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Polymerization of α-Hydroxy Acids by Ribosomes

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Over 30 years ago, Fahnestock and Rich reported intriguing data showing the capability of the ribosome to polymerize phenyllactic acid. Although the polymerization was initiated and terminated randomly on polyuridic acids, the given data convincingly suggested that the generated polymer was composed of an approximately 7:3 mixture of phenyllactic acid and phenylalanine. Despite the fact that Fahnestock's conclusion was very likely correct, there have been no reports to follow up the ribosome-catalyzed polymerization of α-hydroxy acids until very recently. At the end of 2007, we reported messenger RNA (mRNA)-directed polyester synthesis by using the new emerging method of genetic-code reprogramming in which α-hydroxy acids with various kinds of side-chains are assigned to arbitrarily chosen codons. In this work, we have achieved the ribosomal synthesis of polyesters with the sequence composition and length in a fully controlled manner according to the sequence of mRNA. This Concept article describes the background of the method development and its application to the synthesis of polyesters.

Introduction

Can ribosomes polymerize an α-hydroxy acid?

Over 30 years ago, Fahnestock and Rich published a report entitled "Ribosome-catalyzed polyester formation".[1] This report describes a landmark experiment in which it was shown that the ribosome is capable of polymerizing a non-α amino acid substrate, phenyllactic acid (F^{lac}). The classic Nirenberg method, which led to decoding the universal genetic code, was modified for their experiment. Instead of translating a synthetic polyuridic (poly-U) acid by using Phe–$tRNA^{Phe}$, [^{14}C]-labeled F^{lac}–$tRNA^{Phe}$ was added to the translation system, which was composed of a cell-free *E. coli* S-100 and minimal organic/inorganic components, such as GTP and buffer (Figure 1 A). The [^{14}C]-F^{lac}–$tRNA^{Phe}$ was chemically prepared by deamination of [^{14}C]-Phe–$tRNA^{Phe}$ by using nitrous acid. After the translation reaction, the polymerized products were precipitated with trichloroacetic acid (TCA) and the radioactivity of the precipitates was counted to quantify the yield of polymer. It was shown that the TCA-insoluble matter was formed only in the presence of all translation components, that is, S-100, poly-U, and GTP, and its recovery yield was nearly 10% of the poly-Phe synthesis. Upon treating the precipitate with alkaline, the resulting products were analyzed by paper electrophoresis. Supposedly, alkaline digestion would cleave ester bonds between F^{lac}–F^{lac}, but not amide bonds such as F^{lac}–Phe or Phe–Phe. In fact, the paper electrophoresis separated the products that originated from F^{lac}–Phe and F^{lac} with a ratio of approximately 3:7. The authors suggested that Phe likely originated in S-100, and thus such a minimal contamination of amino acids could not avoided. Nonetheless, this observation gave indirect but convincing support for the idea that the polymerization of F^{lac} occurred consecutively three or four times followed by random incorporation of Phe into the poly-F^{lac} chain.

Despite the limitations of the analytical techniques available at that time, the data convincingly suggested that the ribosome is capable of catalyzing the polymerization of F^{lac}; however, many technical as well as scientific questions were left unresolved by this demonstration. Regarding technical issues, the polyester formation was only confirmed by indirect evidence, that is, by detecting the [^{14}C]-radioisotope of the alkaline-digested sample of the acid-insoluble precipitants. Therefore, it was still unknown how long F^{lac} could be consecutively polymerized. Ideally, the polyester should be directly detected as an intact polymer. Moreover, due to unavoidable contamination of Phe in S-100, deacylated $tRNA^{Phe}$ generated by the hydrolysis of [^{14}C]-F^{lac}–$tRNA^{Phe}$ would be recharged by phenylalanyl–tRNA synthetase (PheRS). This resulted in the random incorporation of Phe into the poly-F^{lac} chain after every three or four residues. Thus, the contamination-free polymerization of F^{lac} was not, unfortunately, achieved. Moreover, it is of scientific interest whether other types of α-hydroxy acids (α-ha) can be accepted by ribosomes for polymerization. Along the same line, instead of the random initiation and termination demonstrated by Fahnestock's experiment, it is critical to utilize the full capability of the translation system, that is, to show fully controlled initiation, elongation, and termination along the mRNA sequence that leads to the generation of a defined length of polyester. Finally, it would be the most critical challenge to demonstrate the ribosomal synthesis of a variety of polyesters that contain distinct side-chains designated by mRNA templates. To this end, we set a project to challenge the ribosomal polymerization of α-hydroxy acids that would simultaneously address all of the above issues.[2]

