THE PROTEINS OF DONOR tRNA-BINDING SITE OF *ESCHERICHIA COLI* RIBOSOMES

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Received 1 October 1981

1. Introduction

A new photoreactive derivative of $tRNA^{Phe}$ containing several arylazido-groups scattered statistically over the tRNA's guanine residues was proposed for photoaffinity labeling of tRNA-binding sites of ribosomes from *Escherichia coli* [1,2]. It was obtained in two steps:

(i) Statistic alkylation of N7 atoms of guanosine in tRNA by 4-(*N*-2-chloroethyl-*N*-methylamino)-benzylamine up to the average extent of modification 3–4 mol reagent/mol tRNA;

(ii) Selective attaching of photoreactive groups to the aliphatic amino groups of reagent residues by treating of alkylated tRNA with 2,4-dinitro-5-fluorophenylazide [1].

This derivative is competent for non-enzymatic, poly(U)-dependent binding with ribosomes and being bound may be crosslinked with ribosome by UV-irradiation [1,2].

Here, we show this photoreactive derivative of $tRNA^{Phe}$ (I) to bind only at the ribosomal P-site in the presence of the excess of ribosomes and poly(U). After UV-irradiation of the ternary complex 70 S · poly(U) · I the derivative of $tRNA^{Phe}$ was covalently linked to 30 S and 50 S ribosomal subunits. We have found that the proteins S5, S9, S11, S12, S13, S19 and S21 were labeled in the 30 S subunits and the proteins L11, L13, L14 and possibly L27 in the 50 S ones. No modification of 16 S and 23 S RNAs was observed.

2. Materials and methods

Ribosomes were isolated from *E. coli* MRE-600 as in [3]. 4-(*N*-2-Chloroethyl-*N*-methylamino)-[^{14}C]-benzylamine (25 mCi/mmol) was synthesized as in [4,5]. 2,4-Dinitro-5-fluorophenylazide was prepared as in [6]. Poly(U) with av. M_r 30 000 was from SCTB of biologically active compounds (USSR). [^{14}C]-Phenylalanine (360 mCi/mmol) was from UVVVR (Czechoslovakia), ATP was from Reanal (Hungary), RNases A and T_1 were from Sankyo Co. (Japan) and $tRNA^{Phe}$ was from Sigma. Phenylalanyl-tRNA-synthetase was a kind gift from Dr S. Khodyreva. Aminoacylation of $tRNA^{Phe}$ was done as in [7]. For isolation of Phe-$tRNA^{Phe}$ 1 ml reaction mixture was passed through the column with DEAE (DE-52)-cellulose (0.2 ml). The column was washed with 0.2 M NaCl and then Phe-$tRNA^{Phe}$ was eluted from the DEAE-cellulose with 1 M NaCl.

Alkylation of $tRNA^{Phe}$ under the conditions of the lability of the tRNA tertiary structure and following attaching of arylazido-groups to the aliphatic amine residues was performed as in [1]. The ternary complex 70 S ribosome · poly(U) · Phe-$tRNA^{Phe}$ was obtained in buffer A (0.05 M Tris–HCl (pH 7.3), 0.1 M NH_4Cl, 0.02 M $MgCl_2$) using 3–5-fold excess of ribosomes to tRNA at 0°C. In the experiments on inhibition of binding of [^{14}C]Phe-$tRNA^{Phe}$ to 70 S · poly(U) complex by unlabeled I both tRNA species were added to the complex simultaneously. In the experiments with tetracycline (Tet) ribosomes were preincubated with Tet (4×10^{-5} M) at 0°C for 30 min. The ternary complex 70 S · poly(U) · I was formed in the buffer A at 0°C for 2 h. Concentrations of I, ribosomes and poly(U) were 10^{-6} M, 3.5×10^{-6} M and 0.1 mg/ml, respectively. To obtain the covalent bond between I and the ribosome the solution containing ternary complex was passed through a cuvette cooled to 13°C at a rate of ~2.4 ml/h. The cuvette was irradiated with a high pressure mercury lamp (500 W), $\lambda \geqslant 350$ nm (glass and water filters). After irradiation the ribosomes were precipitated by adding 0.8 vol. ethanol and subsequent separation into 30 S

