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REVIEW

Recent advancements in enzyme engineering via site-specific incorporation of unnatural amino acids

Hang-Qin Zhu[1,2] · Xiao-Ling Tang[1,2] · Ren-Chao Zheng[1,2] · Yu-Guo Zheng[1,2]

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Abstract

With increased attention to excellent biocatalysts, evolving methods based on nature or unnatural amino acid (UAAs) mutagenesis have become an important part of enzyme engineering. The emergence of powerful method through expanding the genetic code allows to incorporate UAAs with unique chemical functionalities into proteins, endowing proteins with more structural and functional features. To date, over 200 diverse UAAs have been incorporated site-specifically into proteins via this methodology and many of them have been widely exploited in the field of enzyme engineering, making this genetic code expansion approach possible to be a promising tool for modulating the properties of enzymes. In this context, we focus on how this robust method to specifically incorporate UAAs into proteins and summarize their applications in enzyme engineering for tuning and expanding the functional properties of enzymes. Meanwhile, we aim to discuss how the benefits can be achieved by using the genetically encoded UAAs. We hope that this method will become an integral part of the field of enzyme engineering in the future.

Keywords Unnatural amino acids · Site-specific incorporation · Enzyme engineering · Orthogonal translation system

Introduction

Enzymes are important biocatalysts that catalyze a variety of chemical reactions found in nature, which have been extensively applied in the field of fine chemical, agrochemical and pharmaceutical (Naowarojna et al. 2021; Zheng and Kwon 2012). Owing to the growing market demand for biocatalysts with ideal properties such as high activity, stability and selectivity, enzyme engineering techniques are going on to be applied to modulate their properties for industrial applications (Zeymer and Hilvert 2018). Although numerous achievements have been made through traditional techniques such as directed evolution, they are limited to only 20 proteinogenic amino acids building blocks for changing biochemical properties and physiological functions of enzymes (Gargiulo and Soumillion 2021). To overcome such limitations, the development of more innovative methodologies is essential to give rapid access to made-to-order biocatalysts with desired functions.

With unlocking of two rare proteinogenic amino acids (selenocysteine, and pyrrolysine) in some organisms, the concept that proteins composed of only 20 natural amino acids (NAAs) was rethought (Nikic-Spiegel 2020). Replacing the NAAs with unnatural amino acids (UAAs) containing various chemical groups (such as bipyridine, alkynyl, azide) has emerged as a powerful mean to evolve enzymes with ideal physicochemical properties and biological functions (Drienovská et al. 2020; Gao et al. 2019; Jin et al. 2019; Mayer 2019). Generally, incorporation of UAAs can be achieved by two different manners including residue-specific incorporation and site-specific incorporation (Won et al. 2019), which are based on "misacylation" of tRNA with UAAs (Agostini et al. 2017).

The residue-specific manner exploits endogenous translation machinery of auxotrophic host cells to mischarge UAAs into proteins in the absence of the cognate amino acid (Link et al. 2006). Notably, the most important prerequisite for

✉ Ren-Chao Zheng
zhengrc@zjut.edu.cn

1 Key Laboratory of Bioorganic Synthesis of Zhejiang Province, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China

2 Engineering Research Center of Bioconversion and Biopurification of Ministry of Education, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China

residue-specific incorporation is that UAAs must be recognized by endogenous aminoacyl tRNA synthetase (aaRS), enabling its use by the native translational machinery; meanwhile, intracellular cognate amino acids of auxotrophic host cells is removed extremely to avoid the competition with the UAAs (isostructural analogs) (Agostini et al. 2017). The residue-specific manner is straightforward *via* selective pressure incorporation and allows the global replacement of one cognate amino acid with one UAA. The most prominent characteristic of this manner is the multi-site incorporation of UAA into the enzyme, resulting in the synergistic effects (favorable or detrimental). To date, many successful cases have been reported through residue-specific manner to evolve enzymes. For example, the fluorinated ω-transaminase (FY-ω-TA) displayed improved thermostability and organic solvent tolerance by the global replacement of tyrosine with 3-fluorotyrosine (Deepankumar et al. 2014). Although the residue-specific incorporation of UAAs seems as an amazing strategy for manipulating biological functions and properties, its utility for engineering enzymes is still limited since it often cause large perturbations in the folded structure (Won et al. 2019). In addition, residue-specific incorporation is restricted to the chemical structure of UAAs that must be highly similar to NAAs and required available auxotrophic host cells that can use these UAAs in the absence of cognate NAAs (Agostini et al. 2017).

A powerful alternative is the site-specific manner, which allows diverse chemistries to be precisely introduced into a protein of interest (Wang and Schultz 2002). This method relies on introducing an orthogonal translation system (OTS) into the host cells for the incorporation of UAAs (Ravikumar et al. 2015). The OTS requires an orthogonal aaRS/tRNA pair which is specific for UAAs in response to the reassigned codon. Compared with residue-specific manner, the site-specific manner enables the incorporation of a UAA into the target protein with high fidelity. For instance, the incorporation of O-methyl-L-tyrosine into dihydrofolate reductase exhibited the fidelity of translation up to over 99% (Wang et al. 2001). The site-specific incorporation of UAAs was an innovative way for engineering of enzymes. In this review, we offered a brief introduction of site-specific incorporation of UAA and highlighted its application in enzyme engineering.

Site-specific incorporation of UAAs

In the native translation machinery, a transfer RNA (tRNA) is aminoacylated specifically with its cognate amino acid by the corresponding aaRS and then delivered to the ribosome for peptide chain extension (Liu and Schultz 2010). The site-specific incorporation of UAAs relies on an engineered translation system which is orthogonal to the native one to warrant a reassigned codon to specify the target UAAs (Fig. 1) (Wals and Ovaa 2014). In the orthogonal system, the exploitation of the desirable orthogonal aaRS/tRNA pair with reassigned codon has been a challenge. An ideal orthogonal aaRS/tRNA pair is that imported aaRS can effectively charge cognate tRNA with a given UAA in response to a unique codon while not cross-react with native pairs (Nikic-Spiegel 2020). To avoid the competition with endogenous tRNA, the reassignment of the stop codons including

