STRUCTURAL CHANGE OF THE Phe-tRNA$^{Phe}_{(CCCA)}$ AND THE EFFECT ON THE RATE OF PEPTIDE FORMATION

M.N. THANG, L. DONDON and B. RETHER

Institut de Biologie Physico-chimique, rue P. et M. Curie, Paris 75005, France
and
Institut de Biologie Moléculaire et Cellulaire, 67000 Strasbourg, France

Received 27 December 1973

1. Introduction

Previous studies [1] have shown that the presence of a pCpCpCpA 3′-terminus in Phe-tRNA$^{Phe}_{yeast}$ induces a structural change in this artifical tRNA which results in an alteration of the stability of the EFT$_u$–GTP–Phe-tRNA ternary complex evidenced by a shift to higher optimum Mg^{2+} concentration for the binding of this complex to ribosomes, as well as in a marked decrease of the rate and extent of polyhenylalanine synthesis. On the other hand, the presence of an additional pC moeity at this end of the tRNA does not abolish, but only reduces, the activity of peptide formation in all the combinations between Phe-tRNA, N-Ac-Phe-tRNA, Phe-tRNA$_{(CCCA)}$ and N-Ac-Phe-tRNA$_{(CCCA)}$. In order to further understand structural requirements controlling interactions of tRNA and of the ribosomal decoding sites, a comparative kinetic study has been undertaken on the entry of tRNAs at the P and A sites and its interaction with peptidyl-transferase; part of these results have been presented at an EMBO workshop [2].

2. Materials and methods

The products used originated as follows: Puromycin, PL Biochemicals (USA); Streptomycin sulfate: Specia (France). [^{14}C]phenylalanine: CEA, Saclay (France). Purified tRNA$^{Phe}_{yeast}$ and purified Phe-tRNA-ligase from yeast were obtained from Institut de Biologie Moleculaire, Strasbourg (France). Nitrocellulose membranes: Millipore (USA), and ethylene acetate: Merck (Germany).

The tRNA$^{Phe}_{(CCCA)}$ was synthesized as previously described [1]. The polyuridylic acid was synthesized by pure *E. coli* polynucleotide phosphorylase. Preparations of *E. coli* EFT, EFG, IF, and washed ribosomes, as well as the acetylation of Phe-tRNA have been previously described [1, 3], except that IF and one sample of ribosomes were isolated from the EFG thermosensitive mutant G1 [4].

Binding of phe-tRNA to the A site was always performed under optimal conditions [5]: namely pre-fixation of N-Ac–Phe-tRNA in the presence of IF and GTP at the P site and subsequent addition of Phe-tRNA and EFT. To avoid any contaminant effect of translocation induced by residual EFG actually present in washed ribosomes, the latter were isolated from mutant G1.

Polymerization was also carried out in two steps: pre-fixation, at 25°C, of the initiator-tRNA–IF complex at the P site, and beginning of polymerization at 37°C by addition of Phe-tRNA, EFT and EFG.

Puromycin reactivity with N-Ac-Phe-tRNA was followed by the formation of puromycine derivative extracted in ethyl acetate, according to Leder and Burzstyn [6].

Chasing of N-Ac-Phe-tRNA from initiation complex was performed under the conditions described by Zagorska et al. [7].

3. Results

3.1. *Rates of polymerization of phenylalanine from normal and artificial Phe-tRNA*

