INTERACTION OF TRANSFER RNA WITH THE 30 S SUBUNITS OF RIBOSOMES IN THE ABSENCE OF MESSENGER

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1. Introduction

The method of measurement of tRNA binding to ribosomes by means of ultracentrifugation described in our preceding paper [1] was used for the study of tRNA adsorption on 30 S ribosomal subunits of *E. coli*. We found about one molecule of bound deacylated tRNA per subunit. The dissociation constant K of this reversible binding was measured and found to be equal by order of magnitude both in case of isolated subunits and joined with 50 S to form 70 S ribosomes. The enthalpy of binding was evaluated from a plot of $\log K$ versus $1/T$. Cleavage of two or three nucleotides from the 3′-end of tRNA does not affect its adsorption to a significant degree. The interaction of phenylalanyl-tRNA with 30 S subunits in the presence of poly U prevented a subsequent adsorption of labeled total tRNA.

2. Materials and methods

2.1. *Preparation of ribosomes*

Cells of the strain *E. coli* MRE 600 grown on a glucose mineral medium were harvested in the middle of exponential phase [2]. After opening the cells ribosomes were sedimented (2.5 hr at 40 000 rpm) and stored frozen.

2.2. *Preparation of subunits*

The precipitate was resuspended in the buffer (0.01 M Tris-HCl pH 7.4, 0.2 mM $MgCl_2$, 0.05 M NH_4Cl, 0.1 mM EDTA, 6 mM β-mercaptoethanol) and the subunits were separated by ultracentrifugation (as described in [3]), in a sucrose gradient containing the same buffer. Then the concentration of Mg^{2+} was increased to 0.015 M, the subunits were concentrated in a centrifuge and stored at concentration of 75–150 units of A_{260} per ml in liquid nitrogen in small portions. In all experiments the 30 S subunits were annealed before use during 15 min at 37°C in a buffer containing 0.02 M Tris-HCl pH 7.4, 0.1 M NH_4Cl, 0.03 M $MgCl_2$, 0.002 M EDTA.

2.3. *Preparation of uniformly labeled deacylated [^{32}P]tRNA* and its purification were described in paper [1].

[^{14}C]phe-tRNA^{phe} was a preparation of tRNA from *E. coli*, aminoacylated by [^{14}C]phenylalanine with a specific activity of 240 cpm/pmole and enriched 15–20-fold. It was kindly supplied by Dr. S.V. Kirilov.

2.4. *Cleavage of 3′-end groups from [^{32}P]tRNA* was effected by means of a purified phosphodiesterase from snake venom (Koch Light Lab.) according to [4]. Ascending paper chromatography was used as control. The elution solvent was a mixture (79:19:2) of a saturated $(NH_4)_2SO_4$ solution, 0.1 M phosphate buffer pH 6 and isopropanol. The spots of AMP and CMP contained together 4% of the total radioactivity.

2.5. *Ribosomal RNA (E. coli) and poly U* were purified by means of a Sephadex G-200 column. Only those fractions were used which were not retarded on the column. Oligonucleotide $(Ap)_5Up$ was supplied by Dr. V. Chernajenko.

2.6. *Measurement of [^{32}P]tRNA adsorption on ribosomes* was performed as described in [1]. This method allows to measure in a quantitative way the reversible binding of tRNA by the subunits. The incubation mixture contained a standard buffer (0.01 M Tris-HCl pH 7.4; 0.05 M NH_4Cl; 0.015 M $MgCl_2$; 1 mM EDTA), 30 S subunits and [^{32}P]tRNA. The samples (0.05–0.1 ml) were centrifuged in a bucket rotor SW-50 or SW-25 of Spinco L-65 in glass tubes of 3 mm diameter, inserted into plastic blocks. The time and speed were chosen just to achieve complete sedimentation of the subunits.

Then the content of the tubes was divided into two parts. About 80% of the supernatant was pipetted, the remaining 20% at the bottom contained the subunits The radioactivity and optical density (A_{260}) of both layers were measured. From these data the precise amount of [^{32}P]tRNA, bound by the subunits was computed. A small correction has to be introduced for the sedimentation of free [^{32}P]tRNA. Radioactivity was measured by means of a scintillation counter on nitrocellulose filters after the precipitation of ribosomes by trichloroacetic acid. We took in all calculations that one unit of A_{260} corresponds to 75 pmoles of 30 S subunits or 1600 pmoles of tRNA.

