The effect of poly(U) on the arrangement of $tRNA^{Phe}$ in donor tRNA-binding site of *Escherichia coli* ribosomes

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1. INTRODUCTION

Codon–anticodon interaction is known to occur both at the A- and P-sites of ribosomes [1,2]. However, contrary to the binding of tRNA at the A-site, P-site binding of aminoacyl- and acylaminoacyl-tRNA proceeds in the absence of a template as well as in its presence as revealed from puromycin reactivity [1,3]. The same is known for deacylated tRNA as has been demonstrated from competition tests with aminoacyl-tRNA [1]. To understand the role of codon–anticodon interaction in the P-site it seems essential to learn whether template influences the arrangement of tRNA at the P-site.

One of the most informative approaches to elucidate the arrangement of tRNA in the ribosome is the affinity-labelling of ribosomes with tRNA derivatives bearing photoreactive groups scattered-statistically over tRNA guanosine residues. These reagents (azido-tRNA) may be prepared in two steps: (i) statistical alkylation of N7 atoms of guanosines of tRNA with 4-*N*-(2-chloroethyl-*N*-methylamino)-benzylamine; and (ii) selective attachment of arylazidogroups to aliphatic amino residues by treatment of alkylated tRNA with 2,4-dinitro-5-fluorophenylazide [4].

In this paper with the use of the above mentioned reagents we have demonstrated the pattern of modification points to differ significantly for ribosomes labelled by azido-$tRNA^{Phe}$ in the presence and absence of poly(U), namely: (i) only the 30 S subunit was labelled by azido-tRNA being bound to 70 S ribosomes without poly(U) whereas both subunits were labelled by azido-$tRNA^{Phe}$ in the presence of poly(U); (ii) proteins S5, S9, S11, S12, S19 and S21 were labelled by azido-$tRNA^{Phe}$ both in the presence and absence of poly(U), whereas protein S13 was labelled in the presence of poly(U) only.

2. MATERIALS AND METHODS

70 S ribosomes were isolated from *E. coli* MRE-600 as described in [5] with activity in poly(U)-directed binding of Phe-$tRNA^{Phe}$ about 50%. ^{14}C-labelled 4-(*N*-2-chloroethyl-*N*-methylamino)-benzylamine (25 mCi/mmol) was synthesized as in [6,7]. 2,4-Dinitro-5-fluorophenylazide was prepared according to [8]. Aminoacylation of $tRNA^{Phe}$ was performed as in [9]. Poly(U) was from Reanal (Hungary). RNases A and T_1 were from Sankyo Co. (Japan), $tRNA^{Phe}$ was from Sigma. Alkylation of tRNA with following attachment of arylazido groups was carried out as in [9]. The extent of tRNA modification was 1.8–4.0 mol of the reagent per mole of tRNA. Binary complexes tRNA · 70 S and ternary complexes $tRNA^{Phe}$ · 70 S · poly(U) were obtained at 0°C for 2 h in buffer A (0.05 M Tris–HCl, pH 7.4, 0.1 M NH_4Cl, 0.02 M $MgCl_2$) using tRNA preincubated in the same buffer at 37°C for 15 min and 1.5–3-fold excess of the ribosomes towards tRNA. In the experiments on inhibition of noncovalent and covalent binding of azido-tRNA to ribosomes the excess (5–100-fold) of unmodified tRNA was added simultaneously with azido-tRNA. Irradiation of the complexes and separation of ribosomes into subunits with subsequent ethanol precipitation was carried out as in [9]. Ribosomal proteins (un-

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labelled and with cross-linked tRNA) were extracted from the ribosomal subunits by 2 M LiCl in 4 M urea at 4°C for 100 h according to [10]. Hydrolysis of tRNA bound covalently to proteins with RNases A and T_1 and subsequent preparation for electrophoresis was carried out as in [9]. Gel electrophoresis of the probe and analysis of the labelled proteins were performed as in [11]. Analytical separation of ribosomal subunits into rRNA and proteins was carried out as in [9] by centrifugation in sucrose density gradient in the presence of sodium dodecyl sulphate and EDTA.