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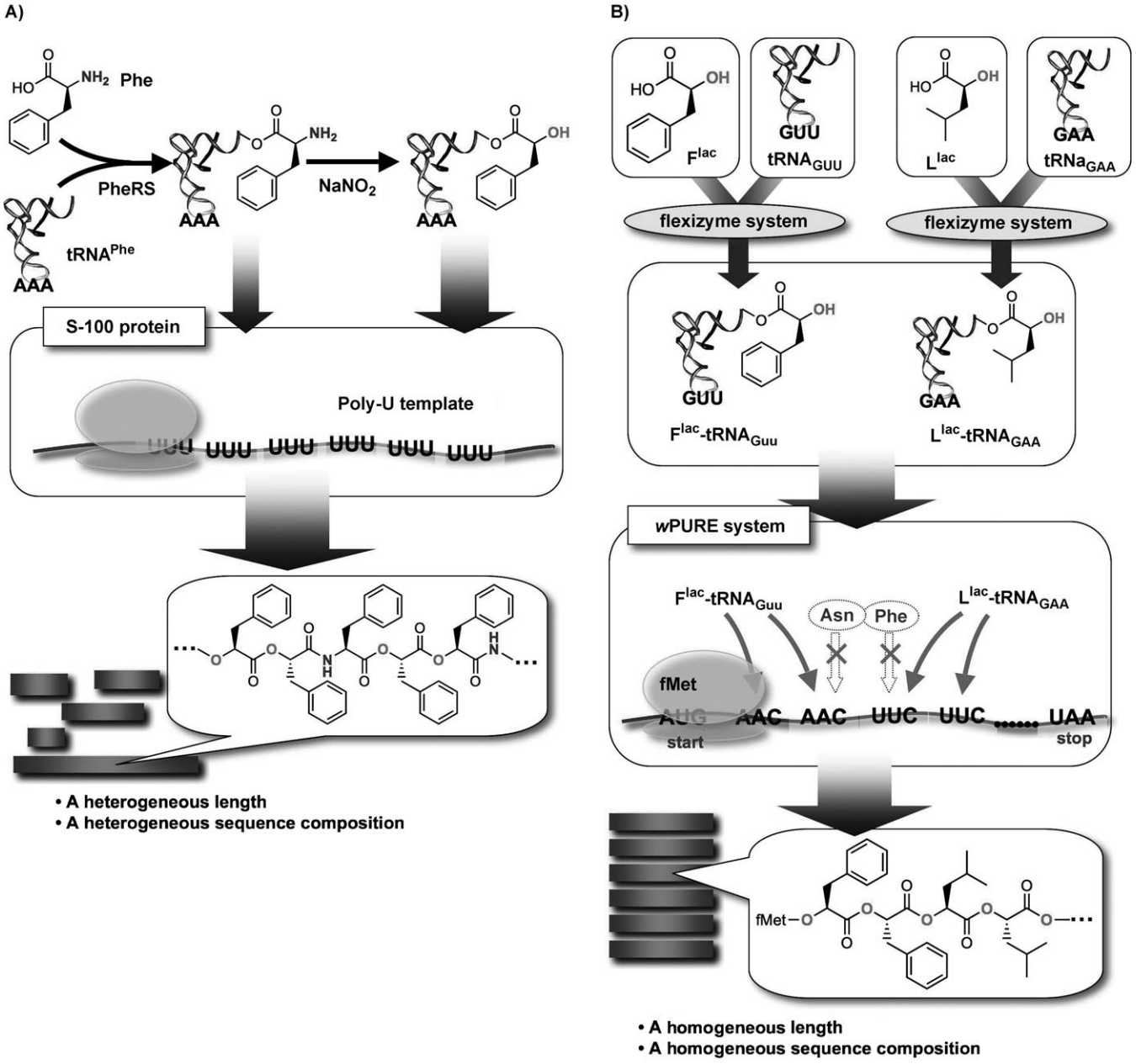

