Properties of $tRNA^{Phe}$ from yeast carrying a spin label on the 3'-terminal. Interaction with yeast phenylalanyl-tRNA synthetase and elongation factor Tu from Escherichia coli

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ABSTRACT

The 2-thioketo function of $tRNA^{Phe}$-C-s^2C-A in which the penultimate cytidine residue is replaced by 2-thiocytidine can serve as a site of specific attachment of spin label. By alkylation of $tRNA^{Phe}$-C-s^2C-A with iodoacetamide or its spin label derivatives $tRNA^{Phe}$-C-(acm)s^2C-A or $tRNA^{Phe}$-C-(SL)s^2C-A are formed. The enzymatic phenylalanylation of these $tRNAs^{Phe}$ revealed that the 2-position of the penultimate cytidine can be modified without impairing this enzymatic reaction but there exists a sterical limitation for the substituent on this position beyond which the $tRNA^{Phe}$:phenylalanyl-tRNA synthetase recognition is not possible. Both Phe-$tRNA^{Phe}$-C-(acm)s^2C-A as well as Phe-$tRNA^{Phe}$-C-(SL)s^2C-A form ternary complexes with EF-Tu·GTP. The part of the 3'-terminus of $tRNA^{Phe}$ where the additional substituents are attached is therefore not involved in the interaction with this elongation factor. This could be also demonstrated by ESR measurements of spin labelled $tRNAs^{Phe}$. The correlation times, τ_c, for $tRNA^{Phe}$-C-(SL)s^2C-A, Phe-$tRNA^{Phe}$-C-(SL)s^2C-A and Phe-$tRNA^{Phe}$-C-(SL)s^2C-A·EF-Tu:GTP are essentially identical indicating that the structure of the 3'-end of $tRNA^{Phe}$ is not influenced significantly by aminoacylation or ternary complex formation.

INTRODUCTION

Transfer ribonucleic acid interacts during its functional cycle in protein biosynthesis with several different proteins. Despite the available information about the structure of tRNA (1,2) there is little known about these interactions at a molecular level. Until co-crystallization of tRNA and its interacting enzymes becomes possible allowing a direct X-ray crystallographic study of the complexes, the physicochemical, physical and chemical methods remain the only way to approach the problem of protein-nucleic acid interactions.

The 3'-terminal C-C-A end of the tRNA plays an important role in the tRNA function (3). By modification of this part of the molecule a substantial amount of information was collected allowing some insight into the recognition of chemical processes in which the C-C-A end is involved.

It was shown previously that the cytidine residues of the C-C-A end can be replaced by 5-iodocytidine or 2-thiocytidine without impairing the ability of the tRNA for participation in the in vitro ribosomal protein biosynthesis (4). 2-Thiocytidine incorporated into the polynucleotide chain can be also used as a specific site for further modification by alkylation reactions and is also a suitable site for introduction of spectroscopic reporter residues (5).

In the present work we investigated the ability of $tRNA^{Phe}$-C-s^2C-A alkylated on the thioketo function of the s^2C 75 residue for participation in the enzymatic aminoacylation reaction and formation of Phe-$tRNA^{Phe}$·EF-Tu·GTP complexes. Further, the motion of a nitroxyl radical spin label attached to s^2C 75 residue of $tRNA^{Phe}$-C-s^2C-A was investigated by electron spin label resonance, ESR, measurements. Comparison of the data obtained for non aminoacylated tRNA, aminoacylated tRNA and tRNA in the ternary complex with EF-Tu·GTP provided informations about the structure of the C-C-A end during different functional states in the tRNA cycle.

MATERIALS AND METHODS

$tRNA^{Phe}$-C-s^2C-A was prepared as previously described (6,7), $tRNA^{Phe}$-C-C-A was isolated (8) from bulk tRNA from yeast (Boehringer, Mannheim, Germany). One A_{260} unit $tRNA^{Phe}$ was taken as 1.5 nmol. Phenylalanyl tRNA synthetase (9) (EC 6.1.1.20), specific activity 800 units/mg protein (one unit is defined as the capacity to aminoacylate 1 nmol of $tRNA^{Phe}$ per min), was obtained from Dr. F. von der Haar, Göttingen. Elongation factor Tu from Escherichia coli (10), specific activity 22 000 units/mg, was obtained from Dr. D. Miller, Roche Institute for Molecular Biology (Nutley, N.Y., U.S.A.), pyruvate kinase 10 mg/ml (EC 2.7.1.40), phosphoenol pyruvate and GMP-P(NH)P were products of Boehringer (Mannheim, Germany).

[^{14}C]Phenylalanine, specific activity (522 Ci/mol), [^{3}H]Phenylalanine (12 Ci/mol) and iodo[^{14}C]acetamide (57 Ci/mol) were obtained from Radiochemical Centre (Amersham, England). N-(1-oxyl-2,2,6,6 -tetramethyl-4-piperidyl)iodoacetamide and N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide were products of Syva (Palo Alto, California, U.S.A.), Ultrogel AcA 44 from LKB (Bromma, Sweden), Tris buffer from Sigma (St. Louis, U.S.A.), Aquasol scintillation fluid from NEN (Boston, U.S.A.) and all other chemicals from Merck (Darmstadt, Germany).

Alkylation of $tRNA^{Phe}$-C-s^2C-A with iodoacetamide.