As has been shown previously (ref. [1], table 2),

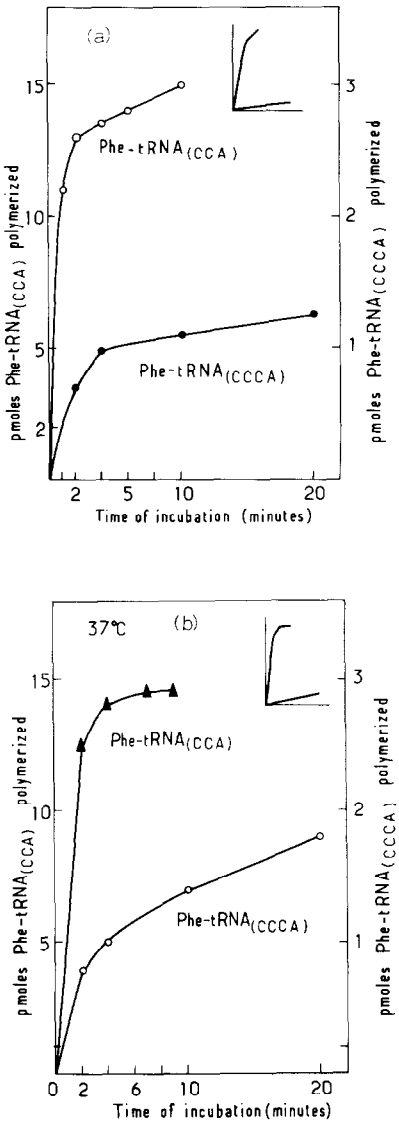

Fig. 1. Polymerization of polyphenylalanine from the Phe-tRNA and Phe-tRNA$_{(CCCA)}$. N-Ac-Phe-tRNA (10 pmoles/100 μl) was first bound to ribosomes in a Basic Medium (final volume 100 μl) containing: Tris–HCl, pH 7.5, 50 mM; NaCl, 100 mM; Mg acetate, 5.5 mM; GTP, 0.5 mM; β-mercaptoethanol, 7 mM; poly U, 0.09 A_{260} units; ribosomes, 0.9 A_{260} units; IF, 11 μg. Preincubation at 25°C for 20 min. The mixture was cooled before addition of [^{14}C]Phe-tRNA, 18 pmoles, or [^{14}C]Phe-tRNA$_{(CCCA)}$, 12 pmoles; EFT, 21 μg. Polymerization was started at 37°C. The polyphenylalanine synthesized was precipitated by TCA at 90°C, and the radioactivity measured in a scintillation counter, as usual. (a) with MRE 600 ribosomes; (b) with G1 ribosomes. The insertion shows curves in the same scale.

the synthesis of polyphenylalanine with Phe-tRNA$_{(CCCA)}$ is promoted by the presence of initiation factors and of N-Ac-Phe-tRNA. We thus compared the rates of polymerization of Phe-tRNA and Phe-tRNA$_{(CCCA)}$ using N-Ac-Phe-tRNA as initiator. The rate obtained with the homologous system is about 20 times higher than that of the heterologous one, whether the ribosomes isolated from MRE 600 (fig. 1a) or those from mutant G1 (fig. 1b) were used. We then examined each step individually during the polyphenylalanine synthesis.

3.2. *Binding of EFT–GTP–Phe–tRNA complex to ribosomes*

The EFT-dependent binding of tRNA to ribosomes was performed under optimal conditions for Phe-tRNA and Phe-tRNA$_{(CCCA)}$. In order to ascertain that no trace of EFG could contaminate the ribosomes used, we employed ribosomes, as previously described, from mutant G1. Surprisingly, the rate of binding of the ternary complex to the A site with Phe-tRNA$_{(CCCA)}$was even higher than with Phe-tRNA (fig. 2). The IF-mediated binding of initiator tRNAs was also examined. The fixation of N-Ac-Phe-

