Simple and quick chemical aminoacylation of tRNA in cationic micellar solution under ultrasonic agitation

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A simple and quick method for direct aminoacylation of a tRNA with a non-natural amino acid was developed by using an *N*-protected amino acid cyanomethyl ester as a substrate solubilized in CTACl micelle under ultrasonic agitation.

Aminoacylation of tRNA with a non-natural amino acid is a key step for its incorporation into proteins. The non-natural aminoacylation has been carried out by a variety of sophisticated methods. Hecht[1] first reported chemical aminoacylation synthesizing aminoacylated pdCpA dinucleotide followed by its enzymatic ligation to a tRNA that lacks a 3′-terminal pCpA dinucleotide unit, tRNA(-CA). Suga[2] selected a ribozyme that brings an amino acid activated ester close enough to form an ester linkage with the 3′ end of tRNA. Schultz[3] and Yokoyama[4] have been successful in alteration of the substrate specificity of several aminoacyl tRNA synthetases to accept non-natural amino acids and to transfer them to orthogonalized tRNAs. We have shown that an amino acid thioester that is linked to a peptide nucleic acid can aminoacylate selectively its complementary tRNA in an E.coli in vitro protein synthesizing system.[5] Those methods for non-natural aminoacylation, however, need sophisticated syntheses or repeated selection processes for each non-natural amino acid.

In a previous communication, we reported a simple method for aminoacylation of a pdCpA dinucleotide in cationic micellar solution.[6] But the aminoacylated pdCpA still needs to be ligated with tRNA(-CA). We report here that the micellar method can apply directly to tRNAs when it was carried out under ultrasonic agitation.

The micellar aminoacylation system consists of an *N*-protected amino acid activated ester as the substrate, a target tRNA, and a cationic detergent (hexadecyltrimethylammonium chloride, CTACl) in a buffer at pH 7.5. The cationic micelle solubilizes the hydrophobic *N*-protected amino acid ester into its hydrophobic core and concentrates negatively charged tRNA onto its cationic surface. *N*-Pentenoyl-L-2-naphthylalanine cyanomethyl ester (Pen-2napAla-OCM) was used as a standard substrate. A full-length synthetic yeast phenylalanine tRNA with a CCCG four-base anticodon was used as the target tRNA.[5]

Typical procedure is as follows. The reaction mixture (total volume 20 μL) contains 18 mM CTACl, 0.01 mM tRNA and 5 mM Pen-aa-OCM in 90 mM imidazole buffer at pH 7.5. The mixture was periodically sonicated with an ultrasonic homogenizer (Branson250 sonifier) under 18 W at 25% duty ratio for 5 min at 25 °C. After the sonication, the mixture was acidified by adding 60 μL of acidic AcOK solution [a mixture of 1.5 M aqueous AcOK and AcOH (pH 4.5)] to prevent spontaneous hydrolysis, and washed with 80 μL of acidic AcOK-saturated phenol and then with 80 μL of chloroform. The aqueous layer was taken and 360 μL of ethanol was added to precipitate the tRNA. After storing at −30 °C for 1 h, the ethanol suspension was centrifuged at 15 000 rpm for 30 min at 4 °C and the supernatant was removed. By this procedure, most of CTACl and the amino acid derivatives were removed. The precipitate was dried under vacuum and stored at −30 °C.

The yield and the positions of the aminoacylation were checked by HPLC analysis of the digestion products of aminoacylated tRNA after treatment with nuclease S1 (Fig. 1).[5]

If the aminoacylation took place correctly, the S1 digestion gives 2′- and 3′-aminoacyl AMPs [AMP-2′(3′)-(Pen-napAla)] that appear at 28.8 min and 29.2 min under the present HPLC conditions.[5] In fact, large peaks were observed at these positions. These peaks disappeared when the S1-digested product was treated with $NaHCO_3$ (pH 9) for 30 min, indicating *O*-aminoacylation rather than *N*-aminoacylation. The HPLC peak intensity was converted to the yield of aminoacyl AMPs by using an authentic sample.[5] The yield reached to 74% after ultrasonic agitation for 5 min then gradually decreased.

Fig. 2 shows dependence of the yield of AMP-(Pen-napAla) on the average power of the sonicator (solid circles). The yield increased from 0% to 74% with the increase of the power from 0 to

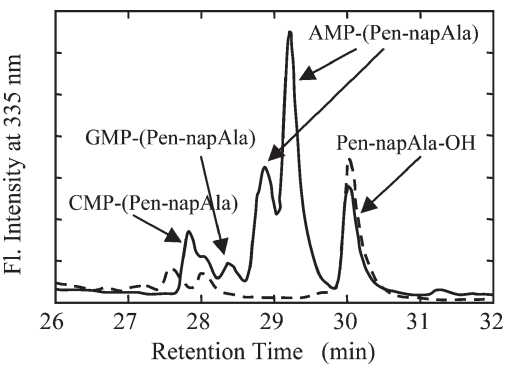

Fig. 1 HPLC charts of the products of micellar aminoacylation with Pen-2napAla-OCM on yeast phenylalanine tRNA (solid line) and on the tRNA in which the 3′-terminal 2′,3′-diol group has been removed by periodate oxidation (dashed line), after S1 nuclease treatment. HPLC conditions: column; ODS-3 (GL Sciences), eluents; A: 0.1M ammonium acetate, pH 4.5, B: acetonitrile, A/B: 1–100% (50 min) λ_{ex} = 285 nm, λ_{em} = 330 nm.

