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A Flexible, Scalable Method for Preparation of Homogeneous Aminoacylated tRNAs

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Abstract

Transfer RNAs (tRNAs) are cellular courier molecules that decipher the genetic code in messenger RNAs and enable the transfer of appropriate esterified amino acids to the growing peptide chain. The preparation of biophysical quantities of homogeneous aminoacylated tRNAs has remained a significant technical challenge. This is primarily due to the difficulty in removing contaminating non-aminoacylated tRNAs that have very similar properties overall, as well as the hydrolytic instability of the aminoacyl linkage. We describe a flexible, scalable method to prepare homogeneous aminoacylated tRNAs that is also broadly compatible with mutant, misacylated or otherwise aberrant tRNAs and other RNAs. This method combines ribozyme-mediated aminoacylation with reversible N-pentenoylation of the esterified amino acid, which not only protects against spontaneous deacylation but also provides a hydrophobic purification handle. This protocol makes it straightforward to produce biophysical quantities of natural and unnatural aminoacylated tRNAs, and has proven essential for mechanistic investigations of the T-box riboswitches.

1. INTRODUCTION

Transfer RNAs (tRNAs) are cellular non-coding RNAs that carry out essential adaptor functions during protein synthesis. Working in conjunction with aminoacyl-tRNA synthetases (aaRSs) that covalently attach appropriate amino acids to their 3' termini, tRNAs serve as an information conduit and structural medium that converts the genetic information in trinucleotide units (or codons) into amino acid sequences, which prescribe the structure and function of cellular proteins (Banerjee, Chen, Dare, Gilreath, Praetorius-Ibba et al., 2010). Beside their canonical roles in translation, it is increasingly apparent that tRNAs have evolved to execute a wide range of non-canonical cellular functions in transcriptional regulation, post-translational protein modification, cellular signal transduction, stress response, etc (Geslain & Pan, 2011; Phizicky & Hopper, 2010).

The availability of highly purified components is a prerequisite for quantitative biochemical and biophysical analyses in many *in vitro* systems. The preparation of various aminoacylated tRNAs (aa-tRNAs) have traditionally required laborious cloning, expression, and purification of individual cognate aaRSs, as these enzymes are highly specific towards

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their tRNA substrates (Walker & Fredrick, 2008). Aminoacylation reactions using aaRSs almost invariably produce a heterogeneous mixture of aa-tRNAs and (uncharged) non-aa-tRNAs. Such mixtures can adequately support protein synthesis, albeit the kinetics and thermodynamics of aa-tRNA utilization may be difficult to establish. Importantly, in the study of other systems that discriminate between charged and uncharged tRNAs, such as the bacterial T-box riboswitches (Grundy & Henkin, 1993; Zhang & Ferré-D'Amaré, 2013) and eukaryotic GCN2 kinases (Dong, Qiu, Garcia-Barrio, Anderson & Hinnebusch, 2000), it is necessary to separate aa-tRNAs from non-aa-tRNAs. The small differences in size, charge, and composition between these tRNAs make it a significant technical challenge to achieve satisfactory separation, in particular for tRNAs charged with small amino acids such as glycine and alanine. Significantly, the aminoacyl bond between tRNA 3' termini and esterified amino acids are prone to rapid hydrolysis at even slightly alkaline conditions (Hentzen, Mandel & Garel, 1972). The resulting unavailability of homogeneous aa-tRNAs has hampered, for instance, the functional studies of T-box riboswitches for two decades. Thus far, the method of choice to isolate aa-tRNAs took advantage of selective binding of aa-tRNAs to immobilized translation factor EF-Tu, but suffered from generally low efficiency (5–30%) of EF-Tu activation by GTP as well as tRNA deacylation during purification (Asahara & Uhlenbeck, 2005; Louie, Masuda, Yoder & Jurnak, 1984; Nissen, Kjeldgaard, Thirup, Polekhina, Reshetnikova et al., 1995; Ohtsuki, Yamamoto, Doi & Sisido, 2010).

In this chapter, we describe a simple, broadly applicable protocol to prepare biophysical quantities of highly purified (>95%) aa-tRNAs. This flexible method does not require proteins such as aaRS or EF-Tu, and is compatible with mutant or misacylated tRNAs and tRNAs charged with unnatural or modified amino acids. Application of this method has enabled detailed mechanistic investigations of the T-box riboswitches (Zhang & Ferré-D'Amaré, in press). Further, this procedure can also be used for preparation of aminoacylated RNAs other than tRNAs as long as the RNA has a single-stranded 3' terminus.

