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Role of the 5'-terminal phosphate of tRNA for its function during protein biosynthesis elongation cycle

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ABSTRACT

The 5'-terminal phosphate of $tRNA^{Phe}$ from yeast was removed using $tRNA^{Phe}$ lacking its 3'-terminal adenosine. After regeneration of the C-C-A terminus this tRNA was investigated in following reactions: aminoacylation, spontaneous hydrolysis of the amino acid from aminoacyl-tRNA, aminoacyl-tRNA·EF-Tu·GTP ternary complex formation and poly(U)-dependent synthesis of poly(Phe). The absence of the 5'-terminal phosphate of $Phe\text{-}tRNA^{Phe}$ does not influence the rate of hydrolysis of the amino acid or the ability of this tRNA to participate in complex formation with EF-Tu·GTP. The translation of the polyuridylic acid is slightly inhibited whereas the rate and extent of the enzymatic aminoacylation is not affected.

INTRODUCTION

The enzymatic or chemical modification of tRNA is still a useful tool to investigate the structural requirements of this substrate in various enzymatic reactions. In connection with our studies on the interaction of aminoacyl-tRNAs with elongation factor Tu we described recently the role of the side chain of the aminoacyl residue on the efficiency of this interaction (1). The position of attachment of the aminoacyl residue on the 3'-terminal adenosine of tRNA was also found to influence the binding of aa-tRNA to EF-Tu·GTP (2,3). As a result of the hypothesis that an intramolecular salt bridge between the α-amino group of the amino acid and the 5'-terminal phosphate of the tRNA determines the conformation of the aminoacyl residue on the aminoacyl-tRNA (4,5), we decided to perform experiments by which the formation of this salt with non isomerisable aminoacyl-tRNA should be investigated. We found, however, in the initial stage of our experiments that the above mentioned hypothesis in incorrect and report here on these results.

MATERIALS

$tRNA^{Phe}$-C-C lacking the 3'-terminal adenosine was prepared from bulk yeast tRNA (Boehringer, Mannheim, Federal Republic of Germany) by purification on

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benzoylated DEAE-Cellulose column (6). Yeast phenylalanyl-tRNA synthetase (7) and ATP (CTP) tRNA nucleotidyl transferase (8) were obtained from Drs. F. von der Haar and H. Sternbach (Göttingen), respectively. Alkaline phosphatase (E.C. 3.1.3.1) from E. coli was a product of Sigma (St Louis, USA), the AcA 44 gel support was from LKB (Bromma, Sweden). [^{14}C] Phenylalanine of a specific activity 50 Ci/mol was obtained from Amersham-Buchler (Braunschweig, Federal Republic of Germany), that of specific activity 486 Ci/mol was from Amersham-Buchler (Braunschweig, Federal Republic of Germany). All other biochemicals were from Boehringer (Mannheim, Federal Republic of Germany). Elongation factor Tu from E. coli was prepared according to Arai et al. (9) and had a GDP binding activity of 19.500 pmol/mg protein.

METHODS

Hydrolysis of the 5'-terminal phosphate from $tRNA^{Phe}$: $tRNA^{Phe}$-C-C from yeast (50 A_{260} units) was incubated in 2 ml 50 mM Tris-HCl buffer at 60°C with 0.25 units alkaline phosphatase from E. coli. At selected time intervals, 200 µl aliquots were removed, treated with water-saturated phenol, intensively stirred and centrifuged. The aqueous phase was removed and the tRNA was precipitated with 400 µl ethanol. After centrifugation the pellet was washed with ethanol and dried in desiccator. For analysis of these aliquots, a sample was incubated in 250 µl 100 mM phosphate buffer pH 7.0 for 3 h at 37°C with 50 units ribonuclease T_1 and the digest was analyzed by high performance liquid chromatography (HPLC). Under these conditions a 20 min incubation with alkaline phosphatase was sufficient to remove the 5'-terminal phosphate.