3. RESULTS AND DISCUSSION

At first specificity of binding and cross-linking of azido-tRNA to 70 S ribosomes without template was tested. Binding of azido-tRNA is inhibited by addition of an excess of unmodified tRNA (see fig.1A). On the other hand, template-independent binding of Phe-tRNA^{Phe} is inhibited by addition of an excess of azido-tRNA (see fig.1B). Both experiments indicate that azido-tRNA binds at the P-site.

To obtain tRNA-ribosome cross-links azido-tRNA . 70 S complexes were irradiated with UV-light ($\lambda \geq 350$ nm) and destroyed by centrifugation in sucrose density gradient under dissociating conditions (0.5 mM Mg^{2+}). To prove the specificity of the photoreaction between azido-tRNA and 70 S ribosomes without template, the 70 S azido-tRNA complexes in the control experiment were irradiated in the presence of an excess of unmodified tRNA. The data on the distribution of the label between ribosomal subunits after irradiation in the presence and in the absence of an excess of unmodified tRNA are given in table 1. It is evident that in the presence of unmodified tRNA photocross-linking of azido-tRNA to 70 S ribosomes decreases considerably indicating the specificity of the photoreaction.

As one can see from table 1 only 30 S subunit is labelled by azido-tRNA^{Phe} and azido-tRNA (unfractionated) in the absence of a template. In a previous paper [9] the labelling of both subunits by azido-tRNA^{Phe} was reported in the presence of poly(U). These data were obtained using azido-tRNA^{Phe} with rather high (4 mol of reagent per mole of tRNA^{Phe}) level of tRNA modification and a single ribosome preparation. In the present

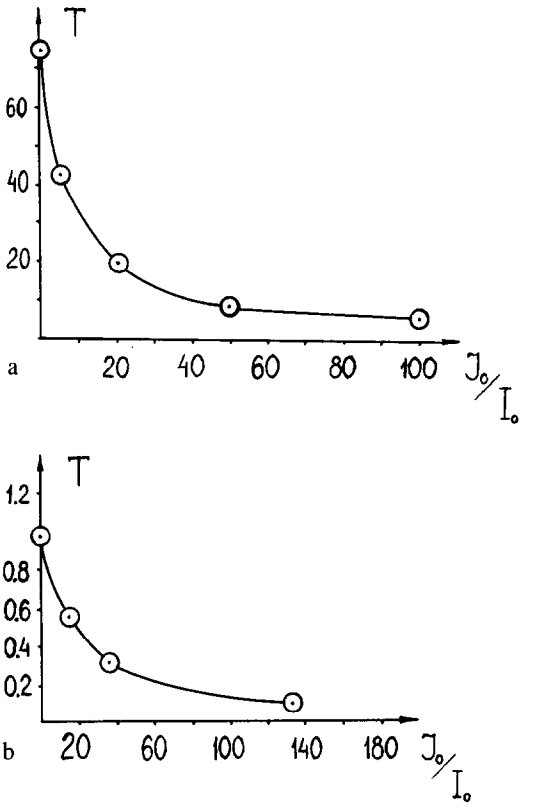

Fig.1. (a) Dependence of the amount of [^{14}C]azido-tRNA bound to 70 S ribosomes on the concentration of unmodified tRNA. Each reaction mixture contained in 0.28 ml of the buffer A 380 pmol of 70 S ribosomes, 250 pmol of azido-tRNA with the modification level about 3 mol of the reagent per mole of tRNA and 0–25 000 pmoles of unmodified tRNA. (J_o) Starting concentration of unmodified tRNA; (I_o) starting concentration of [^{14}C]azido-tRNA; (T) amount of azido-tRNA bound to 70 S ribosomes, pmoles. (b) Dependence of the amount of [^{14}C]Phe-tRNA^{Phe} bound to 70 S ribosomes on the concentration of unlabelled azido-tRNA with the modification level about 3. Each reaction mixture contained in 0.2 ml of the buffer A 3 pmol of [^{14}C]Phe-tRNA^{Phe}, 8 pmol of 70 S ribosomes and 0–400 pmol of [^{12}C]azido-tRNA. (I_o) Starting concentration of [^{14}C]Phe-tRNA^{Phe}; (J_o) starting concentration of [^{12}C]azido-tRNA; (T) amount of azido-tRNA bound to 70 S ribosomes, pmoles.

