THE BEHAVIOUR OF PHENYLALANINE TRANSFER RIBONUCLEIC ACID WITH 3′-TERMINAL FORMYCIN IN PROTEIN BIOSYNTHESIS USING A RABBIT RETICULOCYTE CELL-FREE SYSTEM

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1. Introduction

The interaction between ribosomes and tRNA has been the subject of many investigations. Ribosomes have at least two tRNA specific binding sites. The interactions between tRNA and the ribosomes is a multisite interaction [1]. One of the approaches to study the ribosome–tRNA interaction is the use of tRNAs modified at those nucleotides which are directly involved in this process.

The 3′-terminal trinucleotide C-C-A plays an important role in the binding of tRNA to the ribosomes [2]. Recently tRNAs have been prepared with a modified terminal adenosine nucleotide. Some of these tRNAs can be aminoacylated and the aminoacyl-tRNAs have been used in studies on the specificity of the tRNA-ribosome interaction [3–6].

By introducing formycin (7-amino-3(β, D-ribofuranosyl)pyrazolo[4,3-d]pyrimidine) into the 3′-end of $tRNA^{Phe}$ from yeast by tRNA-nucleotidyl transferase [7], $tRNA^{Phe}$-C-C-F was obtained. The structure of modified 3′-end of this tRNA was studied by physical and chemical methods [7] and it was found that a stacking of the terminal formycine to the penultimate cytidine in $tRNA^{Phe}$-C-C-F is weaker than the stacking of the terminal adenosine in the native $tRNA^{Phe}$-C-C-A. This difference led to pronounced effects on the interaction of $tRNA^{Phe}$-C-C-F with its cognate synthethase as compared to native $tRNA^{Phe}$-C-C-A [7].

The behaviour of Phe-$tRNA^{Phe}$-C-C-F in a ribosomal system from reticulocytes has been studied and the results are the subject of this paper.

2. Materials and methods

$tRNA^{Phe}$-C-C-F was prepared from yeast $tRNA^{Phe}$, and aminoacylated as described [7]. Acetylation of [^{14}C]Phe-$tRNA^{Phe}$-C-C-A and [^{14}C]Phe-$tRNA^{Phe}$-C-C-F was performed according to Rappaport and Lapidot [8] except that the product was precipitated by alcohol instead of dichloroacetic acid.

Washed ribosomes from rabbit reticulocytes were prepared as previously described [9]. An EF-I preparation, free of EF II activity, was prepared from reticulocyte S-100 fraction by hydroxylapatite chromatography as described by Hardesty et al. [10].

Binding assays were performed by the nitrocellulase filter technique [11]. Standard buffer contained 60 mM Tris-HCl buffer, pH=7.4; 70 mM KCl; 1 mM DTT and $MgCl_2$ as specified.

Oligophenylalanine synthesis was measured as incorporation of [^{14}C]phenylalanine into hot trichloroacetic acid insoluble material using Whatman No. 3 MM filter papers.

3. Results and discussion

Fig.1 shows the kinetics of the poly U dependent incorporation of [^{14}C]Phe from Phe-$tRNA^{Phe}$-C-C-A

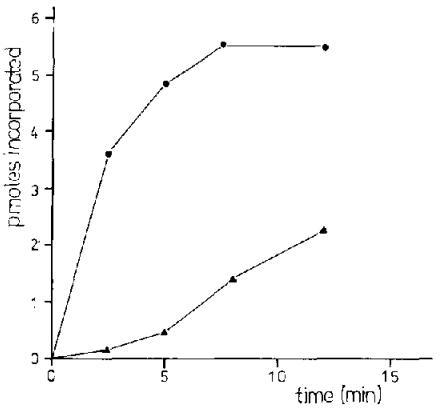

Fig.1. Poly U-directed oligo Phe-tRNA synthesis. Incubation mixture contains (final vol 0.1 ml): standard buffer with 6 mM $MgCl_2$; 60 μg poly U; 0.5 mM GTP; 100 μg ammonium sulfate (40–70%) fractionated S-100 fraction; 0.2 A_{260} units ribosomes and 20 pmol of either [^{14}C] Phe-tRNA^{Phe}-C-C-A (•), or [^{14}C] Phe-tRNA^{Phe}-C-C-A (▲). Incubation at 37°C.

and [^{14}C] Phe-tRNA^{Phe}-C-C-F into 5% hot TCA insoluble material catalysed by a ribosomal incorporation system from reticulocytes. It is clear that an oligo Phe-tRNA is formed from Phe-tRNA^{Phe}-C-C-F. Therefore, Phe-tRNA^{Phe}-C-C-F is capable of undergoing all the individual steps of the elongation cycle, namely binding to the ribosomes and functioning both as acceptor and as donor substrate. The kinetics of oligo Phe-tRNA^{Phe} formation with Phe-tRNA^{Phe}-C-C-F as

