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Participation of X47-fluorescamine modified E. coli tRNAs in in vitro protein biosynthesis

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ABSTRACT

The reaction of fluorescamine with primary amino groups of tRNAs was investigated. The reagent was attached under mild conditions to the 3'-end of $tRNA^{Phe}$-C-C-A(3'NH_2) from yeast and to the minor nucleoside X in E.coli $tRNA^{Arg}$, $tRNA^{Lys}_2$, $tRNA^{Met}$, $tRNA^{Ile}$ and $tRNA^{Phe}$. The primary aliphatic amino groups of these tRNAs react specifically so that the fluorescamine dye is not attached to the amino groups of the nucleobases. E.coli tRNA species modified on the minor nucleoside X47 can all be aminoacylated. An involvement of the minor modified nucleoside X47 in the tRNA : synthetase interaction is detected. Native $tRNA^{Lys}$-C-C-A from E.coli can be phenylalanylated by phenylalanyl-tRNA synthetase from yeast, whereas this is not the case for fluorescamine treated $tRNA^{Lys}$-C-C-A(XF47). Phe-$tRNA^{Phe}$-C-C-A(XF47) forms a ternary complex with the elongation factor Tu:GTP from E.coli, binds enzymatically to the ribosomal A-site and is active in poly U dependent poly Phe synthesis. Fluorescamine-labelled E.coli tRNAs provide new substrates for the study of protein biosynthesis by spectroscopic methods.

INTRODUCTION

Naturally fluorescent or fluorescence-labelled tRNA species have been widely used for investigations of the mechanisms of protein biosynthesis. $tRNA^{Phe}$ from yeast containing the fluorescent minor nucleoside wybutine in the anticodon loop has been the subject of spectroscopic studies of the interaction with the cognate synthetase (1) and with E.coli elongation factor Tu:GTP (2). The wybutine of $tRNA^{Phe}$ from yeast can also be chemically replaced by other fluorescent residues without impairing the biological activity of tRNA (3,4). The minor nucleoside Q in the anticodon loop of $tRNA^{Tyr}$ from E.coli has been specifically modified with fluorescein isothiocyanate (5). Enzymatic (6) modification of the C-C-A end of $tRNA^{Phe}$ from yeast has also been used for the insertion of a fluorescent label.

The incorporation of a spectroscopic label into the variable loop of various tRNAs results in differing effects on the biological activity of the nucleic acid. Acylation of the minor nucleoside X, 3-N(3-amino-3-carboxypro-

pyl)uridine, (7) leads to complete (8,9), partial (10) or no loss (11-14) of of the acceptor activity of the modified tRNAs. In fact, there is no satisfactory explanation available whether the inactivation of X-base modified tRNA is associated with the participation of the variable loop of tRNA in the interaction with the cognate aminoacyl-tRNA synthetase or if this effect is due to unspecific side reactions or irreversible inactivation of the tRNA during the modification procedures.

Fluorescamine, 4-phenylspiro[furan-2(3H), 1'-phthalan]-3,3'dione, has recently been introduced as a reagent for the fluorimetric quantitation of primary amino groups in proteins and amino acids (15). This nonfluorescent compound reacts at room temperature and in aqueous solution with primary amines giving rise to strongly fluorescent products.

In some tRNAs primary aliphatic amino groups occur as the side chain of the minor nucleoside X (16), Fig. 1; or an amino group can be introduced by enzymatic incorporation of modified nucleosides (17,18).

We investigated the reaction of fluorescamine with these native and modified tRNA species and determined the activity of the fluorescamine labelled tRNAs in an in vitro protein synthesizing system.

MATERIALS AND METHODS

Unfractionated tRNA from E. coli MRE 600 cells was obtained from Boehringer, Mannheim, Germany. The specific tRNAs were isolated by a combination of standard chromatographic procedures. Chromatography on benzoylated DEAE-cellulose column at pH 5.2 (19) was the first purification step. The appropriate fractions were rechromatographed on a Sepharose 4B column at pH 4.5 and room temperature essentially according to Holmes et al. (20). The 3 x 60 cm column was developed with a 2 x 1500 ml linear gradient of an ammonium sulfate solution in a 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM $MgCl_2$. The ammonium sulfate concentration in the mixing chamber was 2 M; in the reservoir 1 M.

The fractions from Sepharose 4B chromatography containing the appropriate tRNA were rechromatographed on an RPC-5 column (21). The 1 x 100 cm column was developed with a 2 x 1000 ml gradient of NaCl, from 0.1 M to 1 M in 50 mM sodium acetate, pH 5.6, containing 10 mM $MgSO_4$. The amino acid acceptance of tRNAs is given in Table 1 in the Results section.

$tRNA^{Phe}$-C-C-A(3'NH_2) was prepared from yeast $tRNA^{Phe}$-C-C (22) as described by Fraser and Rich (17).

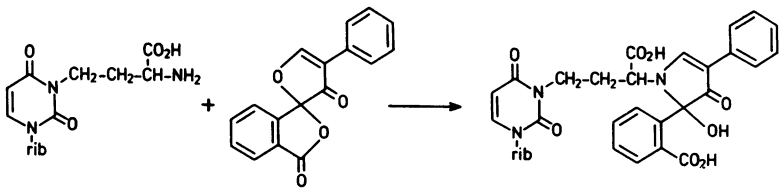

Fig. 1: Modification of E. coli tRNAs with fluorescamine at the side chain of the minor nucleoside X. The structure of the reaction product is taken from ref. 15.

Purified phenylalanyl-tRNA synthetase from E. coli of specific activity 800 units/mg protein was a generous gift from Dr. E. Holler, Regensburg, phenylalanyl-tRNA synthetase from yeast, specific activity 1820 units/mg, was from Dr. F. von der Haar, Göttingen. (1 unit = incorporation of 1 nmol phenylalanine into $tRNA^{Phe}$/min). Partially purified aminoacyl-tRNA synthetases from E. coli were prepared as described (23).

Fluorescamine and RPC-5 adsorbent were obtained from Serva (Heidelberg, Germany), Sepharose 4B was from Pharmacia (Uppsala, Sweden). Benzoylated DEAE-cellulose (BD-cellulose) was a product of Boehringer (Mannheim, Germany). [^{14}C]-labelled amino acids (50 Ci/mol) and HEPES buffer were from Schwarz/Mann (Orangeburg, USA).

