EQUILIBRIUM SCREENING-DIALYSIS INVESTIGATION OF THE NUCLEOTIDE SEQUENCES IN THE $tRNA^{Phe}$ RECOGNIZED BY PHENYLALANYL-tRNA SYNTHETASE (*ESCHERICHIA COLI*)

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1. Introduction

Isoleucyl-tRNA synthetase was shown to specifically interact with trinucleotide UAG from yeast $tRNA^{Ile}$ [1]. This finding stimulated us to investigate the interaction of oligonucleotides originated from *Escherichia coli* $tRNA^{Phe}$ with PheRSase* in order to identify the sequences recognized by this enzyme.

A simple method for identification of nucleotide sequences strongly interacting with polymers was proposed [2,3]. The idea was to use for the binding experiments mixtures of oligonucleotides of various sequences and to isolate the oligonucleotides tightly bound to the polymer under investigation from the noninteracting oligonucleotides by gel-filtration. The isolated oligonucleotides may be sequenced in the usual way. Application of the method is restricted by the systems where strong interactions are observed.

We propose here another approach for identification of nucleotide sequences in tRNAs interacting with ARSases. The approach is based on the investigation of the interaction of oligonucleotide mixtures with the enzymes by equilibrium dialysis (screening dialysis). In this way it is possible to identify oligonucleotides interacting with the enzymes in one experiment. As oligonucleotide mixtures, enzymatic digests of the cognate tRNAs can be used.

We present here results of the investigation of interaction of oligonucleotides from pyrimidyl-ribonuclease digest of $tRNA^{Phe}$ with phenylalanyl-tRNA synthetase (*E. coli*). Three oligonucleotides interact with the enzyme: AGGGGAψp, GGA s^4Up and AGAGCp.

* *Abbreviations*: ARSase, aminoacyl-tRNA synthetase; PheRSase, phenylalanyl-tRNA synthetase

2. Materials and methods

$tRNA^{Phe}$ was isolated from *E. coli* MRE 600 by a combination of chromatographies on BD-cellulose [4] and DEAE–Sephadex [5]. The tRNA prepared accepted 1000 pmol phenylalanine/$A_{260\,nm}$ unit. Phenylalanyl-tRNA synthetase (EC 6.1.1) was isolated from *E. coli* MRE 600 as in [6]. The purity of the enzyme was 80% as estimated by polyacrylamide gel electrophoresis.

To obtain oligonucleotides for binding experiments, $tRNA^{Phe}$ was digested with RNase A and the digest was treated with bentonite.

The dialysis cell material was teflon. The membrane was clamped between two teflon plates with recesses (depth 0.5 mm) forming two (30 μl) chambers. In the experiments one of the chambers contained PheRSase (4 mg/ml) in 20 μl 0.01 M cacodylate (pH 6.5), 0.005 M $MgCl_2$, 2×10^{-4} M EDTA. The second chamber contained 0.15 A_{260} units of the oligonucleotide mixture in 20 μl of the same buffer. The cell was incubated for 14 h at 0°C. Then the solution from the first chamber was chromatographed on Sephadex G-50 (0.8 × 200 mm) equilibrated with 0.01 M Tris–HCl (pH 8.0), 2×10^{-4} M EDTA at 25°C to separate oligonucleotides from the enzyme The oligonucleotide solution from the second chamber was treated in the same way. As a control, the starting oligonucleotide mixture was treated identically. Oligo-

nucleotides were analysed by micro-column chromatography using multi-wavelength microspectrophotometer as in [7,8].

3. Results and discussion

ARSases most probably recognize fragments of the polynucleotide chain of tRNAs specially oriented in space due to the tertiary structure of tRNAs [9–11]. However until recently no experimental evidences for the recognition of nucleotide sequences by ARSases were reported. It was found [1] that the isoleucyl-tRNA synthetase specifically interacts with the fragment from $tRNA^{Ile}$ oligonucleotide UAG. The interaction was found to be strong enough to be detected by equilibrium dialysis. This makes possible the identification of sequences in tRNAs recognized by ARSases by investigation of the binding of various oligonucleotides to these enzymes. To avoid the repetitive mechanistic testing of all the nucleotide sequences present in tRNAs it is important to develop an approach to simplify the procedure. For the strongly interacting oligonucleotide–polymer complexes the problem can be solved by using mixtures of oligonucleotides and by isolation of complexes of polymers with oligonucleotides by gel filtration [2,3]. However the interaction of a single recognized nucleotide sequence with ARSases will be weak since the binding of whole tRNA molecules to the enzymes is not too tight under physiological conditions.