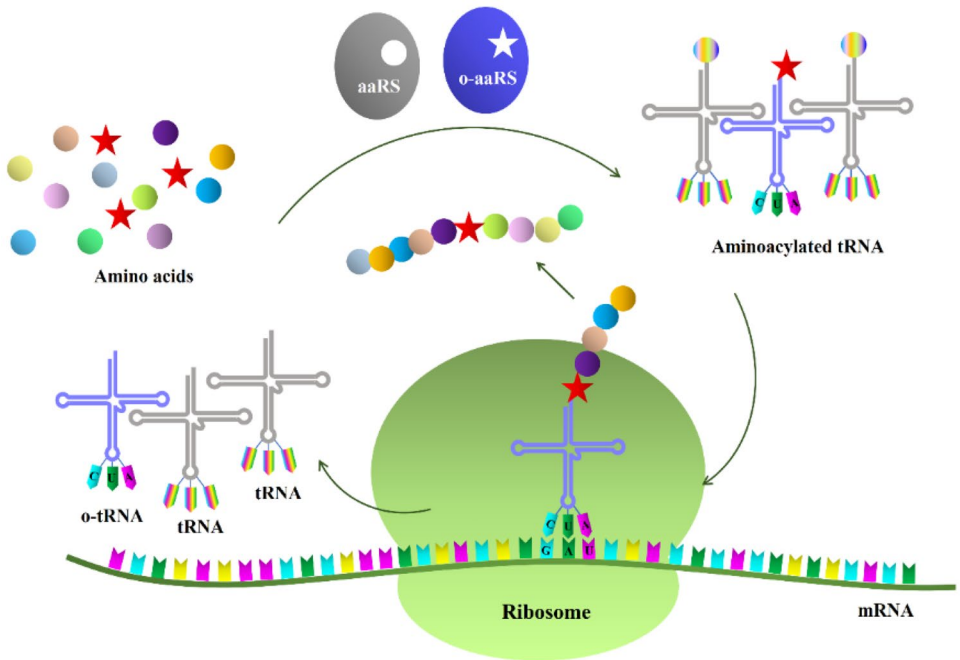

Fig. 1 Schematic representation of UAA incorporation using an orthogonal translation system by amber codon suppression. aaRS, aminoacyl tRNA synthetase; o-aaRS, orthogonal aminoacyl tRNA synthetase; o-tRNA, orthogonal tRNA; Amino acids, natural (circle) and unnatural (star)

amber (TAG), ochre (TAA) and opal (TGA) is regarded as a good strategy which provide the stop signal to terminate the translation process (Kisselev et al. 2003) but not used for encoding any of the NAAs in the endogenous translation systems (Dumas et al. 2015). Typically, the amber codon has attracted a great deal of attention for UAAs incorporation due to low abundance in both prokaryotes and eukaryotes. In addition, the use of opal and ochre stop codons, as well as quadruplet codons has also been reported, especially in the application for incorporation of multiple distinct UAAs into one protein (Neumann et al. 2010; Wan et al. 2010; Yu et al. 2015).

The orthogonal aaRS/tRNA pair is a prerequisite to achieve high efficiency and specificity for UAAs incorporation. To date, numerous orthogonal aaRS/tRNA pairs such as the TyrosylRS/tRNA pair from *Methanocaldococcus jannaschi* (MjTyrRS/$tRNA_{CUA}$ pair), the TyrosylRS/tRNA pair from *E. coli* (EcTyrRS/$tRNA_{CUA}$ pair), the LeucylRS/tRNA pairs from *E. coli* (EcLeuRS/$tRNA_{CUA}$ pair) and the pyrrolysylRS/tRNA pair from *Methanosarcina mazei* (MmPylRS/$tRNA_{CUA}$ pair) and *Methoansoarcina barkeri* (MbPylRS/$tRNA_{CUA}$ pair) have been commonly applied (Chin 2017; Krauskopf and Lang 2020). In general, the orthogonal aaRS/tRNA pairs are only applicable to the corresponding hosts. The MjTyrRS/$tRNA_{CUA}$ pair is suitable for *E. coli* system whereas the EcTyrRS/$tRNA_{CUA}$ pair and EcLeuRS/$tRNA_{CUA}$ pair are fit for eukaryotes. Comparatively, the PylRS/$tRNA_{CUA}$ pair manifests more flexibility due to its orthogonality in both prokaryotes and eukaryotes (Hancock et al. 2010; Wan et al. 2014). In addition, the orthogonal pairs can be engineered for UAAs incorporation by successive rounds of positive selection (based on the chloramphenicol-resistance gene) and negative selection (based on lethal barnase gene) (Young and Schultz 2010). With these wild-type or evolved orthogonal aaRS/tRNA pairs, more than 200 UAAs could successfully be introduced into proteins, which now is becoming a valuable tool kit in the application of protein engineering and enzyme engineering (Vargas-Rodriguez et al. 2018).

Enzyme engineering via site-specific incorporation method

In addition to traditional enzyme engineering techniques, genetically encoded UAAs incorporation has emerged as a powerful technology to open new avenues for improving enzyme catalytic properties, exploring enzyme mechanisms and even creating enzymes with new catalytic activity, explicitly delivering their values for a variety of desired applications in biocatalysis (Gargiulo and Soumillion 2021; Mayer et al. 2019). In the following text, an overview of many successful attempts of UAAs mutagenesis-based engineering enzymes is presented and these examples are grouped based on the effect on enzyme properties such as enhanced activity, enhanced stability and altered selectivity, which are also displayed in Table 1. The chemical structures of UAAs used in these examples are mentioned by number (bold font); structures 1–24 is depicted in Fig. 2. In addition, we described a schematic representation of the distribution of introduced UAAs into different positions of enzymes (I–VI) which are also displayed in Fig. 3.

Engineering of enzyme with improved activity

The catalytic capacity of enzymes has attracted great interest for chemists and biologists during the past decades. The main source of catalytic power of an enzyme is its active site, the pocket (often buried) in which the catalytic reaction occurs, involving in a small subset of residues that participate in substrates (and/or cofactor) binding and the catalytic reaction (Holliday et al. 2009). The conformation of active site is susceptible to changes in the global protein structure, and sometimes even imperceptible changes caused by residues remotion from the active site. Introducing genetically encoded UAAs will endow the enzymes with unprecedented catalytic behavior by altering the active site environment such as steric and electronic interactions either directly or indirectly, which may not be achieved by substitution of any of NAAs.