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00145793/81/0000–0000/$02.75

and 50 S subunits as in [8]. Separation of modified subunits into rRNA and proteins was done as in [9]. [^{14}C]Protein fractions were dialysed against buffer B (0.02 M Tris–HCl, (pH 7.3), 0.01 M EDTA, 3 M urea) and concentrated against Sephadex G-75 or against 30% polyethyleneglycol in the same buffer. After this RNase T_1 (up to 20 units/ml) and RNase A (up to 0.04 mg/ml) were added with incubation following for 24 h at 40°C to hydrolyse tRNA covalently bound to the proteins. Then the reaction mixture was concentrated against 30% polyethyleneglycol in the buffer 0.02 M Tris–H_3BO_3 (pH 8.6), 7 M urea to 0.5 ml. Gel electrophoresis of the probe was performed as in [10]. Protein spots were cut out, followed by elution of proteins from the gel with 0.5% SDS at 40°C for 24 h and counting in a dioxan scintillator.

3. Results and discussion

One can assume the photoreactive tRNA derivative to bind at the ribosomal P-site with excess ribosomes [11]. Phe-tRNA^{Phe} is really bound at the P-site under these conditions because the presence of 4×10^{-5} M Tet (inhibitor for binding of tRNA at the A-site [11]) results in decreasing the amount of ribosome-bound Phe-tRNA^{Phe} < 10%. To prove the binding of I at the ribosomal P-site we used the competition assay between [^{14}C]Phe-tRNA^{Phe} and unlabeled I at the presence of poly(U) and Tet with excess ribosomes. Excess of I results in considerable decrease of binding of Phe-tRNA^{Phe} at the P-site (fig.1). This indicates that both tRNA species bind to 70 S · poly(U) complex at the same P-site.

In the presence of poly(U) and Tet up to 0.3 mol I can bind to 1 mol ribosomes (activity of the ribosomes in poly(U)-dependent non-enzymatic binding of tRNA^{Phe} was ~60%).

To obtain tRNA–70 S crosslinks the ternary complex was irradiated and destroyed by centrifugation in a linear sucrose density gradient (10–30%) under the conditions of dissociation into subunits [8]. By this procedure we completely separated modified ribosomal subunits from non-bound I. To evaluate the contribution of unspecific photoreaction of I with ribosomes out of the complex the following experiment was carried out. Complex tRNA^{Phe} · poly(U) · 70 S was incubated with I in the presence of the excess tRNA^{Phe} and then irradiated. The data on the distribution of the ^{14}C-label between the subunits in the presence or absence of non-modified tRNA^{Phe} are presented in table 1. It is seen that the extent of subunit modification from unspecific reaction is nearly 1 order of magnitude lower than for the same photoreaction in the complex for both subunits.

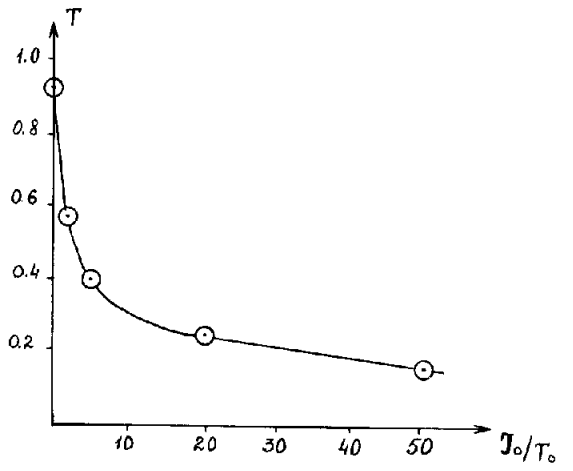

Fig.1. The dependence of amount of [^{14}C]Phe-tRNA^{Phe} bound to 70 S · poly(U) complex in the presence of Tet, upon the concentration of tRNA^{Phe} photoreactive derivative (I). Each reaction mixture contained in 0.2 ml buffer A, 5 pmol 70 S ribosomes, 10 μg poly(U), 5 pmol [^{14}C]Phe tRNA^{Phe}, Tet (4×10^{-5} M) and 0–250 pmol unlabeled I with an extent of modification ~3 mol reagent/mol tRNA^{Phe}; J_0, starting concentration of I; T_0, starting concentration of [^{14}C]Phe-tRNA^{Phe}; T, amount of [^{14}C]Phe-tRNA^{Phe} bound to 70 S · poly(U) complex (pmol).