Aminoacylation

In a standard aminoacylation assay the reaction mixture (100 µl) contained 50 mM Tris-HCl, pH 7.6, 10 mM KCl, 10 mM $MgSO_4$, 2 mM ATP, 0.05 mM amino acid and 3 µM tRNA.

To start the reaction an amount of synthetase was used to achieve complete aminoacylation within 15 min. Highly purified phenylalanyl-tRNA synthetases were used for aminoacylation of $tRNA^{Phe}$ from E.coli and $tRNA^{Phe}$ from yeast, respectively. The extent of aminoacylation of other tRNA species was determined using partially purified synthetase fraction. 10 µl aliquots were withdrawn from the aminoacylation assay after appropriate incubation times and radioactive material insoluble in 5 % trichloroacetic acid, was measured by liquid scintillation counting.

For the determination of Michaelis constants of native and fluorescamine-modified $tRNA^{Phe}$ from E. coli the aminoacylation assay contained 0.2 to 3.0 µM $tRNA^{Phe}$. Misacylation of $tRNA^{Lys}$ from E. coli by phenylalanine using phenylalanyl-tRNA synthetase from yeast was performed at 30°C in a reaction mixture containing 10 mM Tris-HCl, pH 9.0, 10 mM $MgSO_4$, 0.5 mM ATP and

0.05 mM phenylalanine. The reaction was monitored for 60 min by taking aliquots and determining the radioactivity precipitable in 5 % trichloroacetic acid.

Preparative aminoacylation of $tRNA^{Phe}$ from E. coli with [^{14}C]phenylalanine was performed in standard aminoacylation assay. The phenylalanyl-$tRNA^{Phe}_{E.coli}$--C-C-A was isolated by a procedure involving phenol extraction, alcohol precipitation and filtration through a Biogel P2 column (18). The extent of aminoacylation of Phe-$tRNA^{Phe}_{E.coli}$-C-C-A used for ribosomal assays and determination of Phe-$tRNA^{Phe}_{E.coli}$-C-C-A:EF-Tu:GTP ternary complex formation was 1570 pmoles Phe/A_{260} unit tRNA.

Ribosomal Assays

For ribosomal assays, EF-Tu dependent binding to ribosomal A-site and poly U dependent poly Phe synthesis, the tRNAs were preparatively aminoacylated with [^{14}C]phenylalanine (485 Ci/mol) (Amersham-Buchler, Braunschweig, Germany) as described (23). EF-Tu:GDP, (specific activity 22000 units/mg of protein) was isolated from E. coli following the procedure of Arai et al. (24). The formation of Phe-$tRNA^{Phe}$:EF-Tu:GTP ternary complexes was monitored by the gel filtration assay described previously (18).

70 S ribosomes from E. coli A 19 cells were isolated, salt washed and stored as a precipitate in 72 % saturated ammonium sulfate (490 g/liter) at 4°C according to Gavrilova and Spirin (25). Prior to use the ribosomes were diluted to 200-250 A_{260} units/ml with 10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 30 mM NH_4Cl and 1 mM dithiothreitol, then dialysed against a 2x100 fold volume of the same buffer at 4°C.

Reaction of tRNAs with fluorescamine

10 A_{260}-units tRNA were dissolved in 0.5 ml 100 mM borate buffer, pH 9.0, and treated with five successive additions of 10 µl portions of 100 mM, fluorescamine in dry acetone, at 30 second intervals. During the additions the reaction mixture was vigorously stirred and protected from direct light. After 10 min at room temperature 1.5 ml ethanol were added. The reaction mixture was then left for 2 hrs at -20°C and the precipitated tRNA was isolated by centrifugation. Subsequently, the pellet was washed with 1 ml 70 % aqueous ethanol and then with 1 ml absolute ethanol. The pellet was dried in a desiccator and then dissolved in 200 µl of 1 mM potassium phosphate buffer, pH 7.0,containing 100 mM KCl and 10 mM $MgSO_4$.

Fluorescamine-treated tRNA was finally purified by chromatography on a RPC-5 column (0.6 x 50 cm). The column was eluted with a 2x200 ml gradient of

0.4 M to 1.0 M NaCl in a buffer containing 50 mM potassium phosphate, pH 7.0 and 10 mM $MgSO_4$, followed by 100 ml of the same buffer with 1.0 M NaCl. 6 ml fractions were collected at a flow rate of 80 ml/h. The UV-absorbance of the effluent at 254 nm was continuously monitored by an ISCO UV-5 optical unit. The relative fluorescence of each fraction was determined at 485 nm by a Farrand MK-1 spectrofluorometer. The excitation wavelength was 385 nm. Fluorescent fractions were pooled and the volume was reduced by 50 % by evaporation at reduced pressure and 20°C. The solution was then passed through a Biogel P2 column (3.5x60cm) and eluted with water The tRNA fraction was collected, concentrated by evaporation and made 10 mM in potassium phosphate, pH 7.0, and 100 mM in potassium chloride and 10 mM in $MgSO_4$.

The tRNA concentration in final solutions was 20-60 A_{260} units/ml. Solutions of fluorescamine treated tRNAs were kept frozen at -20°C. Under these conditions the aminoacylation activity of the fluorescamine treated tRNAs did not change for several months. A 30 % decay in the fluorescence amplitude was, however, observed after one month storage at -20°C in the dark. Uncorrected fluorescence spectra were recorded in solutions containing 1 A_{260}-unit tRNA/ml in a 10 mM phosphate buffer, pH 7.0, 10 mM $MgSO_4$ and 100 mM KCl, with the instrument described above, using band widths of 5 nm on the excitation and 10 nm on the emission side.