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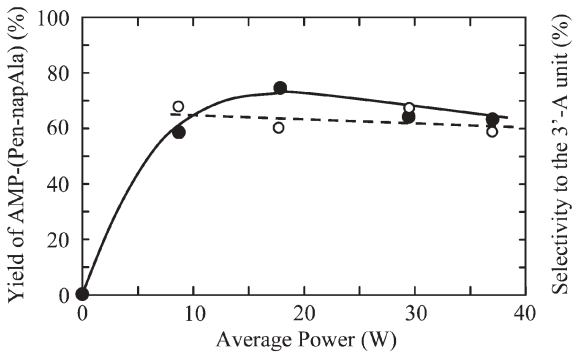

Fig. 2 Dependence of the yields of AMP-(Pen-napAla) (solid circles) and the regioselectivity to the 3′-terminal A unit (open circles) on the average power of ultrasonication.

18W, then gradually dropped at higher powers under constant agitation time (5 min). It must be noted here that the yields from HPLC analysis have been underestimated by 10–20%, due to partial loss of the aminoacyl tRNA during purification processes and also due to partial hydrolysis during S1 treatment.[5] In any case, the yield is practically high enough.

To confirm that the aminoacylation is occurring on the adenosine unit at the 3′-end of tRNA, a tRNA in which the 3′-terminal diol group was oxidized with $NaIO_4$, was prepared.[7] The latter tRNA after the same treatment gave an HPLC profile as shown in Fig. 1 (dashed line). Disappearance of the aminoacyl AMP indicates that the micellar aminoacylation is occurring mostly at the 2′(3′) position of the 3′-terminal adenosine unit. The regioselectivity evaluated from the HPLC peak ratio was about 70%. The selectivity was insensitive to the ultrasonic power as shown by open circles in Fig. 2.

The aminoacylation did not take place when Pen-2napAla-OH was used as the control substrate. Neither anionic (sodium dodecylsulfate), non-ionic (Tween20) detergent nor a mineral oil gave the aminoacylated tRNA. Less efficient aminoacylation was observed with other cationic detergents, like octyltrimethylammonium chloride, dodecyltrimethylammonium chloride and octadecyltrimethylammonium chloride. No aminoacylation took place in the absence of ultrasonic agitation. Presumably, the sonication flutters the micellar structure to increase chances of the encounter between amino acid activated ester and the 2′(3′)-OH group of tRNA, that are otherwise separated inside and outside the micelle.

Polymeric surfactants with cationic charges, like partially dodecylated and fully quarternized polyethyleneimines with different contents of dodecyl groups[8] were examined instead of CTACl. But none of the polymers with 5–25% dodecyl contents gave higher aminoacylation yield than CTACl. Similarly, fully quarternized star-shaped dendrimers[9] gave only 12% aminoacylation yield under optimized conditions. Those polymeric systems may be more rigid than the CTACl micelle and may not efficiently flutter to promote the encounter between the amino acid activated ester and the tRNA.

Besides the correct aminoacylation products, some minor peaks are detected in the HPLC chart of Fig. 1. By using authentic samples synthesized by micellar aminoacylation of CMP, GMP and UMP,[6] the fairly intense peaks at 27.8 min and 28.0 min were assigned to CMP-2′(3′)-(Pen-napAla) and a small peak at 28.4 min is GMP-(Pen-napAla). No peak that is assignable to UMP-2′(3′)-(Pen-napAla) was detected. Since most of the hydroxyl groups are exposed to water in the L-shaped tRNA structure,[10] the 70% regioselectivity to the 3′-terminal adenosine unit is unexpectedly high. Presumably, for steric reason, the 2′-OH group inside the tRNA body cannot be esterified, whereas the terminal *gem*-diol must be more reactive than other OH groups.

Aminoacylation was also successfully carried out with Pen-1-naphthylalanine-OCM at 70% yield with 70% regioselectivity. For Boc-2-acrydonylalanine-OCM,[11] however, only 2% yield was obtained. The yield increased to 32% with 70% regioselectivity under ultrasonication at higher power (100 W at 100% duty ratio). Fortunately, no degradation of tRNA chain was observed in the gel-shift assay of the aminoacylated tRNA under agitation of such high power. The lower yield for 2-acrydonylalanine derivative may be due to reduced solubility of the amino acid and was improved by stronger ultrasonic agitation.