The approach proposed here is based on the investigation of interaction of oligonucleotide mixtures with the enzymes by equilibrium dialysis. After equilibration of the dialysis system the determination of concentrations of individual oligonucleotides in the chamber containing the enzyme, and in the chamber separated from the enzyme by the membrane, provides information about oligonucleotides interacting with the enzyme. The quantitative analysis of oligonucleotides can be performed by column chromatography. As oligonucleotide mixtures one can use enzymatic digests of the cognate tRNAs. We investigated interaction of oligonucleotides of pyrimidyl-ribonuclease digest of $tRNA^{Phe}$ with PheRSase (*E. coli*). Figure 1 and table 1 show results of analysis of oligonucleotides from the dialysis cell. Concentrations of the majority of oligonucleotides including the hexanucleotide originating

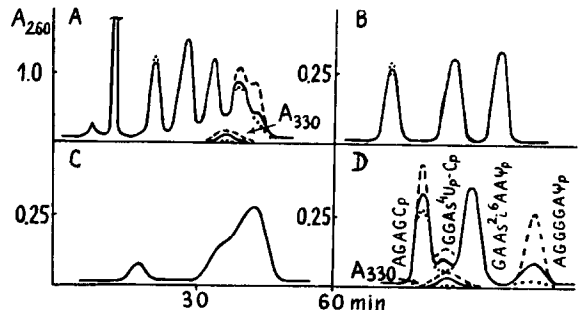

Fig.1. Analysis of oligonucleotides of ribonuclease digest of $tRNA^{Phe}$ after the dialysis experiment. Oligonucleotides were separated on DEAE-cellulose (column 0.6 × 40 mm) in 7 M urea, 0.01 M Tris–HCl (pH 8.0): NaCl 0.00–0.30 M. (A) The isoplith fractions were re-chromatographed on the same column in 7 M urea (pH 3.7) (HCOOH); (B) trinucleotides, NaCl 0.00–0.11 M; (C) tetranucleotides, NaCl 0.00–0.14 M; (D) penta+hexa+heptanucleotide, NaCl 0.00–0.20 M. (– – – –) Oligonucleotides from the chamber with the PheRSase; (· · · · · ·) oligonucleotides from the chamber without enzyme; (——) control digest. The A_{330} characteristic for the oligonucleotide GGAs^{4}Up–Cp is shown on the chromatographic profiles A and D.

from the anticodon loop are equal on the both sides of the membrane, showing that the equilibrium was attained. Three oligonucleotides interact with the enzyme. Two oligonucleotides bind rather tightly: AGGGGAψp and the product of interaction of s^4U_8 with C_{13} GGAs^{4}Up–Cp. The third oligonucleotide AGAGCp does not bind so tightly (fig.2,3). The

Table 1
Screening-dialysis investigation of binding of oligonucleotides from RNase digest of $tRNA^{Phe}$ (*E. coli*) to PheRSase

Oligonucleotide	PheRSase chamber content
	Second chamber content
AGCp	1.0
AGDp+GAUp	0.8
GGDp+GGTp	0.8
pGCp	1.0
GAGUp+GGAUp	1.1
GGGCp	0.9
AGAGCp	1.5
GAAs^{2i6}AAψp	1.0
AGGGGAψp	4.8
GGAs^{4}Up–Cp	2.8

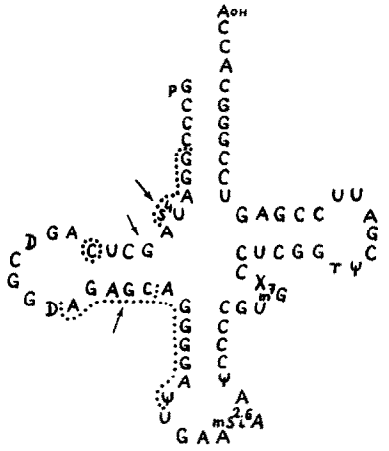

Fig.2. Cloverleaf structure of $tRNA^{Phe}$ (*E. coli*) [16]. Nucleotide sequences interacting with PheRSase are indicated by dotted outlines. Arrows show nucleotides protected by PheRSase from alkylation [12,13].

second oligonucleotide overlaps the sequence identified [1] in $tRNA^{Ile}$ as the sequence interacting with isoleucyl-tRNA synthetase. Oligonucleotide AGAGCp originates from the region proposed to be involved in recognition [14]. The oligonucleotides include nucleoside G_{24} and the product s^4U_8–C_{13} protected by

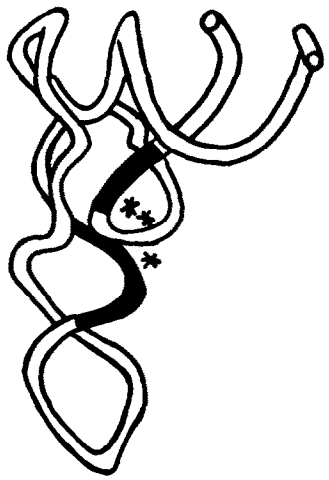

Fig.3. Schematic view of the three-dimensional model of tRNA [15]. Nucleotide sequences interacting with PheRSase are indicated by shading. Asterisks show positions of nucleosides s^4U_8, G_{10}, G_{24} protected by PheRSase from alkylation.

PheRSase from alkylation [12,13]. The oligonucleotides interacting with the PheRSase contain unique sequences GGA and AGA not present in the non-interacting oligonucleotides. The question of the length of the recognized sequences and the question whether the oligonucleotides contain the whole recognized sequences or only parts of them can be solved by investigation of binding to the enzyme of oligonucleotide mixtures of another composition, for example of T1 digest of the $tRNA^{Phe}$. The positions of the oligonucleotides in the tertiary structure of the $tRNA^{Phe}$ fit the general scheme of interaction of tRNA with ARSases [15] (fig.3).

The proposed experimental approach may be useful for investigation of interactions of other macromolecules when it is possible to obtain fragments of macromolecules comparable in size to the recognized sequences and to analyse quantitatively the fragments mixtures.

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