RESULTS

Reaction of tRNA with fluorescamine

The formation of a fluorescent adduct upon reaction of fluorescamine with $tRNA^{Phe}$-C-C-A(3'NH_2) from yeast or $tRNA^{Phe}$-C-C-A from E. coli is demonstrated in Fig. 2. The relative fluorescence intensity of the reaction product is proportional to the amount of tRNA present. Thus using a defined standard and an excess of reagent the number of aliphatic amino groups in a given tRNA can be quantitatively determined. The fluorescamine reacts only with tRNAs containing primary amino groups; the terminal 3'-deoxy-3'-aminoadenosine of $tRNA^{Phe}$-C-C-A(3'NH_2) from yeast or the base X47 of $tRNA^{Phe}$-C-C-A from E. coli. Treatment of native yeast $tRNA^{Phe}$-C-C-A, which does not contain such primary amino groups, with fluorescamine, on the other hand, does not lead to the formation of a fluorescent reaction product.

The observed differences in the relative fluorescence intensities of $tRNA^{Phe}$-C-C-A(XF47) from E. coli and yeast $tRNA^{Phe}$-C-C-A(3'NF), Fig. 2 and Table 1, are probably due to different quantum yields of the fluorophor in the

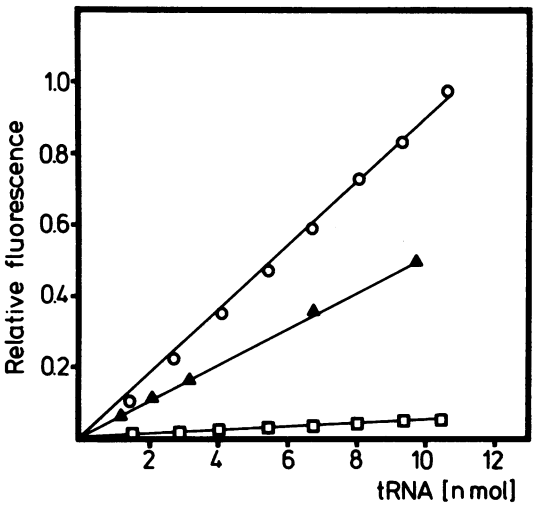

Fig. 2: Reaction of tRNAs with fluorescamine. Given amount of tRNA in 20 µl of 100 mM sodium borate buffer, pH 9.0, was treated at room temperature with three 2 µl portions of 100 mM fluorescamine dissolved in acetone. After 5 min the reaction mixture was diluted to a total volume of 2.5 ml with 10 mM potassium phosphate buffer, pH 7.0, and the fluorescence intensity was determined. Excitation wavelength was 385 nm, emission was measured at 485 nm. $tRNA^{Phe}$-C-C-A from yeast, -□-; $tRNA^{Phe}$-C-C-A(XF47) from E.coli, -▲-; $tRNA^{Phe}$-C-C-A(3'NF) from yeast, -O-.

tRNAs depending on the particular environment or its exposure to deactivating processes. Another possible explanation is that the yield of the chemical reaction may be lower with E. coli $tRNA^{Phe}$ as compared to yeast $tRNA^{Phe}$--C-C-A(3'NH_2) because of the hindered accessibility of the amino group of the base X47. This can be excluded since the chromatography of the reaction products obtained from the respective tRNAs on an RPC-5 column (Fig. 3) shows that about the same relative amount of unlabelled material is present in both preparations.

The fluorescamine labelled tRNAs are eluted from the RPC-5 column at high ionic strength. Other UV absorbing species including unreacted or contaminating tRNA is eluted at lower ionic strength. In the case of E. coli $tRNA^{Phe}$, Fig. 3a, the second peak, which contains covalently bound fluorescamine, is fully aminoacylatable, whereas the earlier peaks cannot be phenylalanylated. This indicates that there is no unreacted, active $tRNA^{Phe}$ from E. coli left in the reaction mixture after treatment with the reagent.

The second, fluorescamine-labelled peak in the elution pattern of yeast $tRNA^{Phe}$-C-C-A(3'NF), Fig. 3b, is completely inert in the aminoacylation reaction, while the first peak has a strongly reduced aminoacylation capacity. The chromatographic procedure shown in Fig. 3 was applied to the purification of all fluorescamine treated tRNAs.

The specificity of the reaction of tRNAs with fluorescamine can be demonstrated by the chromatographic analysis of an RNase T_1 digest of yeast $tRNA^{Phe}$--C-C-A(3'NF), Fig. 4. Compared to the T_1 digest derived from native yeast

Table 1: Aminoacylation and relative fluorescence intensity of fluorescamine treated tRNAs.

tRNA species	Relative fluorescence[a]	Yield[b]	Maximal aminoacylation pmol/A_{260} unit tRNA	
			native	modified[c]
$tRNA^{Arg}_{E.coli}$-C-C-A(XF47)	29.1	0.62	1592	1180
$tRNA^{Lys}_{E.coli}$-C-C-A(XF47)	55.2	0.79	1470	1400
$tRNA^{Lys}_{E.coli}$-C-C-A(XF47)	55.2	0.79	1150[d]	40[d]
$tRNA^{Ile}_{E.coli}$-C-C-A(XF47)	36.4	0.80	1280	1150
$tRNA^{Met}_{E.coli}$-C-C-A(XF47)	31.2	0.55	1340	1120
$tRNA^{fMet}_{E.coli}$-C-C-A	2.4	0.0	1450	800
$tRNA^{Phe}_{E.coli}$-C-C-A(XF47)	37.5	0.72	1440	1610
$tRNA^{Phe}_{E.coli}$-C-C-A(XF47)	37.5	0.72	1367[e]	1539[e]
$tRNA^{Val}_{1\ E.coli}$-C-C-A	1.3	0.0	1300	1290
$tRNA^{Phe}_{Yeast}$-C-C-A	3.6	0.0	1520	1380
$tRNA^{Phe}_{Yeast}$-C-C-A(3'NF)	100.0	0.72	1580	20

a) Relative fluorescence applies to a solution containing one A_{260} unit tRNA/ml. The fluorescence of yeast $tRNA^{Phe}$-C-C-A(3'NF) was arbitrarily taken as 100. Reaction conditions were a s in Fig. 2.

b) Yield was calculated from the RPC-5 chromatograms (Fig. 3) as A_{260} units in the second, fluorescamine labelled, peak / total A_{260} units.

c) The maximal aminoacylation of fluorescamine treated tRNAs was determined after isolation by RPC-5 chromatography.

d) Phenylalanylation with phenylalanyl-tRNA synthetase from yeast under misacylation conditions as described in Methods.

e) Phenylalanylation with phenylalanyl-tRNA synthetase from yeast.

