Expanding the genetic code to study metalloproteins and protein posttranslational modifications

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Genetic code expansion is a useful method to study metalloproteins and protein posttranslational modifications. We constructed several tyrosine and pyrrolysine tRNA synthetase libraries based on tyrosine and pyrrolysine tRNA synthetase/tRNA pairs to screen unnatural amino acids (UAAs) with similar chemical structure to tyrosine or lysine. We had incorporated several unnatural amino acids into proteins, including those with electron and proton transfer mediators, metal-chelating, bio-orthogonal reaction groups, and played a series of bio-orthogonal reactions in vitro and in vivo, such as copper click of azide and alkyne, copper free click of azide and cycloalkyne, photoclick of alkene and tetrazole, which laid the foundation for proteins specifically labeling, protein-protein interaction probing, and biological processes photo-regulation. Besides that, we applied this method to incorporate redox tyrosine analogs to improve the property of fluorescent proteins. We directed Nε-formyllysine into histones to study the impact of protein formylation naturally generated by oxidative damage. We also explored the activities of proteins and downstream signaling pathways by using sulfur or fluorine mimetics of acetylated lysine as probes of protein posttranslational modifications.


Expansion of gene encoding bio-orthogonal unnatural amino acids refers to a genetically modified method that can introduce unnatural amino acids into defined sites of target proteins to study the properties and functions of proteins by using orthogonal synthetase tRNA systems. Without affecting the activity of proteins, this method can direct the unnatural amino acids into any exposed sites of proteins to improve the properties of proteins, to real-timely regulate functions of proteins and to study various post-translational modifications. Up till now, these techniques have been applied in a large number of biological systems, including E. coli, S. cerevisiae, human cell lines, C. elegans, D. melanogaster, A. thaliana, Hepatitis D Virus and so on.

In the active site of cytochrome c nitrite reductase, a thioether covalent bond structure named cysteine tyrosine crosslink (cys-tyr) was crucially important. The sulfur atom of the cysteine side chain could spontaneously form a crosslink with the nearby carbon atom of the tyrosine benzene ring. In order to mimic this crosslink, we biosynthesized a tyrosine analogue MtTyr with the similar structure to this cys-tyr posttranslational modification and incorporated it into a refined scaffold of myoglobin. After several rounds of selections, a functional nitrite reductase enzyme, which could efficiently transfer hydroxylamine reduction to ammonia, was obtained and had greater activity than the one with a tyrosine residue at the corresponding site. It was firstly reported that 3-methylthio tyrosine analogue could be used to increase enzyme activity. This method based on enzyme rational design could be used to accelerate...
directed evolution of other cys-tyr crosslink metalloenzymes, such as cysteine dioxygenase, galactose oxidase and so on. Since this amino acid could be synthesized with over 40% yield, functional myoglobin mutants could be expressed in large quantities by this method, which would be an ideal cys-tyr metalloenzyme model for future study in basic research and industry.\textsuperscript{[10]}

Metal-chelating unnatural amino acids were introduced into the chromophores of several fluorescent proteins to design metalloprotein sensors. After rounds of screening, we fortunately got a tyrosine phenol lyase mutant that could biosynthesize a metal-chelating tyrosine analogue HqAla with high efficiency and then incorporated HqAla into the chromophores of some fluorescent proteins. We found that Zn\textsuperscript{2+} ions could be sensed by HqAla fluorescent protein mutant \textit{in vivo} and sub-femtomolar Cu\textsuperscript{2+} ions could be also detected by HqAla fluorescent protein mutant. Besides that, most transition metal ions could be strongly bound by 8-hydroxyquinoline motif of HqAla as well. Since unnatural amino acid HqAla could be efficiently biosynthesized in one step without further purification, HqAla mutant proteins can be largely produced in any biological labs with our method, which overcame the bottleneck of the usage of metal-chelating unnatural amino acids incorporation. In the future research, we would apply this technology to engineer brighter fluorescent proteins, evolve metalloproteins, and investigate protein function by NMR.\textsuperscript{[11]}

