogen bonds, Sakamoto *et al.* have reported that incorporation of bulky halogenated UAAs viz. 3-chloro-L-tyrosine, **14**, and 3-bromo-L-tyrosine, **15**, at selected positions of glutathione S-transferase improved the thermal stability [97]. These studies proposed a new mechanism of enzyme stabilization that bulky halogens not only occupy the void spaces within the molecules but also cause local structural changes, including additional *Van der Waals* interactions, halogen bonds and side-chain movements.

The formation of halogen bonds between UAAs and amino acid residues of the proteins has been reported to enhance durability of the enzymes that were previously susceptible to heat and organic solvents [96]. Remarkably, as substituted UAAs are similar in structure to natural amino acids, they did not interfere with the native structure of enzymes, so that substrate specificity and enantioselectivity of the enzyme was retained. These results have suggested that incorporation of halogenated UAAs can serve as an effective strategy to enhance the stability of enzymes.

4.2. Applications of non-halogenated UAAs

Incorporation of hydrophobic UAAs is also favored tool in enzyme engineering. Antranikian and co-worker have replaced the hydrophobic amino acids like methionine, proline, and phenylalanine from *Thermoanaerobacter thermohydrosulfuricus* lipase with their more hydrophobic

Table 4. Application of halogenated UAAs

UAAs	Enzyme	Related interaction	Comments	Ref.
10	Lipase B	Halogen bonds (Electrostatic interactions)	Prolonged the shelf life of lipase activity	[93]
11		Van der waals (Dipole-Diploe)		
12				
12	ω-Transaminase	Halogen bonds (Electrostatic interactions)	Enhance stability in harsh environments	[94]
13		Van der waals (Dipole-Diploe)		[95]
14	GST	Halogen bonds (Electrostatic interactions)	Improvement in thermal stability by	[97]
15		Van der waals (Dipole-Diploe)	5.6 kcal/mol	

Table 5. Application of non-halogenated UAAs

UAAs	Enzyme	Related interaction	Comments	Ref.
16	Lipase	Hydrophobic interaction	10-fold increase in catalytic activity	[98]
17	Phosphotriesterase	Hydrogen bonds (Electrostatic interactions)	Promotion of Michaelis complex formation	[99]
18	NAL	Hydrogen bonds (Electrostatic interactions)	Alters substrate selectivity	[100]

structural analogs in a residue-specific manner (Table 5) [98]. Substitution of these structural analogs not only increased the substrate accessibility range, but also resulted in improved catalytic performance. Especially, when methionine residue was replaced with norleucine, **16**, which is a very hydrophobic UAA, the activity of enzyme increased by 10-fold over the wild-type without thermal activation. Similarly, replacement of Tyr309 residue with L-(7-hydroxycoumarin-4-yl)ethylglycine (Hco), **17**, in phosphotriesterase from *Agrobacterium radiobacter,* that catalyzes the hydrolysis of the paraoxon component of the insecticide at high efficiency, resulted in the improvement of enzyme activity by 8-11 fold. The 7-hydroxyl group of Hco promoted the hydrolysis of paraoxone as a result of the interaction between enzyme and substrate during Michaelis complex and product release [99]. Hydrogen bond formation through UAA is strategy of chemical mutagenesis that changes substrate specificity of the enzyme. N-acetylneuraminic acid lyase (NAL) mutants obtained after random incorporation of various UAAs with thiol groups into active site exhibited significantly increased activity towards aldol reactions of erythrose and pyruvate compared to wild type [100]. For instance, replacement of 2,3-dihydroxypropyl cysteine (Dpc), **18**, with phenylalanine at position 190 formed the stronger H-bond with surrounding residues and thus increased the activity.

Altogether, examples of incorporation of UAAs capable of forming non-covalent bonds such as hydrophobic interaction, hydrogen bonds, etc. enhances interaction between substrates and enzymes at the position of protein domain that binds to substrate, enabling fast catalysis.

5. Concluding Remarks

Development of techniques for the incorporation of unnatural amino acids into proteins has accelerated the evolution of enzyme engineering by exponentially expanding the number of building blocks that had never been possibly used for translation. Especially, through incorporation of UAAs, formation of additional non-covalent bonds and covalent bonds in enzymes has extended characteristics of existing enzymes to a wide range through a variety of reactive functional groups. Incorporation of UAAs, which are very similar in structure and size to natural amino acids, leads to the formation of additional non-covalent bonds such as Van der Waals interaction, hydrogen bonds and hydrophobic interaction, etc. The synergistic effects of UAAs introduced globally through residue-specific manner enable to maintain the stability of the enzymes in harsh environmental conditions like high temperature and organic solvents. In addition, non-covalent bonds formed between substrate and enzyme active site also have been reported to extend substrate specificity and rate of enzyme catalysis. The incorporation of UAAs with highly reactive functional groups has been reported to form various covalent bonds with external ligands and residues in proteins. These kinds of crosslinking interactions have paved the way of using antibodies or fluorescent proteins as reporter proteins. Typically, UAAs capable of forming photo-induced cross-linking, are currently being utilized to capture unknown proteins involved in signal pathway of the metabolism by forming irreversible bonds with C-H bonds through radical reaction. UAAs including chemical moieties such as electrophilic or haloalkanes, can form intermolecular and intramolecular bioconjugation, which is longer and more flexible than covalent bonds formed by existing 20 natural amino acids. It is presumed that these enzyme bioconjugates can function as a linker between enzymes involving multiple steps reaction. In addition, UAAs with functional groups capable of immobilizing metal ions have been successfully used to design artificial metalloenzymes with desired catalytic functions. Various research efforts on enzyme engineering using UAAs' special functional groups have been reported in the recent years and are still ongoing. However, immediate applications to the industry are still difficult in terms of the cost of UAA-fusion enzyme engineering. One way to overcome this is to enable the *in vivo* biosynthesis of UAAs in the target expression host. These enzymes can reduce the cost burden by using a relatively inexpensive precursors, and intermediate products of metabolism as substrates [101-103]. Establishing strategies such as efficient introduction methodologies, increased yields of proteins and inexpensive enzymatic synthesis of UAAs will be a driving force in the field of enzyme engineering. In the near future, use of improved engineered enzymes obtained through incorporation of UAAs will serve as a basis for the utilization of bioprocesses in many fields, including pharmaceutical, medical and industrial.

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