Figure 1. Two different approaches for ribosomal polyester synthesis. A) In Fahnestock's approach, in which F^{lac}–$tRNA^{Phe}$ was prepared by chemical deamination of Phe–$tRNA^{Phe}$, polymerization was randomly initiated and terminated on the poly-U template. Due to the lack of an open-reading frame in the poly-U template, the resulting product had nonhomogenous lengths of polyesters. Moreover, a trace amount of Phe–$tRNA^{Phe}$ resulted in the random incorporation of Phe into the polyester chain; B) mRNA-directed polyester synthesis by using genetic-code reprogramming. The flexizyme system facilitates the hydroxyacylation of tRNAs, and these hydroxyacyl–tRNAs were added to a *w*PURE system for the polymerization of the α-hydroxy acids; L^{lac}: isopropyllactic acid; F^{lac}: phenyllactic acid.

Genetic-Code Reprogramming for Polyester Synthesis

About ten years after Rich's report, a new methodology was developed for introducing nonproteinogenic α-amino acids into peptide chains.[3,4] A mischarged $tRNA_{CUA}$ with a nonproteinogenic amino acid was used to suppress a UAG stop codon (amber codon) and the amino acid was incorporated into a specific site of the peptide chain by using a cell-free translation system. This method is also applicable for the incorporation of α-hydroxy acid[5–9] to generate an ester bond in the peptide chain. In fact, it has been utilized to disrupt the backbone hydrogen-bonding network in a protein of interest and to specifically cleave the peptide chain at the ester site. Unfortunately, the mischarged $tRNA_{CUA}$ must inherently compete with the release factor present in the translation apparatus, that is, translation termination, and therefore the incorporation efficiency heavily depends on the kind of side chains in the α-hydroxy acid. This fact also prohibits us from performing consecutive multiple incorporations of an α-hydroxy acid(s).

Clearly, it is necessary to use another methodology that allows us to control the undesirable competition in translation so as to polymerize many kinds of α-hydroxy acids. More recently an alternative technology, referred to as genetic-code reprogramming, has been devised to resolve the above problem.

In genetic-code reprogramming some of the proteinogenic amino acids and/or other components are withdrawn from the translation system so as to break the tight relationship between the amino acids and cognate codons in the genetic code. In 2003, Forster et al. introduced this concept by demonstrating that three kinds of nonproteinogenic amino acids were reassigned to three different codons and incorporated into a peptide in succession by sense suppression.[10] Despite the fact that this early work used a translation system that was unable to turnover, two significant benefits over the classical method that used amber suppression[3,4] are evident. First, because the proteinogenic amino acids, the codons of which are aimed at reprogramming, are withdrawn from the translation system, there are no direct competitors against the desired suppression. This is in sharp contrast to the amber suppression in which the release factor competes with a suppressor $tRNA_{CUA}$ that is charged with a nonproteinogenic amino acid to terminate the translation. Therefore, it is expected that the efficiency of sense suppression would be higher than that of amber suppression. Second, because the assignment of nonproteinogenic amino acids can be achieved by choosing any desired codons, and is not restricted to stop codons, the number of nonproteinogenic amino acids for the codon reassignments is—in principle—unlimited. If these two concepts were achieved, we expect that nonstandard peptides or even other types of biopolymers could be synthesized with the translation machinery. Stimulated by Foster's experiment, several groups have started work on further development of genetic-code reprogramming.[11–25]

For nearly ten years, we have engaged in a project to develop artificial ribozymes, called "flexizymes", which are capable of charging amino acids onto tRNA.[16,26–31] The latest version of the flexizyme system enables us to charge virtually any amino acid, including nonproteinogenic ones, onto tRNA that bear various anticodons.[16] Importantly, we have found that it is able to charge a variety of α-hydroxy acids onto tRNAs, to yield hydroxyacyl–tRNAs (ha–tRNAs).[2,16] Thus, the use of this system should facilitate the study of the ribosomal synthesis of polyesters to address the questions that were raised earlier when it was combined with the genetic-code reprogramming methodology.

