Enzymatic *N*-methylaminoacylation of tRNA using chemically misacylated AMP as a substrate†

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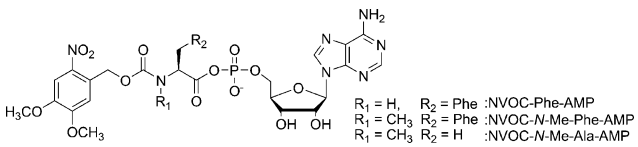
Using "chemically misacylated AMP" which is photogeneratable, tRNA can be enzymatically acylated with *N*-methylamino acids in an efficiency comparable to that of the corresponding natural aminoacylation process with amino acids.

A challenge currently exists in the manipulation of the ribosomal translation system for the sense codon-directed synthesis of nonnatural molecules[1–6] or biopolymers,[7] with possible applications for the systematic selection or evolution of active ligands in combination with ribosome-based display technologies.

Among the multi-step processes of translation, misacylation of tRNA with nonnatural substrates is one of the vital steps. tRNA can be chemically misacylated[8] at the 3′-terminus with a variety of nonnatural substrates without severe structural limitations, allowing incorporation of the latter into proteins and also enabling probing of the substrate acceptability of the ribosomal translation machinery.[9] Nevertheless, enzymatic aminoacylation still has a great advantage in its simplicity, efficiency (catalytic activity), and *in situ* applicability, as revealed by the remarkably efficient protein synthesis in naturally occurring translation systems. Evolution-based engineering of aminoacyl-tRNA synthetases (aaRSs) has allowed the use of many nonnatural amino acids as substrates.[10] A simpler method for sense codon-templated synthesis is to use wild-type aaRS. Previous[7] and recent[3b] studies have suggested that wild-type aaRSs can aminoacylate tRNAs with a variety of substrates beyond the canonical ones, especially when used under highly purified conditions.[3b] Very recently, Wolfson *et al.* have reported that the use of acyl- or hydroxyacyl-adenylate as substrates enables enzymatic tRNA acylation by acid and α-hydroxy acid analogues of amino acids.[11] Independently, we developed a new strategy for "chemically-misacylated AMP" with *N*-methylamino acids, potent candidates as building blocks for ribosomally synthesizable peptides/proteins with protease resistance and membrane permeability.[12] Our approach allows photogeneration of the active (and hence rather unstable) *N*-methylaminoacyl adenylate from *N*-NVOC-protected precursors, which are stable even at neutral pH. Here we show that this strategy relaxes the substrate specificity of aaRS and dramatically enhances the acylation efficiencies of *N*-methyl substrates, enabling us to enzymatically attach *N*-methylphenylalanine onto the 3′-end of the cognate tRNA with efficiency comparable to that of the natural aminoacylation system.

Enzymatic aminoacylation consists of two successive reactions. The aaRS first recognizes ATP and the cognate amino acid (aa) to form an aminoacyl-adenylate (aa-AMP) and releases inorganic pyrophosphate (PPi) (step 1: aa + ATP → aa-AMP + PP_i). The tRNA then binds to the resulting complex of RS-(aa-AMP) and the amino acid is transferred to tRNA at the 3′-end (step 2: aa-AMP + tRNA → aa-tRNA + AMP). The binding affinity of the amino acid to aaRS should be much weaker than that of the resulting aa-AMP, so that step 1 is expected to be primarily responsible for the substrate specificity of aaRS. We hypothesized that if a nonnatural *N*-methylamino acid is converted to an AMP derivative in advance, the misacylated AMP could bind to aaRS by avoiding the strict substrate filtration step 1, allowing it be attached to the tRNA.



We selected *Escherichia coli* PheRS/Phe pair as a target. Based on an early demonstration,[13] we first investigated whether the preacylated Phe-AMP could be practically used as a multi-turnover substrate for acyl-transfer reaction by *E. coli* PheRS. *E. coli* $tRNA^{Phe}$ was run-off transcribed by T7 RNA polymerase from the PCR-generated dsDNA template. Putative substrate Phe-AMP was initially synthesized by a simple DCC coupling of phenylalanine with AMP.[14] However, the Phe-AMP was unstable against hydrolysis and found to be unsuitable for purification by HPLC (half-life ≈ 60 s in an acylation buffer (100 mM Tris-HCl, pH 7.5 containing 15 mM $MgCl_2$, 40 mM KCl, and 1 mM DTT) at 37 °C) (Fig. S1†). We found that protection of the amino group of aa-AMP enhances its stability against hydrolysis.[15] We thus prepared the Phe-AMP substrate as an *N*-NVOC-protected derivative, which can be deprotected by photo-irradiation at 365 nm just prior to or during the course of the aminoacylation reaction. The half-life of the resulting NVOC-Phe-AMP reached 40 min ($k_{decomp} = 1.7 \times 10^{-2}$ min^{-1}) in the acylation buffer.

