40 S subunits from rat liver ribosomes contain two codon-dependent sites for transfer RNA

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40 S subunits from rat liver ribosomes are able to bind, after heat activation, two molecules of either Phe-tRNA^{Phe}, Ac-Phe-tRNA^{Phe} or deacylated tRNA^{Phe}. Addition of 60 S subunits to the quaternary complex 40 S · poly(U) · (Phe-tRNA^{Phe})$_2$ results in quantitative formation of (Phe)$_2$-tRNA^{Phe}. This indicates that the two binding sites for tRNA on 40 S subunits should be considered as the constituent of P and A sites of 80 S ribosomes.

Eukaryotic ribosome *40 S subunit* *tRNA* *Binding site* *Poly(U)* *Association constant*

1. INTRODUCTION

There is now considerable evidence for the existence of 3 tRNA-binding sites on ribosomes involved in protein biosynthesis [1–8] extending the classical two-site model of Watson [9]. In this respect, it is of interest how many binding sites for tRNA exist on isolated small ribosomal subunits.

In earlier experiments thermodynamic and kinetic quantities of tRNA-30 S subunit interactions were determined [10–14]. From *E. coli* highly active small ribosomal subunits were isolated which were able to bind 2 molecules of tRNA [15,16]. The 2 tRNA-binding sites were proved to be constituents of the P and A site of 70 S ribosomes.

Information concerning the interaction of tRNA with eukaryotic 40 S subunits is much scarcer. It is only known that the binding of tRNA to 40 S subunits is codon-dependent [17] and 40 S subunits were suggested to contain one decoding or A site [18].

Here, we have investigated quantitatively the interactions of both mRNA and tRNA with 40 S ribosomal subunits from rat liver. Using similar criteria to those which have been valuable in analogous studies with 30 S subunits [15], we have shown that: (i) 40 S subunits bind fractionated poly(U) (about 60 nucleotides in length) with a stoichiometry of 1:1, the association constant of this interaction being equal to 2×10^6 M^{-1}; (ii) at saturating concentrations of poly(U) each 40 S subunit binds 2 molecules of either Phe-tRNA^{Phe}, Ac-Phe-tRNA^{Phe} or deacylated tRNA^{Phe}; (iii) the 2 sites for tRNA binding found on 40 S subunits are constituents of the P and A sites of 80 S ribosomes since addition of 60 S subunits to the preformed 40 S · poly(U) · (Phe-tRNA^{Phe})$_2$ complex resulted in almost quantitative formation of (Phe)$_2$-tRNA^{Phe} per 40 S subunit in the complex.

2. MATERIALS AND METHODS

Livers from adult male Wistar rats were homogenized in 3 vols ice-cold medium containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, 10 mM 2-mercaptoethanol and 5% glycerol. The homogenate was centrifuged twice for 15 min at $12000 \times g$ at 0°C, and the resulting postmitochondrial supernatant was mixed with Triton

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X-100 and sodium deoxycholate to final concentrations of 2.0 and 1.3%, respectively. Ribosomes were pelleted by centrifugation at 0°C for 120 min at 100 000 × *g* and resuspended in 5 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 500 mM KCl and 10 mM 2-mercaptoethanol. Puromycin was added to 0.3 mM and the mixture incubated for 20 min at 0°C and 15 min at 37°C to achieve dissociation of ribosomes into subunits. After centrifugation for 15 min at 12 000 × *g* at 20°C, 4 ml of the mixture were loaded onto a linear glycerol gradient (10–40%) containing 5 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 500 mM KCl and 10 mM 2-mercaptoethanol. Centrifugation was for 15 h at 16 000 rpm in a Spinco SW 25.2 rotor at 20°C. 60 S and 40 S subunits were pooled according to the A_{260} pattern and pelleted by centrifugation at 100 000 × *g* for 15 h at 2°C. The pellets were resuspended in 5 mM Tris-HCl, pH 7.5, 9 mM $MgCl_2$, 50 mM KCl, 10 mM 2-mercaptoethanol and 5% glycerol to concentrations of 200–300 A_{260} units per ml, centrifuged for 15 min at 12 000 × *g* at 0°C and stored in sealed 30–50 μl aliquots in liquid nitrogen. Before use the subunits were activated by incubation for 5 min (if not stated otherwise) at 40°C in TAM buffer (20 mM Tris-HCl, pH 7.4, 200 mM NH_4Cl, 20 mM $MgCl_2$, 1 mM EDTA). The activation step did not produce any notable change in the sedimentation pattern of the 40 S and 60 S subunits when compared to 40 S or 60 S subunits that had not been activated (not shown).

Analysis of 40 S and 60 S subunits on 5–20% sucrose gradients containing 0.5% SDS, 5 mM Tris-HCl, pH 7.0, and 2 mM EDTA in the SW 40 rotor of a Spinco L5 for 17 h at 30 000 rpm at 10°C revealed the absence of any cross-contamination, i.e. only symmetric peaks corresponding to 18 S and 28 S ribosomal RNAs were observed. However, while 40 S subunits sedimented as a single 40 S peak through sucrose gradients containing TAM buffer, 60 S subunits were found to contain about 50% of faster-sedimenting material, presumably aggregrated 60 S. In this work we assumed that 1 A_{260} unit was equal to 50, 25 and 18 pmol 40 S, 60 S and 80 S ribosomes, respectively.