$tRNA^{Phe}$-C-C-A (data not shown), a disappearance of one peak, corresponding to the pentanucleotide C-A-C-C-A was observed in the chromatogram of T_1 fragments from $tRNA^{Phe}_{yeast}$-C-C-A(3'NF). A new peak, identified as C-A-C-C-A(3'NF) and exhibiting fluorescence excitation and emission spectra, typical for the condensation product of fluorescamine and primary aliphatic amines (15)

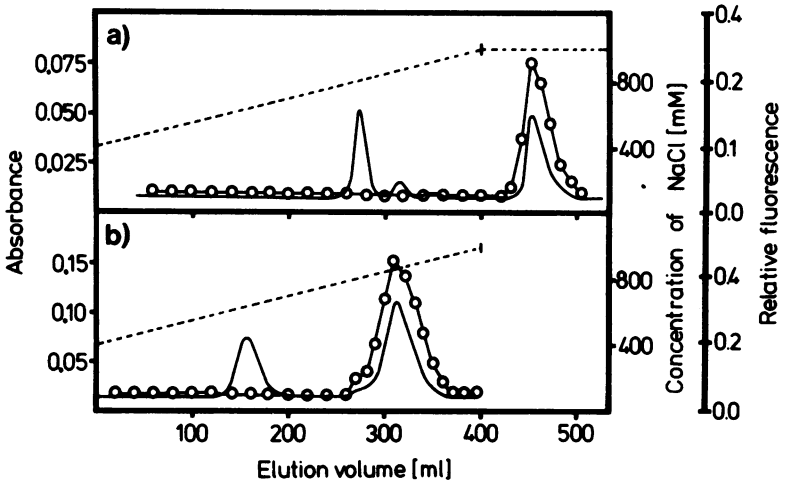

Fig. 3: Chromatography of fluorescamine treated tRNA species on RPC-5 columns. The conditions are given under Methods a) $tRNA^{Phe}$-C-C-A(XF47) from E. coli, b) $tRNA^{Phe}$-C-C-A(3'NF) from yeast. Relative fluorescence, -O-; UV absorbance of the eluant at 254 nm, ——; NaCl concentration, ,---.

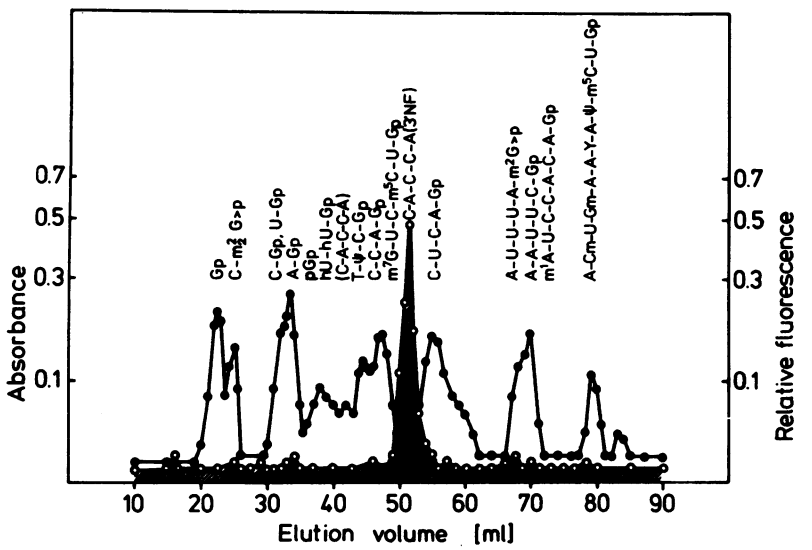

Fig. 4: Separation of T_1 oligonucleotide fragments obtained by digestion of yeast $tRNA^{Phe}$-C-C-A(3'NF). Relative fluorescence, -o-; UV absorbance ,-●-. 58 A_{260} units $tRNA^{Phe}_{yeast}$-C-C-A(3'NF) were incubated in 1 ml of 100 mM potassium phosphate buffer, pH 7.0, with 250 units ribonuclease T_1 for 16 hrs at 37°C. The digest was chromatographed on a DEAE-cellulose DE 52 column (0.5x120 cm) using a linear gradient of 2x300 ml from 10 mM to 300 mM NaCl in 10 mM sodium acetate, pH 5.8 and 7 M urea.

appeared in later fractions. An aliquot of this peak was analyzed for nucleoside composition revealing a ratio of U:G:A:C = 0.14:0.2:1.0:2.8. No fluorescent oligonucleotide other than the terminal C-A-C-C-A(3'NF) sequence was found in the RNase T_1 digest. Similar analyses were performed for the modified E. coli tRNAs (data not shown). Using the method described by Friedman et al (26), the only fluorescent oligonucleotide isolated, contained m^7G and must have originated from the variable loop region of tRNA (16).Thus, the position of fluorescamine modification is the aliphatic amino group of the side chain of the minor nucleoside X47.

As shown in Table 1, tRNAs which do not contain the base X in their variable loop region, like $tRNA^{fMet}$ and $tRNA_1^{Val}$ from E. coli, do not produce a fluorescent product. These two tRNAs were used to demonstrate the application of fluorescamine as an analytical agent to allow the fast and selective distinction between pairs of isoaccepting tRNAs such as E. coli $tRNA^{Met}$(X47) and $tRNA^{fMet}$(U47) or E. coli $tRNA_1^{Val}$(U47) and $tRNA_2^{Val}$(X47), (16).

Aminoacylation of fluorescamine treated tRNAs

The data on maximal aminoacylation of the investigated tRNA species before and after treatment with fluorescamine are summarized in Table 1. Yeast $tRNA^{Phe}$-C-C-A(3'NH_2) which can be quantitatively aminoacylated (18), is completely inactivated by the incorporation of fluorescamine. Native $tRNA^{Phe}$-C-C-A from yeast which was treated with the reagent in the same manner is not impaired in its acceptor activity. This is in support of our finding that the reaction exclusively occured at the amino group of the terminal ribose of $tRNA^{Phe}$-C-C-A(3'NH_2). The substrate properties of the tRNA species which were modified with fluorescamine on the minor nucleoside X47 are not significantly influenced by the presence of the fluorescence label and the tRNAs are aminoacylated with the corresponding amino acid to about the same extent as the native tRNAs. The aminoacylation of $tRNA^{fMet}$ and $tRNA_1^{Val}$, which do not contain the base X, is similarly not impaired by the fluorescamine treatment.