The site-specific incorporation of UAAs into substrate binding regions of enzymes may be beneficial for enzyme activity. For example, replacing phenylalanine (Phe) 124, a critical residue for substrate binding, with a diverse set of UAAs in the prodrug activator nitroreductase (NTR) prominently increased its catalytic properties. Among those variants, the NTR containing *p*-nitrophenylalanine, **1**, (pNF-NTR) exhibiting more than 30-fold and 2.3-fold improvement in the catalytic efficiency (k_{cat}/K_M), over that of the native NTR and the best natural mutants at the same site, respectively. The improved activity of pNF-NTR might be attribute to the effect of pi-stacking interaction between polarized aromatic ring of pNF and aromatic substrate (Jackson et al. 2006). In addition, Tyr309, a residue lies in substrate binding region of *Agrobacterium radiobacter* phosphotriesterase (arPTE), was replaced by the L-(7-hydroxycoumarin-4-yl) ethylglycine (Hco), **2**, generating an over 8-fold improvement in turnover rate towards the paraoxon hydrolysis compared to the native one. The electrostatic repulsion between negative charge associated with Hco and the product (4-nitrophenolate) results in an accelerated release of product, revealing the importance of a trade-off between efficient binding and turnover (Ugwumba et al. 2011).

Moreover, the adjustment of metal coordination environments in tunable protein scaffolds through incorporating

Table 1 Altering/enhancing enzyme properties through site-specific incorporation

Functional property	Enzyme	UAAs	Positions	Characteristics	Reference
Activity	Nitroreductase	1	IV	Improved catalytic efficiency (k_{cat}/K_M): > 30-fold over the native NTR; > 2.3-fold over the best natural mutants at position 124	(Jackson et al. 2006)
	Phosphotriesterase	2	IV	> 8-fold improved in the turnover rate	(Ugwumba et al. 2011)
	Ascorbate peroxidase	3	VI	~5-fold increased total turnover number	(Green et al. 2016)
	Sperm whale myoglobin (Mb)	3	VI	3.7-fold improved catalytic efficiency	(Pott et al. 2018)
	A myoglobin-based functional oxidase model	4; 5	III	> 2.0-fold increased turnover number	(Yang et al. 2015; Yu et al. 2015)
	TEM-1 β-lactamase	6	I	~8.0-fold improved catalytic efficiency	(Xiao et al. 2015)
Stability	Glutathione S-transferase	4; 7	II	> 5.0 kcal/mol increased melting enthalpy	(Ohtake et al. 2015)
	T4 lysozyme	4	II	3 kcal/mol increased melting enthalpy	(Carlsson et al. 2018)
	Homoserine O-succinyltransferase	8	II	21 °C increased melting temperature	(Li et al. 2018)
	ZSPA affibody (Afb)	9	II	Increased melting temperature from 46 °C up to 60 °C	(Xiang et al. 2014)
	β-lactamase	10	II	9 °C increased melting temperature	(Liu et al. 2016)
Altering regio- and stereo- selectivity	Cytochromes P450	11–14	I	Alters regioselectivity	(Kolev et al. 2014)
	Diketoreductase	15–18	II	Alters enantioselectivity	(Ma et al. 2014a)
	Lipase	11; 16; 19; 20	I	Alters diastereoselectivity	(Yu et al. 2021)
Altering substrate specificity	Murine dihydrofolate reductase (mDHFR)	13	I	7.6-fold increased catalytic efficiency ratio ((k_{cat}/K_M for FOL)/ (k_{cat}/K_M for DHF))	(Zheng et al. 2015)
	The mDHFR	13; 21	I	a significantly reduced binding affinity toward the inhibitor	(Zheng and Kwon 2013)
Expanding reaction scope	Multidrug transcriptional regulator (LmrR)	11	III	Enables abiological hydrazone and oxime formation reaction	(Drienovská et al. 2018)
	Superfolder yellow fluorescent protein	8	III	High CO_2/CO conversion quantum efficiency of 2.6%	(Liu et al. 2018)
	Mb (H64V, V68A)	3	VI	Enables abiological olefin cyclopropanation reactions (convert styrene without reductant and under aerobic conditions)	(Hayashi et al. 2018)
	Mb (H64V, V68A) [Fe (DADP)]	3	VI	Enables abiological olefin cyclopropanation reactions (convert a series of electron-rich and electron-deficient alkenes)	(Carminati and Fasan 2019)
	LmrR	22	V	Novel catalytic activity: asymmetric vinylogous Friedel–Crafts alkylation reactions	(Drienovská et al. 2015)
Enzyme immobilization	Lipases	23	II	> 6.0-fold improved catalytic activity and better thermo-stability	(Wang et al. 2016)
	ω-Transaminases	24	II	A great reusability for 10 cycles	(Deepankumar et al. 2015)