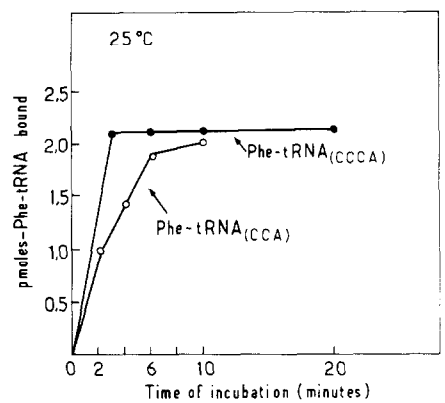

Fig. 2. EFT and GTP-dependent binding of Phe-tRNA. The experimental conditions and the pre-fixation of the non-radioactive N-Ac-Phe-tRNA initiation complex were identical to those described for fig. 1b. After cooling, [^{14}C]Phe-tRNA, 16 pmoles, or [^{14}C]Phe-tRNA$_{(CCCA)}$, 15.5 pmoles, were added in the presence of a soluble fraction from G1, 0.27 A_{260} units. The mixture was then incubated at 25°C as a function of time. The Phe-tRNA bound to ribosomes was retained on nitrocellulose membranes. After washing, the radioactivity was determined in liquid scintillator.

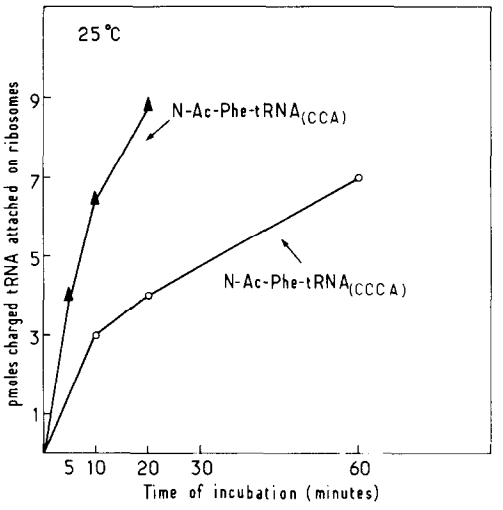

Fig. 3. IF and GTP-dependent binding of N-Ac-Phe-tRNA. 20 pmoles of N-Ac-[^{14}C] Phe-tRNA or N-Ac-[^{14}C] Phe-tRNA$_{(CCCA)}$ were incubated in 200 μl of Basic Medium (described under fig. 1a) at 25°C for the time indicated.

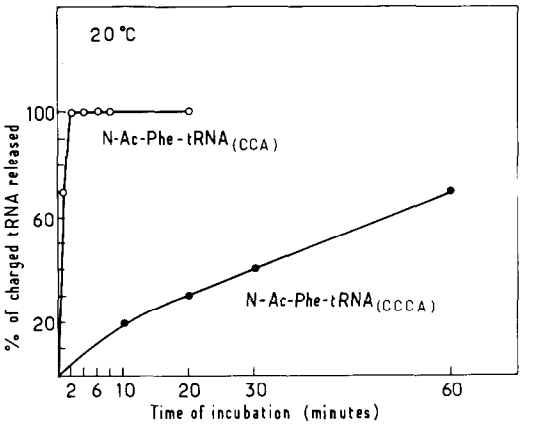

Fig. 4. Puromycin reaction. N-Ac-[^{14}C] Phe-tRNA, and N-Ac-[^{14}C] Phe-tRNA$_{(CCCA)}$ were pre-fixed to initiation complex under conditions identical to those described under fig. 3. After 20 min incubation at 20°C, trichloric acid was added to one sample of each series for determination of control value of tRNA bound. Other samples were immediately cooled, and diluted by addition of 200 μl of cooled Tris-HCl, pH 7.5, 50 mM, containing 100 mM NaCl, 7 mM β-mercaptoethanol, 5 mM Mg acetate, and 100 μg puromycin. The reaction was started at 25°C; the puromycin derviative of N-Ac-[^{14}C] Phe was extracted by ethyl acetate in Na-acetate buffer, 0.1 M, pH 5.5, according to Leder and Bursztyn [6]. Radioactive N-Ac-Phe-puromycin was measured with 1 ml of the organic phase in 10 ml of Bray solution. The amount of N-Ac-Phe transferred is expressed as percentage of the control value of N-Ac-Phe-tRNA bound.

tRNA to the P site proceeded with a higher rate than that with N-Ac-Phe-tRNA$_{(CCCA)}$ (fig. 3). However, the 2-fold differences in the rate of binding of these tRNAs to both the P and A sites cannot be accounted for the 20-fold difference in the rate of polymerization (fig. 1).