For a preparative scale reaction, the $tRNA^{Phe}$-C-C was incubated under the same conditions with alkaline phosphatase for 30 min. After phenolisation and ethanol precipitation, the $tRNA^{Phe}_{-5'p}$-C-C was further purified by chromatography on Sephadex A25 column as described previously (10). Starting with 50 A_{260} units native $tRNA^{Phe}$-C-C, 46 A_{260} units $tRNA^{Phe}_{-5'p}$-C-C were obtained.

The aminoacylation of $tRNA^{Phe}$ was performed in the presence of an excess of ATP (CTP) tRNA nucleotidyl transferase as described (2). The 3'-terminus of $tRNA^{Phe}_{-5'p}$-C-C was fully regenerated under these conditions to $tRNA^{Phe}_{-5'p}$-C-C-A. Native $tRNA^{Phe}$ accepted 1320 pmol phenylalanine/A_{260} unit tRNA, the activity of $tRNA^{Phe}_{-5'p}$-C-C-A was 1350 pmol phenylalanine/A_{260} unit tRNA. The reaction conditions for determination of K_m and V_{max} were the same as in ref. 1.

The determination of the rate of hydrolysis of phenylalanine from [^{14}C]Phe-$tRNA^{Phe}$ was performed according to ref. 11. The gel filtration of [^{14}C]Phe-$tRNA^{Phe}$ EF-Tu·GTP ternary complexes and the measurement of poly (U)-dependent poly(Phe)

synthesis of E. coli ribosomes was made according to ref. 1.

Analysis of the T_1 ribonuclease digest of $tRNA^{Phe}$ for the presence of guanosine-3',5'-diphosphate was performed on a high performance liquid chromatographic system using the Du Pont Model 850 liquid chromatograph equipped with Du Pont Zorbax ODS column (4.6 mm x 250 mm). Chromatography was performed at 35°C with a flow rate of 2 ml/min. The column was eluted with 50 mM ammonium sulfate buffer pH 5.5 and after each run (20 min) regenerated with the same buffer containing 20 % methanol (15 min).

RESULTS

The specific removal of the 5'-terminal phosphate from $tRNA^{Phe}$-C-C can be accomplished with alkaline phosphatase from E. coli under mild conditions without cleavage of the tRNA polynucleotide chain. A simple analysis of the reaction product on a HPLC system can be used to monitor the reaction (Fig. 1) and to control the integrity of the reaction product (12).

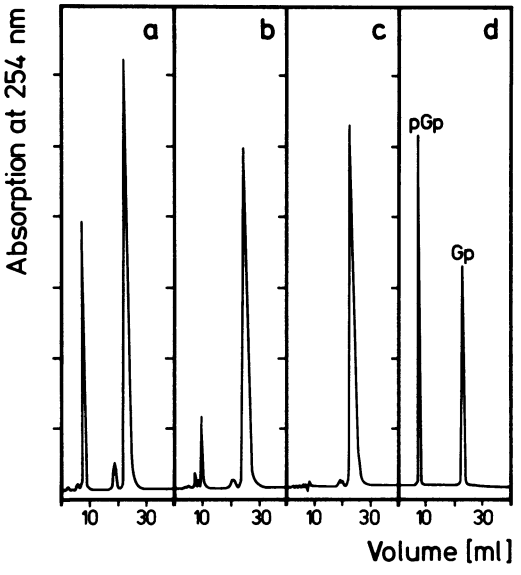

Fig. 1: Part of the HPLC chromatogram of T_1 RNase hydrolysates of $tRNA^{Phe}$-C-C from yeast demonstrating the course of the hydrolysis of 5'-terminal phosphate from this tRNA. Details are described in the Methods section. Analysis was performed (a) prior to incubation of $tRNA^{Phe}$ with alkaline phosphatase from E. coli (b) after 10 min and (c) after 20 min incubation time. The chromatogram of the authentic standards (P-L Biochemicals, St. Goar, FRG) are shown in (d).