Reaction mixture contained 0.05 mM $tRNA^{Phe}$-C-s^2C-A, 1.0 mM [^{14}C] iodoacetamide and 10 mM phosphate buffer pH 7.2. Incubation was performed for 5 hrs at 37°C. The course of the reaction was monitored by spotting aliquots onto paper discs and determination of the remaining radioactivity after washing the filter discs in 5 % aqueous trichloracetic acid (5). Under the conditions applied no further progress of the reaction could be detected after 5 hrs. The reaction mixture was treated with a twofold volume of ethanol, chilled at -20°C for 2 hrs, centrifuged and the pellet washed twice with ethanol. The tRNA pellet was then dissolved in 10 mM phosphate buffer pH 6.0 and stored at -20°C.

Preparation of spin labelled $tRNAs^{Phe}$

Reaction mixture contained in 20 % (v/v) ethanol 0.1 mM $tRNA^{Phe}$-C-s^2C-A, 20 mM sodium acetate buffer pH 5.2 and 2 mM appropriate iodoacetamide spin label. Incubation was performed either at room temperature for 10 hrs or at 37°C for 5 hrs. Spin labelled tRNA was precipitated with ethanol. The pellet obtained by centrifugation was washed with 70 % aqueous ethanol, then twice with ethanol and dried in a desiccator. Spin labelled $tRNAs^{Phe}$ were dissolved in 5 mM acetate buffer pH 5.2 and stored at -20°C. Phe-$tRNA^{Phe}$-C-s^2C-A was spin labelled under the same conditions except that incubation was performed for 3 hrs at 37°C. As could be demonstrated in model experiment in which [^{3}H]Phe-$tRNA^{Phe}$-C-s^2C-A was alkylated with [^{14}C]iodoacetamide, under these conditions the extent of alkylation is 80 % whereas about 20 % phenylalanine is hydrolysed from the $tRNA^{Phe}$ during the reaction. The amount of tRNA which contained both the alkyl group on the s^2C 75 residue as well as the amino acid on the terminal adenosine was 64 %. It is assumed that also the Phe-$tRNA^{Phe}$-C-(SL)s^2C-A preparations contain about 64 % of the expected species, and 16 % uncharged $tRNA^{Phe}$-C-(SL)s^2C-A.

Aminoacylation

Aminoacylation assay for $tRNA^{Phe}$ was performed as described previously (6), except that, unless otherwise indicated, the 2-mercaptoethanol was omitted. For preparation of Phe-$tRNA^{Phe}$ species (up to 5 A_{260} units) the appropriate $tRNA^{Phe}$ (0.015 mM) was incubated in a mixture containing 150 mM Tris-HCl pH 7.6, 100 mM KCl, 50 mM $MgCl_2$, 2 mM ATP and 0.05 mM phenylala-

nine with phenylalanyl-tRNA synthetase (3.0 units enzyme/nmol tRNA) at 37°C for 20 min. The mixture was extracted with the same volume of water saturated phenol, centrifuged, the aqueous phase separated, then the tRNA was precipitated wtih ethanol and centrifuged. The pellet was dissolved in 0.5 ml 5 mM sodium acetate buffer pH 4.5 and the solution applied on to a Sephadex G-25 column (0.5 x 20 cm). The column was washed with 1 mM sodium acetate buffer pH 4.5 and the Phe-$tRNA^{Phe}$, eluted in the exclusion volume of the column, was collected and concentrated by ethanol precipitation.

Ternary complex formation

EF-Tu·GDP (600 pmol) was incubated in 25 µl solution containing 50 mM HEPES buffer pH 7.0, 50 mM NH_4Cl, 50 mM KCl and 10 mM $MgCl_2$ with 18 nmol GMP-P(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 Phe-$tRNA^{Phe}$ 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. After adding 2.5 ml Aquasol scintillation fluid to each sample, the radioactivity was determined in a scintillation counter.

For the ternary complex formation with tRNAs carrying a spin label, 6 nmol EF-Tu·GDP was incubated in 20 µl of the same buffer as given above with 100 nmol GMP-P(NH)P, 50 nmol phosphoenolpyruvate and 5 µg pyruvate kinase at 37°C for 10 min., then 2.5 nmol spin labelled [^{3}H]Phe-$tRNAs^{Phe}$ in 20 µl buffer were added and ESR spectra were taken immediately. After measurements the existence of the complex formation was demonstrated by gel filtration. Each second fraction of the eluate was used alternatively for determination of radioactivity and for the determination of the nitroxyl radical by ESR. Tubes containing the fractions for ESR measurements were placed in a desiccator and the solvent was removed by evaporation in vacuum at room temperature. Subsequently the samples were dissolved in 25 µl water and the intensity of the central signal of the ESR spectrum was determined using 20 µl aliquots.

ESR measurements

Electron spin resonance spectra were measured with a Varian E3 spectrometer at 9255 MHz (x-band). The spectrometer was equipped with a 100 kHz

modulation unit. The modulation amplitude was set equal to 0.5. Since the correlation time was found to depend strongly on the microwave power, τ_c being about doubled by increasing the power from 5 to 100 mW, the power was kept below 5 mW for all measurements. A Varian E-4540 Temperature Controller was used to thermostat the cavity, and the temperature was measured by a thermocouple. All samples were placed in glass capillaries about 1 mm of diameter and sealed with a teflon stopper at the lower end. The samples were dissolved in 30 µl solution containing 50 mM HEPES pH 7.0, 50 mM NH_4Cl, 50 mM KCl and 10 mM $MgCl_2$. The concentration of spin labelled component for ESR measurements was in the range of 0.05 to 0.5 mM.

RESULTS

Preparation of modified tRNAs

Cytidine residues of the -C-C-A end of tRNA can be specifically modified using a combination of enzymatic and chemical reactions. Thus the penultimate cytidine residue, C 75 of $tRNA^{Phe}$ from yeast was replaced by 2-thiocytidine using AMP(CMP):tRNA nucleotidyl transferase catalysed incorporation of this modified nucleoside into $tRNA^{Phe}$ lacking its C-C-A end. $tRNA^{Phe}$-C-s^2C-A was then alkylated on the 2-thioketo group of the s^2C residue using iodoacetamide or its spin label derivatives. The chemical structure of the penultimate residue in the particular modified tRNA is shown in Fig. 1.

