# INVOLVEMENT OF THE D-STEM OF $tRNA^{Phe}$ *(E. COLI)* IN INTERACTION WITH PHENYLALANYL-tRNA SYNTHETASE AS SHOWN BY CHEMICAL MODIFICATION

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## 1. Introduction

Alkylation with a water soluble reagent MepURCl** was proposed by us for investigation of the macromolecular structure of tRNAs. Using low extents of modification (1–2 mol reagent attached per 1 mole tRNA) it was possible to compare the reactivities of the separate regions of the tRNA towards the reagent in a structure undisturbed by modification [1,2].

In the present paper the same reagent was used to study the complex of *E. coli* $tRNA^{Phe}$ with phenylalanyl-tRNA synthetase (Phe-RSase) and it was demonstrated that the $G_{24}$ residue is protected against alkylation in the presence of enzyme thus showing that $G_{24}$-containing part of D-stem of $tRNA^{Phe}$ participates in the interaction with the cognate synthetase.

## 2. Materials and methods

$tRNA^{Phe}$ was isolated from *E. coli* MRE 600 by a combination of chromatography on BD-cellulose [3], and DEAE-Sephadex A-50 [4]. The tRNA prepared accepted 800 pmol of phenylalanine per optical density unit (260 nm).

MepURCl was synthesized as described earlier [5].

T1- and T2-RNAases were from Sankyo Co. (Tokyo). Enzyme units were taken as specified by the producer.

Purified phenylalanyl-tRNA synthetase (EC 6.1.1) was isolated according to [6].

All chromatographic experiments were performed using a microspectrophotometer and equipment for micro-column chromatography, elaborated in our institute [7].

Complex of $tRNA^{Phe}$ and Phe-RSase was obtained by mixing the solutions of $tRNA^{Phe}$ and the enzyme in 0.025 M potassium acetate, pH 5.8, 0.005 M $MgCl_2$, $1 \times 10^{-4}$ EDTA. Final concentrations of tRNA and enzyme were $2 \times 10^{-5}$ M, final vol 0.1 ml. The mixture was incubated for 5 min at 20°C and then the reagent was added to a concentration of 5 mM, as determined from the absorption at 350 nm after acidic treatment [8]. The mixture was incubated 15 h at 20°C and then the complex was separated from unbound tRNA and all low-molecular compounds by gel-chromatography on Sephadex G-100 (Superfine, 5 × 500 mm), equilibrated with 0.025 M potassium acetate, pH 5.8 0.005 M $MgCl_2$, $1 \times 10^{-4}$ EDTA. Then the complex was dissociated by adjusting the pH to 7.7 with 1 M Tris buffer and the modified tRNA was separated from the enzyme by chromatography on the same Sephadex column, equilibrated with 0.02 M Tris–HCl, pH 7.7. The modified tRNA was concentrated by absorption on a DEAE-cellulose column (0.7 × 100 mm) and elution with 1 M NaCl. Then 0.5 M acetate, pH 3.8, 1/5 (v/v) was added, and the mixture incubated for 40 min at 40°C to split off the nucleotide group of the reagent [8]. Modified tRNA was desalted by gel-filtration

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**_Abbreviations:_ MepURCl, 2',3'-O-[4-(*N*-2-chloroethyl-*N*-methyl-amino)-benzylidene]-uridine-5'-methylphosphate; Phe-RSase, phenylalanyl-tRNA synthetase (EC 6.1.1.); ARSase, aminoacyl-tRNA synthetase.



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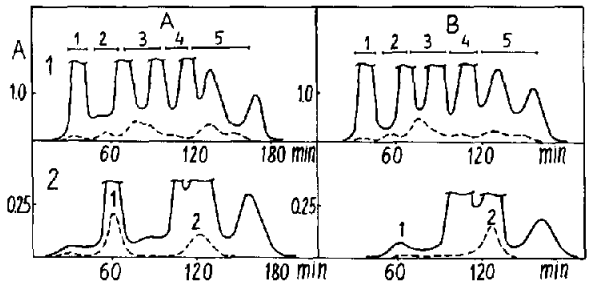