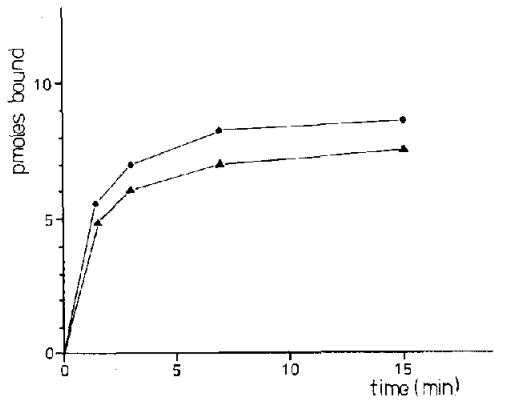

Fig.2. Non-enzymatic binding of Phe-tRNA^{Phe} species to ribosomes. Incubation mixture contains (in 50 μl): standard buffer with 20 mM $MgCl_2$; 40 μg poly U; 1 A_{260} units ribosomes and 12 pmol of either [^{14}C] Phe-tRNA^{Phe}-C-C-A (•), or [^{14}C] Phe-tRNA^{Phe}-C-C-F (▲). Incubation at 37°C.

substrate differs, however, significantly from the rate of oligo Phe-tRNA^{Phe} formation with Phe-tRNA^{Phe}-C-C-A as substrate. Not only is there in the case of Phe-tRNA^{Phe}-C-C-F a definite lag phase, but also, the rate of incorporation after the lag period is over, is much slower than the rate of oligo Phe-tRNA formation from Phe-tRNA^{Phe}C-C-A.

Therefore, we carried out some experiments on the kinetics of the intermediate reactions in oligo Phe-tRNA^{Phe} synthesis with Phe-tRNA^{Phe}-C-C-F and acetyl Phe-tRNA^{Phe}-C-C-F and compared the results with those obtained with the corresponding unmodified substrates.

Fig.2 shows that both the rate and the extent of non-enzymatic binding of the two substrates are nearly the same. Non-enzymatic binding is rather sensitive to the presence of uncharged tRNA and the relatively small difference in the rate of binding and in the final amount which was bound can be explained by some difference in the extent in which the tRNAs are charged.

Although the kinetics of Phe-tRNA^{Phe}-C-C-F binding did not differ from that of the normal substrate, Phe-tRNA^{Phe}-C-C-F binds less tightly to the ribosomes than the unmodified Phe-tRNA^{Phe}, as uncharged tRNA^{Phe}-C-C-A displaces Phe-tRNA^{Phe}-C-C-F much faster from the ribosomes than the 'normal' Phe-tRNA (results not shown).

Donor activity of Ac Phe-tRNA^{Phe}-C-C-A and Ac Phe-tRNA^{Phe}-C-C-F were measured by following the rate of Ac Phe-puromycin formation. The Ac Phe-tRNA^{Phe} species were prebound in the presence of EF II to assure maximal binding to the donor site, prior to the addition of puromycin.

The donor activity of Ac Phe-tRNA^{Phe}-C-C-F is considerably lower than that of Ac Phe-tRNA^{Phe}-C-C-A. (fig.3). When the reaction with Ac Phe-tRNA^{Phe}-C-C-F was continued after 10 min the rate of reaction remained constant for at least another 20 min (results not shown) and the percent of the Ac Phe-tRNA^{Phe}-C-C-F bound which had reacted after 30 min, approached that of the Ac Phe-tRNA^{Phe}-C-C-A. Differences in donor activities of the two substrates, similar to those presented in fig.3 were also obtained when the puromycin reaction was carried out at 37°C and 20 mM $MgCl_2$ or at 16°C and 6 mM $MgCl_2$.

The relative low donor activity of Ac Phe-tRNA^{Phe}-C-C-F can be explained by a change in the conformation, or a conformational instability of the -C-C-F terminal

