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NON-ACYLATED tRNA BINDING ON RAT LIVER 60S SUBUNITS

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Summary

The ability of rat liver ribosomes and subunits to form complexes with non-acylated tRNAs in the absence of mRNA has been studied using nitrocellulose membrane filtration technique. Binding to 60S subunits required the integrity of the pCpCpA end of the tRNA molecule and was not decreased when unpaired guanine had been modified using kethoxal. Scatchard plot analysis suggests that large subunits have two binding sites, whose affinity constant values, relatively high, vary according to the ionic composition of the medium. Thus, the affinity constant of the stronger site (about 3.10^9 M^{-1}) *is from 7 to 21 times higher than that of the weaker. High* Mg^{2+} *and low* K^+ *concentrations stabilized binding to both sites. tRNA is at least partly retained on the subunits by heat-labile bonds.*

We have recently shown that prior binding of non-acylated tRNAs markedly protected 60S subunits against heat and LiCl-mediated inactivation and unfolding (1, 2). It appeared unlikely that tRNAs had a protective effect against ribonuclease since rRNAs behaved differently. Up till now binding of non-acylated tRNAs has been the subject of few studies, concerning principally procaryotic ribosomes. It has been shown that there is one site for this binding on the 70S E. coli ribosome, located on the 50S subunit (3). This site coincides at least partially with the peptidyl (donor) site of the ribosome and the affinity constant for tRNA has been determined (4, 5). Binding of non-acylated tRNAs to the P site of eucaryotic 80S ribosomes was ascertained in an indirect way by determining the inhibitory effect of tRNAs on the binding of fmet-$tRNA_F$ which was shown to react with puromycin (6). Further, there have not so far been any estimates of either the number or of the affinity constants of these ribosomal sites for tRNAs ; such estimates would be of particular value for a better understanding of the mechanism of protein synthesis since a ribosome-deacylated tRNA complex is produced during protein synthesis each time a peptide bond is formed.

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In this paper we first compared the tRNA binding ability of Rat liver ribosomal subunits. For the 60S subunits, which were the most efficient, we determined, by Scatchard plot analysis (7), the number of their binding sites and the values of their affinity constants under a variety of ionic conditions. The contribution of ionic and hydrogen bonds which are responsible for the interaction were evaluated tentatively.

Methods

Preparations

Preparation of ribosomal subunits was carried out as previously described (8). Concentration of 60S ribosomal subunits was calculated from the absorbance at 260 nm with $E^{1\%}_{1cm}$ of 140. To estimate the number of 60S molecules an average molecular weight of 2.9 x 10^6 daltons was adopted. We were unable to detect any endogeneous tRNA remaining in 60S subunits by polyacrylamide gel electrophoresis. The [^{14}C] uniformly labelled rat liver tRNAs, unfractionated and deacylated, were incubated with the supernatant fraction and ATP plus CTP before the experiments (3). They had a specific radioactivity of 170-255 cts/min/µg. tRNA concentration was calculated from the absorbance at 260 nm with $E^{1\%}_{1cm}$ of 240 and an average molecular weight of 27 000 daltons.

Assays

Samples contained in a constant volume of 0.05 ml : 10 mM Tris HCl pH 7.4, 6 mM β-mercaptoethanol, 286 µg of subunits, $MgCl_2$ and monovalent salts as indicated, and 20-500 µg of [^{14}C] tRNA. The measurement of binding was performed, after incubation for 20 min at 20°, according to the slightly modified procedure of Niremberg and Leder (9). Reaction mixtures were filtered at the incubation temperature through nitrocellulose membranes (Millipore, type HA, 25 mm) previously soaked in the buffer. The filters were washed 4 times with a small volume of buffer, dried and analyzed for radioactivity. Control experiments indicated that binding changed neither when the incubation time was increased to 4 h, nor when the washing steps were increased. These results proved that equilibrium was reached during incubation, and further that filtration did not significantly perturb this equilibrium. Scatchard analysis of nitrocellulose filter assays is valid only when ratios of bound : free tRNA are $\ll 1(10)$ as is the case in these experiments (see fig. 2).