The $tRNA^{Phe}_{-5'p}$ was aminoacylated to the same extent as the native $tRNA^{Phe}$. The Michaelis constant of aminoacylation, using phenylalanyl-tRNA synthetase is identical for both tRNA species (K_m = 3.03 x 10^{-6} M). The maximal velocity of aminoacylation, V_{max}, is reduced by 20 % in the case of $tRNA^{Phe}_{-5'p}$.

The rate of non enzymatic hydrolysis of [^{14}C]Phe-$tRNA^{Phe}$ species was measured at pH 7.6 and pH 9.0. In both cases there is no difference observed in rate of spontaneous hydrolysis of the amino acid from [^{14}C] Phe-$tRNA^{Phe}$ and [^{14}C] Phe-$tRNA^{Phe}_{-5'p}$ (Fig. 2).

The formation of the ternary complexes between [^{14}C]Phe-$tRNA^{Phe}$·EF-Tu and GTP was investigated by gel filtration. It is evident from the experiment illustrated by Fig. 3, that the [^{14}C]Phe-$tRNA^{Phe}_{-5'p}$ is interacting with the elongation factor Tu from E. coli. In the presence of the protein and GTP the tRNA is eluted earlier from the column of AcA 44 gel than in the absence of EF-Tu·GTP. This result was confirmed by additional experiments in which the protective effect of EF-Tu·GTP on the rate of spontaneous hydrolysis of phenylalanine from [^{14}C] Phe-$tRNA^{Phe}$ species was determined (Fig. 4a). Using an excess of EF-Tu, both the native Phe-$tRNA^{Phe}$ as well as Phe-$tRNA^{Phe}_{-5'p}$ are protected. By lowering the EF-Tu·GTP concentration to an extent at which only a part of the

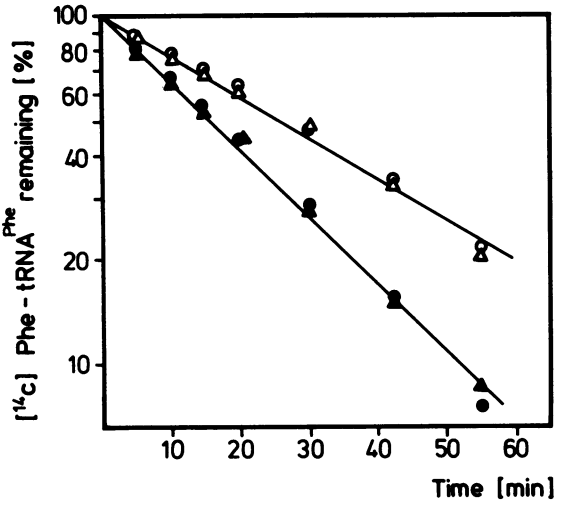

Fig. 2: The rate of non-enzymatic hydrolysis of phenylalanine from $Phe\text{-}tRNA^{Phe}$ (triangles) and $Phe\text{-}tRNA^{Phe}_{-5'p}$ (circles) at pH 9.0 (open symbols) and pH 7.6 (full symbols). 15 µM [^{14}C]Phe-$tRNA^{Phe}$ species were incubated at 36°C in a buffer containing 50 mM Tris-HCl of a given pH and 5 mM $MgSO_4$. At given times aliquots were removed and the radioactivity precipitable in 5 % trichloroacetic acid was determined.

