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Phenylalanyl-tRNA synthetase from *Thermus thermophilus* can attach two molecules of phenylalanine to $tRNA^{Phe}$

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Phenylalanyl-tRNA synthetase from the extreme thermophilic bacterium *Thermus thermophilus* can incorporate more than one molecule of phenylalanine into the $tRNA^{Phe}$. It is shown that the 'hyperaminoacylated' $tRNA^{Phe}$ is the bis-2',3'-*O*-phenylalanyl-$tRNA^{Phe}$, and its formation is typical for the thermophilic enzyme but does not occur for *E. coli* phenylalanyl-tRNA synthetase under the same conditions.

Phenylalanyl-tRNA synthetase; $tRNA^{Phe}$; tRNA aminoacylation

1. INTRODUCTION

When tRNAs are aminoacylated in vitro, the limiting substrate is usually tRNA itself because the concentrations of ATP and phenylalanine are higher than that of tRNA. The amount of aminoacyl-tRNA formed during the aminocylation reaction must be equal to the amount of the limiting substrate (tRNA). However, deacylation of aminoacyl-tRNA (both enzymatic and nonenzymatic) which occurs simultaneously with tRNA aminoacylation can markedly complicate the situation; for example, a dependence of aminoacylation plateaux on the enzyme concentration has been observed [1–4]. We have found analogous dependence for a *Thermus thermophilus* phenylalanylation system but the most intriguing fact is that, at high enzyme concentrations, the incorporation of more than one molecule of phenylalanine into $tRNA^{Phe}$ ('hyperaminoacylation' of tRNA) has been detected. The purpose of the present work was to establish the nature of this phenomenon.

2. MATERIALS AND METHODS

Homogeneous PheRSase from *Thermus thermophilus* HB8 (264 kDa) was isolated as described in [5]. PheRSase from *E. coli* MRE-600 (267 kDa) was isolated as described in [6]. Electrophoretically pure $tRNA^{Phe}$ from *Thermus thermophilus* was prepared as in [7].

Synthesis of aminoacyl-$tRNA^{Phe}$ was carried out at 37°C for 20 min. The reaction mixtures contained 5 mM ATP, 9 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.5), 12 μM L-[^{14}C]phenylalanine (UVVVR, 225 Ci/mol), 0.7 A_{260} U/ml (1.3 μM) of $tRNA^{Phe}$, 5 μg/ml PheRSase from *E. coli* MRE-600 or 45 μg/ml PheRSase from *Thermus thermophilus* HB8. The reaction was stopped by the addition of sodium acetate (to pH 5.0). Then the enzymes were removed by phenol extraction. Aminoacyl-tRNA was precipitated by the addition of ethanol and centrifugation.

N-Acetylaminoacyl-tRNA was prepared according to [8] by incubation of 0.3 A_{260} units (560 pmol) of aminoacyl-tRNA with 20 μl 0.3% (v/v) acetic anhydride in 50 mM sodium barbiturate (pH 9.5) at 4°C during 30 min. *N*-Acetylaminoacyl-tRNA was precipitated by the addition of ethanol and centrifugation.

Hydrolysis of aminoacyl-tRNA and *N*-acetylaminoacyl-tRNA with RNase A from bovine pancreas (Sigma) was performed at 37°C. The reaction mixtures (20 μl) contained 5 μg/ml RNase A, 0.3 A_{260} units (560 pmol) of aminoacyl- or *N*-acetylaminoacyl-tRNA, 50 mM sodium acetate (pH 5.0). After 10 min of incubation large fragments of tRNA were precipitated by the addition of ethanol and centrifugation. The supernatant was concentrated by evaporation and was analysed on a Merck RP-$18F_{254S}$ thin-layer plate. The length of the tRNA fragments formed during RNase A hydrolysis was determined by gel electrophoresis on 10% polyacrylamide–7 M urea gels.

Bis-2',3'-*O*-(*N*-acetyl-L-[^{14}C]phenylalanyl)-adenosine was synthesized and purified according to [9]. *N*-acetyl-L-[^{14}C]phenylalanine was prepared as in [10], 5'-*O*-dimethoxytrityladenosine was prepared as in [11]. The esterification of 5'-*O*-dimethoxytrityladenosine was carried out in dry dimethyl formamide by imidazolide of *N*-acetyl-L-[^{14}C]phenylalanine overnight at room temperature. The dimethoxytritylic group was removed by addition of HCl to pH 3.0. Adenosine diester was isolated using a PepRPC Pharmacia reversed-phase column (C_2/ C_{18} phase).

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Abbreviations: PheRSase, phenylalanyl-tRNA synthetase (EC 6.1.1.20); hyper-Phe~tRNA, tRNA phenylalanylated above 100%; Phe~tRNA, normally phenylalanylated tRNA; RNase A, ribonuclease A.