To use the aminoacylated tRNA for translation in an *E.coli in vitro* system, the pentenoyl protecting group in the aminoacylated tRNA was removed by I_2 treatment.[12] The aminoacylated product was dissolved in water (10 μL) and 100 mM I_2 solution in THF/water (1 + 1) mixture (1 μL) was added under shaking. The mixture was stored at room temperature for 10 min and the pH of the mixture was adjusted to 5.3 by adding 3M sodium acetate. The tRNA was precipitated with ethanol and the precipitate was dried under vacuum.

The *in vitro* translation system (total 10 μL) contained 55 mM Hepes-KOH, pH 7.5, 210 mM potassium glutamate, 6.9 mM ammonium acetate, 12 mM magnesium acetate, 1.7 mM DTT, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9% polyethyleneglycol-8000, 35 μg/ml folinic acid, 0.1 mM each amino acid, 80 μM mRNA of streptavidin, and 2 μL of E.coli S-30 extract (Promega). Concentration of the aminoacylated $tRNA_{CCCG}$ was about 140 μM irrespective of the type of non-natural amino acids. The position of the non-natural amino acid on a protein was assigned by a CGGG four-base codon at the 83rd position of streptavidin.[13] *In vitro* protein synthesis was conducted in the absence and presence of the aminoacyl tRNA. The protein products were analyzed on Western blotting using anti-T7 tag antibody/alkaliphosphatase-labeled anti-mouse IgG. Results are shown in Fig. 3. Full-length protein was successfully obtained when the aminoacylated tRNA was added to

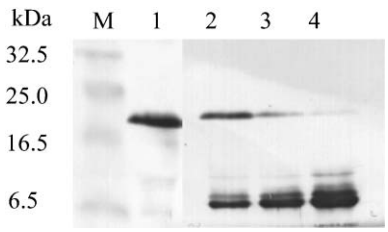

Fig. 3 Western blot analysis of the translation products of mRNA for wild-type streptavidin and for ^{83}CGGG mutant, in the absence and the presence of the aminoacylation product of $tRNA_{CCCG}$. The band at 19kDa is a streptavidin monomer: lane 1, wild-type mRNA; lane 2, mRNA containing ^{83}CGGG + micelle-aminoacylated $tRNA_{CCCG}$ with 2napAla; lane 3, mRNA containing ^{83}CGGG + free $tRNA_{CCCG}$; lane 4, mRNA without $tRNA_{CCCG}$. Lane M contained prestained molecular weight marker.

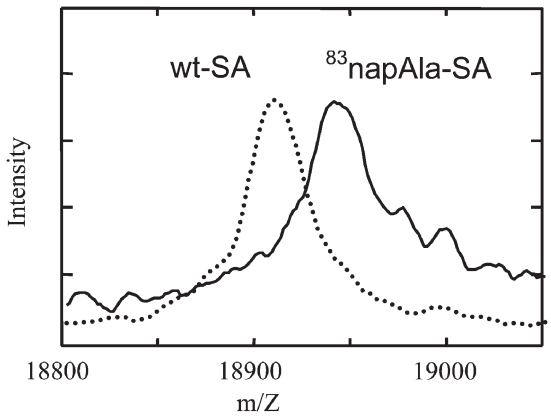

Fig. 4 TOF mass charts for wild-type streptavidin (wt-SA) synthesized in *E.coli in vitro* system and for ^{83}napAla-streptavidin (^{83}napAla-SA) synthesized in the presence of ^{83}CGGG mRNA of streptavidin and $tRNA_{CCCG}$ aminoacylated in micellar solution with Pen-napAla-OCM under ultrasonication.

the *in vitro* system. The yield of the mutant was about 20% with respect to the yield of wild-type streptavidin under the same conditions.

TOF-mass charts of the wild-type and the ^{83}napAla streptavidin are shown in Fig. 4. A single peak was observed at 18906 *m*/*z* for the wild-type protein and a single peak at 18938 *m*/*z* for the ^{83}napAla mutant, respectively. These values are in consistent with the theoretical masses, 18910 u and 18943 u, respectively. The successful translation ensures that most of the aminoacylation is occurring at correct positions and the incorrectly aminoacylated products at C and G units did not cause serious inhibition in the translation process. Indeed, when the aminoacylation products of a tRNA with an oxidized 3′ A unit were added to the *in vitro* translation system for wild-type streptavidin, no clear decrease of the protein yield was observed. The little inhibition by the incorrectly aminoacylated tRNAs may be due to inefficient binding of the latter to EF-Tu.

The yield of mutant protein (25%) in the Western blotting was, however, lower than that when the aminoacylation was carried out by the standard Hecht method (70%).[13] This may be due to CTACl molecules that are remained bound onto negatively charged tRNA. Indeed, when the aminoacyl tRNA prepared by the Hecht method was treated in CTACl micelle, the yield decreased to the same level as that from micellar aminoacylation. Further purification of the aminoacyl-tRNA would increase the protein yield, but will decrease its total quantity. Total protein yield will be better increased by enlarging total scale of the *in vitro* system, because larger amount of aminoacyl-tRNA is now easily available.

To conclude, a simple and quick method for direct tRNA aminoacylation has been developed. This method will make non-natural amino acid mutagenesis a routine technique for common biochemists.

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