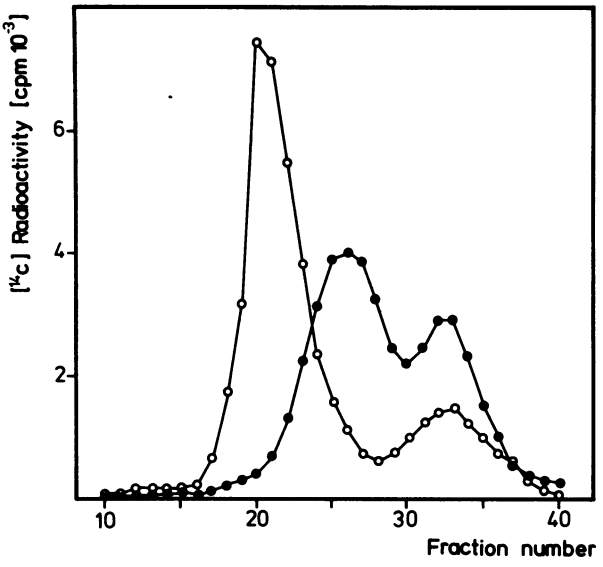

Fig. 3: Gel filtration of [^{14}C]Phe-tRNA$^{Phe}_{-5'p}$·EF-Tu·GTP ternary complex (o) and [^{14}C]Phe-tRNA^{Phe} (●) on Ultragel Aca 44 column. [^{14}C]Phe-tRNA$^{Phe}_{-5'p}$ (200 pmol) was mixed with 50 µl EF-Tu·GTP which was prepared by incubation of 10 mmol EF-Tu·GDP in 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 50 mM KCl, 50 mM NH_4Cl, 1.0 mM phosphoenolpyruvate, 0.05 mM GTP and 2 units pyruvate kinase for 10 min at 37°C. The specific activity of [^{14}C]phenylalanine was 480 Ci/mol. The mixture was applied onto a Ultragel Aca 44 column (0.8 x 25 cm) which was then eluted at 4°C by a buffer solution containing 10 mM Tris-HCl pH 7.4, 10 mM KCl and 10 mM $MgCl_2$. Fractions of 0.55 ml were collected. Radioactivity of 0.5 ml aliquots was determined in a scintillation counter.

Phe-tRNA^{Phe} is complexed with EF-Tu·GTP, no significant differences in the rates of hydrolysis were observed (Fig. 4b). It therefore follows that the binding constants of Phe-tRNA^{Phe} and Phe-tRNA$^{Phe}_{-5'p}$, respectively, to EF-Tu·GTP are very similar or identical.

The absence of the 5'-terminal phosphate in the Phe-tRNA^{Phe} affects the rate of poly(U)-dependent synthesis of poly(Phe). This can be concluded from the results shown in Fig. 5, where activities of [^{14}C]Phe-tRNA^{Phe} and [^{14}C]Phe-tRNA$^{Phe}_{-5'p}$ were compared in an E. coli poly(U)-dependent ribosomal system. There is a significant reduction of the rate of poly(Phe) synthesis after tRNA modification by removal of the 5' phosphate. This suggests that the 5'-terminal phosphate of tRNA is either involved in an interaction with ribosomal components at some stage of the elongation process or the absence of the phosphate affects the tRNA structure in such a way that its function in the elongation process is