Results

Binding of non-acylated tRNAs to subunits and ribosomes

40S and 60S subunits, either separately or in equimolecular combination, were incubated under standard conditions in the presence of [^{14}C] labelled tRNAs. Table I shows that free 60S subunits formed about twice as much complex as the 40S subunits. Under our conditions (15 mM $MgCl_2$) the binding of tRNA to the 80S couple was equal to the sum of the individual subunit responses. In our subsequent experiments, described hereunder, only 60S subunits were used.

Factors affecting the stability of the tRNA-subunit complex

We first observed that old tRNA preparations did not bind to subunits. Binding ability was restored by incubating old preparations with the

Table I : Binding of [^{14}C]tRNAs to Rat liver ribosomal subunits.

40S	60S	tRNA bound (cts/min).
+	-	186
-	+	368
+	+	554
-	-	20

Samples containing equimolar amounts of 40S and/or 60S subunits (143 and/or 286 µg respectively) were incubated with 50 µg of [^{14}C] tRNA (8 500 cts/min) in 15 mM $MgCl_2$ and 30 mM KCl (see "Methods").

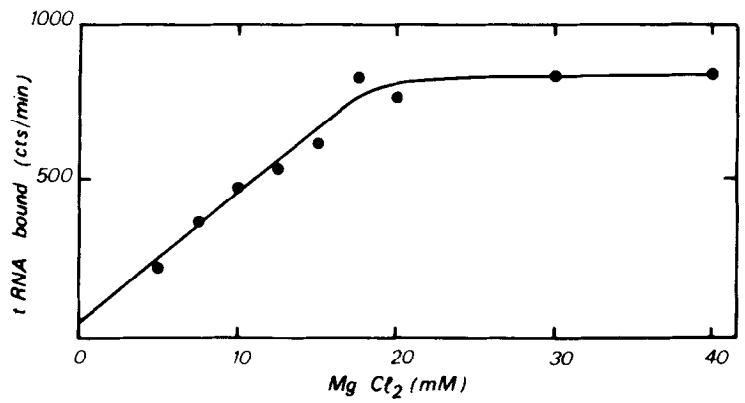

Figure 1 : Binding of non-acylated tRNAs to 60S subunits at increasing concentrations of magnesium chloride.

The binding mixtures as described under "Methods" contained 286 µg of 60S subunits, 50 µg of [^{14}C] tRNA (12 750 cts/min), 30 mM KCl and varying amounts of $MgCl_2$.

supernatant fraction and ATP plus CTP. This strongly suggested that the terminal pCpCpA sequence of tRNA, which was damaged during long term storage, is involved in the binding of deacylated tRNAs. The same phenomenon has been observed by Cannon (3) using procaryotic ribosomes. Prior modification of the non base-paired guanine residues in tRNA, using kethoxal, did not influence binding.

The binding of non-acylated tRNAs to rat liver 60S subunits was characterized by a marked dependence on $MgCl_2$ concentrations up to 20 mM. With higher concentrations, a plateau was reached (fig. 1). Since the difference in tRNA binding at low and high concentration of $MgCl_2$ can be explained either in terms of differences in affinity or in the number of 60S binding sites, we analyzed the binding of tRNAs to 60S subunits, at 10, 15 and

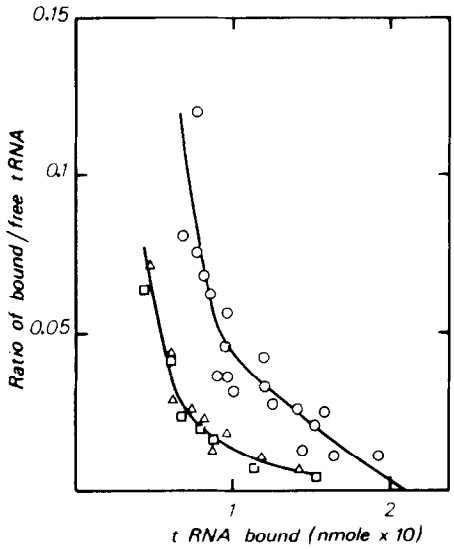

Figure 2 : Representative Scatchard plots of binding of non-acylated $|^{14}C|$ tRNAs to 60S ribosomal subunits.