The kinetics of aminoacylation was determined for $tRNA^{Phe}$-C-C-A(XF47) from E. coli and compared to native E. coli $tRNA^{Phe}$-C-C-A, Fig. 5. The Lineweaver-Burk plot for the modified species is linear only up to 1 µM concentration of tRNA. A substrate inhibition was observed above this concentration. The native $tRNA^{Phe}$-C-C-A from E. coli does not show this effect, Fig. 5. This finding indicates that the fluorescence label attached to base X, although not preventing aminoacylation, in some way affects the tRNA: aminoacyl-tRNA synthetase interaction. The effect of X-base modification upon aminoacylation is more

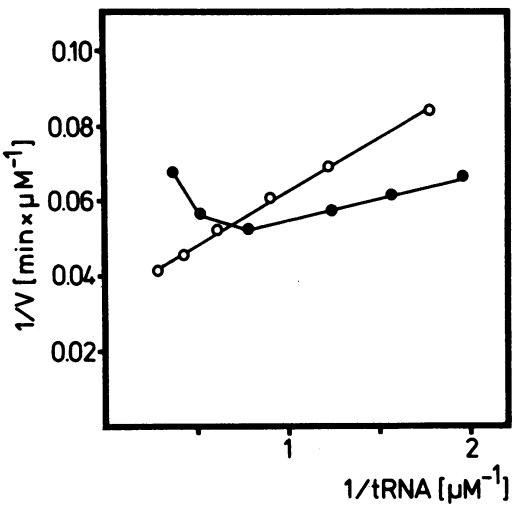

Fig. 5: Lineweaver-Burk plot of aminoacylation of E. coli $tRNA^{Phe}$-C-C-A, -O-, and E. coli $tRNA^{Phe}$-C-C-A(XF47), -●-, respectively, with phenylalanine using E. coli phenylalanyl-tRNA synthetase. Experimental conditions are described in Methods.

obvious under mischarging conditions. Native $tRNA^{Lys}_{E.coli}$-C-C-A can under these conditions be phenylalanylated by phenylalanyl-tRNA synthetase from yeast up to 1150 pmol per A_{260} unit tRNA, Table 1. Whereas fluorescamine modification of E. coli $tRNA^{Lys}$-C-C-A has little if any effect on the aminoacylation with cognate, homologous lysyl-tRNA synthetase from E. coli the phenylalanylation of modified E. coli $tRNA^{Lys}$-C-C-A(XF47) by phenylalanyl-tRNA synthetase does not take place.

The involvement of the fluorescamine label of E. coli $tRNA^{Phe}$-C-C-A(XF47) in the interaction with phenylalanyl-tRNA synthetase from E. coli could be further demonstrated by static fluorescence measurements. Titration of $tRNA^{Phe}_{E.coli}$-C-C-A(XF47) with increasing amounts of Phe-tRNA synthetase from E. coli is associated with a significant enhancement of fluorescence intensity reaching 27 % at saturation (Fig. 6). An enhancement of fluorescence was not observed when E. coli $tRNA^{Lys}$-C-C-A(XF47) was treated with yeast Phe-tRNA synthetase (data not shown) or when $tRNA^{Phe}_{E.coli}$-C-C-A(XF47) was treated with yeast phenylalanyl-tRNA synthetase. This is surprising since the E. coli $tRNA^{Phe}$-C-C-A(XF47) similarly as the native species (3), can be phenylalanylated with phenylalanyl-tRNA synthetase from yeast (Table 1). These results suggest that although both the yeast and the E. coli synthetase aminoacylate $tRNA^{Phe}_{E.coli}$-C-C-A(XF47) the nature of the enzyme : tRNA complex with respect to the XF47 residue has to be different in both cases.

Protein Biosynthesis

Phe-$tRNA^{Phe}_{E.coli}$-C-C-A(XF47) interacts with the elongation factor Tu from

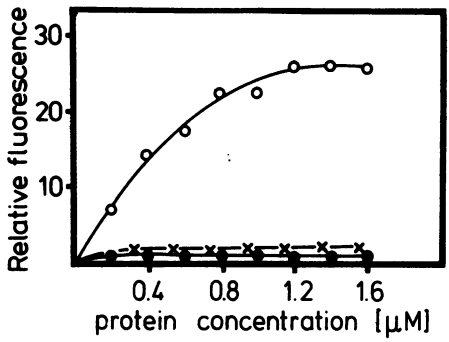

Fig. 6: Interaction of $tRNA^{Phe}$-C-C-A (XF47) from E. coli with phenylalanyl-tRNA synthetase from E. coli, -O-, and phenylalanyl-tRNA synthetase from yeast, -●-, and interaction of Phe-$tRNA^{Phe}$-C-C-A(XF47) from E. coli with EF-Tu: GMPP(NH)P, -X-. The solution of tRNA species (1.12 μM) in a 20 mM potassium phosphate buffer, pH 7.0, 200 mM KCl, 5 mM $MgCl_2$ was titrated with the respective proteins indicated in the figure. The relative fluorescence intensity was determined after each addition. Dilution, which was smaller than 10 %, has been taken into account. The titration was performed at 20°C within a period of 30 min. After the titration with EF-Tu:GMPP(NH)P the formation of the ternary complex was detected by gel filtration (Fig. 7c).