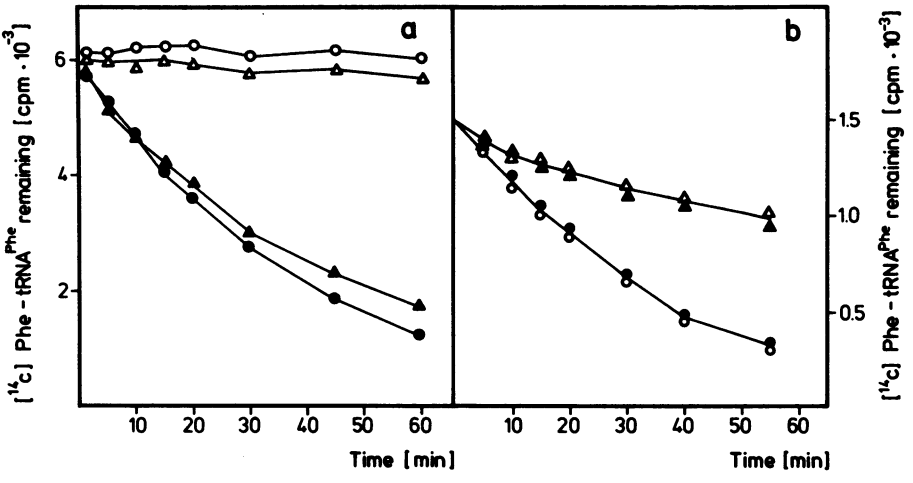

Fig. 4: Rate of hydrolysis of [^{14}C]phenylalanine from [^{14}C]Phe-tRNA^{Phe} (triangles) and [^{14}C]Phe-tRNA$^{Phe}_{-5'p}$ (circles) in the presence of EF-Tu·GTP (open symbols) or in the absence of the elongation factor (full symbols). The procedure used for the formation of Phe-tRNA^{Phe}·EF-Tu·GTP ternary complexes is given in Fig. 3. The specific activity of [^{14}C]phenylalanine was 486 Ci/mol. The reaction mixture was incubated at 36°C and the radioactivity present in 10 µl aliquots which is precipitable in 5 % trichloroacetic acid was determined. The concentration of tRNA and EF-Tu·GTP was 0.80 µM and 13 µM in (a), respectively. In the experiments shown in (b) the concentration of tRNA was 0.2 µM, that of EF-Tu·GTP was 0.2 µM.

inhibited. Such structural changes, however, could not be identified by UV-monitored thermal-melting experiments or by measurement of the high field nuclear magnetic resonance spectra of tRNA^{Phe} and tRNA$^{Phe}_{-5'p}$ at various temperatures (13) (data not shown).

DISCUSSION

Earlier it was suggested that there might exist an intramolecular salt bridge between the protonated α-amino group of the amino acid bound to the tRNA and its 5'-terminal phosphate residue. This was substantiated by experiments indicating that the removal of the 5'-phosphate from tRNA interferes with the aminoacyl-tRNA·EF-Tu·GTP ternary complex formation (4). Furthermore, the binding of oligonucleotides complementary to the A-C-C-A terminus of tRNA^{Phe} was investigated using aminoacylated and nonaminoacylated tRNA^{Phe} (5). The results of this work also supported the above hypothesis.

In light of the rapid progress towards the elucidation of the structure

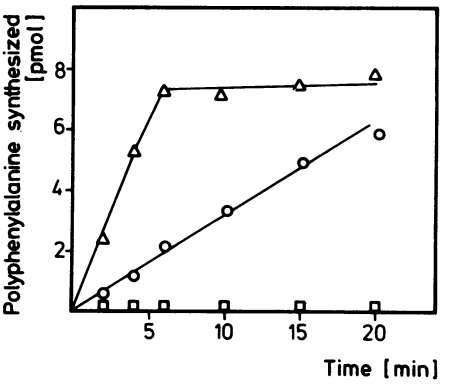

Fig. 5: Poly(U)-dependent synthesis of poly(Phe) (1) using Phe-tRNA^{Phe} (Δ) and Phe-tRNA$^{Phe}_{-5'p}$ (o) and a control without EF-Tu·GTP (□) which is identical with both tRNAs.

of elongation factor Tu from E. coli (14,15) it is necessary to obtain information regarding the structural features which determine the aminoacyl-tRNA·EF-Tu·GTP interaction. Therefore the problem of the function of the 5'-terminal phosphate of tRNA in the aa-tRNA·EF-Tu·GTP ternary complex formation was reinvestigated. Surprisingly we were unable to observe any effects resulting from the removal of the 5'-terminal phosphate on the efficiency of this ternary complex formation. It is conceivable that the recently available analytical methods may have allowed the enzymatic modification to occur under milder conditions than previously and without damage to the rest of the tRNA molecule. Furthermore, there now exist more reliable assays (gel filtration (2), and hydrolysis protection (11)) than the cellulose acetat membrane filtration assays of the previous studies (4), which were used to detect the aa-tRNA·EF-Tu·GTP ternary complex formation.