Fig.1. Chromatographic analysis of pancreatic-RNase digests of the modified $tRNA^{Phe}$ *(E. coli)*. (A) Control, (B) complex. 1 (A,B) Chromatography of the digests on DEAE-cellulose. Column 0.8 × 70 mm, total volume of the gradient ~600 μl. Total amount of tRNA per analysis - 1.0 optical density unit (260 nm). Solid line, $A_{260}$. Dashed line, $A_{350}$. 5 fractions of oligonucleotides, absorbing at 350 nm were collected. Gradient NaCL 0.01 M–0.24 M, Tris–HCl, pH 8.2. 2 (A, B) rechromatographies of penta- and hexanucleotides (peak 5, chromatographies 1 (A,B). 7 M urea, NaCl 0.01 M–0.20 M, pH 3.7 (HCOOH).

through Sephadex G-50 (2 × 150 mm), equilibrated with 0.02 M Tris–HCl, pH 7.7, and digested with pancreatic RNAase. In control experiments $tRNA^{Phe}$ was alkylated in the same way, but addition of the enzyme and G-100 chromatography were omitted. The oligonucleotides obtained were analyzed as described earlier [1] (See legend for fig.1).

For digestion with T1-RNAase, the enzyme ($5 \times 10^{-3}$ units) was added to the fraction containing oligonucleotides, and the solution incubated for 2 h

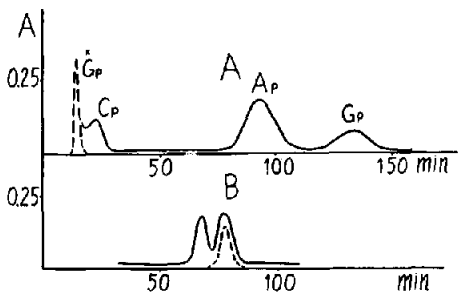


Fig.2. Analysis of the modified oligonucleotide (peak 1, chromatographies 2, fig.1). (A) Chromatography of the T2-RNase digest of the oligonucleotide on Dowex 1 × 8 (0.7 × 50 mm). $NH_4COOH$ pH 4.2, 0.01–1.2 M. (B) Chromatography of the T1-RNase digest of the oligonucleotide on DEAE-cellulose. 7 M urea, NaCl 0.01 M–0.20 M, Tris–HCl, pH 8.2. Solid line, $A_{260}$. Dashed line, $A_{350}$.

at 37°C. The digest was chromatographed on DEAE-cellulose (fig.2). For digestion with T2-RNAase, the pH of solution containing oligonucleotides was brought to pH 4.6 by addition of 1/10 (v/v) of 0.5 M ammonium acetate, pH 4.6. Then the T2-RNAase was added (0.04 units) and the mixture was incubated for 1 h at 37°C. The nucleotides were separated by Dowex 1 column chromatography (See legend for fig.2).

The rate of aminoacylation of tRNA by Phe-RSase was measured as described in [9]. The extent of Phe-RSase inactivation during modification of the complex was determined from the ratio of the rates of aminoacylation of tRNA catalyzed by the modified (isolated from the complex after the modification) and by the unmodified enzyme.

## 3. Results

The main approach of the investigation was to compare the points of alkylation of $tRNA^{Phe}$ in complex with Phe-RSase with those of free tRNA. Alkylation of the complex was performed at 20°C, pH 5.8, where the complex is particularly stable. After the modification, the enzyme possessed not less than 70% of the starting aminoacylating activity. After the recovery of $tRNA^{Phe}$ from the reaction mixture, the nucleotide moieties (MepU) of the reagent, attached to the $tRNA^{Phe}$, were split off to produce benzaldehyde residues on the modified guanosines, absorbing UV light at 350 nm. Then the modified tRNA was subjected to ribonuclease digestion. The obtained oligonucleotides were analyzed by microcolumn chromatography on DEAE cellulose (fig.1). The elution patterns of oligonucleotides in the course of the microcolumn chromatography were in accordance with those expected from the known primary structure of $tRNA^{Phe}$ [10].

The oligonucleotides, containing modified guanosines, have practically the same chromatographic properties, as the unmodified ones at pH 8.2, and are eluted earlier than the unmodified oligonucleotides at pH 3.7 due to appearance of positive charge on the alkylguanosine at acidic pH [1]. Therefore, it is possible to identify the modified oligonucleotides according to their chromatographic behavior. They are easily detected spectrophotometrically due to absorbancy of modified guanosine residues at 350 nm [8].