Tobacco mosaic virus (TMV) was a RNA virus that would infect tobacco and other plants. We reported that an azide unnatural amino acid was efficiently introduced into the TMV coat protein (TMVCP). TMVCP azide mutant could form functional virus particles by itself, which was firstly found that UAA\textregistered modified TMVCP could assemble into functional structure. TMVCP biotin mutant was modified by copper-free cycloaddition reaction between azide group of TMVCP azide mutant and dibenzocyclooctyne group of dibenzocyclooctyne-PEG4-biotin, which could also self-assemble into functional disk-like or rod-like virus particles. This virus labeling technology could be used to study the mechanism of self-assembly of virus particles and develop new drugs to interfere virus reproduction, which showed great potential to be applied to bio-imaging and drug therapy as well.\textsuperscript{[12]}

In order to design new fluorescent sensors, photo-induced electron transfer (PET) is a most commonly used theory. Because of the lack of ways to introduce PET probes site-specifically into target proteins, there was an urgent need to develop genetically-encoded PET sensors. By genetically incorporating a series of superior PET quenchers into a fluorescent flavoprotein iLov, we succeeded in developing a number of acid turn-on fluorescent mutants. By using \textit{E. coli} which carried acid turn-on fluorescent sensors with unnatural amino acids, we monitored pH changes during the phagocytosis process \textit{in vivo}. Besides that, we also obtained Mn\textsuperscript{5+} fluorescent sensors by expanding the chromophores of iLov mutants with UAA\textregistered, which were the first genetically-encoded sensors that could detect biogenic Mn\textsuperscript{3+}. Moreover, we provided a well-established toolbox for monitoring conformational changes of proteins and rational design of metalloenzymes with redox activity.\textsuperscript{[13]}

Reduction potential and pK\textsubscript{a} of phenol ring were two most concerned issues in oxidase research for years. Although there was a conserved tyrosine in the active sites of these enzymes, the specific functions of this conserved tyrosine in a series of oxidases reactions were not well understood, which was a large obstacle to understand oxygen reduction mechanisms. We succeeded in creating an exquisite linear dependent system between oxidase activity and pK\textsubscript{a} of phenol ring of tyrosine analogs by genetically introducing a family of tyrosine analogs, whose reduction potentials were gradually increasing but phenol ring pK\textsubscript{a}s were decreasing, into a refined myoglobin oxidase model. It was firstly reported that oxidase activity could be enhanced by introducing tyrosine derivatives into the active sites of enzymes, and proton-coupled electron transfer (PCET) ability of the conserved tyrosine residue was increased. Moreover, we found that a tyrosine radical was generated by a special splitting pattern in the reaction with hydrogen peroxide, by incorporating tyrosine and its analogs into the same position of myoglobin.\textsuperscript{[14]}

For further increasing the enzyme activity of our myoglobin model mimicking cytochrome c oxidase (CeO), a tyrosine analogue named 3-methoxy tyrosine (OMeY), which had similar pK\textsubscript{a} to tyrosine but a lower reduction potential, was efficiently biosynthesized by a thermo-stable tyrosine phenol lyase (TPL) mutant and site-specifically incorporated into our refined myoglobin models. Since OMeY was more stable than other redox active amino acids in oxidative environment, it showed a larger application potential for oxidases study. Besides that, OMeY CeO model generated less reactive oxygen species but showed satisfying oxygen reduction activity, which enlarged our toolbox for engineering oxidative enzymes with conserved tyrosine residue.\textsuperscript{[15]}

Protein formylation was recently found in histones and several proteins in nucleus, which was recognized as a posttranslational modification (PTM) generated by oxidative damage. The exact role of this PTM was poorly understood due to lack of good methods to introduce N\textsuperscript{ε}-formyllysine (ForK) site-specifically into target proteins. We firstly
reported that ForK was genetically incorporated into histones and other proteins in *E. coli* and human cells with high efficiency, which would promote protein formylation research. We showed that lysine formylation was a totally different posttranslational modification to lysine acetylation, which indicated that ForK would affect methylation or acetylation modification of the same lysine residues. Besides that, this technology would commercially be applied to produce anti-Nε-formyllysine antibody for epigenetics study [16].

In conclusion, we synthesized interesting unnatural amino acids in biological or chemical ways, constructed and screened tRNA synthetases libraries to get specific mutants, and incorporated UAAs into target sites of proteins to improve the function of enzymes, selectively introduce novel chemical functional groups, and regulate biological processes. We could introduce posttranslational modifications residues, such as Nε-acetyllysine [17] and Nε-crotonyllyine [18], into cells to study their effects on epigenetics. Recently, we also used sulfur or fluorine mimetics of acetylated lysine as probes of protein posttranslational modifications and explored downstream signaling pathways.

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**References**