3. RESULTS AND DISCUSSION

Aminoacylation of $tRNA^{Phe}$ was performed in the presence of varied concentrations of PheRSases from *Th. thermophilus* and *E. coli* (Fig. 1). At low concentrations of *Th. thermophilus* PheRSase, the aminoacylation of $tRNA^{Phe}$ was incomplete; however, using high en-

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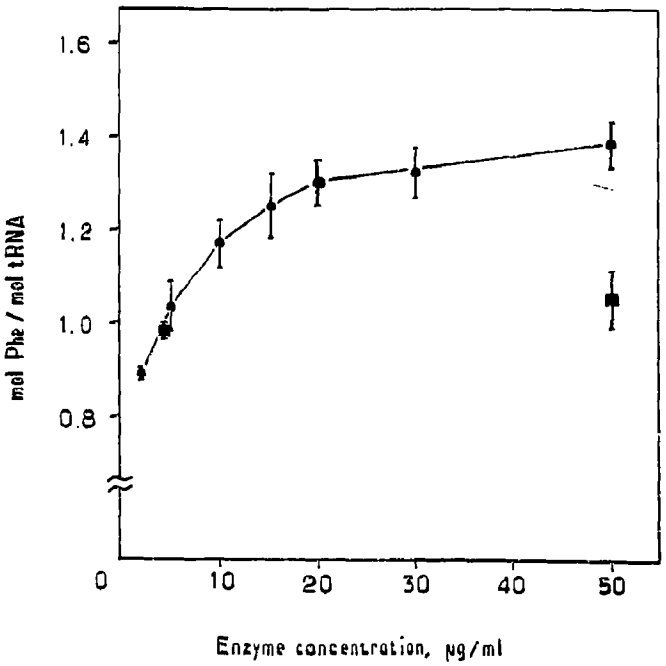

Fig. 1. Dependence of the *Th. thermophilus* $tRNA^{Phe}$ phenylalanylation plateaux on enzyme concentration. For every enzyme concentration the kinetics of aminoacylation at 37°C were carried out. The reaction mixtures contained 5 mM ATP, 9 mM $MgCl_2$, 50 mM Tris-HCl (ph 8.5), 12 μM L[^{14}C]phenylalanine, 1 mM EDTA, 0.6 A_{260} U/ml (1.1 μM) of $tRNA^{Phe}$ from *Th. thermophilus*, PheRSase from *Th. thermophilus* (●) or *E. coli* (■). The aliquots were removed at 5, 10, 15, 20, 25, 30, 40, 50, 55, 60, 70 min of incubation and spotted on FN-16 filters impregnated with 5% solution of trichloroacetic acid.

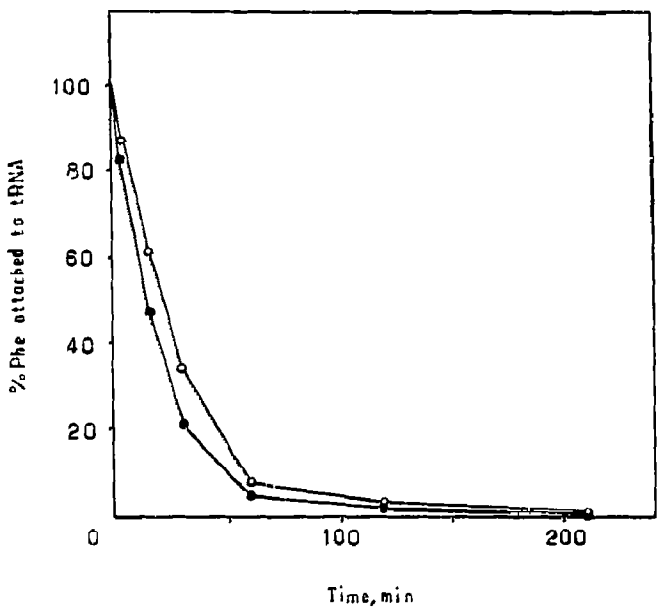

Fig. 2. Kinetics of hyper-[^{14}C]Phe~tRNA (○) and [^{14}C]Phe~tRNA (●) deacylation in 50 mM Tris-HCl (pH 8.5) at 37°C.

zyme concentrations, we achieved a plateau level of amino acid incorporation equal to 1.4 mol Phe per mol tRNA. Aminoacyl-$tRNA^{Phe}$ containing an extra phenylalanyl residue was marked as 'hyperaminoacylated tRNA' (hyper-Phe~tRNA).

It is of interest that, in the case of PheRSase from *E. coli* which can utilize $tRNA^{Phe}$ from *Th. thermophilus* as substrate, the aminoacylation level was approximately 1 mol Phe per mol tRNA at high and low enzyme concentrations. Therefore we used $tRNA^{Phe}$ aminoacylated by PheRSase from *E. coli* ('normally aminoacylated tRNA', Phe~tRNA) as control.

As regards the site of additional phenylalanyl residue attachment two possibilities were analyzed: (1) hyper-Phe~tRNA has a dipeptide PhePhe on its 3′-terminus, (2) both 2′- and 3′-hydroxyl groups of 3′-terminal adenosine are esterified by phenylalanine.