Enzymatic aminoacylation of *E. coli* $tRNA^{Phe}$ (4 μM) by PheRS (50 nM) was carried out in 100 mM Tris-HCl buffer (pH 7.5) containing 15 mM $MgCl_2$, 40 mM KCl, 0.04 units μL^{-1} inorganic pyrophosphatase, and 1 mM DTT at 37 °C. Aminoacylation reactions were analyzed by 9% acid PAGE with SYBR green staining (Fig. 1a). The presence of both of phenylalanine (100 μM) and ATP (4 mM) produced distinct bands for phenyalanyl-tRNA (Phe-tRNA) and intact $tRNA^{Phe}$ (lane 5). The absence of ATP

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† Electronic supplementary information (ESI) available: General details, synthesis of chemically misacylated AMP, enzymatic aminoacylation of tRNA, translation and mass analysis conditions, and Fig. S1–S4. See DOI: 10.1039/b806965d

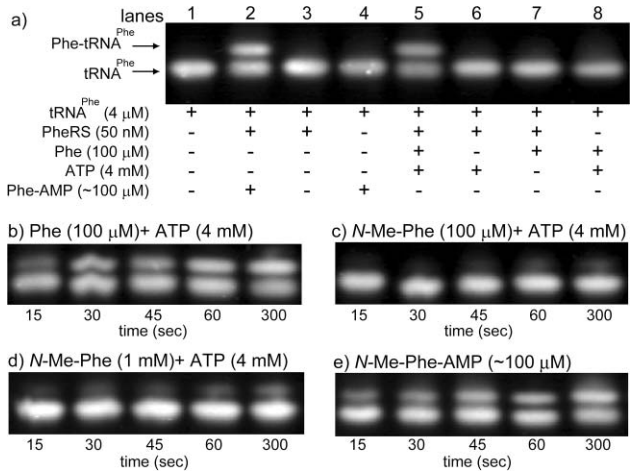

Fig. 1 a) Acid PAGE analysis of aminoacylation (5 min) of $tRNA^{Phe}$ by *E. coli* PheRS using Phe-AMP (lanes 2–4) and Phe + ATP (lanes 5–8) as substrates and time courses of respective aminoacylation reactions of $tRNA^{Phe}$ using b) 100 μM Phe + ATP, c) 100 μM *N*-Me-Phe + ATP, d) 1 mM *N*-Me-Phe + ATP, and e) ~100 μM *N*-Me-Phe-AMP. The reactions were carried out in a 100 mM Tris-HCl buffer (pH 7.5) containing 15 mM $MgCl_2$, 40 mM KCl, 0.04 units μL^{-1} inorganic pyrophosphatase, 1 mM DTT, and 10% DMSO (except for lanes 5–8 in Fig. 1a) at 37 °C using substrates as indicated.

completely suppressed the production of acylated tRNA (lane 7), indicating that other components were completely free from ATP. Following the validation of the reaction conditions for enzymatic acylation, we proceeded to enzymatic aminoacylation using Phe-AMP as a substrate. When Phe-AMP (~100 μM,[16] final concentration), generated by deprotection of NVOC-Phe-AMP by 365 nm photo-irradiation in the solution of 0.33 mM KOAc pH 5.0 (33% DMSO),[17] was used in place of phenylalanine and ATP, a band for Phe-tRNA was clearly observed on the gel in a yield of approximately 50% (lane 2), indicating that preacylated Phe-AMP can work as a practical substrate of *E. coli* PheRS to transfer the phenylalanine moiety to $tRNA^{Phe}$.

We then moved on to the acylation reaction using AMP chemically misacylated with an *N*-methylamino acid. The ATP-based normal aminoacylation reaction using ATP (4 mM) and *N*-methylphenylalanine (*N*-Me-Phe, 100 μM) produced no detectable band of *N*-Me-Phe-$tRNA^{Phe}$ within 5 min under our experimental conditions (Fig. 1c). A higher concentration of *N*-Me-Phe (1 mM) allowed the production of *N*-Me-Phe-$tRNA^{Phe}$. However, incubation for 5 min was not sufficient to give a distinct product (Fig. 1d), suggesting the potency of wild-type *E. coli* PheRS to accept *N*-Me-Phe; however, the efficiency was much lower than that for natural substrate Phe. This was consistent with recent findings that *N*-Me-Phe is a poor substrate for PheRS.[3b] On the other hand, as shown in Fig. 1e, a distinct band of acylated $tRNA^{Phe}$ was observed in the new system using *N*-Me-Phe-AMP (~100 μM).[16,18] The acylation yield reached about 60% within 5 min (Fig. 1e), which was comparable to that of the natural *E. coli* Phe/PheRS system (Fig. 1b). Ten-fold higher concentrations of PheRS (500 nM) and *N*-Me-Phe substrate (1 mM) afforded acylated tRNA in a yield of 50%; however, it required longer (15 min) incubation time (Fig. S3c). Furthermore, the acylated tRNAs obtained turned out to contain a substantial amount of Phe-$tRNA^{Phe}$ (Fig. S4c). The present *N*-Me-Phe-AMP system reached 50% acylation within 10 s (Fig. S3d) under the same *E. coli* PheRS concentration with much lower concentration of substrate *N*-Me-Phe-AMP (~100 μM).