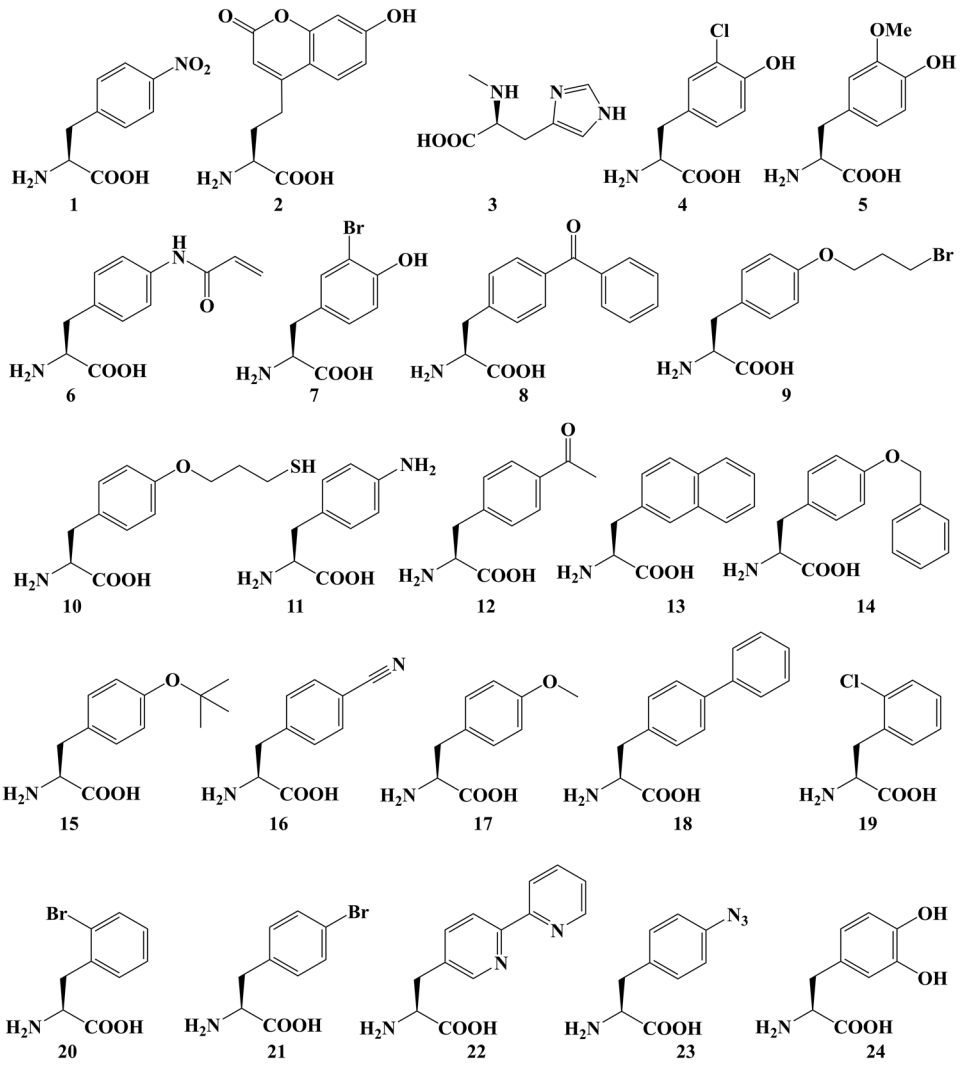

Fig. 2 The structures of UAAs discussed in this Minireview. *p*-nitrophenylalanine (pNF),1; L-(7-hydroxycoumarin-4-yl) ethylglycine (Hco), 2; Nδ-methyl histidine (NMH), 3; 3-chloro-L-tyrosine (ClTyr), 4; 3-methoxytyrosine (OMeY), 5; *p*-acrylamido-phenylalanine (AcrF), 6; 3-bromo-L-tyrosine, 7; 4-benzoyl-L-phenylalanine (pBzF), 8; O-(3bromoethyl)-L-tyrosine (BprY), 9; O-(4-mercaptobutyl)-L-tyrosine (SbuY), 10; *p*-amino-phenylalanine (pAmF), 11; *p*-acetyl-phenylalanine (pAcF), 12; 3-(2-napthyl) alanine (NapA), 13; O-benzyl-tyrosine (OBnY), 14; O-tert-butyl-L-tyrosine (BuOF), 15; 4-cyanophenylalanine (CNF), 16; 4-methoxy-L-phenylalanine (OMeF), 17; 4-phenyl-L-phenyalanine, 18; *o*-chlorophenylalanine, 19; *o*-bromophenylalanine (oBrF), 20; *p*-bromophenylalanine (pBrF), 21; (2,2'-bipyridin-5yl) alanine (BpyA), 22; *p*-azido-L-phenylalanine, 23; L-3,4-dihydroxyphenylalanine (DOPA), 24

UAAs can contribute to improving the catalytic properties of the enzyme. Histidine is an important residue in the catalysis mechanisms of many enzymes such as serine peptidases (Polgar 2005), arylamine N-acetyltransferase (Sim et al. 2008) and heme enzymes (Roach et al. 2000). It participates in forming the important interactions with neighboring functional residues and substrates, and also act as a metal ion chelating ligand (Green et al. 2016; Hongsthong et al. 2004). The site-specific incorporation of UAAs into heme peroxidases, which catalyze various oxidative transformations with a histidine as the axial ligand, can improve the catalytic properties of the enzyme. For example, the introduction of Nδ-methyl histidine (NMH), **3**, into the engineered ascorbate peroxidase (APX2) at position 163 and sperm whale myoglobin (Mb) at position 93 lead to the substantial increase in total turnover number (5-fold) (Green et al. 2016) and catalytic efficiency (k_{cat}/K_M) (3.7-fold) (Pott et al. 2018), respectively, toward oxidation of guaiacol compared to the parent enzymes. Notably, manipulating coordination ligand environments in metalloenzymes through genetically encoded UAAs could be realized to evolve biocatalysts with augmented catalytic properties.

In addition, tyrosine (Tyr) is found to be highly conserved in numerous enzymes and is a crucial residue at active center of enzyme due to its ability to donate electron and proton in catalytic mechanism (Jackson et al. 2007; Kong et al. 1992). It's reported that fine-tuning Tyr residues redox potential by genetically incorporating Tyr analogs may enhance the oxidase activity. For example, the introduction of 3-chlorotyrosine (ClTyr), **4**, in the active site of a myoglobin-based functional oxidase models (Phe33Tyr-Cu_BMb) at positon 33 caused a significant increase of the turnover number (>2.0 fold) (Yu et al. 2015). Similarly, the substitution of 3-methoxytyrosine (OMeY), **5**, also led to an improvement of turnovers (>2.0 fold) (Yang et al. 2015). The results may be attribute to a change in proton-donating or electron-donating ability of the phenol ring by the substitution of ClTyr or OMeY.