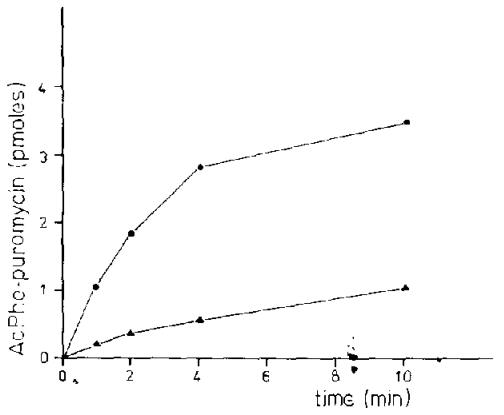

Fig.3. Kinetics of the reaction between puromycin and acetylated Phe-tRNA^{Phe} species. Reaction mixture contains (final vol 0.2 ml): standard buffer with 20 mM $MgCl_2$; 200 μg poly U; 0.5 mM GTP; 30 μg EF-II; 5 A_{260} units of ribosomes; 120 pmol of either Ac[^{14}C]Phe]tRNA^{Phe}-C-C-A (•), or Ac[^{14}C]Phe-tRNA^{Phe}-C-C-F (▲). Reaction mixtures were incubated at 37°C for 20 min; 30 μl samples were taken for binding determination. Reaction mixtures were cooled to room-temperature (16°) and puromycin added (final concentration 0.5 mM). 30 μl samples were withdrawn at the indicated times and Ac[^{14}C]phenylalanyl-puromycin formation was determined by extraction into ethyl-acetate [12]. Binding of Ac[^{14}C]Phe-tRNA^{Phe}-C-C-A and Ac[^{14}C]Phe-tRNA^{Phe}-C-C-F were 7.1 pmol and 6.9 pmol respectively.

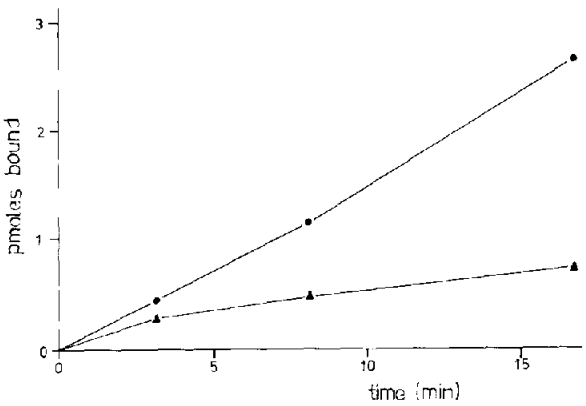

Fig.4. The rate of enzymatic binding of Phe-tRNA^{Phe}, species to ribosomes. Reaction mixture contains standard buffer with 6 mM $MgCl_2$ 100 μg poly U, 0.5 mM GTP, 1.5 A_{260} units ribosomes, 15 μg EF-I and 17 pmol of either [^{14}C]Phe-tRNA^{Phe}-C-C-A (•), or [^{14}C]Phe-tRNA^{Phe}-C-C-F (▲). Incubation was at 37°C. Reaction mixtures not containing EF-I were run in parallel. Results are expressed as the difference between enzymatic and non-enzymatic binding.

portion of the tRNA molecule. As a result the proper alignment of the ester bond, to be cleaved, along the active site of the peptidyl-transferase is disturbed.

The kinetics of the EF-I promoted binding with limiting amount of EF-I is shown in fig.4. The rate of Phe-tRNA^{Phe}-C-C-F binding is significantly lower than the rate of Phe-tRNA^{Phe}-C-C-A binding.

The results obtained from the study of the participation of Phe-tRNA^{Phe}-C-C-F in the particular steps of ribosomal protein biosynthesis allows an interpretation of the observation shown in fig.1. Oligo Phe formed is detectable under the standard assay conditions only when a sufficient chain length is achieved, which makes the product precipitable with 5% TCA. Therefore, the kinetics of oligo Phe-tRNA formation has an obligatory lag period which normally is not observed. However, because of the weaker enzymatic binding and because of the smaller donor activity of the tRNA-C-C-F derivatives the lag period of oligo Phe-tRNA^{Phe} formation is extended and can be easily detected. The presence of the lag period observed in fig.1 could also be explained by inhibition of the initiation of the poly Phe synthesis. As shown in fig.3 the interaction of the Ac Phe-tRNA^{Phe}-C-C-F with the ribosomal P-site is obviously weaker as in the case of non-modified tRNA. Assuming a rigid -C-C-A end is required for the P-site interaction, then the conformationally labile Ac Phe-tRNA^{Phe}-C-C-F is a poorer substrate for this site making the earlier stages of the reaction slower. As the polypeptide chain grows the poly Phe chain could provide additional binding and increase the proper alignment of the petidyl-tRNA in the P-site. When a certain critical poly Phe chain length is achieved the reaction can proceed in its normal speed.

tRNAs modified on the 3′-terminal ribose moiety cannot function in the whole ribosomal elongation process [3–6]. Especially the presence of the 3′-terminal, vicinal, cis-diol group of the ribose residue which allows a migration of the aminoacyl group is necessary [3]. However as shown for a first time in this communication, a modification of the 3′-terminal base of the tRNA can be tolerated by the ribosomes probably because the stacking pattern of the -C-C-A end is not severely disturbed. Replacement of the native bases in this part of the tRNA molecule by modified bases, therefore, allows an introduction of spectroscopic labels [7] or reactive

groups for affintiy labeling experiments in studies of tRNA–ribosome interaction.

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