To further confirm the attachment of *N*-Me-Phe on the 3′-end of tRNA in an active form, we then isolated $tRNA^{Phe}$ after the reaction using *N*-Me-Phe-AMP (100 μM in the presence of 50 nM PheRS with 5 min incubation) and subjected it to a translation reaction in a reconstituted cell-free translation system free from PheRS and Phe. An mRNA encoding 16-mer oligopeptide fMDYKDDDDKQKL**F**LTH (oligopep) was prepared for this assay. The *N*-terminal 9-mer is identical with the FLAG-tag sequence (underlined) and allowed isolation of the translated peptide for direct mass analysis. If *N*-Me-Phe was incorporated at Phe codon (bold), the resulting peptide should give a mass of $[M + H]^+ = 2053.95$. The translated peptide indeed gave a mass peak of 2053.88 (Fig. 2), indicating that *N*-Me-Phe was precisely attached at the 3′-end of $tRNA^{Phe}$. An additional mass peak at 2005.84 was also observed (Fig. 2). This was assigned as the peptide containing Leu or Ile (calcd. 2005.95) incorporated at the Phe codon by $tRNA^{Leu}$ or $tRNA^{Ile}$, as demonstrated by production of peptide with the same mass value in the translation reaction using nonacylated $tRNA^{Phe}$ (Fig. S4a). Interestingly, preliminary experiment suggested that the present aa-AMP/RS system still possesses the original side-chain selectivity. When the acylation reaction (500 nM PheRS, 30 s incubation) was carried out in the presence of *N*-Me-Phe-AMP (~100 μM) and *N*-Me-Ala-AMP (~200 μM),[19] *E. coli* PheRS selectively recognized *N*-Me-Phe-AMP to give *N*-Me-Phe-tRNA, as revealed by the mass spectrum showing the presence of *N*-Me-Phe-containing oligopeptide (found 2053.14) and the absence of *N*-Me-Ala-containing oligopeptide (calcd. 1977.92) (Fig. S4b).

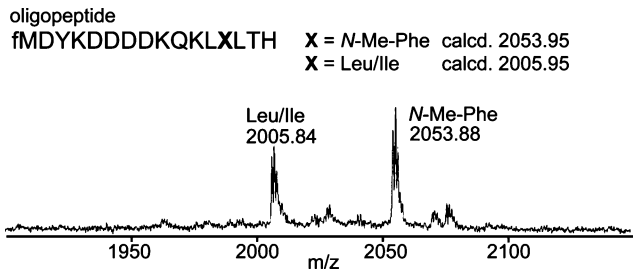

Fig. 2 MALDI-TOF mass spectrum for the oligopeptide produced in the presence of $tRNA^{Phe}$ preacylated by *N*-Me-Phe-AMP (~100 μM) with *E. coli* PheRS (50 nM).

In conclusion, we have demonstrated a strategy of chemically misacylated AMP for catalytic attachment of *N*-methylamino acids to tRNA with an efficiency comparable to that of the natural parent. The advantage of the present strategy is that otherwise active (and hence unstable) *N*-Me-aa-AMP can be photo-generated from stable NVOC-protected derivatives that can be present even at physiological pH without significant decomposition. Therefore, in principle, the present strategy can be directly used for *in situ* application in the translation system without lowering pH;[11] this is a notable merit of this system in view of possible pH-dependence of the activity of protein translation machinery.[20] In addition, the active *N*-Me-aa-AMP could be gradually generated simply by controlling light intensity. This may be another merit of our strategy. Production of sequence-programmed

N-Me peptides or proteins is also an intriguing application in view of retaining inherent side-chain selectivity. Further work is now underway along these lines.

Acknowledgements

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17 The reaction solution contained a final 10% volume of DMSO. Fig. S2 shows that addition of DMSO (10%) leads to slight acceleration of aminoacylation reaction kinetics.

18 The half-lives of NVOC-*N*-Me-Phe-AMP and *N*-Me-Phe-AMP were 95 min ($k_{decomp} = 7.3 \times 10^{-3}$ min^{-1}) and ~4.0 min, respectively, in the acylation buffer at 37 °C.

19 *N*-Me-Ala-AMP was found to be hydrolyzed faster than *N*-Me-Phe-AMP in the acylation buffer. Therefore, we used twice the concentration of *N*-Me-Ala-AMP (~200 μM) relative to *N*-Me-Phe-AMP (~100 μM). HPLC analysis indicated that *N*-Me-Ala-AMP ≥ *N*-Me-Phe-AMP in molar ratio can be assured during the acylation reaction for 30 s under these conditions.

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