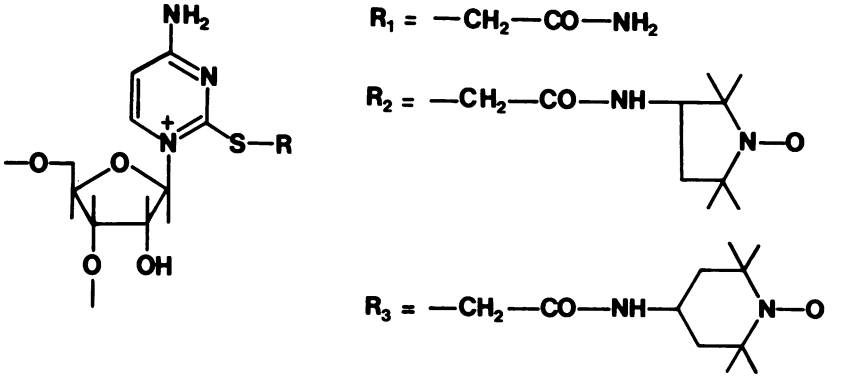

Fig. 1. Structure of the residue 75 in the $tRNA^{Phe}$ from yeast obtained by alkylation of $tRNA^{Phe}$-C-s^2C-A with iodoacetamide (R_1), N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl) iodoacetamide (R_2) and N-(1-oxyl-2,2,6,6-tetramethyl-3-piperidyl)iodoacetamide (R_3). R_1 is abbreviated in the text as acm, R_2 as 5SL and R_3 as 6SL.

After appropriate enzymatic digestion the modified $tRNA^{Phe}$ were analysed for their nucleoside composition using a chromatographic separation of the components (Fig. 2). A new peak corresponding to 2-thiocytidine appears in the elution profile of the enzymatic digest of $tRNA^{Phe}$-C-s^2C-A (Fig. 2b) which is not present in the elution profile of native $tRNA^{Phe}$ digest (Fig. 2a). After treatment of $tRNA^{Phe}$-C-s^2C-A with iodoacetamide the 2-thiocytidine peak disappeared and a new peak in a later elution volume, corresponding to alkylated 2-thiocytidine (for formula see Fig. 1) appears. Since the order of elution under the chromatographic conditions applied reflects the degree of protonation of the nucleosides it is not surprising that an alkylated 2-thiocytidine pocessing a positive charge is eluted in the later elution volume. The assignment of the peaks in the elution profile was also proved by independent experiments with model monomeric substances (6,11). By treatment of $tRNA^{Phe}$-C-(acm)s^2C-A with 2-mercaptoethanol the 2-thioalkyl group can be easily removed under formation of $tRNA^{Phe}$-C-C-A (Fig. 2a). The

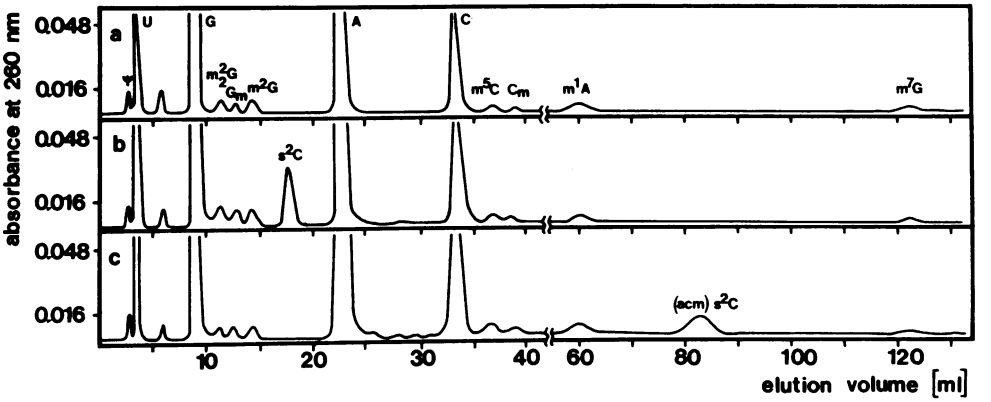

Fig. 2. Analysis of the minor nucleosides in native and modified $tRNA^{Phe}$ from yeast by nucleoside analyser (7). One A_{260} unit $tRNA^{Phe}$ was incubated in 50 µl solution containing 100 mM ammonium acetate buffer pH 8.5, 10 mM $MgCl_2$, 5 µg alkaline phosphatase and 5 µg snake venom phosphodiesterase at 45°C for 4 hrs. The mixture of nucleosides formed by enzymatic digest was applied on the column of Beckman M 71 cation exchanger resin (40 x 0.5 cm) and chromatographed at 50°C using a 0.4 M ammonium formate buffer pH 4.15 as an eluant at a flow rate of 0.45 ml/min. The UV absorbance at 260 nm was monitored. $tRNA^{Phe}$-C-C-A (a), $tRNA^{Phe}$-C-s^2C-A (b), $tRNA^{Phe}$-C-(acm)s^2C-A (c). $tRNA^{Phe}$-C-(acm)s^2C-A after treatment with 10 mM solution of 2-mercaptoethanol for 1 hr at 37°C was desalted on a Sephadex G-25 column as indicated in Fig. 3 and digested as described above. The chromatographic pattern of its digest was identical with that obtained from native $tRNA^{Phe}$-C-C-A (a).

