In situ generation of aminoacyl-tRNAs assisted by ribozymes in translation apparatus

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ABSTRACT

Flexizymes are artificial RNA catalysts that enable us to readily prepare aminoacyl-tRNAs with a variety of amino acid and tRNA kinds. On the other hand, because their flexibility feature lacking high specificities toward amino acids and tRNAs, the in situ aminoacylation in a translation apparatus have not been able to executed. We here present a novel strategy to overcome this specificity problem to tRNA using a 65-acting flexizyme–tRNA construct, called a catalytic precursor tRNA, combining with a naturally occurring ribozyme, ribonuclease P (RNase P). In this coupling system of two RNA enzymes, self-aminoacylation occurs on the catalytic precursor tRNA for specific charging of amino acids at the 3′-end of the tRNA domain in the presence of the cognate amino acid substrates. Subsequently, the aminoacylated catalytic precursor tRNA is specifically cleaved at the 5′-linker region of the tRNA domain, giving the mature aminoacyl-tRNA. Most importantly, the generated flexizyme does not function in trans to tRNAs present in the translation apparatus, indicating that this two-ribozyme coupling system would potentially act as an orthogonal aminoacylation system in the translation apparatus.

INTRODUCTION

The in vitro translation apparatus requires not only ribosome but also various translation protein factors, tRNAs, amino acids, and energy sources in order to express peptides. Among them, a set of protein enzymes, called aminoacyl-tRNA synthetases (ARSs), are responsible to specifically charge the cognate amino acids to tRNA bearing specific anticodons. Thus, this results in the assignment of amino acids to the genetic code. On the other hand, ribosome accepts all proteinogenic amino acids with various sidechains, i.e. ribosome itself does not strictly discriminate against the kinds of sidechains on the proteinogenic amino acids. Apparently, such a feature of ribosome could be utilized to expand repertoire of amino acid kinds to non-proteinogenic amino acids. For instance, when a suppressor tRNA_{UUA} mischarged with a non-proteinogenic amino acid is subjected to the translation, it can read the amber stop codon (UAG) encoded on mRNA and the amino acid is then incorporated into the polypeptide chain during the elongation. Clearly, the expanding the genetic code as such, or even further steps toward reprogramming the genetic code where multiple non-proteinogenic amino acids are assigned to desired codons, would rely on the means of how such aminoacyl-tRNAs charged with non-proteinogenic amino acids are prepared.

A classical method for the preparation of mischarged tRNA is the chemical aminoacylation that has been utilized in the majority of related approaches. Although this method in principle is applicable to a variety of amino acids, it is laborious and technically difficult due to the involvement of multiple steps in the synthetic process. The other approach is an engineering of ARS by directed evolution or rational designs, in which appropriate mutations are introduced, resulting in relaxation or alteration of the specificities to amino acid substrates. However, this approach is adaptable to only a certain set of ARSs or kinds of non-proteinogenic amino acids that are structurally resemble to natural ones. Moreover, evolution is required to alter the specificity for new amino acid substrates because the individual mutant ARS is generally specific to an amino acid substrate. Thus, it has been still arduous to obtain ARS mutants applicable for a wide variety of pairs of non-proteinogenic amino acids and tRNAs.

As an alternative method, we have devised a highly flexible tRNA aminoacylation system consisting of artificial ribozymes, called flexizymes. Flexizymes recognizes the 3′-end of tRNA (RCCA-3′, R = G or A as the discriminator base) as well as a benzylic moiety on the leaving group, and catalyze tRNA aminoacylation. The flexibility of this de novo acylation system enables us to choice wide variety of acids, including non-proteinogenic ω-amino acid, D-amino acids, α-N-methyl amino acids, β-amino acids and ω-hydroxy acids. Although the flexizyme system provide technical advantages for the versatility to both structures of substrates and kind of tRNAs, the specific acylation of a tRNA by flexizymes could not be carried out under the condition existing multiple kinds of
RESULTS AND DISCUSSION

Previous investigation indicated that RNase P effectively digested the precursor tRNA possessing ribozyme sequences in 5'-leader regions. We focus on this processing pathway of tRNAs for constructions of a specific aminocatalytic system of a tRNA in translation apparatus.

Firstly, the variant of flexizyme (mdFx) that had accessory domain for exhibiting catalytic activity at lower Mg²⁺ concentration was covalently linked to the 5'-end of tRNA via penta-adenines, designating in cis aminocatalysis onto this precursor tRNA. Its aminocatalytic efficiency under the conditions of the PURE system was analyzed by biotinylation of the aminocatalyzed precursor tRNA followed by streptavidin-dependent mobility-shift PAGE. The in cis aminocatalytic efficiency of the catalytic precursor tRNA charged by N⁶-acetyl-lysine 3,5-dinitro-benzyl ester (DBE) was 13% in 5 min under the above conditions. In contrast, the catalytic activity of mdFx in trans generated by the RNase P digestion of the mdFx-tRNA was not observed at all under the same conditions. This study suggests that the coupling of the catalytic precursor tRNA with RNase P is able to generate only the desired aminocatalytic tRNA designated by the cis system.

Next, we extended our attempt to couple the catalytic precursor tRNA with the RNase P in PURE system toward the in situ generation of the aminocatalytic tRNA. Incorporation of non-proteinogenic amino acids into a peptide by the combination of catalytic precursor tRNA and RNase P was analyzed by sense-suppression methods. We chose CUC codon for reassignments of the N⁶-acetyl-lysine. The translation was performed in the presence of catalytic precursor tRNA, N⁶-acetyl-lysine DBE, RNase P, along with the minimal members of amino acids for peptide synthesis, Met, [¹⁴C]-Asp, Tyr and Lys. Four control experiments were also carried out; the translation in the absence of one of three additional factors (catalytic precursor tRNAs, non-proteinogenic amino acids substrates and RNase P) and Leu as negative controls. The tricine-PAGE analysis for these samples showed that a discrete band corresponding to the full-length peptide was observed when all three additional factors are present in translation system. On the other hand, the absence of one of the three components gave no band corresponding to the full length peptide. The incorporation of N⁶-acetyl-lysine was also confirmed by the MALDI-TOF analysis of the expressed peptide. These experiments indicate that self-aminocatalysis of catalytic precursor tRNAs with by N⁶-acetyl-lysine followed by its RNase P digestion successfully generates the N⁶-acetyl-lysine-tRNA that is used further used for the site-specific incorporation into the nascent peptide chain.

CONCLUSION

We here demonstrate the in situ generation of non-proteinogenic aminocatalytic tRNA based on the catalytic precursor tRNA–RNase P coupling system followed by site-specific incorporation of the amino acid into nascent peptide chain. This in situ expression system of non-canonical peptide would add a new dimension to the flexizyme-translation coupled system for further development.

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REFERENCES


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