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The phenylalanine tRNA from Mycoplasma sp. (Kid): a tRNA lacking hypermodified nucleosides functional in protein synthesis

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ABSTRACT

Phenylalanine tRNA from *Mycoplasma* sp. (Kid) was purified and characterized. The tRNA can be aminoacylated by phenylalanyl-tRNA synthetase from both *Mycoplasma* and *E. coli*. In a tRNA-dependent cell-free *E. coli* amino acid incorporating system programmed with poly U pure *Mycoplasma* $tRNA^{Phe}$ was fully active in promoting phenylalanine incorporation, even in direct competition with homologous *E. coli* $tRNA^{Phe}$. Since the *Mycoplasma* tRNA lacks isopentenyladenosine, or any related hypermodified nucleoside, it appears that the presence of such nucleosides in tRNA is not an absolute requirement for protein synthesis.

INTRODUCTION

Isopentenyladenosine or close derivatives of it have been found to be present in the tRNA of almost all organisms investigated (1). Its occurrence among the tRNA species is restricted to those which correspond to codons beginning with U (2,3). Sequence analysis of a number of those tRNAs showed that in each case the hypermodified nucleoside is found adjacent to the 3'-terminal nucleotide of the anticodon (2,3).

A variety of experiments designed to elucidate the role of this hypermodified nucleoside in the functions of tRNA have been done. There is evidence that this modified nucleoside is involved in the interaction of tRNA with the messenger RNA on the ribosome. Chemical reactions of pure yeast tRNAs with iodine (4,5) or sodium bisulfite (6) lead to specific modifications of the isopentenyladenosine. The altered tRNA species show a reduced affinity for forming the mRNA:ribosome:aminoacyl-tRNA complex. Isopentenyl deficient tRNA was shown to be less efficient in *in vitro* protein synthesis: in studies with *E. coli* su_3^+ amber suppressor tyrosine tRNA species in which the isopentenyladenosine moiety was modified to varying degrees the fully modified tRNA had the highest activity (7). On the other hand, it was shown that unfractionated *Lactobacillus acidophilus* tRNA which lacked one half of its normal content of isopentenyladenosine

functions normally in *in vitro* protein synthesis (8).

tRNA from *Mycoplasma* sp. (Kid) has an unusually low content of modified nucleosides and in particular lacks isopentenyladenosine and its derivatives (9). Furthermore, unfractionated tRNA from this strain supports protein synthesis directed by poly U or f2 RNA in a cell-free *E. coli* amino acid incorporating system. In this paper we describe the isolation of pure phenylalanine tRNA from *Mycoplasma* sp. (Kid) which contains m^1G instead of i^6A (10). This tRNA promotes phenylalanine incorporation in a tRNA-dependent cell-free *E. coli* system directed by poly U.

MATERIALS AND METHODS

General. Uniformly labelled L-[^{14}C]phenylalanine and L-[^{3}H]phenylalanine were obtained commercially and had specific activities of 0.383 and 7.6 Ci/mM, respectively.

tRNA and Aminoacyl-tRNA Synthetases. The growth of *Mycoplasma* sp. (Kid) has been described earlier (9). The preparation of tRNA and aminoacyl-tRNA synthetases (free from tRNA) from Kid or *E. coli* K12 (CA244) was performed as reported previously (9,11). *E. coli* K12 $tRNA^{Phe}$ (specific activity 460 pmoles per A_{260} unit) was the gift of Dr. S.K. Yang.

Assay for Amino Acid Acceptor Activity. The incubation mixture contained per ml: 0.05 - 10 A_{260} units of tRNA, 50 µmoles sodium cacodylate (pH 7.2), 10 µmoles magnesium acetate, 10 µmoles KCl, 2 µmoles ATP, 2-4 nmoles radioactive amino acid, and aminoacyl-tRNA synthetase. In addition, 80 µmoles of ammonium chloride were present in reactions with the *Mycoplasma* enzymes. The incubation temperature was 37° for the reactions with *E. coli* enzyme, or 30° for those with *Mycoplasma* enzymes.

Preparation of Mycoplasma $tRNA^{Phe}$. Pure $tRNA^{Phe}$ was isolated from unfractionated *Mycoplasma* sp. (Kid) tRNA on benzoylated DEAE-cellulose by the method of Gillam, *et al.* (12) as described by Roy, *et al.* (13), using the phenoxyacetyl ester of N-hydroxysuccinimide as derivatizing agent. A sham derivatization was performed before the aminoacylation step. 60 A_{260} units of $tRNA^{Phe}$ (specific activity 1550 pmoles per A_{260} unit) were obtained from 7500 A_{260} units of unfractionated *Mycoplasma* tRNA.