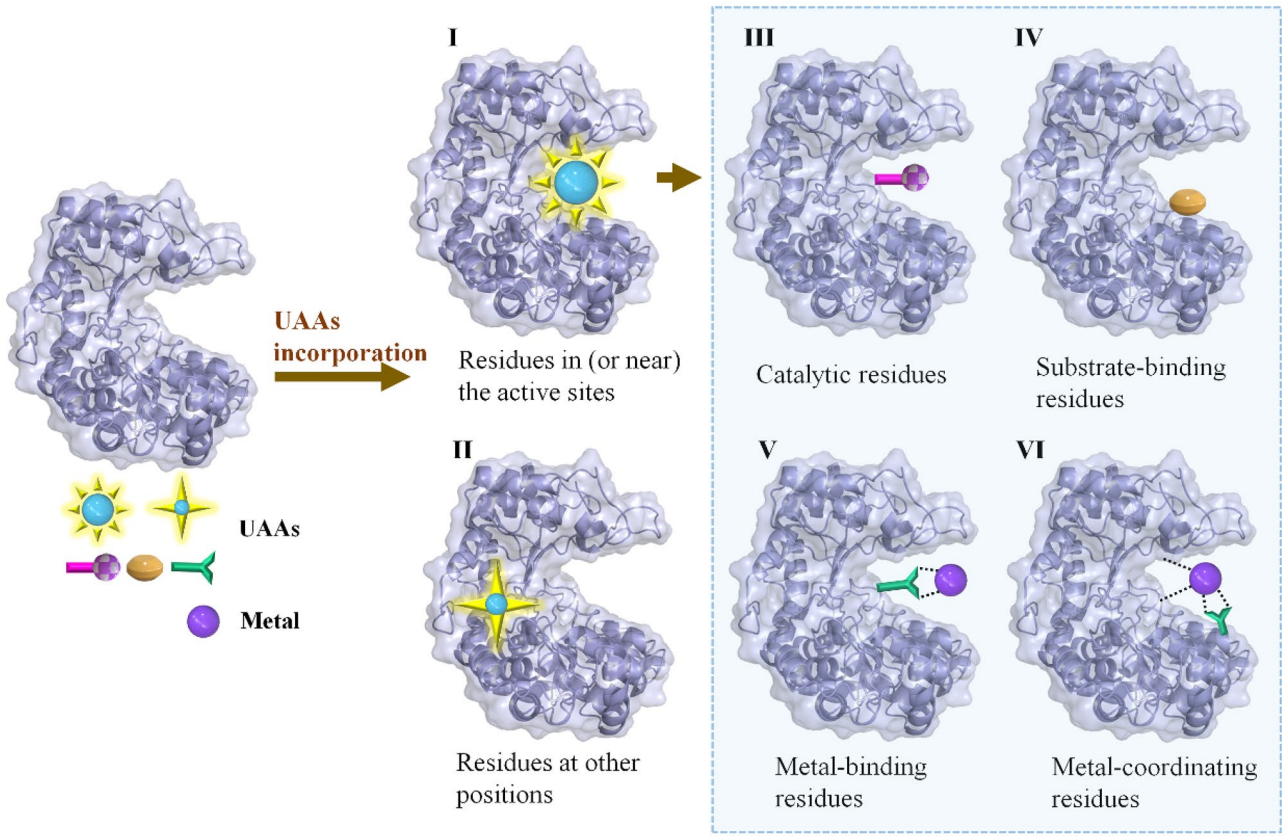

Fig. 3 Schematic representation of the distribution of genetically encoded UAAs in different positions of enzymes

Except for the introduction of UAAs at predetermined positions, the genetic code expansion is considered as a potential tool for use in directed enzyme evolution. In term of catalytic activity, a single substituted mutation library of TEM-1 β-lactamase variants with 10 structurally distinct UAAs randomly throughout the protein was reported (Xiao et al. 2015). The β-lactamase variant with *p*-acrylamido-phenylalanine (AcrF), **6**, at position of 216 (near the active site) was obtained through a growth-based screening, leading to a substantial improvement in catalytic efficiency (~8.0-fold). In comparison, it was not available with any of NAAs at this position to achieve similar improvement.

Engineering of enzyme with enhanced stability

The stability of protein relies on plenty of van der Waals, electrostatic interactions, hydrogen bonding and a handful of disulfide bonds (Gromiha 2010). The ability to maintain enzyme stability under harsh conditions is one of major challenges in the enzyme engineering. Despite a lot of efforts on enhancing enzyme stability through the traditional approaches such as directed evolution have been achieved, the application of genetically encoded UAAs by introducing unusual noncovalent or covalent interactions has provided a new avenue to evolve enzyme with improved stability.

The incorporation of halogenated amino acids has proved to be a very useful strategy for modifying the properties of proteins, especially in terms of stability (Ohtake et al. 2015). The UAAs with the bulky halogens such as chlorine and bromine can be site-specific incorporated into proteins, and several successful cases have been reported. For instance, Ohtake et al. (2015) have reported that incorporation of ClTyr, **4**, and 3-bromo-L-tyrosine, **7**, into glutathione S-transferase at seven selected positions exhibited significantly enhanced structural stability with an increase of 5.2 and 5.6 kcal/mol, respectively. A tightly packed protein interior was observed from crystal structure of the variants, most likely deriving from the additional halogen bond interactions between inter-residue and the bulky halogen atoms. Similarly, engineering the T4 lysozyme by site-specific introducing ClTyr, **4**, offered an increased thermal stability (~1 °C for melting temperature and 3 kcal/mol for the melting enthalpy) (Carlsson et al. 2018). Hence, the site-specific incorporation of halogenated UAAs seem to be an effective strategy for enhancing enzyme stability as larger halogens may fill the void to exert stabilizing effects and form a distinctive halogen bond interaction with adjacent residues.

The disulfide bond that generated between two cysteine (Cys) residues is vital for the function and stability of numerous enzymes (Rietsch and Beckwith 1998). However, the reversibility and inherent redox sensitivity, as well as the constraints of distance and dihedral angle restrict its applications in enzyme engineering (Berkmen 2012). As such, selective introduction of unusual covalent bonds provides new avenues to address such issues. Owning the unique reactivity of cysteine thiol, the design of a new covalent bond between a genetically encoded UAA and an adjacent Cys residues can be readily achieved. More recently, various achievements have been witnessed by using this strategy. For instance, an evolved homoserine *O*-succinyltransferase (*metA*) equipped with (*p*-benzoylphenyl) alanine (pBzF), **8**, at position 21, resulting in a 21 °C improvement of melting temperature. Phe 21 lies in an extremely flexible N-terminal domain and its substitute with pBzF may lead to a stabilization of the native dimeric configuration of *metA* due to the cross-link between the aryl keto group of pBzF 21 and thiol group of Cys 90 (Li et al. 2018). The schematic diagram of the engineered cysteine cross-links was shown in Fig. 4a. In addition, the site-specific introduction of haloalkane UAAs into ZSPA affibody (Afb) also exhibited significantly improved thermal stability, because of the formation of intramolecular covalent linkage between alkyl halides and an adjacent Cys (Fig. 4b). Among those variants, the replacement of Phe 30 with O-(3bromoethyl)-L-tyrosine (BprY), **9**, offered an obvious increased melting temperature, from 46 ^{o}C up to 60 ^{o}C (Xiang et al. 2014). Similarly, the β-lactamase mutants equipping with UAAs with long side-chain thiols manifested markedly improved thermostability due to the formation of an unusual disulfide bond between Cys and UAAs (Fig. 4c). In particular, the variant R65C/A184SbuY containing O-(4-mercaptobutyl)-L-tyrosine (SbuY), **10**, showed a 9 °C enhancement in thermal stability, which was higher than most of the engineered Cys disulfide bonds with a common Tm increase around 5 °C (Liu et al. 2016). These results suggested that introducing an unusual covalent bond between a genetically encoded UAA and a NAA exhibit a tremendous potential for the enhancement of enzyme stability.

Engineering of enzyme with altered regioand stereo-selectivity

Apart from enhancement of activity and stability, altering regioand stereo-selectivity is also important for the application of enzymes. To date, controlling regioand stereo-selectivity of enzymes remains a great challenge in the field of biocatalysts. In terms of technologies, the site-specific incorporation of UAAs is considered as an effective complement in enzyme engineering for altering such properties. For example, incorporating four Tyr analogs including *p*-aminophenylalanine (pAmF), **11**, *p*-acetyl-phenylalanine (pAcF), **12**, 3-(2-napthyl) alanine (NapA), **13**, and O-benzyl-tyrosine (OBnY), **14**, into a cytochromes P450 enzymes (P450s) at 11 active-site positions resulted in large shifts in regioselectivity of these engineered variants (Kolev et al. 2014). The native enzyme converts (*S*)-ibuprofen methylester to benzylic alcohol (62%) and allylic alcohol (38%) derivatives. Comparatively, the variant (Ala78pAcF) containing pAcF at position 78 obtained 88% of benzylic alcohol and 12% of allylic alcohol derivatives, while another variant (Ala328NapA) incorporated with NapA at position 328 produced 5% of alcohol and 95% of allylic alcohol derivatives. As such, introducing UAAs specifically into the active sites of enzymes seem to be a useful strategy in altering regioselectivity of the enzyme.