Despite the fact that some α-hydroxy acids were successfully incorporated into a peptide (protein) chain at a specific site by the nonsense suppression,[5–9,32,33] to the best of our knowledge there is no report for the successive incorporations of α-hydroxy acids by any means except for Fahnestock's experiment. To successfully achieve the polymerization of α-hydroxy acids by ribosome catalysis, two technical improvements turned out to be critical. First, we needed to use a special reconstituted *E. coli* cell-free translation system,[25,34–37] referred to as *w*PURE, in which both amino acids and cognate aminoacyl–tRNA synthetases (ARSs) for the reprogramming codons were *withdrawn* from the ordinary PURE translation system. Because α-hydroxy acids are intrinsically poorer substrates for ribosome than α-amino acids,[38–40] the *w*PURE system that lacked only α-amino acids was insufficient to control the background level of competitive incorporation of α-amino acids into the polyester chain. Second, we developed two types of engineered, orthogonal tRNAs that are not aminoacylated by *E. coli* ARSs, $tRNA^{Asn\text{-}E}$ and $tRNA^{MLAsn}$ (Figure 2A and B), to carry the α-hydroxy acids—in the experiments, we used three tRNAs, but two of these belonged to the family of $tRNA^{Asn\text{-}E}$ and behaved virtually the same. We performed the reassignment of seven codons to seven α-hydroxy acids (Figure 3A), but one of the codons, CAG (Gln), suffered from minor misincorporation of Lys as the LysRS catalyzed the mischarge of Lys onto $tRNA^{Asn\text{-}E}_{CUG}$, the body sequence of which was our standard scaffold for orthogonal tRNAs with various anticodons (Figure 2A). We thus screened potential body sequences of orthogonal tRNA and found $tRNA^{MLAsn}$, derived from mycobacteriophage L5 $tRNA^{Asn}$.