2. METHODS

2.1. tRNA aminoacylation using the flexizyme

To achieve broad compatibility with mutant or misacylated tRNAs and other RNAs, aminoacylation is performed using an *in vitro* selected ribozyme termed flexizyme (Fx, 46 nucleotides, Fig. 1A) instead of proteinaceous aaRS enzymes (Goto, Katoh & Suga, 2011; Lee, Bessho, Wei, Szostak & Suga, 2000; Xiao, Murakami, Suga & Ferré-D'Amaré, 2008). Unlike aaRSs, flexizyme requires pre-activated amino acids as donors for RNA aminoacylation. All natural amino acids and many non-natural amino acids and hydroxy acids can be employed by flexizyme. Depending on the chemical nature of the amino acid, an appropriate leaving group (cyanomethyl ester, 3,5-dinitrobenzyl ester, 4-chlorobenzyl thioester, or 4-[(2-aminoethyl)carbamoyl]benzyl thioester) and a matching flexizyme variant (aFx, dFx, or eFx) are used (Goto, Katoh & Suga, 2011). Generally, for aromatic amino acids, the enhanced flexizyme (eFx) and cyanomethyl ester substrates are used. For non-

aromatic amino acids, the dinitroflexizyme (dFx) and 3,5-dinitrobenzyl ester is the most versatile combination.

To illustrate the use of the flexizyme as a broadly applicable aminoacylation system, we describe a representative protocol using *in vitro* transcribed *Bacillus subtilis* tRNA^{Gly}, dFx, and dinitrobenzyl glycine as the acceptor, catalyst, and substrate for glycylation, respectively (Fig. 1).

1. **Synthesis of dinitrobenzyl glycine.** Chemical synthesis of glycine dinitrobenzyl ester is performed essentially as described with minor modifications (Murakami, Ohta, Ashigai & Suga, 2006). Briefly, 1.05 g of α -N-Boc-Glycine (6 mmol) and 1.08 g of 3, 5-dinitrobenzyl chloride (5 mmol) are dissolved in 1.4 mL triethylamine (10 mmol) and 1.0 mL dimethylformamide, and stirred for 16 hours at 21 °C. Subsequently, 90 ml diethylether is added and the mixture is washed three times with 30 mL 0.5 M HCl, three times with 30 mL 4 % NaHCO₃, and once with 50 mL brine (saturated NaCl solution in water). The organic layer is extracted and mixed with anhydrous MgSO₄ powder for drying and subsequently concentrated under reduced pressure using a rotary evaporator, before being incubated with 20 mL 4 M HCl/ethyl acetate for 20 min at 21 °C. The mixture is concentrated, washed three times with 30 mL of diethylether and dried. The residue is then dissolved in 1:3 (v/v) methanol-ethyl acetate and crystallized by slow addition of hexanes and mixing by manual shaking. The product is verified by mass spectrometry and NMR.
2. ***In vitro* transcription of tRNA^{Gly} and dFx.** *B. subtilis* tRNA^{Gly} (75 nt) and dFx (46 nt) are transcribed *in vitro* using DNA templates produced by PCR and recombinant T7 RNA polymerase as described (Milligan, Groebe, Witherell & Uhlenbeck, 1987), purified by electrophoresis on 8% polyacrylamide, 8 M urea TBE gels (29:1 acrylamide:bisacrylamide), electroeluted using a Whatman Elutrap system, concentrated using Amicon Ultra centrifugal filters (10 kD molecular weight cut off), washed once with 1 M KCl, desalted 4 times with DEPC-treated water, and stored at –20°C before use.
3. **Ribozyme-mediated aminoacylation.** Aminoacylation using flexizyme is performed as previously described (Murakami, Ohta, Ashigai & Suga, 2006). Briefly, to 200 μ L buffer containing 5 mM HEPES-KOH (pH 7.5) in diethylpyrocarbonate (DEPC)-treated water, tRNA^{Gly} and dFx are added to 40 μ M and 60 μ M, respectively. Using a thermocycler, the mixture is heated to 90 °C for 2 min and slowly cooled to 21 °C over a course of 8 min. A stock solution of 2M MgCl₂ is then added to this RNA mixture to produce 600 mM MgCl₂. This mixture is kept at 21 °C for 5 min and then on ice for 3 min. To initiate the aminoacylation reaction, 25 mM dinitrobenzylglycine dissolved in 100% DMSO is added to produce a final concentration of 5 mM and the reaction is allowed to proceed on ice for 2–6 hours (Fig. 1B). NaOAc and ethanol are added to 100 mM and 70% respectively to quench the reaction and precipitate the RNAs. The RNAs are subsequently washed with 70% ethanol, dried in a centrifugal vacuum concentrator, dissolved in 10 mM NaOAc pH 5.5, and stored at –80 °C.