Binding assays were performed as described in "Methods" using 1.10^{-1} nmole of 60S subunits and increasing amounts of non-acylated $|^{14}C|$ tRNAs (20-500 µg). The mixtures contained 10 mM $MgCl_2$, 30 mM KCl (□), or 15 mM $MgCl_2$ and either 30 mM KCl (o) or 100 mM KCl (△).

20 mM $MgCl_2$ using Scatchard plots (7) to determine the affinity constant and the number of sites per molecule of subunits. The results of these experiments are listed in table II (experiments 1, 2, 3). Figure 2 (□, o) is an exemple of the results achieved in experiments 1 and 2. This figure shows that the experimental points fall on a nonlinear curve, which suggests that there are different classes of binding sites. In spite of the difficulty of extrapolation to determine accurately both abscissa and ordinate intercepts, it can be calculated that, at the lowest tRNA concentrations using a molar ratio of tRNA to subunits ranging from 7 to 30, non-acylated tRNAs bound to one site, with a high constant affinity ($2.75\ 10^9\ M^{-1}$ in 15 mM $MgCl_2$, 30 mM KCl). At higher tRNA concentrations, using molar ratios of tRNA to subunits varying from 30 to 188, non-acylated tRNAs also bound to a second site, with an affinity constant which was, under these conditions, about seven times lower. The gradual increase of the binding of tRNAs with increasing Mg^{2+} concentrations (10-20 mM, experiments 1, 2, 3 — see also fig. 1 —) appeared to be due not to a consecutive saturation of more sites at higher Mg^{2+} concentration but rather to a stabilization of the binding, mainly to the low-affinity site. Indeed, the value of the constant for this site was increased by a factor of 3.8 from 10 to 20 mM $MgCl_2$, whereas the constant of the high affinity site was only

Table II : Affinity constants and binding site number of 60S subunits for non-acylated tRNA.

No	$MgCl_2$ mM	KCl mM	K x 10^{-9} M^{-1}	number of sites per 60S subunits
1	10	30	2.35	0.8
			0.11	1.9
2	15	30	2.75	1.1
			0.40	2.1
3	20	30	3.78	1.2
			0.42	2.3
4	15	6	3.55	1.2
			0.40	2.1
5	15	100	2.37	0.8
			0.11	1.9

Varying concentrations of non-acylated [^{14}C] tRNA were incubated with 1.10^{-1} nmole of 60S subunits in the binding assays described in "Methods". Values for the affinity constant (K) and for the number of binding sites per 60S subunits were obtained from Scatchard plots (7) Figure 2 gives the graphs for experiments 1, 2 and 5.

enhanced by a factor of 1.6 under the same conditions. tRNA was released from subunits when the magnesium concentration was lowered to 0.1 mM.

The effect of increasing concentrations of KCl on complex formation was studied. Compact active subunits retained the maximal amount of non-acylated tRNAs when assayed in 6-30 mM KCl. In contrast, the K^+-free subunits, which were inactive and unfolded (11, 12), showed only 45-65 % of the binding activity of the control subunits when tested in 30 mM KCl buffer. For KCl concentrations higher than 140 mM, the binding of non-acylated tRNAs to compact subunits decreased to 68 % of the maximal value. Data in table II (experiments 2, 4, 5) and in fig. 2 show that increasing concentrations of KCl diminished the binding to both sites. Affinity constant of the stronger site decreased by 33 % and that of the second site by 73 %, when the KCl concentration was increased from 6 mM to 100 mM. We also investigated the effect of another monovalent cation, Li^+ in place of K^+, on complex formation. Subunits dialyzed against 20 mM LiCl and no KCl were 50 % inactivated as compared with the control subunits tested using 20 mM KCl. When the incubations were performed in presence of increasing concentrations of LiCl and a small amount of KCl (6 mM), exactly the same ionic-strength dependence was obtained with LiCl as with KCl.