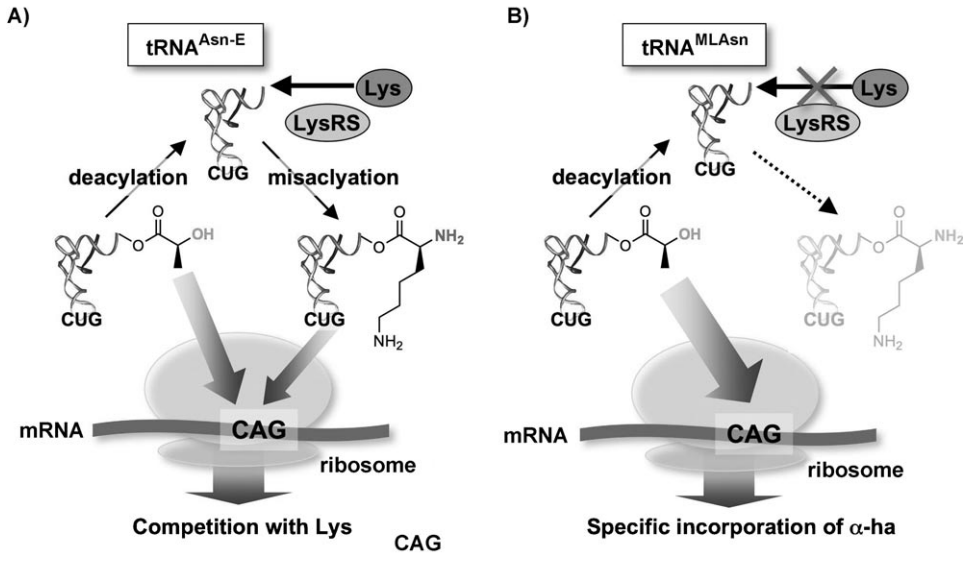

Figure 2. A strategy for controlling the competition with undesirable misincorporation into the polyester chain. A) Competition between α-hydroxy acid and Lys for $tRNA^{Asn\text{-}E}$ at the CAG codon. Because the deacylated $tRNA^{Asn\text{-}E}$ suffered from mis-lysinylation catalyzed by Lys–tRNA synthetase (LysRS), the resulting Lys–$tRNA^{Asn\text{-}E}$ competed with ha–tRNA for CAG decoding; this resulted in misincorporation of Lys into the polyester chain. B) Specific incorporation of α-hydroxy acid on $tRNA^{MLAsn}$ at the CAG codon. The deacylated $tRNA^{MLAsn}$ was inert to LysRS, which prevented its mis-lysinylation. Thus, the CAG codon was exclusively decoded by ha–$tRNA^{MLAsn}$; this led to the programmed incorporation of the α-hydroxy acid.