3. Results

3.1. *Reversibility of [^{32}P]tRNA adsorption on 30 S subunits*

If adsorption follows a Langmuir's isotherm characteristic of a reversible equilibrium, $m = C/(K + C)$, where m = the amount of bound [^{32}P]tRNA (moles per mole of subunits), C = the concentration of free [^{32}P]tRNA, M = the number of sites on a 30 S subunit, K = the dissociation constant of the complex, then the experimental points must fit a linear plot of 1/m versus 1/C (like a Lineweaver–Burk plot). A straight line was drawn by the least squares method (fig. 1) From the intersection with the axis of ordinate we obtain $M = 0.85 \pm 0.2$. We see that the number of sites per subunit is near to unity. The most probable conclusion is that most of the subunits participate in binding and contain one site for every active particle. From the slope we find $K = 1.3 \pm 0.2$ μM at 2°C. Adsorption is much weaker than on

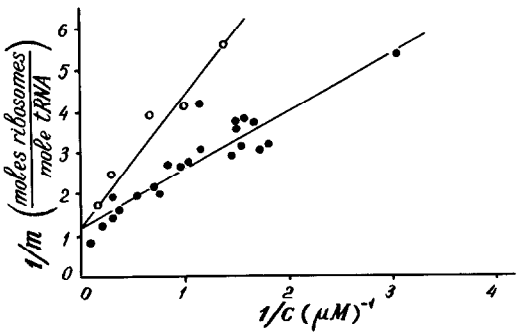

Fig. 1. Concentration dependence of [^{32}P]tRNA adsorption on 30 S subunits: (•—•—•) at 2°C, (o—o—o) at 20°C.

50 S subunits [1] and is manifested only at 30 times higher tRNA concentrations.

The scattering of experimental points allows the possibility of a slight inhomogeneity of the adsorbing 30 S subunits. The experiments give us the average binding constant K and the range of possible variations. Reversibility of adsorption is confirmed by the independence of its final value of the initial concentration. Table 1 shows that taking a high initial tRNA concentration, which yields a high value of adsorption, we come to much lower values after dilution of the reaction mixture with buffer. The adsorption decreases precisely as predicted by the isotherm on fig. 1.

Table 1
Reversibility of [^{32}P]tRNA adsorption on 30 S subunits.

	No. of probe	$\frac{1}{C}(\mu M)^{-1}$	$\frac{1}{m}\left(\frac{\text{Moles 30 S}}{\text{Mole tRNA}}\right)$
Initial adsorption	–	0.77	2.1
Adsorption after dilution	1	3.0	5.4
	2	3.8	5.6

The initial probe contained 375 pmoles of subunits, 570 pmoles of [^{32}P]tRNA (330 000 cpm) in a volume 0.25 ml. After sedimentation of subunits (55 min, 40 000 rpm, 2°C) a part of the precipitate and of the supernatant was used for the measurement of adsorption, another part of the precipitate was diluted with a standard buffer at 2°C (probe 1 = 13.5-fold, probe 2 = 19-fold) and centrifuged anew. Finally, the adsorption after dilution was measured.

3.2. *Dependence of adsorption on temperature*

From the isotherm (plot of 1/m versus 1/C) at 20°C (fig. 1) we obtain $K_{20°} = 3 \times 10^{-6}$ M. From the temperature dependence of K assuming that the number of sites remains constant we can evaluate the enthalpy of adsorption $H = (RT^2/\Delta T) \ln (K_{20°}/K_{2°}) = (8 \pm 2)$ × kcal · mole^{-1}. This value is near to that found for the adsorption of tRNA on 50 S subunits (8.8 ± 1) × kcal · mole^{-1} [1].

3.3. *Effect of 3′-end group modification*

Enzymatic cleavage of 2–3 nucleotides from the 3′-end of tRNA does not affect adsorption to a significant degree (m = 0.33 at C = 1.2 μM comparing to m = 0.41 at the same C for native [^{32}P]tRNA). Obviously the interacting part of tRNA is distant from the 3′-end of the chain. This makes a great difference with the adsorption of tRNA on 50 S subunits. In the latter case even the absence of one adenosine at the chain end of tRNA prevented adsorption totally [5]. In our control experiments a sample of [^{32}P]tRNA modified by phosphodiesterase revealed a binding on 50 S subunits at least 10 times weaker than a native one.

3.4. *Effect of tetracycline*

This antibiotic at 10^{-4} M does not affect in the range of errors the adsorption of [^{32}P]tRNA.