Amino Acid Incorporating System. The preparation of a tRNA-dependent cell-free amino acid incorporating system from *E. coli* K12 was described

previously (14). Polypeptide synthesis was assayed as the incorporation of radioactive amino acid into acid-insoluble material as described by Morgan *et al.* (15).

Sedimentation of Aminoacyl-tRNA. Zone sedimentation was performed as described by Burgi and Hershey (24). A linear concentration gradient of 20 to 80% deuterium oxide (v/v) in 0.01 M sodium acetate (pH 5.0), 0.005 M $MgCl_2$ was used. The sample (4000 cpm *E. coli* [^{14}C]Phe-tRNA and 3500 cpm *Mycoplasma* [^{3}H]Phe-tRNA) in 0.1 ml of buffer was layered onto a 5.2 ml gradient and spun in an SW65 rotor in a Spinco L2-65B ultracentrifuge at 4° and 65,000 rpm for ten hours. The polyallomer tube was punctured and fractions collected from the bottom. An aliquot of each fraction was applied onto a Whatman 3 MM filter disc, dried, and counted.

RESULTS

Purification of Mycoplasma tRNA^{Phe}. In all phenylalanine tRNA species with known nucleotide sequence isopentenyladenosine or a fluorescent nucleotide (Y or derivatives) are found next to the anticodon (21). Since *Mycoplasma* sp. (Kid) tRNA does not contain isopentenyladenosine (9) nor any fluorescent bases we attempted to isolate pure phenylalanine tRNA from this organism in order to study the properties of such an isopentenyladenosine deficient tRNA.

In order to assay and purify the tRNA we needed a preparation of good phenylalanyl-tRNA synthetase. From earlier studies we anticipated difficulties with *Mycoplasma* aminoacyl-tRNA synthetase preparations as they are not very stable and do not charge to high levels. However we knew that there was complete cross-chargeability of *Mycoplasma* tRNA with *E. coli* aminoacyl-tRNA synthetases (22,23). In a preliminary survey we found a phenylalanine acceptor activity of unfractionated *Mycoplasma* tRNA when assayed with *E. coli* enzyme of 42 pmoles/A_{260} unit, while the homologous enzyme preparation charged only up to a quarter of that amount. Since we could not improve the homologous charging reaction in many trials (addition of CTP, terminal nucleotidyl transferase, or pyrophosphatase) we decided to use the *E. coli* phenylalanyl-tRNA synthetase for purification of the tRNA. Using a partially purified *E. coli* phenylalanyl-tRNA synthetase the tRNA^{Phe} from *Mycoplasma* was isolated by chromatography on benzoylated DEAE-cellulose using the general procedure of Gillam, *et al.* (12). The isolated tRNA was over 90% pure as judged by the phenylalanine acceptor activity of 1550

pmoles/A_{260} unit (16). A further criterion for the purity may be the fact that in charging with the 19 other amino acids less than 30 pmoles could be esterified to the tRNA.

Since the heterologous *E. coli* enzyme was used for the purification we had to establish that the tRNA purified was really *Mycoplasma* $tRNA^{Phe}$. Purified tRNA could be charged with phenylalanine by a crude mixture of *Mycoplasma* aminoacyl-tRNA synthetases to the extent of 410 pmoles/A_{260} unit. (This is similar to the charging of unfractionated tRNA by the homologous synthetase, where only one quarter of the charging level obtainable with the *E. coli* aminoacyl-tRNA synthetase was reached.) Therefore we decided to check by column chromatography whether the *E. coli* and the *Mycoplasma* enzymes charge the same phenylalanine isoacceptor species. Unfractionated *Mycoplasma* tRNA was acylated with [^{14}C] phenylalanine catalysed by the *Mycoplasma* synthetase, and the purified $tRNA^{Phe}$ preparation was acylated with [^{3}H] phenylalanine by the *E. coli* enzyme. The two tRNA preparations were co-chromatographed on a reversed phase chromatography system (RPC-5). If the *E. coli* synthetase acylates other tRNA species, then there should be several peaks of [^{3}H] radioactivity, only one coincident with the peak of [^{14}C] labelled material. Figure 1 shows the results of the experiment.