loss of radioactivity from $tRNA^{Phe}$-C-([^{14}C]acm)s^2C-A upon treatment with 2-mercaptoethanol was demonstrated further by a gel filtration experiment shown in Fig. 3. The radioactively labelled carbamoylmethyl residue elutes in the same position where the UV absorbance of $tRNA^{Phe}$-C-([^{14}C]acm)s^2C-A appears whereas after the detachment due to 2-mercaptoethanol treatment almost the whole radioactivity is eluted in the later fractions. This reaction, the mechanism of which was investigated previously on the corresponding nucleotide (11) can thus be performed also at the polynucleotide level. 2-Thiocytidine residues, which are also present in the anticodon loop of some tRNA species (12) can therefore be converted via iodoacetamide alkylation and 2-mercaptoethanol treatment to cytidine under very mild conditions. (See accompanying paper, Ref. 21).

Aminoacylation

$tRNA^{Phe}$-C-(acm)s^2C-A can be aminoacylated by phenylalanyl-tRNA synthetase (Table 1). Due to the lability of the 2-thioalkyl group of this tRNA in the presence of thioalcohols as 2-mercaptoethanol or dithiothreitol, which are usually present in a common enzyme preparation, a careful analysis of the

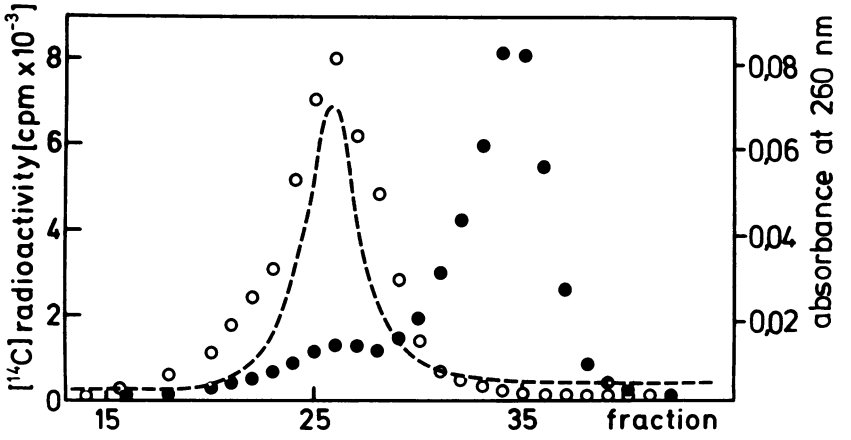

Fig. 3. Gel filtration of $tRNA^{Phe}$-C-([^{14}C]acm)s^2C-A before (open circles) and after (full circles) treatment with 10 mM 2-mercaptoethanol for 1 hr at 37°C. Five A_{260} units appropriate tRNA were applied on to a column of Sephadex G-25 (30 x 1 cm) and eluted with 5 mM sodium acetate buffer pH 4.5. The UV absorbance at 260 nm (line) was continuously monitored and the radioactivity present in 0.3 ml fractions was determined (circles).

Table 1 Enzymatic aminoacylation of native and modified $tRNA^{Phe}$ species. Assays are described in (6).

$tRNA^{Phe}$ species	maximal aminoacylation [pmol/A_{260} unit tRNA]	Km μM	V rel
$tRNA^{Phe}$-C-C-A	1525	2.0	100
$tRNA^{Phe}$-C-s^2C-A	1520	2.80	45
$tRNA^{Phe}$-C-(acm)s^2C-A	1508	2.02	40
$tRNA^{Phe}$-C-(5SL)s^2C-A	184	no comp. inhibit.	
$tRNA^{Phe}$-C-(5SL)s^2C-A[a]	1320	not determined	

a) The tRNA was incubated for 10 min with 14 mM 2-mercaptoethanol at 37°C prior to aminoacylation.

product of aminoacylation was performed to exclude the possibility of the loss of the acetamido residue leading to native $tRNA^{Phe}$-C-C-A during the enzymatic reaction.

$tRNA^{Phe}$-C-s^2C-a was therefore first alkylated with iodo[^{14}C]acetamide and subsequently the enzymatic aminoacylation with [^{3}H]phenylalanine was investigated. Data obtained from this double labelling experiments (Table 2) demonstrate that in the absence of 2-mercaptoethanol there is no hydrolysis of the [^{14}C]carbamoylmethyl residue from the tRNA during the enzymatic aminoacylation and in the same time this modified $tRNA^{Phe}$ is phenylalanylated by [^{3}H]phenylalanine. On the other hand in the presence of 2-mercaptoethanol a substantial decrease of [^{14}C]acetamide radioactivity was observed. This reaction leading to conversion of $tRNA^{Phe}$-C-([^{14}C]acm)s^2C-A to $tRNA^{Phe}$-C-C-A did not influence the extent of enzymatic aminoacylation, which remains the same if the alkylgroup on the C 75 residue is present or not.