paper we have examined the distribution of the label between ribosomal subunits after irradiation of the ternary complexes azido-tRNA^{Phe} . 70 S . poly(U) and binary complexes azido-tRNA . 70 S using azido-tRNA with varying modification levels

Table 1

Covalent linking of azido-tRNA to 70 S ribosomes under the different conditions

tRNA derivative	Unmodified tRNA	Poly(U)	Moles of [^{14}C]azido-tRNA bound to mole of subunit	
			30 S	50 S
Azido-tRNA^{Phe}	–	–	0.06	< 0.005
Azido-tRNA^{Phe}	–	+	0.09	0.05
Azido-tRNA (unfractionated)	–	–	0.08	0.01
Azido-tRNA (unfractionated)	+	–	0.01	< 0.005

Each reaction mixture contained in 0.2 ml of the buffer A 500 pmoles of 70 S ribosomes, 200 pmol of azido-tRNA or azido-tRNA^{Phe} (with the extent of modification about 3 mol of the reagent per mole of tRNA) and if mentioned 1500 pmol of unmodified tRNA or $2A_{260}$ of poly(U). The reaction mixtures were irradiated and analyzed by sucrose density centrifugation in the presence of 0.5 mM Mg^{2+}.

Table 2

Distribution of the label between the ribosomal subunits after irradiation of the complexes [^{14}C]azido-tRNA^{Phe} . 70 S . poly(U) and [^{14}C]azido-tRNA^{Phe} . 70 S with different extents of tRNA^{Phe} modification

The extent of tRNA^{Phe} modification (moles of the reagent per mole of tRNA^{Phe}	Poly(U)	Moles of [^{14}C]azido-tRNA^{Phe} bound to mole of subunit	
		50 S	30 S
4.0	+	0.05	0.09
	–	< 0.005	0.07
3.0	+	0.04	0.08
	–	< 0.005	0.055
2.5	+	0.032	0.07
	–	< 0.005	0.05
1.8	+	0.02	0.04
	–	< 0.005	0.03

Each experiment was repeated using 3 different ribosome preparations. The table gives the average values for each modification extent. Experimental conditions are the same as in table 1.

and different ribosome preparations (data presented in table 1). One can see that relative distribution of the label between ribosomal subunits does not depend both on ribosome preparation and the level of tRNA modification (up to 4). Only photoreaction yield decreases with lowering of the extent of tRNA modification due to the smaller number of reactive groups per tRNA molecule. Because of the absence of 30 S–50 S cross-links on the sedimentation profiles in the experiments on dissociation of 70 S ribosomes labelled by azido-tRNA one can conclude that the latter links to ribosome mainly by only one azido-group. Taking this into account one can easily calculate the amount of azido-tRNA bound covalently to 70 S ribosomes and ribosomal subunits from the data on distribution of the radioactive label between the subunits.

30 S subunits labelled with and without poly(U) were centrifuged in a sucrose density gradient in the presence of sodium dodecyl sulfate and EDTA. The label was found only in the protein fractions.

Proteins labelled were isolated from the subunits and analysed by two-dimensional gel electrophoresis. 30 S proteins labelled in the presence of poly(U) under the same conditions as without poly(U) were also analysed as a control. The data on the distribution of the label between proteins of the small subunit labelled by azido-tRNA^{Phe} with and without poly(U) within 70 S ribosomes are given in fig.2.

One can see that 30 S proteins S5, S9, S11, S12, S13, S19 and S21 were labelled by azido-tRNA^{Phe} in the presence of poly(U). The same was found earlier using another ribosome preparation and a different extent of tRNA^{Phe} modification [9]. Without poly(U) the same proteins are labelled with the exception of protein S13.