Also, the method allows to fine-tune enantioselectivity of enzymes to an ideal level. Diketoreductase (DKR), a homodimeric protein, is a useful biocatalyst that can reduce various ketones to chiral alcohols. Trp222 of DKR lie in

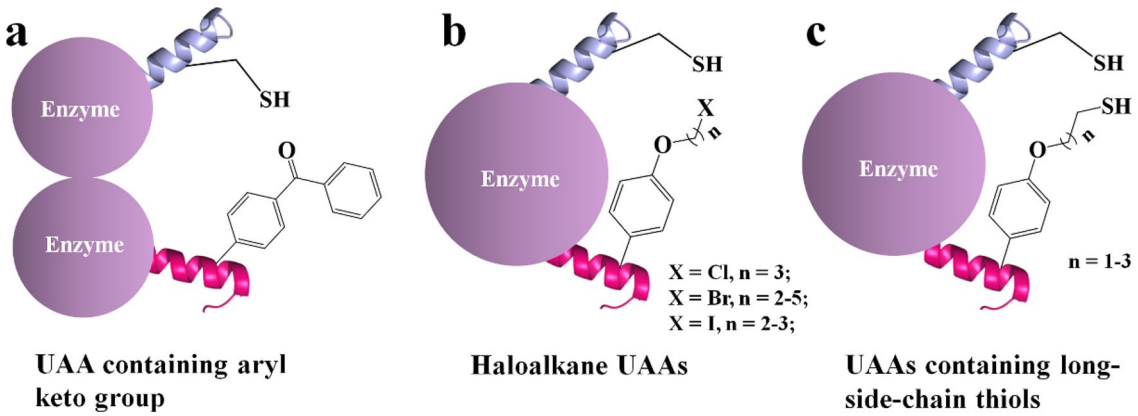

Fig. 4 The schematic diagram of the engineered cysteine cross-links. **a** Genetically encoded UAAs containing aryl keto group. **b** Genetically encoded haloalkane UAAs. **c** Genetically encoded UAAs containing long-side-chain thiols

the C-terminal hydrophobic dimeric interface and is crucial to structural integrity and dimerization of the enzyme (Huang et al. 2012). Replacing Trp222 with Tyr analogs, O-tert-butyl-L-tyrosine (BuOF), **15**, 4-cyanophenylalanine (CNF), **16**, 4-methoxy-L-phenylalanine, **17**, 4-phenyl-L-phenyalanine, **18**, alter the enantiopreference of DKR toward 2-chloro-L-phenylethanone (Ma et al. 2014a). The parent enzyme presented a *Re*-preference that converts 2-chloro-L-phenylethanone into *R*-alcohols with a 9.1% enantiomeric excess (*e.e.*) value. The replacement with BuOF, **15**, causes a notable change in the conformation, leading to an enhanced enantioselectivity of DKR with a 33.7% *e.e.* value. Interestingly, the substitution of Trp222 with CNF, **16**, yields an enantiopreference inversion of DKR from *Re*- to *Si*-preference.

Except for enantioselectivity of enzymes, the diastereoselectivity is also an important property when multiple chiral centers exist in the substrates. Genetically encoded UAAs apply equally to manipulation of enzyme diastereoselectivity. Yu et al. (2021) proposed that the diastereoselectivity of *Pseudomonas alcaligenes* lipase (PaL) may be determined by certain key factors such as the flexibility of the active center and steric hindrance after analyzing the interactions between PaL active sites and the substrate L-menthol propionate with three chiral centers. Thereafter, four UAAs with diverse size and polarity including pAmF, **11**, CNF, **16**, *o*-chlorophenylalanine, **19**, and *o*-bromophenylalanine (oBrF), **20**, were introduced in 9 selected sites at the active center of PaL, generating a series of variants that obviously enhanced diastereopreference. Among those variants, the substitution of Ala253 with all four UAAs exhibited higher conversion rate and concurrently the diastereomeric excess (90%–95%) was higher than parent enzyme over 40%.

Engineering of enzyme with altered substrate specificity

Manipulating the substrate specificity of enzymes has always been the research topic for enzyme engineering (Zheng et al. 2015). In general, the synergism of substrate access and enzyme-substrate recognition interactions within the active center can affect the substrate specificity of the enzymes (Stevenson et al. 2000). Nowadays, various techniques utilizing NAAs or UAAs, particularly genetically encoded UAAs, have been applied to re-design the active site of enzymes to fit new substrates or poor substrates. The murine dihydrofolate reductase (mDHFR) readily catalyzes dihydrofolate (DHF) into tetrahydrofolate, whereas folate (FOL) is regard as a relatively poor substrate for the enzyme. Zheng et al. introduced NapA, **13**, into the active site of mDHFR at position 31 to explored whether the enzyme substrate specificity can be changed (Zheng et al. 2015). The variant exhibits a 2-fold improvement in binding affinity (K_M) toward the substrate FOL. Meanwhile, it also showed a 7.6-fold increase in catalytic efficiency ratio ((k_{cat}/K_M for FOL)/ (k_{cat}/K_M for DHF)) compared with the native mDHFR, demonstrating a dramatically change in the substrate specificity of the variant toward FOL.

The inhibitory effects of substrates, products, and other compounds on enzyme activities are a rather common phenomenon in the field of biocatalysis. In particular, the competitive inhibitors usually compete with the substrates for the active sites of an enzyme due to highly structural similarities between substrate and inhibitor (Alam et al. 2017). To selectively weaken the binding of inhibitors without compromising enzyme substrate binding is a huge challenging. Rationally introducing UAAs into the active sites of enzyme can be a promising strategy to control the binding affinity of inhibitors. For instance, Zheng and his co-workers set a model system including the enzyme mDHFR, its substrate DHF and inhibitor methotrexate to selectively control the inhibitory effect (Zheng and Kwon 2013). Replacing the key phe31 with NapA, **13**, and *p*-bromophenylalanine (pBrF), **21**, resulted in a significantly reduced binding affinity toward the inhibitor with a negligible impact on the catalytic efficiency toward the substrate DHF.