4. Analysis of aminoacylation efficiency using acid-PAGE. The efficiency of aminoacylation (typically 50–60%) is evaluated using acid gel electrophoresis (6.5% polyacrylamide; 29:1 acrylamide:bisacrylamide; Fig. 1C). The gels are cast and run in 100 mM NaOAc pH 5.5 as described (Varshney, Lee & RajBhandary, 1991).

2.2. Chemical protection of the aminoacyl bond

The hydrolytic instability of the aminoacyl bond partly stems from the protonation of the free α -amine group of the esterified amino acid near neutral pH. The resulting α -ammonium is more positively charged and has a higher propensity to draw electrons from the neighboring carbonyl group, making it a better electrophile for hydrolysis (Walker & Fredrick, 2008). To stabilize the labile aminoacyl bond against spontaneous hydrolysis, the aminoacylation mixture is reacted with N-pentenoyl succinimide (Fig. 2A). N-pentenoylation of the α -amino group of esterified amino acid significantly stabilizes the aminoacyl bond (Lodder, Wang & Hecht, 2005). Similarly, peptidyl-tRNAs that carry substituted α -amines are much more hydrolytically stable (Strickland & Jacobson, 1972; Walker & Fredrick, 2008). Although N-pentenoylation requires alkaline pH under which the aminoacyl bond is rapidly hydrolyzed, under the following experimental conditions, N-pentenoylation occurs faster than deacylation, thus converting most aa-tRNAs into protected aa-tRNAs that are stable at alkaline pH (Fig. 2C). In addition, N-pentenoylation adds five hydrophobic carbons to the aa-tRNA, which can serve as an effective purification handle using reversed-phase high-performance liquid chromatography (RP-HPLC).

1. Chemical synthesis of N-pentenoyl succinimide. N-pentenoyl succinimide is synthesized essentially as described with minor modifications (Lodder, Golovine, Laikhter, Karginov & Hecht, 1998; Lodder, Wang & Hecht, 2005). Briefly, 5.0 mL pentenoic acid and 5.6 g *N*-hydroxysuccinimide are dissolved in 95 mL dichloromethane (CH_2Cl_2), to which 10.3 g of *N,N'*-dicyclohexylcarbodiimide is added. The reaction mixture is stirred at 21 °C for 90 min, filtered to remove *N,N'*-dicyclohexylurea, and concentrated under reduced pressure (~380 mBar) at 40 °C using a rotary evaporator. The concentrated oily mixture is then loaded onto a silica gel column and eluted isocratically with 7:3 hexane–ethyl acetate (v/v) using an Isolera One purification system. The fractions containing N-pentenoyl succinimide are pooled, dried under reduced pressure (100 mBar), dissolved in ethyl acetate, and crystallized by gradual addition of hexanes and mixing by manual shaking. The product (6.6 g, 68% yield) is verified by mass spectrometry and NMR.
2. N-pentenoylation of aa-tRNA. The aminoacylation mixture containing 100 μM tRNA is mixed 1:1 (v/v) with 100 mM N-pentenoyl succinimide previously dissolved in 100% dioxane. Next, 1/10 volume of 1M NaHCO_3 is added to raise the pH to ~8.5 to initiate the reaction, which is allowed to proceed for 16 hours at room temperature with gentle stirring (Fig. 2B). The reaction is quenched by adding NaOAc pH 5.5 to a final concentration of 300 mM, precipitated by adjusting to 70% (v/v) ethanol, dried, and stored at –80 °C. The N-pentenoylation is typically near-quantitative, converting essentially all aa-tRNAs to protected-aa-tRNAs (Fig. 2C).