The slight decrease of the rate of aminoacylation of $tRNA^{Phe}$-C-(acm)s^2C-A (Table 1) is common to almost all $tRNA^{Phe}$ species which are modified on their C-C-A end (3) but similarly as in other cases the Km remained essentially unchanged. $tRNA^{Phe}$-C-(5SL)s^2C-A bearing larger group on the s^2C 75 residue on the other hand cannot be aminoacylated by phenylalanyl-tRNA synthetase. After treatment of this tRNA with 2-mercaptoethanol, leading to removal of the bulky thioalkyl residue from C 75 the ability of this tRNA

Table 2 Aminoacylation of $tRNA^{Phe}$-C-([^{14}C]acm)s^2C-A in the presence and absence of 2-mercaptoethanol. In these cases 14 mM 2-mercaptoethanol was added to the incubation mixture which was then preincubated at 37°C for 8 min. The aminoacylation was started by adding the phenylalanyl-tRNA synthetase.

incubation time	without 2-mercaptoethanol		with 2-mercaptoethanol	
	[^{14}C]acm attached	aminoacylation	[^{14}C]acm attached	aminoacylation
	[pmol/A_{260} unit tRNA]	[pmol/A_{260} unit tRNA]	[pmol/A_{260} unit tRNA]	[pmol/A_{260} unit tRNA]
0	1145	-	1140	-
5	1128	-	873	-
10	1110	1508	636	1435
15	1107	1408	409	1490
20	1131	1500	369	1506

for accepting phenylalanine is restored (Table 1). $tRNA^{Phe}$-C-(5SL)s^2C-A is not a competitive inhibitor of the synthetase. In fact no inhibition was observed by this tRNA if the aminoacylation assay contained as much as a 6 fold excess of the modified species over the native substrate.

Phe-$tRNA^{Phe}$-C-(5SL)s^2C-A and Phe-$tRNA^{Phe}$-C-(6SL)s^2C-A necessary for ESR measurements of the EF-Tu·GTP Phe-$tRNA^{Phe}$ ternary complexes were prepared by alkylation of Phe-$tRNA^{Phe}$-C-s^2C-A with corresponding iodoacetamide spin labels. Optimal conditions for spin labelling were elucidated by investigation of the rate of phenylalanine hydrolysis from [^{3}H]Phe-$tRNA^{Phe}$-C-s^2C-A during its alkylation with iodo[^{14}C]acetamide (data not shown). By this route it was possible to obtain the spin labelled Phe-tRNA-C-(SL)s^2C-A species in 65 % yield which were used without further purification for ESR measurements.

Phe-$tRNA^{Phe}$·EF-Tu·GMP-P(NH)P ternary complexes

Formation of the ternary complexes between Phe-$tRNAs^{Phe}$, EF-Tu and GMP-P(NH)P was assayed by gel filtration of the appropriate mixtures on AcA 44 columns (Fig. 4 and 5). Native Phe-$tRNA^{Phe}$-C-C-A if applied onto the column alone or in mixture with EF-Tu·GDP is slightly retarded (Fig. 4a).

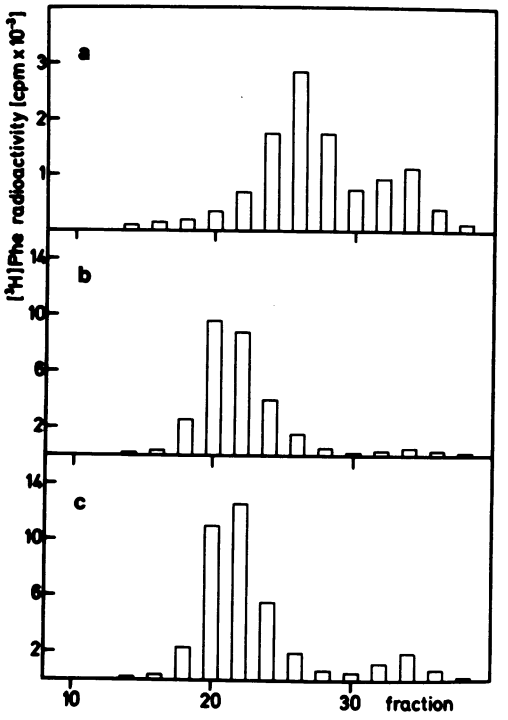

Fig. 4.

Determination of Phe-tRNA^{Phe}·EF-Tu·GMP-P(NH)P ternary complex formation by a gel filtration experiment as described under Methods. Elution profile of [^{3}H]Phe-tRNA^{Phe} alone or in a mixture with EF-Tu·GDP (a), [^{3}H]Phe-tRNA^{Phe} with EF-TU·GMP-P(NH)P (b), and [^{3}H]Phe-tRNA^{Phe}-C-s^{2}C-A with EF-Tu·GMP-P(NH)P.

After conversion of EF-Tu·GDP to EF-Tu·GTP by pyruvate kinase and phosphoenol pyruvate Phe-tRNA^{Phe}·EF-Tu·GMP-P(NH)P ternary complex is formed which is eluted in the void volume of the column (Fig. 4b). Phe-tRNA^{Phe}-C-s^{2}C-A containing 2-thiocytidine in the position 75 of the polynucleotide chain forms a ternary complex with the same efficiency as the native Phe-tRNA^{Phe}-C-C-A (Fig. 4c). Replacement of the 2-oxo function of the cytidine 75 by the 2-thioketo group therefore does not influence the interaction of tRNA with EF-Tu. Furthermore it could be demonstrated that even the presence of carbamoylmethyl residue on s^{2}C 75 is not influencing the ability of EF-Tu for interaction with this modified tRNA. During the filtration of a mixture of [^{3}H]Phe-tRNA^{Phe}-C-([^{14}C]acm)s^{2}C-A, EF-Tu and GMP-P(NH)P on the AcA 44 column (Fig. 5b) both the [^{3}H]phenylalanine as well as [^{14}C]carbamoylmethyl radioactivity appeared in the void volume of the column together with the elongation factor. The ternary complex is therefore formed with Phe-tRNA-C-(acm)s^{2}C-A and there is no loss of the alkyl residue during the complex formation, since no [^{14}C]radioactivity is eluted in the range where the small molecules should appear. Similarly if a larger substituent is attached to s^{2}C 75 as in the case of spin labelled tRNAs the ternary complex