Expanding the reaction scope of enzyme

The posttranslational modification (PTM) such as glycosylation, acetylation, phosphorylation and methylation of the amino acid side chains is a conventional approach to expand the catalytic repertoire and regulate the function of enzymes (Okeley and van der Donk 2000; Walsh et al. 2005; Zhang et al. 2017; Zheng et al. 2018). However, it is difficult to introduce the specific modifications into the desired sites of the enzymes *via* PTM. Catalytic promiscuity, an inherent property of natural enzymes, is a catalytic ability of enzyme for markedly different chemical transformations (Bornscheuer and Kazlauskas 2004). Making use of the catalytic promiscuity provides access to create reactivities unrelated to the native function of an enzyme and even generate new biocatalysts with the ability to catalyze abiological transformation. The catalytic promiscuity of enzymes can sometimes be augmented with the re-design of the hydrophobic pore through enzyme engineering technology. Since site-specific incorporation allow various UAAs with diverse functional groups into enzymes at any desired site, it is considered as a promising approach for mimicking nature PTM and affording novel functions that would not be readily achievable with NAAs.

Embedding genetically encoded UAAs as a catalytic residue into the enzyme may endow it with novel catalytic ability that are naturally unavailable (Drienovská and Roelfes 2020). For instance, the incorporation of pAmF, **11**, into the hydrophobic pore of *Lactococcus lactis* multidrug

transcriptional regulator (LmrR), enables the enzyme to catalyze the abiological hydrazone and oxime formation reaction. The catalytic proficiency is attribute to a promiscuous binding pocket generating from the combination effects of the reactive pAmF residue (a potentially novel catalytic residue) and the hydrophobic interactions in proteinaceous scaffold (Drienovská et al. 2018). Repurposing solar energy into chemical energy has attracted a tremendous interest. However, it is hard to improve and expand the functions of photosensitizers through enzyme engineering. Liu et al. (2018) utilize the genetic codon expansion technology to incorporate a UAA pBzF, **8**, into a superfolder yellow fluorescent protein (sfYFP) at position 66. Tyr66 is an important chromophore residue in the native sfYFP that generate a highly fluorescent p-hydroxybenzylidene-5-imidizolinone species through the spontaneous transformation of its tripeptide, Gly65-Tyr66-Gly67. Then, the variant was followed by multisite mutations and combined with organic nickel-terpyridine complex and the final variant PSP2T2 was obtained. This designed CO_2-reducing enzyme exhibited a CO_2/CO conversion quantum efficiency of 2.6%, which is over most reported CO_2 photoreduction catalysts.

Cyclopropane compounds can be chemically synthesized through the metal-catalyzed cyclopropanation of olefins with diazo compounds (Nakagawa et al. 2015). More recently, several engineered enzymes such as haem proteins have emerged as promising alternatives for catalyzing the abiological reactions such as carbene-transfer reaction (Carminati and Fasan 2019). In terms of enzyme engineering technology, the genetically encoded UAAs has been proved to be effective to realize the non-natural reactions. For example, the substitution of a His residue of the myoglobin variant Mb (H64V, V68A) at position 93 with the NMH, **3**, augments the enzyme's promiscuous carbene-transfer chemistry, endowing the ability of the enzyme to convert the electron-rich styrene to the cyclopropane product without reductant and under aerobic conditions (Hayashi et al. 2018). The use of NMH as the axial haem ligand enables to capture an unusual bridging Fe (III)–C(carbene)–N(pyrrole) configuration, which participates in the cyclopropanation of substrate. The electron-rich olefins can be readily converted to cyclopropane compounds by using these engineered biocatalysts. However, the cyclopropanation of electron-deficient olefins remains an unmet goal in nature. To overcome such limitation, introducing unnatural components (cofactors and UAAs) into the enzyme may enable it to be a best-of-both-worlds catalyst (Agostini et al. 2017). A variant Mb (H64V, V68A) was designed by site-specific incorporating a UAA NMH and an unnatural iron-porphyrin cofactor to form a variant (Mb (H64V, V68A, H93NMH) [Fe (DADP)]). The variant exhibited high efficiency for the asymmetric cyclopropanation of electron-rich olefins, even for the electron-deficient alkenes (Carminati and Fasan 2019). These results suggest that introducing a metal coordinating residue into the active sites of the enzyme by modulating metal coordination environments may be a practical strategy to extend the capabilities of enzymes for abiological chemical transformations.

Apart from a metal coordinating residue, incorporating a metal-binding residues may also be another strategy to achieve this goal (Drienovská and Roelfes 2020; Drienovská et al. 2015) designed an artificial metalloenzymes by introducing of a metal-binding amino acid (2, 2'-bipyridin-5yl) alanine (BpyA), **22**, at various positions, which was able to bind a transition metal ion into the LmrR. The resulting artificial metalloenzymes offered a novel catalytic activity, enabling the asymmetric vinylogous Friedel–Crafts alkylation reactions of indoles derivatives with α, β-unsaturated 2-acylimidazoles. The best variant (LmrR_LM_M89X_F93W) showed an 83% *e.e.* value for the product. It contained a BpyA at position 89, which lie in the hydrophobic pore of the enzyme.

Overall, incorporating UAAs into enzymes through the genetically encoded technology has a considerable effect on the field of enzyme engineering to acquire make-to-order biocatalysts with novel catalytic properties that have no equivalent in nature nowadays.

Application of site-specific incorporation in enzyme immobilization

Amino acids featuring reactive functional groups offer a chemical handle to immobilize it to a solid support. Immobilizing enzymes with various advantages such as improved longevity, enhanced stability, excellent reusability and easy separation from the reaction mixtures, have received great attention on industrial applications. In general, the enzyme immobilization methods are divided into four types including adsorption, covalent bonding, entrapment and cross-linking (Nguyen and Kim 2017). Among them, covalent immobilization is the preferred method to form covalent bonds leading to the stability of enzyme. Although the conventional covalent immobilization has been frequently applied, its randomization may cause unwanted covalent linkage between enzyme and matrix. To overcome this issue, site-specific immobilization by incorporating UAAs into the enzyme is easy to install the reactive handle at a predefined position (Wang et al. 2016). For example, *p*-azido-L-phenylalanine (pAzF), **23**, was firstly inserted at different sites of lipase to make it easy to be coupled with support using strain-promoted azide-alkyne cycloaddition. Among these rational immobilized lipases, several variants presented better thermo-stability and higher relative activity such as AzPhe-Lip243 (6.0-fold improvement) and AzPhe-Lip274 (6.8-fold improvement) than those lipases with traditional immobilization using glutaraldehyde (Wang et al. 2016).