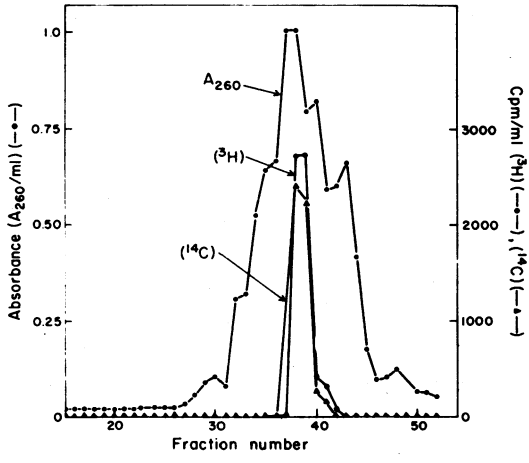

Fig. 1. Reversed phase chromatography of *Mycoplasma* Phe-tRNA charged by *E. coli* or homologous aminoacyl-tRNA synthetases. The sample contained 15 A_{260} units of unfractionated Kid tRNA charged with [^{14}C] phenylalanine catalyzed by Kid synthetase (27,500 cpm total), and 0.07 A_{260} units of Kid $tRNA^{Phe}$ charged with [^{3}H] phenylalanine catalysed by the *E. coli* enzyme (80,000 cpm total). The RPC-5 column (1 x 40 cm) was eluted at room temperature with a 400 ml linear gradient of NaCl (0.4 to 0.7 M) in 0.01 M magnesium chloride - 0.01 M Tris-chloride (pH 5.0).

The [^{3}H] and [^{14}C] radioactivity eluted together indicating that the *Mycoplasma* synthetase and the *E. coli* synthetase acylate the same material with phenylalanine. In addition, it indicates that there is only one $tRNA^{Phe}$

species in this *Mycoplasma* strain. The most convincing proof for it being homogeneous $tRNA^{Phe}$ was the radioactive fingerprinting of this sample with the aid of polynucleotide phosphorylase and polynucleotide kinase which showed it to consist of a single RNA species with the anticodon GAA (17).

Physical Properties of Mycoplasma $tRNA^{Phe}$. Thermal denaturation curves of pure *Mycoplasma* $tRNA^{Phe}$ were determined in the presence and absence of Mg^{++} and are shown in Figure 2. As is generally observed (18) the

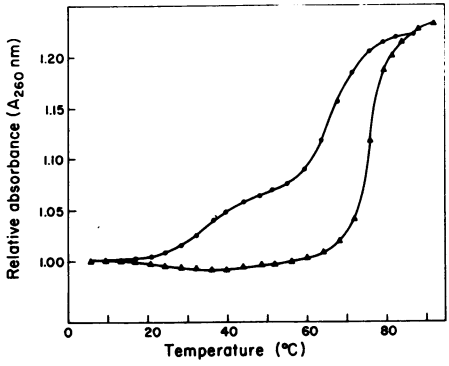

Fig. 2. Thermal denaturation curve of *Mycoplasma* $tRNA^{Phe}$ measured in 0.001 M disodium EDTA - 0.01 M sodium cacodylate - 0.001 M sodium phosphate (pH 7.0) - and 0.15 M sodium perchlorate with (▲) or without (●) 0.002 M magnesium chloride. There is no correction for the expansion of water due to heating.

melting profile in the absence of Mg^{++} was much broader and more structured. The extent of hyperchromicity was the same as that of *E. coli* $tRNA^{Phe}$ (19). For a comparison of their sedimentation behavior, *Mycoplasma* $tRNA^{Phe}$ and partially purified *E. coli* $tRNA^{Phe}$ were charged by the *E. coli* aminoacyl-tRNA synthetase with [^{3}H] phenylalanine and [^{14}C] phenylalanine, respectively. Then the two Phe-tRNA preparations were cosedimented in a deuterium oxide-water gradient in the preparative ultracentrifuge. It is clearly seen from Figure 3 that the *Mycoplasma* and *E. coli* phenylalanyl-tRNA have the same sedimentation behavior.

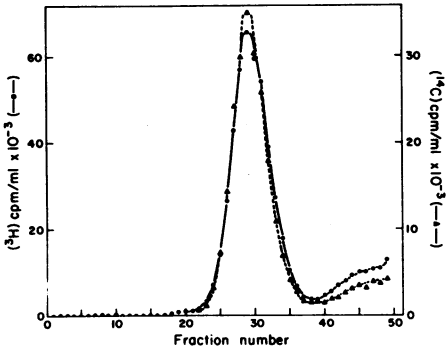

Fig. 3. Sedimentation of a mixture of [^{14}C]Phe-tRNA from *E. coli* (▲) and [^{3}H]Phe-tRNA from *Mycoplasma* (●). For details see Materials and Methods.