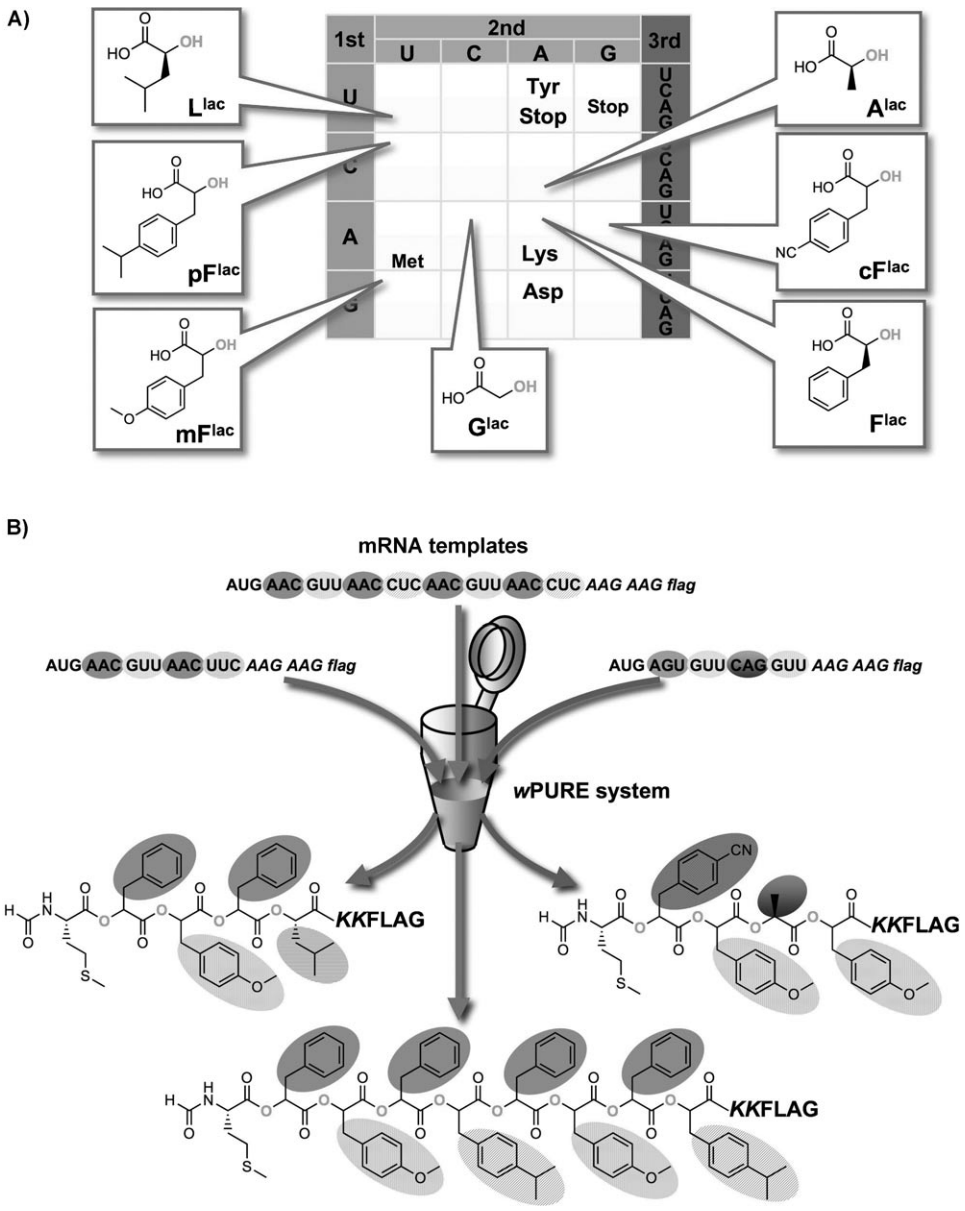

Figure 3. mRNA-directed polyester synthesis by using genetic-code reprogramming. A) The genetic-code reprogrammed by seven kinds of α-hydroxy acids; B) mRNA-directed polyester synthesis. Each template yielded respective polyester sequences under the control of the reprogrammed genetic code shown in A).

This $tRNA^{MLAsn}$ was inert to LysRS and other *E. coli* ARSs, and therefore the CAG codon could also be used for reprogramming (Figure 2B).

Messenger RNA-Directed Polyester Synthesis

Using the above-described *w*PURE and flexizyme systems, we attempted to express polyesters in a mRNA-dependent manner (Figure 1B). We designed the mRNA template sequence to encode a polyester–peptide hybrid. In this polymer, the polymerization was initiated from formylated methionine assigned by AUG, elongated with α-hydroxy acids assigned by seven different codons, and further elongated by three amino acids (K, D, and Y) yielding a modified sequence of flag peptide, KKDYKDDDDK (Figure 3B). This flag peptide facilitated isolation as well as detection/quantification of the products by means of tricine-SDS-PAGE when [^{14}C]-D was included in the translation mixture to yield the [^{14}C]-flag peptide at the C terminus of polyester. It should be noted that *w*PURE is a coupled transcription–translation system, and therefore the actual template is the corresponding DNA that bears a T7 promoter sequence. Thus, the DNA template was added to the *w*PURE system along with ha–tRNAs that were prepared by the flexizyme system, and the resulting product was assayed by MALDI-TOF mass spectrometry and tricine-SDS-PAGE.

We demonstrated the expression of a trimer-polyester and four tetramer-polyesters with a variety of compositions of α-hydroxy acids. In the case of these polyesters, their expression level was comparable to wild-type peptide expression, and gave a quantity that ranged from 5–15 pmol per 5 μL. Most importantly, in all cases the MALDI-TOF analysis revealed a single major peak that was consistent with the molecular mass (MS) of the anticipated composition of polyesters. Therefore, this was the first direct evidence that showed ribosomal synthesis of a polyester, the sequence and length of which were fully controlled by the mRNA template. We also extended the polymerization of α-hydroxy acids to pentamer, hexamer, octamer, and dodecamer. In all cases, we observed a band on tricine-SDS-PAGE in the individual expression; this suggests that the product contained the corresponding [^{14}C]-flag peptide, that is, it is likely that the polyester-flag hybrids were expressed. The MALDI-TOF analysis of the polyester-flag hybrids, however, gave no peak; we examined a variety of analytical conditions by altering the supporting matrixes and/or laser powers, but the expected MS that corresponded to the full-length polyester–flag hybrid was not observed. We assumed that the product was lost during sample manipulation, for example, by aggregation or precipitation of polyesters. We thus treated the crude product under basic condition to hydrolyze it, and analyzed the resulting sample by MALDI-

TOF and found the peaks for $(ha)_2$–flag and ha–flag in all cases. Most importantly, the observed MS for each of the $(ha)_2$–flag was consistent with the MS value that was expected from that expressed from the respective mRNA sequence. Therefore, we concluded that it was very likely that the full-length polyesters were expressed in accordance with the mRNA sequences.