Fig.1 shows the results of analysis of oligonucle-

otides of pancreatic RNAase digests of $tRNA^{Phe}$, modified with MepURCl (A) and of $tRNA^{Phe}$, modified with MepURCl in the presence of Phe-RSase (B). It is seen that the main difference in the chromatographic patterns is a significant decrease in the amount of modified oligonucleotide 1 of the rechromatography at pH 3.7 of the pentahexanucleotide fraction (fraction 5 of the chromatography of the digests at pH 8.2) from $tRNA^{Phe}$ modified in the presence of Phe-RSase (fig.1). Therefore we analyzed only this peak. Fraction 5 contains two oligonucleotides – the pentanucleotide A–G–A–G–Cp and the hexanucleotide G–A–A–$mS^2i^6A$–A–Ψp as expected from the known sequence of $tRNA^{Phe}$ and as it proved by the relative position and spectral ratio of oligonucleotides obtained from unmodified $tRNA^{Phe}$. According to its position, the modified oligonucleotide was identified as modified A–G–A–G–Cp. Further evidence for the structure of this oligonucleotide was obtained by digestion of the oligonucleotide, after purification by additional chromatography at pH 8.2, with T1-RNAase and T2-RNAase. The T1-RNAase digestion gave modified trinucleotide and dinucleotide with spectral ratios consistent with those calculated for A–Gp and modified A–G–Cp. The T2-RNAase hydrolysis gave nucleotides Gp, Ap and Cp in proportion 1:1.8:1 and a component, absorbing at 350 nm (fig.2). Therefore we concluded, that the modified oligonucleotide was A–G–A–G–Cp from the D-stem of the $tRNA^{Phe}$, modified at the second guanosine residue ($G_{24}$ in $tRNA^{Phe}$).

## 4. Discussion

Up to now, little work has been done on the structure of the tRNA: ARSase complexes. The complexes of tRNAs with the cognate ARSases were subjected to nuclease digestion [11–13]. H. G. Zachau observed that the D-loop and anticodon of $tRNA^{Phe}$ (yeast) in the complex with Phe-RSase are protected from nuclease digestion. In $tRNA^{Ser}$ (yeast) the complex formation protects the CCA-end from nucleases, but not the anticodon [11]. P. R. Schimmel found that isoleucyl-tRNA synthetase protects the anticodon and the 3'-end of cognate tRNA from nuclease digestion [12]. S. K. Dube showed that methionyl-RSase protects the anticodon, the variable loop and acceptor stem of $tRNA_f^{Met}$ from nuclease attack [13]. P. R. Schimmel concluded that the nucleases are not adequate instruments for investigation of complexes tRNA:ARSase due to their own large dimentions [12].

It was found also that the complex formation prevents binding of complementary oligonucleotides to the anticodon and CCA-end of $tRNA^{Ile}$ [14].

The other approach, proposed by Schimmel, provides the possibility of identifying regions in tRNA, located in the contact area in tRNA:ARSase complexes. This approach consists in cross-link formation between tRNA and ARSase induced by UV-irradiation. Schimmel found that in the $tRNA^{Tyr}$: synthetase complex the cross-links appear between the protein and the anticodon, D-stem and variable loop of the tRNA [15].

There is evidence for involvement of the D-stem of tRNAs in interaction with synthetases [16–18]. Thus, Dudock and co-workers found that in five tRNA's from *E. coli* and yeast, aminoacylated by Phe-RSase (yeast) there is only one common region of structure, located in the D-stem [17,18].

According to A. Rich, the ARSases interact with tRNAs along their diagonal side, therefore all the helical regions of tRNA, and the D-stem among them, should be involved in interaction [19]. It is not clear now, whether the ARSases cover some parts of tRNA's or not.

We found that Phe-RSase protects nucleotide $G_{24}$ from chemical modification. The modification was performed under mild conditions and the enzyme retained its activity in the course of the reaction. Relatively small molecule of the reagent used possesses high penetrating ability, therefore we believe, that the protection of $G_{24}$ in the $tRNA^{Phe}$: synthetase complex really reflects the involvement of the D-stem of this tRNA in the interaction with the enzyme.

The study of the reactivities of separate regions of tRNAs in the complexes of tRNAs with cognate ARSases provides a useful tool for investigation of the structure of these complexes.

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