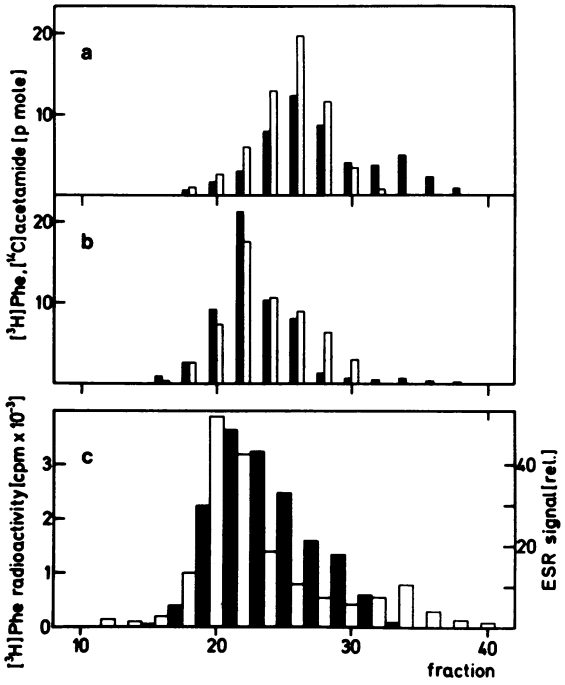

Fig. 5.

Gel filtration of [^{3}H]Phe-tRNA^{Phe}-C-([^{14}C]acm)s^{2}C-A alone or in a mixture with EF-Tu·GDP (a) [^{3}H]Phe-tRNA^{Phe}-C-([^{14}C]acm)s^{2}C-A·EF-Tu·GMP-P(NH)P (b) on AcA 44 column as described under Methods. Open columns represent the [^{14}C] radioactivity, the full columns the [^{3}H] radioactivity found in appropriate fraction. The gel filtration of spin labelled [^{3}H]-Phe-tRNA^{Phe}-C-(5SL)s^{2}C-A·EF-Tu·GMP-P(NH)P ternary complex (c). Open columns represent the [^{3}H] radioactivity, full columns the relative ESR signal.

still can be formed (Fig. 5c). In this experiment a mixture of [^{14}C]Phe-tRNA-C-(5SL)s^{2}C-A, EF-Tu and GMP-P(NH)P were passed through a gel column. Fractions were assayed for both radioactivity as well as for the presence of nitroxyl radical. Comparison of both the profiles indicates that although the total amount of tRNA^{Phe} which carries [^{3}H]phenylalanine is complexed with EF-Tu, some part of the total spin labelled tRNA is not aminoacylated and therefore not able to participate in the complex. This is obvious from the observation that the ESR profile having a maximum at fraction 21 is not symmetrical and is tailing in further fractions due to the presence of non aminoacylated tRNA^{Phe}-C-(5SL)s^{2}C-A. The amount of this tRNA in the total mixture is however not higher than 20 % as can be estimated from the total amount of free amino acid which is eluted around the fraction 34. Clearly the spin labelled tRNA which lost its amino acid during the spin labelling of [^{14}C]Phe-tRNA^{Phe}-C-s^{2}C-A is not in the ternary complex.

Further it is evident from the experiments in Fig. 5 that also in the case of Phe-tRNA^{Phe}-C-(acm)s^{2}C-A preparation the aminoacylation is not complete. The maximal phenylalanine acceptance activity of native tRNA^{Phe} from yeast as determined by incorporation of radioactive amino acid is around

1500 pmol Phe/A_{260} unit tRNA. Although in the case of $tRNA^{Phe}$-C-(acm)s^2C-A similar maximal aminoacylation was achieved as for native $tRNA^{Phe}$ or $tRNA^{Phe}$-C-s^2C-A (Table 1) the Fig. 5b indicates that there is still non aminoacylated $tRNA^{Phe}$-C-([^{14}C]acm)s^2C-A present in this preparation which is not able to form ternary complex. This can be concluded from the slightly different profiles of [^{3}H]Phe where all radioactivity is centred around fraction 20 and that of [^{14}C]carbamoylmethyl group giving an asymmetrical tailing due to a presence of non complexed and uncharged tRNA appearing around fraction 24. It must therefore be assumed that a $tRNA^{Phe}$ from yeast which accepts 1500 pmol Phe/A_{260} unit tRNA is not completely aminoacylated but such preparation still contains at least 10 % non aminoacylated species.

ESR measurements

Fig. 6. shows typical ESR spectrum of some spin labelled compounds which were taken at 28°C.As in our previous communication (5) the ESR spectra obtained at 4°C and at room temperature respectively, were evaluated for the correlation time, τ_c, of the reorientation of the spin label in its environment and for the motional anisotropy parameter, ε. These results are summarised in Table 3. The attachment of piperidyl or pyrrolidinyl spin labels