Amino Acid Incorporation Studies with Mycoplasma tRNA^{Phe}. Since *Mycoplasma* tRNA does not contain hypermodified nucleosides (9), pure *Mycoplasma* tRNA^{Phe} was tested for its efficiency in *in vitro* protein synthesis. Polyphenylalanine formation was studied in a cell-free, tRNA dependent *E. coli* amino acid incorporating system programmed with poly U. As found earlier with unfractionated *Mycoplasma* tRNA (9), preliminary experiments showed pure tRNA^{Phe} to be as effective in this system as *E. coli* tRNA (data not shown). In order to compare the two tRNAs directly, a competition experiment was performed in which the *Mycoplasma* tRNA^{Phe} was acylated with [^{3}H] phenylalanine, and the *E. coli* tRNA was acylated with [^{14}C] phenylalanine. The tRNA samples were extensively dialysed to remove any free amino acid, and a hundred-fold excess of unlabelled phenylalanine was added to the reaction to dilute out any labelled phenylalanine released from the tRNAs by deacylation. No additional radioactive phenylalanine was added to the reaction. If one tRNA was able to react preferentially at the ribosome in transferring its amino acid, then the initial rate of incorporation of its label would be higher than that of the other tRNA. Thus it is important to examine the early part of the reaction. In order to magnify this period, the reaction was performed at dilute concentration and lower temperature (18°). The results are shown in Figure 4. Clearly,

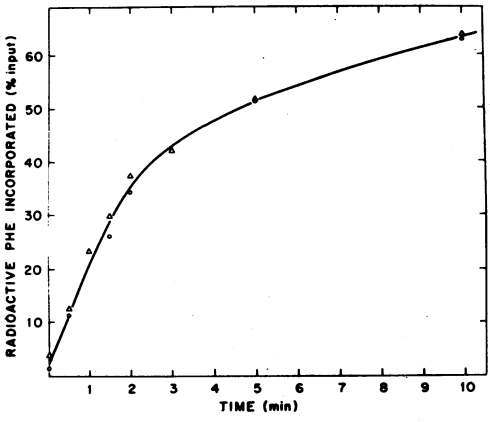

Fig. 4. Phenylalanine incorporation competition experiment with *Mycoplasma* [^{3}H]Phe-tRNA and *E. coli* [^{14}C]Phe-tRNA. The reaction contained per ml: 0.07 A_{260} units (13,000 cpm) of *Mycoplasma* [^{3}H] Phe-tRNA - 3.33 A_{260} units (10,150 cpm) of unfractionated *E. coli* [^{14}C]Phe-tRNA - 50 nmoles [^{12}C] phenylalanine - 2 A_{260} units of ribosomes and 0.2 mg poly U. All other components as described in Materials and Methods. Incubation was at 18°C. Aliquots of 50 µl were taken at the times indicated. A control experiment in which poly U was omitted was done concurrently, with no measurable incorporation of radioactivity above the zero time level.

radioactive phenylalanine from *E. coli* Phe-tRNA and from *Mycoplasma* Phe-tRNA are incorporated at the same initial rate. Thus, in direct competition, *Mycoplasma* $tRNA^{Phe}$ is fully equivalent to *E. coli* in phenylalanine incorporation in *in vitro* protein synthesis.

DISCUSSION

The important difference between the *Mycoplasma* and *E. coli* tRNAs is in the number and kinds of modified nucleosides present. *E. coli* $tRNA^{Phe}$ has ten modified nucleotides; *Mycoplasma* $tRNA^{Phe}$ contains only five (10). As expected, the gross physical properties of the tRNA which were measured are not affected by this. Moreover, *Mycoplasma* $tRNA^{Phe}$ can be fully aminoacylated by the *E. coli* phenylalanyl-tRNA synthetase; so the tRNA must contain the recognition site for this enzyme. The paucity of modified bases in this tRNA does not seem to alter grossly the heterologous charging reaction. However, more detailed enzyme kinetic studies may reveal subtle differences in the kinetic parameters as was found in the reaction of the yeast enzyme with *E. coli* tRNA (20).

In *Mycoplasma* $tRNA^{Phe}$ the nucleoside next to the anticodon is not hypermodified; instead it is 1-methyl-guanosine (21). The change from the bulky methylthioisopentenyladenosine to a single methylated guanosine might be expected to drastically alter the ability of the tRNA to function in protein synthesis. However, our results indicate no detectable difference in the efficiency of the two tRNAs in *in vitro* protein synthesis. There may be more subtle interactions between the components of protein synthesis *in vivo* in which the extent of modification becomes important. Possibly miscoding or premature chain termination may be consequences of isopentenyladenosine deficiency in tRNA. However, *in vivo Mycoplasma* tRNA must function satisfactorily and the presence of isopentenyladenosine in tRNA is no absolute requirement for the function of this macromolecule.

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