Deacylation of hyper-Phe~tRNA at 37°C, pH 8.5 was near complete after 2 h of incubation (Fig. 2). This means that the stability of the bond between the extra phenylalanyl residue and tRNA is comparable with that of the ester bond in Phe~tRNA.

The analysis of low-molecular-weight products of hyper-Phe~tRNA deacylation by reversed-phase chromatography did not show any phenylalanylphenylalanine (data not shown).

To check the second assumption, limited hydrolysis of aminoacyl-tRNA was performed. The 3′-terminal adenosine of tRNA can be removed by RNase A. The most active cleavage site in the $tRNA^{Phe}$ from *Th. thermophilus* is ...C_{75}–A_{76} (deduced from the structure of *Th. thermophilus* $tRNA^{Phe}$ [12] and specificity of RNase A [13]) therefore oligonucleotides with [^{14}C]phenylalanine on the 3′-terminus do not accumulate.

In the course of RNase hydrolysis of hyper-Phe~tRNA a compound was formed which was not observed in the case of Phe~tRNA hydrolysis (Fig. 3A,B). This compound was unstable and destroyed during chromatography.

It is known that the ester bond of a phenylalanyl residue with tRNA terminal adenosine can be stabilised by *N*-acetylation of the phenylalanyl moiety of Phe~tRNA [14]. By analogy we acetylated hyper- Phe~tRNA to stabilize a possible 3′-terminal diester. By reversed-phase TLC analysis of the RNase A hydrolysate of acetylated hyper-Phe~tRNA we have detected a compound with the mobility corresponding to that of bis-2′,3′-*O*-(*N*-acetylphenylalanyl)-adenosine (Fig. 3D,E).

On the basis of these data we conclude that PheRSase from *Th. thermophilus* can esterify simultaneously 2′- and 3′-hydroxyl groups of the tRNA 3′-terminal adenosine. The [^{14}C]Phe transfer on the other groups of tRNA (nucleotides residues 1–75) was not observed. The high-molecular-weight fraction of RNase A hydrolysate of hyper-Phe~tRNA contained no radioactivity and short oligonucleotides covalently bound to [^{14}C]Phe were not formed (data not shown).

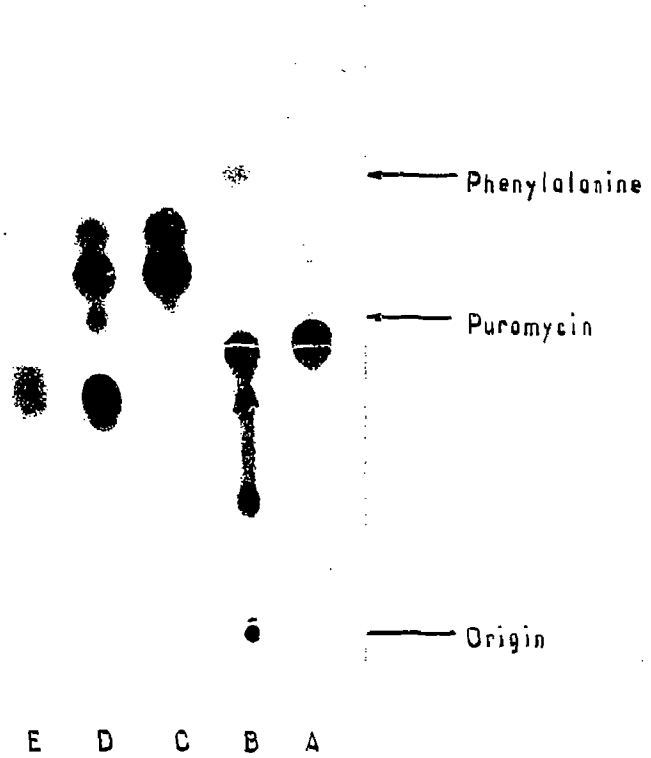

Fig. 3. Autoradiogram of RNase hydrolysates of [^{14}C]Phe~tRNA (A), hyper-[^{14}C]Phe~tRNA (B), acetylated [^{14}C]Phe~tRNA (C) and acetylated hyper-[^{14}C]Phe~tRNA (D) separated on reversed-phase thin-layer plate Merck RP-18F_{254S}. Bis-2'3'-*O*-(*N*-acetyl[^{14}C]phenylalanyl)-adenosine (E), puromycin (as analog of 3'-*O*-phenylalanyladenosine) and phenylalanine were used as markers. Chromatography was done in 65% (v/v) methanol containing 50 mM triethylammonium acetate (pH 6.0) at room temperature.

All the experiments above were performed at 37°C for more convenient manipulations; however, we have observed the hyper-Phe~-tRNA formation in the temperature interval 25–80°C.

It was shown earlier that for bacterial PheRSases 2'-hydroxyl group of $tRNA^{Phe}$ is the main target of aminoacylation (see [15]). The existence of bis-2',3'-*O*-phenylalanyl-tRNA has not been detected before. The formation of this product may be due to rebinding of aminoacyl-tRNA to the enzyme at high concentration of aminoacyl-tRNA. It is of interest to check whether the hyperaminoacylation takes place in vivo or that it is only an in vitro effect.

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