E. coli. This could be demonstrated by the gel filtration of Phe-$tRNA^{Phe}_{E.coli}$-C-C-A(XF47):EF-Tu:GMPP(NH)P ternary complex formed with native Phe-$tRNA^{Phe}_{E.coli}$-C-C-A (Fig. 7b). In the case of Fig. 7c the tRNA present in the ternary complex is fluorescent. The majority of fluorescent compound coincides with the maximum of [^{14}C]phenylalanine radioactivity in the eluant. This proves that the [^{14}C]Phe-$tRNA^{Phe}$-C-C-A(XF47) from E. coli forms ternary complexes and provides further evidence, that $tRNA^{Phe}$-C-C-A(XF47) is indeed aminoacylated with phenylalanine. The small amount of fluorescent tRNA, which appears in the elution volume corresponding to uncomplexed tRNA shows, however, that the Phe-$tRNA^{Phe}_{E.coli}$-C-C-A(XF47) preparation used for the experiment although aminoacylated to an extent of 1570 pmoles phenylalanine/A_{260} unit tRNA still contained some nonaminoacylated $tRNA^{Phe}_{E.coli}$-C-C-A(XF47). Since hydrolysis of the phenylalanine-tRNA ester did not occur during the experiment with the Tu factor (no significant peak of free, hydrolyzed [^{14}C]phenylalanine is observed in Fig. 7c, we have to conclude, that $tRNA^{Phe}_{E.coli}$-C-C-A(XF47) was not completely charged in the aminoacylation reaction. The amino acid acceptance of 1570 pmol phenylalanine/A_{260} unit of tRNA (Table 1) therefore does not correspond to a complete aminoacylation.

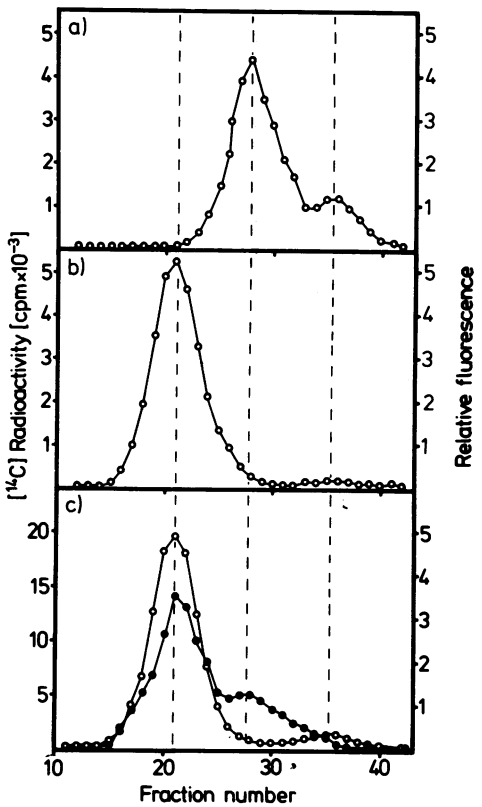

Fig. 7: Formation of Phe-tRNA$^{Phe}_{E.coli}$-C-C-A:EF-Tu:GMPP(NH)P ternary complexes. EF-Tu:GDP (600 pmol) was incubated in 25 µl of a solution containing 50 mM HEPES buffer, pH 7.0, 50 mM NH_4Cl, 50 mM NH_4Cl, 50 mM KCl and 10 mM $MgCl_2$ with 18 nmol GMPP(NH)P, 125 nmol phosphoenol pyruvate and 10 µg pyruvate kinase for 15 min at 37°C. The mixture was then cooled to 0°C and 75 pmol appropriate [^{14}C]Phe-tRNA$^{Phe}_{E.coli}$-C-C-A species were added. The mixture was applied by an injection syringe on to a column of Ultrogel AcA 44 which was eluted with a buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ and 100 mM NH_4Cl. Fractions of 0.35 ml were collected. When [^{14}C]Phe-tRNA$^{Phe}_{E.coli}$-C-C-A(XF47) was used the relative fluorescence intensity, -●-, of the fluorescamine dye was determined in each sample. The radioactivity, -O-, was determined in a scintillation counter after addition of 2.5 ml Aquasol to each sample. a) Filtration of [^{14}C]Phe-tRNA$^{Phe}_{E.coli}$-C-C-A without EF-Tu:GMPP(NH)P, b) [^{14}C]Phe-tRNA$^{Phe}_{E.coli}$-C-C-A:EF-Tu:GMPP(NH)P ternary complex and c) [^{14}C]Phe-tRNA$^{Phe}_{E.coli}$-C-C-A(XF479:EF-Tu:GMPP(NH)P ternary complex.

Although Phe-tRNA$^{Phe}_{E.coli}$-C-C-A(XF47):EF-Tu:GMPP(NH)P ternary complex can be formed, this complex formation does not lead to changes in the fluorescence amplitude of Phe-tRNA$^{Phe}_{E.coli}$-C-C-A(XF47), Fig. 6. In contrast to the interaction with E. coli phenylalanyl-tRNA synthetase where a fluorescence enhancement of the label was observed and an interaction with the enzyme was inferred, the XF47 fluorescence is essentially not affected by the Tu factor upon ternary complex formation. This leads to the conclusion, that the XF47 of tRNA^{Phe} is not involved in direct interaction with this elongation factor.

Furthermore, modification of X47 minor nucleoside does not influence the ability of Phe-tRNA$^{Phe}_{E.coli}$-C-C-A(X47):EF-Tu:GMPP(NH)P ternary complex to interact with the ribosomal A-site. The binding of Phe-tRNA$^{Phe}_{E.coli}$-C-C-A(XF47)

to programmed ribosomes is stimulated to the same extent by EF-Tu:GMPP(NH)P as is the binding of native Phe-tRNA$^{Phe}_{E.coli}$-C-C-A, Table 2. Despite the attached bulky fluorescamine group, the proper orientation of the variable loop of tRNA in the ribosomal A-site is therefore possible.

Phe-tRNA$^{Phe}_{E.coli}$-C-C-A(XF47) also participates as well in the following steps of the elongation process as does the unmodified Phe-tRNA$^{Phe}_{E.coli}$-C-C-A. This is suggested from an experiment where the poly U dependent synthesis of poly Phe was investigated, using phenylalanylated native and fluorescamine modified tRNA species, Fig. 8. No remarkable difference in the enzymatic binding to programmed ribosomes between these two Phe-tRNA^{Phe} derivatives could be detected.

Table 2: EF-Tu dependent binding of native and fluorescamine modified Phe-tRNA^{Phe} to 70S ribosomes[a)]

tRNA species	Binding [pmoles tRNA/A_{260} unit ribosomes]	
	without EF-Tu	with EF-Tu
Phe-tRNA$^{Phe}_{E.coli}$-C-C-A	1.32	10.12
Phe-tRNA$^{Phe}_{E.coli}$-C-C-A(XF47)	1.72	10.32

a) Programmed ribosomes from E. coli were prepared by incubation of 125 pmol 70S ribosomes (5 A_{260} units), 100 µg poly U, 1 A_{260} unit unfractionated tRNA from E. coli in 150 µl of a buffered solution containing 60 mM Tris-HCl, pH 7.8, 30 mM KCl, 30 mM NH_4Cl, 10 mM $MgCl_2$ and 2 mM dithiothreitol at 37°C for 10 min.