Ribosomal proteins were isolated from the modified 30 S and 50 S subunits by centrifugation in a linear sucrose density gradient (5–20%) in the presence of SDS and EDTA [9]. No modification of 16 S

Table 1
Relative distribution of the ^{14}C-labeled tRNA^{Phe} photoreactive derivative (I) between 30 S and 50 S ribosomal subunits after irradiation

Ribosomal subunit	^{14}C incorporation (cpm)	
	With tRNA^{Phe}	Without tRNA^{Phe}
30 S	250	2000
50 S	100	1200

Each reaction mixture contained in 0.5 ml buffer A, 180 pmol I, 700 pmol 70 S ribosomes, 50 μg poly(U) and in one case, 700 pmol tRNA^{Phe}. Incubation at 0°C for 2 h before irradiation

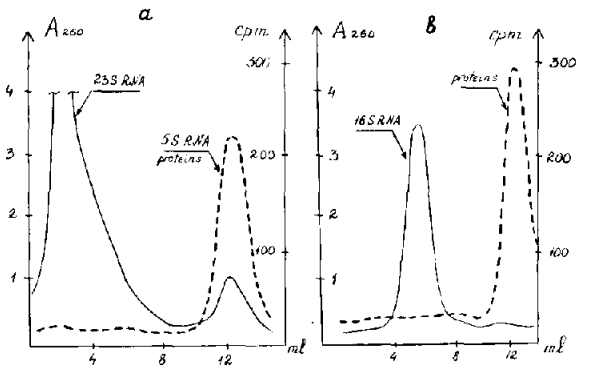

Fig.2. Sedimentation profiles of 50 S (a) and 30 S (b) modified ribosomal subunits in a linear sucrose density gradient (5–20%) in the presence of 0.5% SDS and 2 mM EDTA (Spinco L5-65, rotor SW-40, 40 000 rev./min, at 4°C for 17 h). (——) A_{260}; (– – –) cpm.

and 23 S RNAs was observed (fig.2). This fact should not be due to the inability of the reagent to modify RNA. Arylazido-derivatives of tRNA containing a photoreactive group in the aminoacyl-residue were shown to crosslink with 23 S RNA [12] and on the 4-thiouridine residue with 16 S RNA [13].

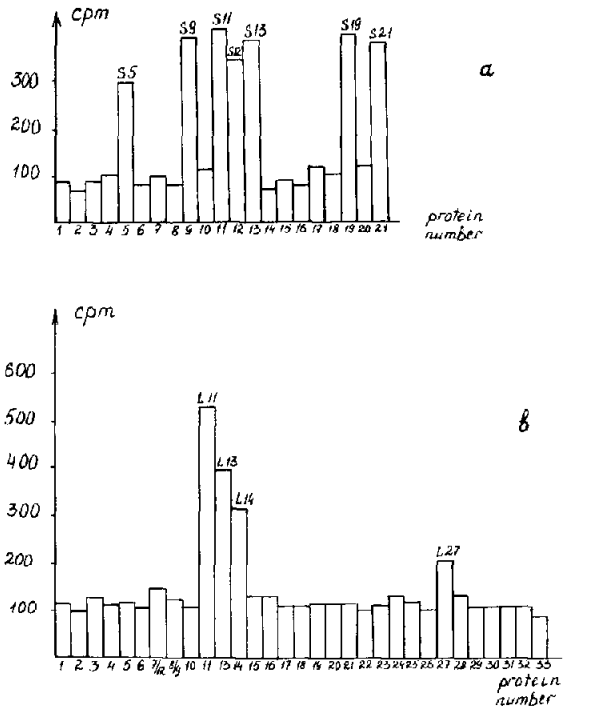

Fig.3. Distribution of the ^{14}C-label between 30 S (a) and 50 S (b) ribosomal proteins.