Outlook

Challenges successfully achieved and still remaining

We have thus far resolved the following questions: 1) Can the polyester sequence be programmed by mRNA? Yes, it can be done by genetic-code reprogramming. We have demonstrated the reassignment of seven different α-hydroxy acids to seven codons and successfully polymerized them to polyesters according to the mRNA templates. 2) How many α-hydroxy acids can be successively polymerized? We have demonstrated polyester synthesis up to tetramer length as confirmed by MS evidence of the full-length products, and up to the dodecamer length as confirmed by MS evidence of the fragmented products.

Although the above two achievements resolved most issues that remained unanswered in the Fahnestock experiment, some issues are still unresolved. The expression level of polyesters longer than dodecamers was very low, which made it difficult to ensure their expression. Moreover, we were only able to detect hydrolyzed products, rather than the full-length polyesters, when their lengths were longer than five. Thus, from a technical point of view, we need to achieve better polymerization efficiency of α-hydroxy acids than the current system, and to develop a method to detect the polyesters as intact full-length products. How can we solve these problems? Unfortunately, we currently do not have a definitive approach. For the former improvement, we might be able to engineer elongation factor Tu (EF-Tu)[41] or orthogonal tRNAs[42,43] for higher affinity of ha–tRNA because it is known that EF-Tu binds F^{lac}–tRNA nearly 300-fold poorer than Phe–tRNA.[39] Also, it would be critical to engineer the ribosome itself[44–47] to increase the acyl-transfer rate for the α-hydroxy acids in the active site, because the transfer rate of α-hydroxy acids has been estimated to be at least tenfold slower than that of α-amino acids.[38,40] By combining these approaches, it might be possible to solve the former problem. The latter problem might be solved by an appropriate manipulation and isolation of the products to avoid their loss, and also by MALDI-TOF analysis under the conditions that are specially designed for the analysis of labile polyesters.

Despite the above technical problems, our technology as a whole has great potential to serve as a platform to new research directions. First, mRNA-programmed polyester synthesis allows us to polymerize multiple α-hydroxy acids that have various side-chains with a desired sequence. Moreover, combining ribosomal polymer synthesis with some in vitro display techniques[48,49] facilitates the selection of functional sequences of polyesters or polyester–peptide hybrids from the corresponding libraries with high complexities. Considering the fact that the ester bond has unique properties—in terms of plasticity and rigidity—that are distinct from the peptide bond, it is of great interest to generate functional polyesters and polyester–polypeptide hybrid biopolymers and investigate their details. Second, α-hydroxy acids are also useful for investigating elongation chemistry in the ribosome. In fact, the use of an α-hydroxy acid (phenyllactic acid) in elongation slows the rate of the peptidyl-transfer reaction in ribosomes;[40] this allows researchers to ask specific questions of the chemical event that are not readily accessible by the use of standard amino acids. Although Rich's classic method was used to prepare the ha–tRNA in the reported work so far,[40] our flexizyme system readily expands the usable repertoire of α-hydroxy acids (and also nonproteinogenic amino acids) without limiting the tRNA species. This would provide researchers a nearly unlimited capability to investigate the elongation chemistry that occurs in the active site of the ribosome.

On the other hand, the methodology developed through this study has already given many fruits. For instance, the same methodology has been fully utilized in our recent work for the synthesis of *N*-methyl-peptides.[22] In this work, because the analytical problem did not exist, we were able to successfully detect up to dodecamer *N*-methyl-peptides by MALDI-TOF. Similarly, we recently reported the ribosomal synthesis of a cyclic peptide that is highly resistant to peptidases, and the methodology used was an adaptation of that developed for the polyester synthesis.[21,23,24] Thus, mRNA-directed polyester synthesis was the first example of a series of studies,[21–24,50] and the methodology described here provides a foundation for new avenues in ribosomal synthesis of biopolymers.

Keywords: genetic code · ribosomes · template synthesis · translation · tRNA

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