In addition, L-3,4-dihydroxyphenylalanine (DOPA), **24**, is regard as a selective chemical cross-linker and the site-specifc incorporation of DOPA into enzymes enables the enzymes to specifically crosslink with polysaccharides or amino-group containing materials. The *ω*-transaminases (*ω*-TA) equipped with DOPA were successfully anchored in chitosan or polystyrene beads, resulting in a great reusability (10 cycles) in the kinetic resolution of chiral amines (Deepankumar et al. 2015). These reports manifested that UAA-mediated bioconjugation can be an efficient and unique tool addressing unmet needs in enzyme engineering, especially rational immobilization.

Challenges and outlook

The incorporation of UAAs into proteins has proved to be a prospective strategy to improve and alter the properties of enzymes such as activity, stability, selectivity. Despite UAAs incorporation for enzyme engineering would increase versatility and possibilities of enzyme catalysis, there are many issues waiting to be coped. How to rapidly and precisely predict the "hot spots" to evolve the enzyme with ideal properties remains a formidable challenge in enzyme engineering. Therefore, it is of great significance to sum up some practical strategies based on the existing successful examples. With these exciting examples, some superficial rules may be helpful for engineering enzymes. For instance, introducing a metal-binding UAA and a UAA possessing a unique reactive side chains as a catalytic residue were demonstrated to be good tactics to extend the reaction scope of enzyme. In addition, introducing halogenated UAAs or UAAs that can spontaneously cross-link with Cys may be beneficial to enhance stability. The positions outside the active sites may fit better for enzyme immobilization.

Nowadays, continued efforts have been invested in accelerating the evolution of enzyme through incorporation of UAAs (Gargiulo and Soumillion 2021; Mayer 2019; Ribeiro et al. 2019). Nevertheless, the applications of UAA mutagenesis-based enzyme engineering are still restricted. There are significant challenges currently being addressed including the expensive and commercial unavailable UAAs and poor protein yields as well as unattainable incorporation of multiple UAAs (Gao et al. 2019).

The high cost of UAAs and their commercial unavailability can preclude researchers from considering the use of this method to engineer enzymes, let alone for further utilization of UAAs in industrial applications. It is reported that the total synthesis cost of green fluorescent protein containing *p*-Propargyloxyphenylalanine is up to USD 0.658 per 100 μg protein (Shrestha et al. 2014). One valuable way to overcome such limitations is exploiting synthetic methodologies for chiral amino acids. Notably, enzymatic asymmetric synthesis of UAAs has attracted extensive attention (Xue et al. 2018). For example, tyrosine phenol lyase exhibits its potential for synthesis of tyrosine analogs, such as L-DOPA (Kim et al. 2018), halogenated tyrosine, methoxytyrosine (Nagasawa et al. 1981) and 2-amino-3-(8-hydroxyquinolin-5-yl) propanoic acid (Liu et al. 2013). With ongoing efforts on asymmetric synthesis of UAAs via chemical and enzymatic methods, it will be a cheap and accessible commodity, which will greatly promote the application of the incorporation of UAAs into proteins. Another way to address such limitation is designing the host cells to enable biosynthesis of UAAs *in vivo*. Evolving metabolic pathways of host cells to produce UAAs from cheap sources and simultaneously fetching them into desired enzymes will facilitate the applications of engineered enzymes (Chen et al. 2018; Ma et al. 2014b).

Although great value of genetically encoded UAAs has been exhibited in the field of biocatalysis, the limitation of poor protein expression levels was still not be tackled. In view of this problem, the coordinate optimization of aaRS, tRNA, codons, the ribosome and elongation factor may improve the UAAs incorporation efficiency (Jin et al. 2019; Park et al. 2011; Young and Schultz 2010). Of particular importance to incorporate UAAs is the optimization of o-aaRS/tRNA pairs and codon utilization (Gao et al. 2019). The common OTS have depended on the reassignment of 'non-sense' codons, however the competitions are unavoidable between this commonly used system and termination machinery of the host cell, leading to truncated proteins and lower protein yields. In prokaryote, the incorporation efficiency of UAAs at the amber codon is robustly repressed by termination machinery, due to recognition of amber stop codon by release factor 1 (RF1) in protein translation (Yanagisawa et al. 2014). To relieve the dependence on RF1 for termination, Lajoie et al. (2013) engineered a *E. coli* MG1655 strain by converting all 321 amber codons to synonymous ochre codons and concurrently deleting *prfA* gene (encodes RF1). Finally, this genomically recoded strain exhibits a higher UAAs incorporation efficiency than the native one. Another valuable method to improve incorporation efficiency of UAAs is using cell-free protein synthesis system which is a protein synthesis methodology without living cells (Lu 2017). The system requires the purified or unpurified transcriptional and translational components from cells, and also contains other essential components, including energy sources, cofactors, salts, buffers, nucleotides, and amino acids for protein production (Gao et al. 2019). To enable UAAs incorporation into proteins, UAA and its corresponding orthogonal aaRS/tRNA pair are also needed (Cui et al. 2020). The cell-free protein synthesis system has paved the way to efficiently incorporate of UAAs into proteins as it abolishes the transmembrane transport and cytotoxicity of UAAs.

The incorporation of UAAs through genetic codon expansion approach provides a useful toolbox for tailoring the protein properties and biochemical studies of protein function. However, introduction of multiple unique UAAs simultaneously into a single protein is still a formidable obstacle (Dumas et al. 2015). In general, the site-specific manner enables the incorporation of a UAA into one protein relying on the amber codon suppression. To date, tremendous efforts have been focused on introduction of multiple UAAs, and the uses of distinct stop codons and evolved quadruplet codons as well as mining new aaRS/tRNA pairs seem to be potential strategies to increase diversity of protein modifications. For example, Wan et al. (2010) developed a handy system for incorporation of two distinct UAAs (Nε-acetyl-L-lysines and *p*-azido-L-phenylalanine) at two defined sites of one protein in *E. coli* by combining the mutated PylRS–$tRNA^{Pyl}$ pair to suppress the ochre (UAA) codon and an evolved MjTyrRS–$tRNA_{CUA}$ pair.

In conclusion, the site-specific incorporation of UAAs is proved to open a promising new window in enzymes engineering. With future development of such technology, it will act as a standardized toolkits to evolve enzyme with desired properties and has a big impact on industrial applications including biopharmaceuticals, drug discovery and clinical trials.

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Declarations

Conflict of interest The authors have no financial or proprietary interests in any material discussed in this article.

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