Our photoreactive derivative contains arylazido-groups scattered over the tRNA molecule statistically so we can assume that tRNA bound at the ribosomal P-site contacts dominantly with proteins. The proteins isolated from the modified subunits were subjected to complete RNase A and T_1 hydrolysis to remove tRNA residues covalently bound to the proteins. The results of two-dimensional gel electrophoretic analysis of the modified proteins are presented in fig.3 and show that proteins S5, S9, S11, S12, S13, S19, S21 were modified in the 30 S subunit and proteins L11, L13, L14 and possibly L27 in the 50 S one. The protein S9/S11 was shown earlier to be crosslinked with $tRNA^{Phe}$ in the ribosomal P-site after UV-irradiation ($\lambda = 254$ nm) [14]. Using the method of reconstruction of ribosomal subunits from the proteins and rRNA after chemical modification of proteins it was shown that proteins S11 and S21 directly participate in tRNA-binding centre formation

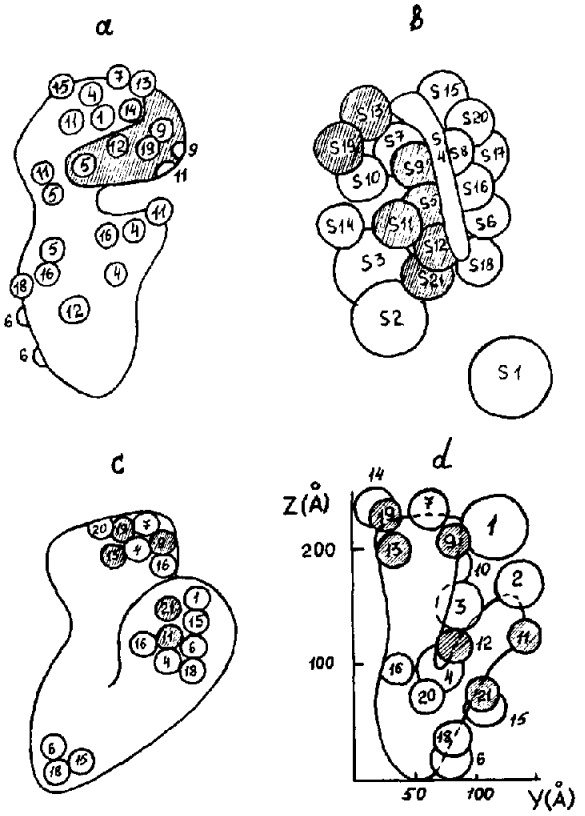

Fig.4. The models of 30 S ribosomal subunit: (a) from [21]; (b) from [22]; (c) from [19]; (d) from [20]. The proteins modified by photoreactive $tRNA^{Phe}$ analog in the ribosomal P-site according to our data are shaded.

[15]. Protein S19 takes part in IF-dependent fMet-$tRNA_f^{Met}$ binding to ribosomes [16]. Participation of the 50 S proteins L11, L13, L14 and L27 in P-site formation was shown using different tRNA derivatives containing reactive groups in the aminoacyl residue [17,18].

To date, several models are known of 30 S ribosomal subunit from *E. coli* obtained by different methods [19–22] for describing positions of proteins on the surface of the 30 S subunit. On the model in [21] which is the most complete for antigen determinants of the ribosomal proteins one can incorporate proteins found in the P-site according to our data into a compact group (see fig.4a). Other models of the *E. coli* 30 S ribosomal subunit (proteins found in the P-site are shaded) are also represented in fig.4: the model of [22] derived from the data on bifunctional crosslinks between the ribosomal proteins (fig.4b); the model of [19] derived from immunoelectron microscopy (fig.4c); the model of [20] (fig.4d). It is evident that the model in [21] is the most convenient for the explanation of our data. We can conclude that the P-site is located on the contact surface of the head of the 30 S ribosomal subunit. As for the 50 S subunit, only the model in [21] is known at present. Antigen determinants of proteins L11 and L14 are located close together on this model, determinant of the protein L13 is unfortunately absent.

It should be noted that >1/2 of our P-site proteins (S5, S9, S11, S13, S21) and none of 50S proteins were earlier found to be near the mRNA-binding centre of ribosomes from the data on affinity labeling of the ribosome by reactive mRNA analogs [23,24]. This implies the considerable overlapping of tRNA-binding P-site and mRNA-binding centre at the surface of the 30 S ribosomal subunit.

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