EF-Tu: GMPP(NH)P was prepared by incubating a 50 µl assay consisting of 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 50 mM NH_4Cl, 800 pmol EF-Tu:GDP, 20 µg pyruvate kinase, 0.8 mM GMPP(NH)P and 5 mM phosphoenol pyruvate at 37°C for 10 min.

The binding reaction was performed by mixing 12.5 pmol of programmed ribosomes (15 µl of incubation mixture), 80 pmol of EF-Tu:GMPP(NH)P (5 µl of incubation mixture) and 20 pmol of [^{14}C]Phe-tRNA$^{Phe}_{E.coli}$-C-C-A or the fluorescamine modified tRNA^{Phe} (in 5 µl 1 mM sodium acetate pH 4.5). In the control assay EF-Tu:GMPP(NH)P was omitted and the binding assay complemented by an equal volume of the corresponding buffer. The total volume of the reaction mixture was thus 25 µl and the final $MgCl_2$ concentration was 8 mM.

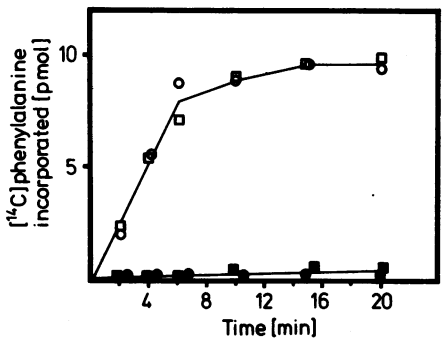

Fig. 8: Poly U dependent synthesis of poly phenylalanine on E.coli ribosomes. The 100 µl reaction mixture contained 60 mM Tris-HCl, pH 7.8, 70 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 0.1 mM GTP, 1 mM phosphoenol pyruvate, 50 µg poly U, 12.5 pmol (0.5 A_{260} units) E.coli S 100 supernatant protein, 10 µg pyruvate kinase and 40 pmol [^{14}C]Phe-$tRNA^{Phe}_{E.coli}$-C-C-A, -o-, or [^{14}C]Phe-$tRNA^{Phe}_{E.coli}$-C-C-A(XF47), -□-. The reaction mixture was preincubated at 37°C for 10 min, then the corresponding [^{14}C]Phe-$tRNA^{Phe}_{E.coli}$ and S 100 protein was added and the mixture was further incubated at 37°C. At the times indicated the radioactive material insoluble in hot 5 % trichloroacetic acid was determined. The control experiments were performed in the absence of S 100 protein, -●- or poly U, -■-.

DISCUSSION

The advantage of the fluorescamine labelling of the X47 residue of tRNA lies in the mild conditions under which the covalent attachment of fluorescamine can be accomplished as compared to other known reactions at this site (8-14) . On the other hand the fluorescamine label is not indefinitely stable and freshly modified tRNA(XF47) species should be used for fluorescence measurements. However, taking into account the simple preparation of tRNA XF47 this is not a serious disadvantage.

E.coli $tRNA^{Phe}$(XF47) can be aminoacylated and participates in all steps of the ribosomal elongation process. This opens the possibility of investigating the interaction of these fluorescent tRNAs with synthetase or ribosomes using fluorescence measurements.

Based on our experiments with X47 fluorescamine labelled E. coli tRNAs a question can be raised as to the interaction of the variable loop of the

particular tRNA with its cognate synthetase. All five tRNA species investigated could be aminoacylated after the modification. The fact, that the side chain of the minor nucleoside X47 is not functionally involved in the tRNA : synthetase interaction seems to be therefore a general phenomenon for this class of tRNAs. The present models for synthetase : tRNA recognition indeed do not involve the extra arm as a recognition site (27). Three facts from our present investigation indicate, however, that the X47 residue of the tRNA is in vicinity to the synthetase during its interaction, since the substituent on the side chain of X47 exhibits some effects on the tRNA : synthetase recognition process: first, E. coli $tRNA^{Phe}$-C-C-A(XF47) is a substrate inhibitor of the phenylalanyl-tRNA synthetase; second, the fluorescence of XF47 is changed after the $tRNA^{Phe}$ binds to this enzyme; and third, the misacylation of $tRNA^{Lys}$ from E. coli catalysed by phenylalanyl-tRNA synthetase from yeast, which can be performed with the native $tRNA^{Lys}$, is not possible after modification of X47 with fluorescamine. It is interesting to note the differences between the phenylalanyl-tRNA synthetases from yeast and from E. coli with regard to their interaction with E. coli $tRNA^{Phe}$-C-C-A(XF47). Although both enzymes catalyse the phenylalanylation of $tRNA^{Phe}$-C-C-A(XF47) from E. coli only the homologous E. coli synthetase gives rise to fluorescence enhancement after binding to $tRNA^{Phe}_{E.coli}$-C-C-A(XF47). The nature of the interaction with the X47 residue must therefore be different for both synthetases, the fluorescence enhancement being characteristic only for the homologous system.

The fluorescence intensity of the fluorescamine label bound to X47 in the variable loop is about 50 % lower than the fluorescence intensity of the dye attached to the 3'-terminal adenosine (Table 1). This implies an interaction of the fluorescamine linked to the side chain of X47 with some parts of tRNA. As apparent from an examination of the threedimensional model of $tRNA^{Phe}$ from yeast an intercalation of the fluorescamine into both main stacked regions of the L-shaped molecule is possible. Taking this into account the enhancement of the fluorescence intensity upon $tRNA^{Phe}_{E.coli}$-C-C-A(XF47) : phenylalanyl-tRNA synthetase complex formation could be due to changes in this stacking interactions. On the other hand the same effect could be achieved by a direct contact of the XF47 in the variable loop with the synthetase.
The present work leads to the understanding of the discrepancies on the aminoacylation activities of X47 modified $tRNA^{Phe}$ from E. coli which have been reported. Using the modification of the amino group of base X by different acylating agents some investigators found the tRNA partly (10) or completely (9)

deactivated after the treatment, while others (11-14) observed a retention of the aminoacylation activity. Clearly, if the minor nucleoside X47 is in close vicinity to the synthetase during the catalytic steps, the nature of the substitution at this site can be important in determining the activity of tRNA during aminoacylation.