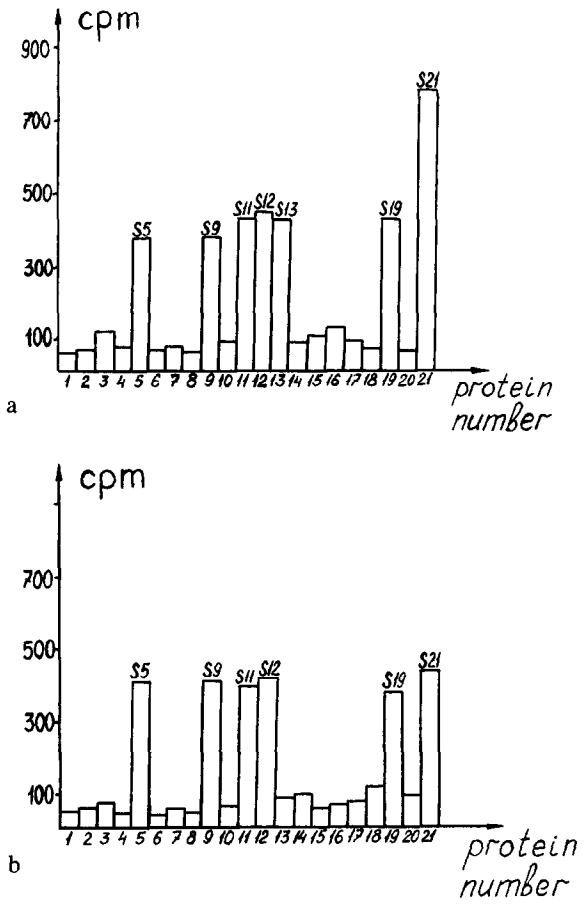

Fig.2. Distribution of the ^{14}C-label among 30 S proteins after (a) poly(U)-dependent cross-linking of [^{14}C]azido-tRNA^{Phe} to 70 S ribosomes; (b) cross-linking of [^{14}C]azido-tRNA^{Phe} to 70 S ribosomes without poly(U). In both cases the reaction mixtures contained in 2.45 ml of the buffer A, 5 nmol of [^{14}C]azido-tRNA with modification level about 3; 9 nmol of 70 S ribosomes and in one case 30 A_{260} units of poly(U).

Only a few data are known on the effect of the template on tRNA arrangement in the complex with ribosome. Hatayama et al. [12] has detected the difference between 30 S ribosomal components labelled by iodoacetyl-Phe-tRNA^{Phe} with and without poly(U). Ofengand et al. [13] used derivatives of tRNA^{Phe} carrying an arylazido group on 47-X (3-*N*-(3-amino-3-carboxypropyl)uridine) residues for the photoaffinity labelling of P-site. In this case 30 S subunits only were labelled without initiation factors and poly(U) whereas both subunits were labelled in the presence of poly(U) and initiation factor. But authors considered poly(U)-independent 30 S labelling as unspecific.

Azido-tRNA contains reactive groups scattered over the whole tRNA molecule statistically with the exception of the guanosine-free CCA-end. Hence affinity labelling by azido-tRNA in one experiment could give information on the arrangement of all guanosine-containing internal parts of tRNA in ribosome. Data obtained indicate a marked difference between the arrangement of tRNA bound at P-site with and without poly(U). Without poly(U) internal parts of tRNA contact proteins S5, S9, S11, S12, S19 and S21 only. In the presence of poly(U) additional contacts of tRNA with 30 S protein S13 and with the large subunit arise. This may be the explanation of the stabilizing role of a template at P-site in binding of tRNA apart from the direct codon–anticodon interaction.

Somewhat surprising is the fact that 30 S subunit is the only one making contacts with internal parts of tRNA within the complex tRNA · 70 S. It is known that ony 50 S but not 30 S isolated subunit can bind tRNA without template [14,15]. Thus, one may conclude that functional conformations of free and 50 S-bound small subunits are rather different. It should be mentioned that some of 30 S proteins labelled by azido-tRNA in the presence of poly(U), namely, S5, S11, S13, were found earlier in the vicinity of the mRNA-binding center by affinity labelling of ribosomes with alkylating analogs of oligouridylates with the reactive group on the 5′-end [11]. These proteins seem to form the 30 S ribosomal decoding region of P-site.

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