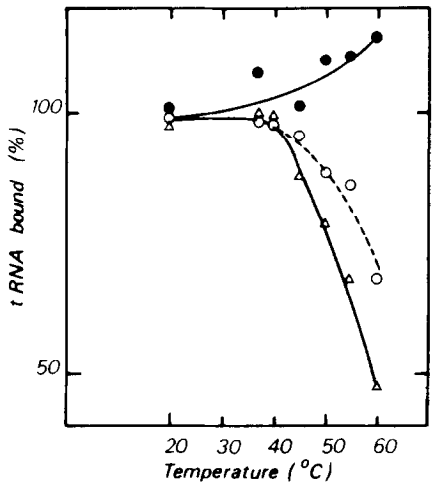

Figure 3 : Influence of the temperature on the stability of the tRNA-60S subunit complex and on the denaturation of each component.

286 µg of 60S subunits (△) and 50 µg $|^{14}C|$ tRNA (●) both suspended in 10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$, 30 mM KCl were maintained 5 min at various temperatures (20°-60°) and then used to form $|^{14}C|$ tRNA-60S subunits complexes, in the same buffer (see "Methods"). In other experiments, preformed $|^{14}C|$ tRNA-60S subunit complexes were heated under the same conditions (o). The amount of bound $|^{14}C|$ tRNA is expressed as a percentage of that determined at 20° (see "Methods").

tRNA molecules bound to 60S subunits could not be removed by centrifuging the complex in LiCl concentrations as high as 1.4 M, which suggests that tRNA is not retained by ionic bonds.

Heating

The degree of stability of tRNA-60S subunit complex subjected to heating was determined in the experiments reported in fig. 3. The dissociation profile of tRNA-subunit complex heated at various temperatures was compared with the thermic denaturation of each component measured under the same conditions. It can be seen that the complex was destroyed only at temperatures higher than 40°. The progressive dissociation of the complex from 40° to 60° was found to reflect not a progressive damage to the tRNA molecules but the inactivation and unfolding of the subunits which occured at these temperatures. The subunits were also shown to be partially protected from inactivation by prior-bound tRNA as previously reported (1) (compare curves o - o and △ - △ in figure 3).

Discussion

Rat liver 60S subunits have been shown above to have two non-equivalent sites for tRNA-binding. In the absence of mRNA either one or two molecules of non-acylated tRNA can be bound to a 60S subunit, according to the molar

ratio of subunits and tRNA in the reaction mixture. Both sites are characterized by different values of the affinity constant depending on the K^+/Mg^{2+} ratio which influences the conformation of the sites. We suggest with others (4 - 6) that the high affinity site correspond to the peptidyl (donor) site. It is not surprising that deacylated tRNA can bind to this site where it is found prior to its release from the ribosome during protein synthesis. The second site, which is characterized by a lower affinity constant, might be part of the A site. The observation that 30 % of the tRNA subunit complex (molar ratio of tRNA to subunit in the reaction mixture : 30) was destroyed on heating 5 min at 60° is in agreement with participation of hydrogen bonds in the binding reaction at the P site. The same conclusion has been reached by others using procaryotic ribosomal subunits (5).In comparison ionic bonds seem to be much less important. It is interesting to note that reactive tRNA analogues interact directly with ribosomal proteins organized in 80S ribosomes (13, 14). We also recently observed that subsequent UV-irradiation of tRNA-subunit complexes resulted in a covalent linkage between bound tRNA and ribosomal protein (unpublished results).

Acknowledgements

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