3.3. *Puromycin reaction*

It is conceivable that modification of the pCpCpA sequence might affect the interaction of the tRNA with the peptide formation site on the ribosome. In fact, it is well known, from experiments with *N*-formylmethionyl oligonucleotides for the reaction with puromycin in the presence of ribosomes and ethanol, that the whole pCpCpA sequence is required, and is sufficient, for the reaction catalyzed by peptidyltransferase whereas a shortened sequence, pCpA is inactive [8]. Fig. 4 shows the formation of puromycin derivatives with both N-Ac-Phe-tRNA and N-Ac-Phe-tRNA$_{(CCCA)}$. It is clear that the latter reacts very slowly with puromycin while the normal initiator tRNA is totally released within 2 min. The difference in rates, about 20-fold, can easily account for the different polymerization rates. This expected reduction in the rate of the puromycin reaction can either be solely due to the topological position of N-Ac-Phe- on the elongated tRNA terminus sequence, or reflect a mis-adjustment of the tRNA on the P site as a consequence of a structural change of the molecule. This can be verified by the action of streptomycin since the site of action of this antibiotic is located on the 30 S ribosomal subunit [9, 10] on which the portion of the tRNA opposite to the 3′ terminus interacts.

3.4. *Streptomycin reaction*

In fact, it has been shown that streptomycin releases fMet-tRNA from the ribosome initiation complex, but only under conditions of proper positioning of the tRNA at the P site [7].

Results on the release of N-Ac-Phe-tRNA and N-AC-Phe-tRNA$_{(CCCA)}$ prefixed on the ribosome in the presence of initiation factors and GTP (fig. 5) show that a long lag phase occurs before the release of modified tRNA by streptomycin, indicating a mis-

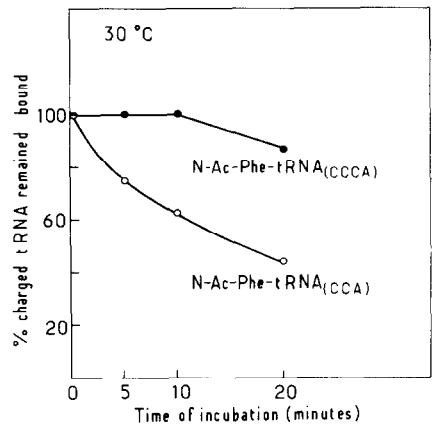

Fig. 5. Streptomycin chasing. N-Ac-[^{14}C] Phe-tRNA (14.5 pmoles) and N-Ac-[^{14}C] Phe-tRNA$_{(CCCA)}$ (16 pmoles) were pre-fixed under the conditions described in fig. 3, with an incubation period of 20 min for the former and 60 min for the latter. Control duplicates were stopped for determination of tRNA bound (control value). Streptomycin was added to give a 3.3 μg/200 μl final concentration, and chasing performed at 30°C as a function of time. The extent of chasing was determined by the amount of N-Ac-[^{14}C] Phe-tRNA which remained bound on the ribosome.

adjustment of N-Ac-Phe-tRNA$_{(CCCA)}$ at the P site, which can be corrected with time.