There is also a difference between the assay described in reference 4 and that used in this study. Schulman et al. utilised an EF-Tu·EF-Ts complex whereas homogeneous EF-Tu·GDP was used in our investigations. It is possible that an unmodified aa-tRNA, that is, with the 5'-phosphate, is necessary to achieve the dissociation of Tu·Ts complex in the presence of GTP to form aa-tRNA·EF-Tu ·GTP ternary complex and free EF-Ts. The aa-tRNA lacking its 5'-terminal phosphate may be unable to participate in this process. In our assays, if preformed EF-Tu·GTP is used for ternary complex formation, differences which are dependent on the presence or absence of the 5'-terminal phosphate of aa-tRNA then could not be observed. Based on our result, it can, however, be concluded that

the lack of the 5'-terminal phosphate of aa-tRNA does not interfere with the ternary complex formation aa-tRNA·EF-Tu·GTP.

Since we did not observe a difference in the rate of hydrolysis of the phenylalanine from Phe-tRNA^{Phe} and Phe-tRNA$^{Phe}_{-5'p}$ we have also concluded that an intramolecular salt bridge between the α-amino group of the amino acid and the 5'-terminal phosphate group of the tRNA is unlikely. If such interaction exists a slower rate of hydrolysis for the protonated and intramolecular bridged amino acid would be expected.

The observation that the rate of the poly(U)dependent poly(Phe) synthesis is slower with Phe-tRNA^{Phe} lacking the 5'-terminal phosphate indicates that there might exist a functional role for this group during the interaction of tRNA with ribosomes. Our results at the present time do not allow any conclusions about the particular step of the protein elongation process in which the 5'-terminal phosphate of tRNA is specifically involved.

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Abbreviation: tRNAs lacking the 5'-terminal phosphate are indexed with -5'p.

REFERENCES

1. Wagner, T. and Sprinzl, M. (1980) Eur. J. Biochem., 108, 213-221.
2. Sprinzl, M., Kucharzewski, M., Hobbs, J.B. and Cramer, F. (1977), Eur. J. Biochem. 78, 55-61.
3. Alford, B.L., Pezzuto, J.M., Hock Tan, K. and Hecht, S.M. (1979) J. Biol. Chem. 254, 6894-6903.
4. Schulman, L.H., Pelka, H. and Sundari, R.M. (1974), J. Biol. Chem. 249, 7102-7110.
5. Pongs, O., Wrede, P., Erdmann, V.A. and Sprinzl, M. (1976) Biochem. Biophys. Res. Commun. 71, 1025-1033.
6. Schneider, D., Solfert, R. and von der Haar, F. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1330-1336.
7. von der Haar, F. (1973) Eur. J. Biochem. 34, 84-90.
8. Sternbach, H., von der Haar, F., Schlimme, E., Gaertner, E. and Cramer, F. (1971) Eur. J. Biochem. 22, 166-172.
9. Arai, K., Kawakita, M. and Kaziro, Y. (1972) J. Biol. Chem. 247, 7029-7037.
10. Sprinzl, M., Sternbach, H., von der Haar, F. and Cramer, F. (1977) Eur. J. Biochem. 81, 579-589.
11. Pingoud, A., Urbanke, C., Krauss, G., Peters, F. and Maass, G. (1977) Eur. J. Biochem. 78, 403-409.
12. McLaughlin, L.W., Cramer, F. and Sprinzl, M. (1980) Anal. Biochem., submitted.
13. Davanloo, P., Sprinzl, M. and Cramer, F. (1979) Biochemistry 18, 3189-3199.
14. Arai, K., Clark, B.F.C., Duffy, L., Jones, M.D., Kaziro, Y., Laursen, R.A., L'Italien, J., Miller, D.L., Nagarkatti, S., Nakamura, S., Nielsen, K.M., Petersen, T.E., Takahashi, K. and Wade, M. (1980) Proc. Natl. Acad. Sci. USA 77, 1326-1330.
15. Morikawa, K., La Cour, T.F.M., Nyborg, J., Rasmussen, K.M., Miller, D.L. and Clark, B.F.C. (1978) J. Mol. Biol. 125, 325-338.