3.5. *Reactivation of 30 S subunits by annealing*

The initial 30 S subunits are poor adsorbents. A plot of 1/m versus 1/C shows that the average number of sites per particle is low (1/3 – 1/5), but the dissociation constant K remains the same. A similar low activity of 30 S subunits towards the binding of phenylalanyl-tRNA in the presence of poly U was found by Zamir et al. [6]. They showed that full activity can be restored by heating in a buffer containing Mg^{2+} and NH_4^+. According to our data optimal conditions of annealing are 15 min at 37°C in a buffer with 0.03 M Mg^{2+} and 0.1 M NH_4^+ (see Methods).

3.6. *Adsorption on 70 S ribosomes*

It is well known that every 70 S ribosome has one site for tRNA on its 50 S subunit [5]. From our preceding results [1] it follows that this site is filled at all tRNA concentrations used in our experiments. Besides on every 30 S an additional site exists, which will be filled at much higher tRNA concentrations. The total amount of tRNA per 70 S ribosome must approach 2 molecules, if adsorption takes place on two different sites. This prediction is confirmed (fig. 2). Comparing adsorption on 70 S ribosomes and the curve calculated as a sum of adsorptions on 30 S and 50 S subunits we can conclude that the isotherms of free 30 S and of 30 S bound to 50 S subunits are roughly similar. It means that the dissociation constants are equal at least by order of magnitude in both cases.

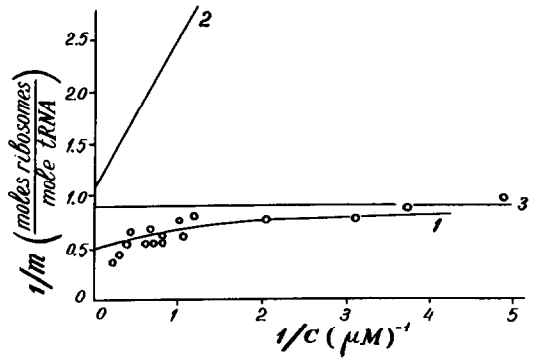

Fig. 2. Adsorption of [^{32}P]tRNA on 30 S subunits bound to 50 S subunits. Adsorption as function of concentration was measured in equimolar mixture of 30 S and 50 S subunits (70 S ribosomes). Experimental data (o) are compared with the adsorption curve 1 calculated as a sum of adsorptions on free 30 S and 50 S subunits. The data for this calculation were taken from fig. 1 for 30 S subunits (curve 2) and obtained in separate experiments for 50 S subunits [1] (curve 3).

3.7. *Specificity of interaction*

Ribosomal RNA (150 μg/ml) and $(Ap)_5$Up (0.3 mM) added in great excess to the incubation mixture do not affect [^{32}P]tRNA binding in the range of errors. Poly U manifests some inhibition (table 2, line 1 and 2). But a prerequisite saturation of the 30 S subunits by a tRNA specific for phenylalanine in the presence of poly U makes the subsequent adsorption of the total [^{32}P]tRNA equal to zero (table 2, lines 3 and 4). Because of this competition test we conclude that the site for adsorption described in the paper is the same site which is involved in the specific tRNA binding in the presence of mRNA.

Table 2
Competition of the adsorption of [^{14}C]phe-tRNA^{phe} and poly U with the subsequent adsorption of [^{32}P]tRNA.

No. of probe	Additions		Amount of adsorbed tRNA	
	Poly U	[^{14}C]phe-tRNA	[^{14}C]phe-tRNA (cpm ^{14}C)	[^{32}P]tRNA (cpm ^{32}P)
1	–	–	–	1460
2	+	–	–	1040
3	–	+	1480	1330
4	+	+	5740	30

The probes contained 80 pmoles of 30 S subunits in a volume 0.07 ml. [^{14}C]Phe-tRNA^{phe} (50 pmoles, 12 000 cpm) and poly U (4 μg) were added as is shown in the table. After preincubation (10 min, 37°C) the probes were cooled, [^{32}P]tRNA (9700 cpm, 50 cpm/pmole) was added with 0.01 ml of water and adsorption of tRNA was measured as described in Methods. During the experiment some cleavage of the ester bonds in [^{14}C]phe-tRNA took place. The total ^{14}C-radioactivity after all manipulations was 7900 cpm in probe 3 and 6600 cpm in probe 4.

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