4. Discussion

Phe-tRNA$_{(CCCA)}$ binds us to the A site, in the presence of EFT and GTP, with a higher rate than that obtained with normal tRNA. On the other hand, the rate of binding to the P site, mediated by IF, is lower with the modified tRNA. Experiments with oligonucleotide fragments have shown that the structural requirement is different at each site. The complete CCA sequence is necessary for reactivity with puromycin [8] whereas the partial sequence CA is able to act as acceptor of the peptide chain [11]. Even an aminoacyl-adenosine is able, in some cases, although with a much reduced rate, to accept the nascent peptide [12]. However, in the cases examined here, the differences in the binding rates of both artificial and normal tRNA to the A or the P site, which might be significant by themselves, are not sufficient to account for the drastic difference in the polymerization rates. Contrarily, the formation of the puromycin derivative of the N-Ac-Phe-tRNA$_{(CCCA)}$ proceedes at a very low rate as compared to the very rapid formation of the same derivative with the normal tRNA. The 20-fold difference in the rate of the puromycin reaction, corresponding to the same order of difference in the overall polymerization, suggests that the rate-limiting step might be the formation of the peptide link. Consequently, two hypotheses might be mainly considered: First, the N-Ac-Phe-tRNA$_{(CCCA)}$ is correctly positioned at the P site, assuming that the introduction of an additional CMP in the terminal sequence does not induce a conformational change of the tRNA which could alter its interaction with the ribosomal decoding site. Under these conditions, the slow reaction with puromycin might reflect the difficulty of the acetylphenylalanine in approaching the transferase center because the additional length of the terminus keeps the amino acid away from the peptide formation site.

Secondly, the modified tRNA would undergo a conformational change in such a way that its correct positioning at the P site would be affected. This appears to be the case, according to the lag phase observed during streptomycin chasing experiment, since the site of action of this antibiotic is the 30 S ribosomal subunit which interacts with a region of the tRNA distal from the 3'terminus. of the tRNA distal from the 3'-terminus. Nevertheless such a mis-adjustment on the ribosome is presumably corrected later on because, despite the very low rate of puromycin reaction, the positive and quantitative reactivity of the N-Ac-Phe-tRNA$_{(CCCA)}$ with puromycin, demonstrates, by definition, that the modified tRNA is indeed positioned at the P site. It is concluded that the decrease in the rate of polymerization of Phe-tRNA$_{(CCCA)}$ might result from a double effect: an initial mis-positioning followed by a re-adjustment at the P site, and topological restriction of the interaction between the amino acid and the peptidyl-transferase.

Acknowledgements

We are grateful to M. Springer for IF and ribosomes from G1 mutant. This work was supported by the following grants: Centre National de la Recherche Scientifique (G.R. No 18); Délégation Générale à la

Rechercher Scientifique et Technique (Conventions No 72.7.0581 et 72.7.0388); Ligue Nationale Française contre le Cancer (Comité de la Seine) and a participation from the Commissariat à l'Energie Atomique.

References

[1] Thang, M.N., Dondon, L., Thang, D.C., and Rether, B. (1972) FEBS Letters 26, 145–150.
[2] Thang, M.N., Dondon, L., and Rether, B., Abstract of EMBO Workshop at Goteborg, Sweden, June 1973.
[3] Thang, M.N., Springer, M., Thang, D.C., Grunberg-Manago, M. (1971) FEBS Letters 17, 221–225.
[4] Tocchini-Valentini, G.P., Mattoccia, E. (1968) Proc. Natl. Acad. Sci. U.S. 61, 146–151.
[5] Springer, M., Biochem. Biophys. Res. Commun. (1972) 47, 477–484.
[6] Leder, P. and Bursztyn, H., Biochem. Biophys. Res. Commun. (1966) 25, 233–238.
[7] Zagorska, L., Dondon, J., Lelong, J.C., Gros, F., and Grunberg-Manago, M. Biochimie (1971) 53, 63–70.
[8] Monro, R.E., Cernà, J., and Marcker, K.A., Proc. Natl. Acad. Sci. U.S. (1968) 61, 1042–1049.
[9] Cox, E.C., White, J.R., and Flaks, J.G. (1964) Proc. Natl. Acad. Sci. U.S. 51, 703–709.
[10] Davies, J.E., (1964) 51, 659–664.
[11] Rychlik, I., Chladek, S., Zemlicka, J., Biochim. Biophys. Acta (1967) 138, 642.
[12] Rychlik, I., Cernà, J., Chladek, S., and Haladovà, Z., J. Mol. Biol. (1969) 43, 13–24.