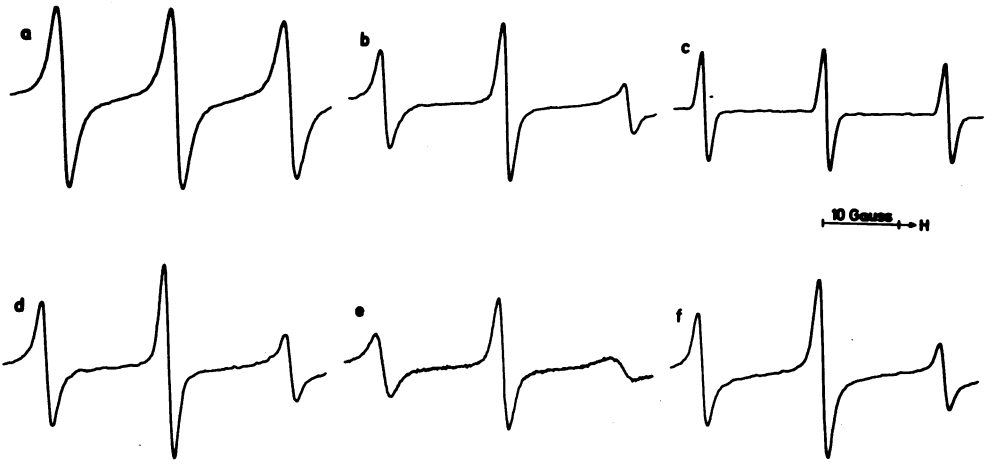

Fig. 6. ESR spectra of spin labelled compounds measured at 28°C as described under Methods. N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (a), $tRNA^{Phe}$-C-(5SL)s^2C-A (b), $tRNA^{Phe}$-C-(5SL)s^2C-A after treatment with 2-mercaptoethanol (c), Phe-$tRNA^{Phe}$-C-(5SL)s^2C-A (d) $tRNA^{Phe}$-C-(5SL)s^2C-A in the presence of 10 mg/ml bovine serum albumin (e), and Phe-$tRNA^{Phe}$-C-(5SL)s^2C-A EF-Tu GMP-P(NH)P ternary complex (f).

to $tRNA^{Phe}$-C-s^2C-A results in increase of τ_c by about an order of magnitude. This effect is the same if the 5-membered or the 6-membered spin label is used. Treatment of spin labelled $tRNAs^{Phe}$ with 2-mercaptoethanol leads to detachment of the spin label from tRNA. The correlation times τ_c of freed spin labels become again shorter and close to a value measured for free iodoacetamide spin label. However the anisotropy factors, ε, measured after treatment of spin labelled tRNAs with 2-mercaptoethanol differs significantly from that of iodoacetamide spin labels. This may be due to formation of disulfide dimers from the hydrolysed spin label compounds (11). Aminoacylation of spin labelled $tRNAs^{Phe}$ does not have a significant influence on the τ_c or ε values. Therefore the presence of phenylalanine on the terminal adenosine does not restrict the motional freedom of the label attached to 2-position of the penultimate cytidine. Also a conformational change of tRNA upon aminoacylation if any, is not obvious if the spin label is attached to this position.

Table 3 Data from ESR spectra of various tRNAs. Conditions are the same as given in the legend to Fig. 6. n.d. = not determined.

Sample	5-membered spin label				6-membered spin label			
	τ_c [sx10^{10}]		ε		τ_c [sx10^{10}]		ε	
	4°C	28°C	4°C	28°C	4°C	28°C	4°C	28°C
free iodoacetamide spin label	n.d.	0.6	n.d.	0.07	n.d.	0.6	n.d.	-0.05
$tRNA^{Phe}$-C-(SL)s^2C-A	11.3	10.5	0.59	0.35	n.d.	13.6	n.d.	0.10
$tRNA^{Phe}$-C-(SL)s^2C-A after 2-mercapto-ethanol treatment	n.d.	1.0	n.d.	0.22	n.d.	0.8	n.d.	0.0
Phe-$tRNA^{Phe}$-C-(SL)s^2C-A	12.5	9.9	0.49	0.40	9.3	7.6	0.21	0.10
EF-Tu·GMP-P(NH)P·Phe-$tRNA^{Phe}$-C-(SL)s^2C-A	9.9	9.1	0.55	0.42	8.7	6.6	0.26	0.18

A slight decrease of the τ_c value of $tRNA^{Phe}$ labelled with the 6-membered spin label which was observed upon aminoacylation may however indicate such phenomena. Due to the difficulties in preparation of Phe-$tRNA^{Phe}$-C-(6SL)-s^2C-A and the relatively small effects observed we did not experimentally follow this finding.

More surprising is the fact that the formation of ternary complexes of spin labelled Phe-$tRNA^{Phe}$ species with EF-Tu·GMP-P(N H)P does not lead to significant changes in the motion of spin label attached to the C-C-A end. The τ_c values for Phe-$tRNA^{Phe}$ species in free form and in complex with elongation factor are essentially the same or even slightly lowered. This can be considered as evidence that during the formation of ternary complexes the 2-position of the penultimate cytidine of tRNA is not in contact with the elongation factor. The possibility that the Phe-$tRNA^{Phe}$-C-(SL)s^2C-A species were not complexed with EF-Tu·GMP-P(NH)P during the ESR measurements could be excluded by gel filtration experiments performed immediately after the ESR measurements (Fig. 5c). There the complex formation with at least 80 % of spin labelled species could be demonstrated.

DISCUSSION

The effect of modification of the C-C-A end of tRNA on enzymatic aminoacylation was extensively investigated in several laboratories (3). Compared with modification of the 3'-terminal adenosine residue it appears that the changes of the penultimate cytidine residue have less effect on the ability of tRNA to accept the amino acid. $tRNA^{Phe}$ from yeast where this cytidine residue is replaced by 2-thiocytidine (6) or 5-iodocytidine (13) are aminoacylated to the same extent as native $tRNA^{Phe}$. Conversion of both CMP residues of the C-C-A end to UMP by sodium bisulphite did not desactivate $tRNA^{Tyr}$ from E. coli (14) or E. coli $tRNA^{Arg}$ (15). $tRNA_f^{Met}$-U-C-A from E. coli, obtained by the same method of modification, can also be aminoacylated, whereas $tRNA_f^{Met}$-C-U-A is inactive (16). On the other hand $tRNA_f^{Met}$-C-C-A containing penultimate 3:N^4-ethenocytidine is still chargeable (17). We have now found that the alkylation on the 2-position of C 75 residue of $tRNA^{Phe}$ from yeast by iodoacetamide does not impair its substrate properties toward phenylalanyl-tRNA synthetase. This together with the previous findings indicates that there is no direct functional interaction of the penultimate nucleoside residue of tRNA with aminoacyl-tRNA synthetase during aminoacylation. However, a large and bulky substituent on the 2-position of C 75 is not tolerated