2.3. Purification of protected aminoacylated tRNA and deprotection

For tRNAs esterified with aromatic or other highly hydrophobic side chains, aa-tRNAs and non-aa-tRNAs can be directly separated using RP-HPLC, based on their difference in hydrophobicity (Cayama, Yepez, Rotondo, Bandeira, Ferreras et al., 2000; Zhang, Liu, Christian, Gamper, Rozenski et al., 2008). For tRNAs esterified with other amino acids, in particular those with small or polar side chains (e.g., glycine, alanine), chromatographic methods are generally unable to achieve satisfactory separation. In these cases, N-pentenoylation of the esterified amino acid adds significantly to the overall hydrophobicity of the aa-tRNA, thus providing a means to effectively separate aa-tRNAs from non-aa-tRNAs.

1. RP-HPLC separation of protected aa-tRNA from non-aa-tRNA. The pentenoylation reaction mixture is diluted into RP-HPLC Buffer A [20 mM NH₄OAc pH 5.5, 10 mM MgOAc₂, 400 mM NaCl, and 5% (v/v) methanol] and fractionated at 0.5 mL/min on a C18 column (Waters Symmetry Shield RP18 3.5 μ m, 4.6 $\times$ 150 mm) previously equilibrated in the same buffer. A linear gradient of methanol from 9% (v/v) to 24% (v/v) over 10 column volumes is used to fractionate the RNA mixture into non-aa-tRNA (fraction 1), flexizyme (fraction 2), and pentenoylated-aa-tRNA (fraction 3; Fig. 3A&3B). Fractions containing N-protected aa-tRNA are pooled, precipitated with 70% ethanol, dried, and resuspended in 10 mM NaOAc pH 5.5 before deprotection.
2. Deprotection of N-pentenoylated aa-tRNA. For deprotection, N-protected aa-tRNA is mixed with 1/4 volume of 50 mM iodine previously dissolved in 1:1 (v/v) tetrahydrofuran:H₂O and allowed to react for 0.5 h at room temperature (Lodder, Golovine, Laikhter, Karginov & Hecht, 1998; Lodder, Wang & Hecht, 2005). Deprotected aa-tRNA is brought up to 0.3 M NaOAc pH 5.5, precipitated by adjusting to 70% (v/v) ethanol, washed, dried, and stored at -80°C . Typical final purity of glycyl-tRNA^{Gly} is better than 95% based on acid gel PAGE and RP-HPLC analysis (Fig. 3D). The very mild condition under which N-pentenoylated aa-tRNA is deprotected safeguards the aminoacyl linkage.

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Figure 1.

RNA aminoacylation by flexizyme. (A) Sequence and secondary structure of dinitroflexizyme (dFx) is depicted bound to tRNA^{Gly} through base pairing. The 3' terminal region of dFx (residues 43–45) and tRNA (residues 73–75) form three base pairs, positioning the tRNA terminal 3'OH to initiate nucleophilic attack on the carbonyl carbon of the dinitrobenzyl glycine ester (arrow). (B) Reaction conditions and chemical changes to the tRNA after aminoacylation. (C) Acid gel analysis showing the gel-mobility change caused by aminoacylation. tRNA aminoacylation and protonation of the α -amine near neutral pH partially neutralizes the negative charge of the tRNA, reducing the gel mobility of aa-tRNA. In contrast, the same non-aa tRNA^{Gly} that carries a terminal 2',3'-cyclic phosphate adds to the overall negative charge and thus exhibits increased gel mobility.

Figure 2.

Pentenoylation of the aminoacylated tRNA. (A) Chemical structure of N-pentenoyl succinimide. (B) Reaction conditions and chemical changes to the aa-tRNA. (C) Acid gel analysis showing the gel-mobility change caused by N-pentenoylation of aa-tRNA. Pentenoylation of the α -amine of the esterified amino acid prevents its protonation near neutral pH, thus increasing its overall negative charge whilst adding 82 Da in molecular weight. This results in an intermediate mobility between aa-tRNA and non-aa-tRNA.

Figure 3. Purification of protected aa-tRNA and deprotection. (A) RP-HPLC separation of non-aa-tRNA (fraction 1), dFx (fraction 2), and pentenoyl-aa-tRNA (fraction 3) using a linear gradient of methanol (9–24%). Pentenoyl-glycyl-tRNA^{Gly} elutes at approximately 16% methanol. (B) Acid gel analysis of the fractions from the chromatogram in (A). (C) Chemical changes during deprotection by aqueous iodine. (D) Final product of purified, deprotected aa-tRNA assayed by acid PAGE.