Since the Phe-$tRNA^{Phe}_{E.coli}$-C-C-A(XF47) forms ternary complexes with E. coli elongation factor Tu and the fluorescence amplitude of the label is not affected by the ternary complex formation we conclude that the X47 of tRNA is probably not involved in this interaction. This is in agreement with the observation made by Pingoud and Urbanke (28) who measured the interaction of different aminoacyl-tRNAs with EF-Tu:GTP complex. As is apparent from their investigation the association constants, although dependent on the nature of the amino acid attached to tRNA, are independent of the structure of the variable loop of the particular tRNA species.

The fluorescamine modification on the variable loop of the aminoacyl-tRNA does not influence its interaction with the ribosomal A-site. The results obtained with $tRNA^{Phe}$-C-C-A(XF47) support the recent data obtained with $tRNA^{Phe}$ species modified on the X47 residue; such tRNAs were active in the induction of the ribosome dependent synthesis of ppGpp (magic spots) (10), which is known to require the binding of tRNA to the ribosomal A-site.

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Abbreviations: $tRNA^{Xxx}$-C-C-A; native $tRNA^{Xxx}$, $tRNA^{Xxx}$-C-C-A(XF47); $tRNA^{Xxx}$-C-C-A with fluorescamine label attached to the minor nucleoside X47 (numbering of the nucleoside residues in tRNA according to ref. 16). $tRNA^{Phe}_{yeast}$-C-C-A(3'NH_2) or $tRNA^{Phe}_{yeast}$-C-C-A(3'NF); $tRNA^{Phe}$ from yeast in which the terminal adenosine 76 is replaced by 3'-deoxy-3'-aminoadenosine, or fluorescamine modified 3'-deoxy-3'-aminoadenosine, respectively. EF-Tu; elongation factor Tu from E.coli; GMPP(NH)P; guanylyl-imidodiphosphate. HEPES = N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid.

REFERENCES

1. Krauss, G., Riesner, D. and Maass, G. (1976) Eur. J. Biochem. 68, 81-93.
2. Beres, L. and Lucas-Lenard, J. (1973) Biochemistry 12, 3998-4002.
3. Wintermeyer, W., Robertson, J.M. and Zachau, H.G. (1976) Proc. Int. Conf. Synthesis, Structure and Chemistry of Transfer RNA and their Components (ed. M. Wiewiórowski) Poznán, Poland, 306.

4. Schleich, H.G., Wintermeyer, W. and Zachau, H.G. (1978) Nucl. Acids Res. 5, 1701-1713.
5. Pingoud, A., Kownatzki, R. and Maass, G. (1977) Nucl. Acids Res. 4, 327-338.
6. Maelicke, A., Sprinzl, M., von der Haar, F., Khwaja, T.A. and Cramer, F. (1974) Eur. J. Biochem. 43, 617-625.
7. Ohashi, Z., Maeda, M., McCloskey, J.A. and Nishimura, S. (1974) Biochemistry 13, 2620-2625.
8. Schiller, P.W. and Schechter, A.N. (1977) Nucl. Acids Res. 4, 2161-2167.
9. Hansske, F., Watanabe, K., Cramer, F. and Seela, F. (1978) Hoppe-Seyler's Z. Physiol. Chem. in press.
10. Ofengand, J. and Liou, R. (1978) Nucl. Acids Res. 5, 1325-1334.
11. Friedman, S. (1972) Biochemistry 11, 3435-3443.
12. Nauheimer, U. and Hedgcoth, C. (1974) Arch. Biochem. Biophys. 160, 631-642.
13. Dugas, H. (1977) Acc. Chem. Res. 10, 47-54.
14. Plumbridge, J.A., Bäumert, H.G., Ehrenberg, M. and Rigler, R. (1978) Abstr. Commun. 12th Fed. Eur. Biochem. Soc. Meet. No. 0130.
15. De Bernardo, S., Weigele, M., Toome, V., Manhart, K., Leimgruber, W., Böhlen, P., Stein, S. and Udenfriend, S. (1974) Arch. Biochem. Biophys. 163, 390-399.
16. Sprinzl, M., Grüter, F. and Gauss, D.H. (1978) Nucl. Acids. Res. 5, r15-r27.
17. Fraser, T.H., Rich, A. (1973) Proc. Nat. Acad. Sci. USA 70, 2671-2675.
18. Sprinzl, M., Kucharzewski, M., Hobbs, J.B. and Cramer, F. (1977) Eur. J. Biochem. 78, 55-61.
19. Roy, K.L., Bloom, A. and Söll, D. (1971) in: Proc. Nucl. Acid Res. Vol. 2, 524-541 (ed. by G.L. Cantoni and D.R. Davies), Harper and Row, New York.
20. Holmes, W.M., Hurd, R.E., Reid, B.R., Rimerman, R.A. and Hatfield, G.W. (1975) Proc. Nat. Acad. Sci. USA, 72, 1068-1071.
21. Egan, B.Z. and Kelmers, A.D. (1972) Prep. Biochem. 2, 265-274.
22. Sprinzl, M., Sternbach, H., von der Haar, F. and Cramer, F. (1977) Eur. J. Biochem. 81, 579-589.
23. Wagner, Th. and Sprinzl, M. (1978) Meth. Enzym. in press.
24. Arai, K., Kawakita, M. and Kaziro, Y. (1972) J. Biol. Chem. 247, 7029-7037.
25. Gavrilova, L.P. and Spirin, A.S. (1974) Meth. Enzym. 30, 452-462.
26. Friedman, S., Li., H.J., Nakanishi, K. and van Lear, G. (1974) Biochemistry 13, 2932-2937.
27. Rich, A. and Schimmel, P.R. (1977) Nucl. Acids Res. 4, 1649-1665.
28. Pingoud, A. and Urbanke, C. personal communication.