by the enzyme, since $tRNA^{Phe}$-C-(SL)s^2C-A is not aminoacylated. The comparison of the activity of this tRNA with that of $tRNA^{Phe}$-C-(acm)s^2C-A allows a speculation about the steric restrictions of the C-C-A end of tRNA during its interaction with the synthetase. The 2-keto function of the penultimate cytidine is probably in the vicinity of the protein but does not participate in the interaction with the enzyme. It seems that there is some empty space of several Å between the two macromolecules, which allows an accommodation of substituents attached to the penultimate cytidine of $tRNA^{Phe}$ without loss of aminoacylation activity. This observation suggests, that the function of this cytidine residue in the C-C-A end is not to provide a specific binding site during the interaction with the synthetase but rather to form a rigid connection between the body of the molecule and its reactive site, namely the hydroxyl functions of the 3'-terminal adenosine. A large substituent on the C-C-A end which cannot be accommodated during interaction with the synthetase without changing the conformation of the C-C-A end, would then lead to the loss of activity of tRNA in the aminoacylation reaction, as in the case of $tRNA^{Phe}$-C-(SL)s^2C-A. It is interesting that $tRNA^{Phe}$-C-(SL)-s^2C-A which is not a substrate for the synthetase is also not a competitive inhibitor of the enzyme. As observed previously the terminal adenosine of $tRNA^{Phe}$ influences the $tRNA^{Phe}$:phenylalanyl-tRNA synthetase interaction by acting as an effector of the enzyme (18,19). It is possible that in the presence of a large substituent on the C 75 residue leading to structural change on the C-C-A end, the terminal adenosine cannot bind properly to the enzyme and act as an effector. This would explain a very similar behaviour of $tRNA^{Phe}$-C-(SL)s^2C-A and $tRNA^{Phe}$-C-C during the interaction with the synthetase.

In contrast to the situation with phenylalanyl-tRNA synthetase the substitution on the 2-position of cytidine 75 of $tRNA^{Phe}$ does not have any effect on the interaction with elongation factor Tu. Both Phe-$tRNA^{Phe}$-C-(acm)-s^2C-A s well as Phe-$tRNA^{Phe}$-C-(SL)s^2C-A form ternary complexes with an efficiency comparable to native Phe-$tRNA^{Phe}$-C-C-A. This would imply that the 2-keto group of C 75 is not involved in any interaction with the protein and is free while tRNA is in complex with EF-Tu. This assumption could be proved by ESR measurements which revealed that the rate of the tumbling of a spin label is essentially identical for free and complexed Phe-$tRNA^{Phe}$. This finding indicates further that there is no large change in the mobility of the C-C-A end of aminoacyl-tRNA in free form and in a complex with EF-Tu. If the 3'-end of Phe-$tRNA^{Phe}$-C-(SL)s^2C-A has a disordered structure in free

form and a rigid one while bound to EF-Tu this should be reflected in the ESR spectra of the appropriate samples. The fact that this is not the case shows that there is a rigid, stacked structure of the 3'-end of tRNA, which is similar for $tRNA^{Phe}$-C-(SL)s^2C-A, Phe-$tRNA^{Phe}$-C-(SL)s^2C-A as well as for Phe-$tRNA^{Phe}$-C-(SL)s^2C-A·EF-Tu·GMP-P(NH)P ternary complex. Such a rigid conformation was already implied for free $tRNA^{Phe}$-C-C-A from study of the chemical reactivity of the C-C-A nucleotides (3).

It was found previously that $tRNA^{Phe}$-i^5C-i^5C-A where the cytidine residues in the C-C-A end are replaced by 5-iodocytidine is active in elongation factor dependent binding to ribosomes (4). This, and the results described in this communication show, that a large modification on the C-C-A end of tRNA is allowed without influence on the EF-TU:aminoacyl-tRNA recognition. A direct interaction between the cytidine residues of the 3'-end of aminoacyl-tRNA and the EF-Tu involving hydrogen bonding of the 2-keto function of the penultimate cytidine can be excluded from our experiments. Schulman et al. reported, however, that a conversion of the C-C-A terminal sequence to U-C-A causes the loss of ability of such modified tRNA to form a ternary complex with EF-Tu·GTP (20). Whether this is due to changes in the conformation of the whole 3'-end of such modified tRNA or reflects the function of the particular cytidylic acid residues of the C-C-A end in this process is not clear at the present.

ABBREVIATIONS

EF-Tu = elongation factor Tu; $tRNA^{Phe}$ = $tRNA^{Phe}$-C-C-A = phenylalanine tRNA; Phe-$tRNA^{Phe}$ = phenylalanyl-$tRNA^{Phe}$; $tRNA^{Phe}$-C-s^2C-A = $tRNA^{Phe}$ in which C 75 is replaced by 2-thiocytidine; $tRNA^{Phe}$-C-([^{14}C]acm)s^2C-A, $tRNA^{Phe}$-C-(SL)s^2C-A, $tRNA^{Phe}$-C-(6SL)s^2C-A and $tRNA^{Phe}$-C-(5SL)s^2C-A = products of alkylation of $tRNA^{Phe}$-C-s^2C-A with iodo[^{14}C]acetamide or spin labels (Fig. 1). TRIS = tris(hydroxymethyl)aminomethane, HEPES = N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid, GMP-P(NH)P = guanylyl-imidodiphosphate.

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