Enriched [^{14}C]Phe-tRNA^{Phe} (1500 pmol/A_{260} unit), Ac-[^{14}C]Phe-tRNA^{Phe} (1590 pmol/A_{260} unit) and [^{14}C]tRNA^{Phe} (1270 pmol/A_{260} unit) from *E. coli* B, as well as fractionated poly(U) (M_r 30 000) were prepared as in [5,15]. Fractionated [^{3}H]-poly(U) (M_r 20 000) and [^{14}C]Phe-tRNA^{Phe} from yeast (1600 pmol/A_{260} unit) were kind gifts from Dr V.I. Katunin. 30 S ribosomal subunits were isolated as in [15].

All experiments were reformed in TAM buffer. The final volume of the samples was 200 μl. Other conditions are given in the figure legends.

3. RESULTS AND DISCUSSION

Fig.1 shows the result of titration of 40 S subunits with [^{3}H]poly(U) in the form of a double-reciprocal plot. Each subunit binds one mRNA molecule when $1/[U] \rightarrow 0$, and the association constant of this interaction is equal to 2×10^6 M^{-1}. At a concentration of [^{3}H]poly(U) corresponding to $\bar{n} = 0.6$ mol poly(U)/mol 40 S, addition of Phe-tRNA^{Phe} stimulates poly(U) binding up to $\bar{n} \cong 1.0$ (fig.1, inset). This means that the presence of cognate tRNA increases the affinity of the mRNA for 40 S subunits. An analogous effect was observed earlier with 30 S subunits [19].

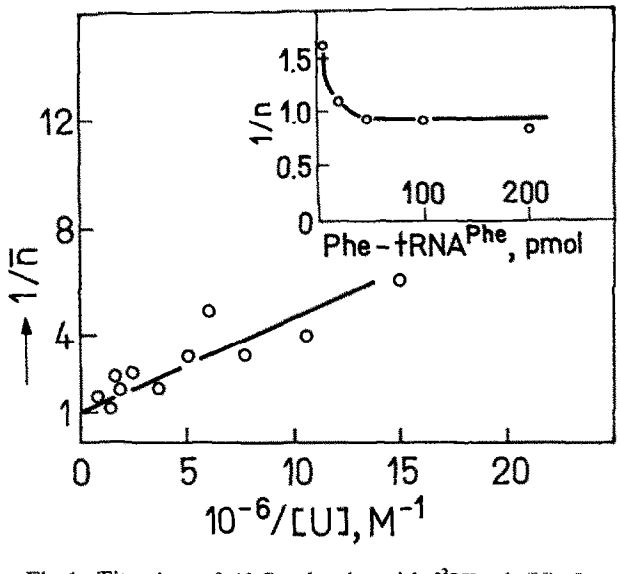

Fig.1. Titration of 40 S subunits with [^{3}H]poly(U). Incubation mixtures contained 10 pmol activated 40 S subunits and 5–150 pmol [^{3}H]poly(U). After 15 min incubation at 0°C, $\bar{n}$ values (i.e. the average numbers of poly(U) molecules bound per subunit) were measured as described in [19]. Inset: stimulation of [^{3}H]poly(U) binding to 40 S subunits with Phe-tRNA^{Phe}. Incubation mixtures contained 10 pmol 40 S subunits, 100 pmol [^{3}H]poly(U) and increasing amounts of unlabeled Phe-tRNA^{Phe}.

40 S subunits as well as 30 S subunits [20] require heat activation to display their maximal activity in binding experiments. After incubation for 5 min at 40°C they are able to bind 2 molecules of enriched Phe-tRNA^{Phe} at 0°C (fig.2A, ○). The binding reaches maximal values after 45–60 min of incubation followed by a progressive decrease of $\bar{\nu}^{\Sigma}$ value (fig.2B, ○). This can be readily explained by the spontaneous deacylation of aminoacyl-tRNA: At elevated temperature (30°C) the hydrolysis velocity increases significantly [21] and the accumulating deacylated tRNA^{Phe} competes with [^{14}C]Phe-tRNA^{Phe} for the binding sites [22]. Obviously it is for this reason that a rapid decrease of the $\bar{\nu}^{\Sigma}$ value occurs after 15 min of incubation at 30°C (fig.2B, ●).

40 S subunits can bind not only 2 molecules of Phe-tRNA^{Phe}, but also 2 of the peptidyl-tRNA analogue, Ac-Phe-tRNA^{Phe}, or deacylated tRNA^{Phe} (fig.2C, ○ and ●). Titration curves for the Phe-tRNA^{Phe} from *E. coli* and yeast are practically indistinguishable (fig.2C, △ and ▲). It is likely, therefore, that the difference in the primary structures of tRNA^{Phe} from both sources does not significantly affect their affinities for the 2 binding sites on the 40 S subunits.

An attempt to characterize functionally the 2 sites on the 40 S subunits is demonstrated in fig.3. Increasing amounts of 60 S subunits were added to the preformed 40 S · poly(U) · (Phe-tRNA^{Phe})$_2$ complex ($\bar{\nu}^{\Sigma}$ = 1.8), and no increase of total aminoacyl-tRNA binding was observed (○). Parallel samples were used to evaluate the number of diphenylalanines synthesized per 40 S subunit in the mixture. This number increases with increase in the 60 S/40 S ratio and reaches a plateau value when an approx. 2-fold excess of 60 S subunits is added (●). This correlates well with the fact that about 50% of the large subunits used here (see section 2) have formed aggregates which are presumably inactive. The remaining 60 S subunits obviously reassociate with the quaternary complex to form 80 S ribosomes of which at least 75% are active in peptide bond synthesis.

Since addition of 60 S subunits to the 40 S · poly(U) · (Phe-tRNA^{Phe})$_2$ complex does not result in the formation of additional binding site(s) on the 80 S ribosomes and, secondly, since both Phe-tRNA^{Phe}

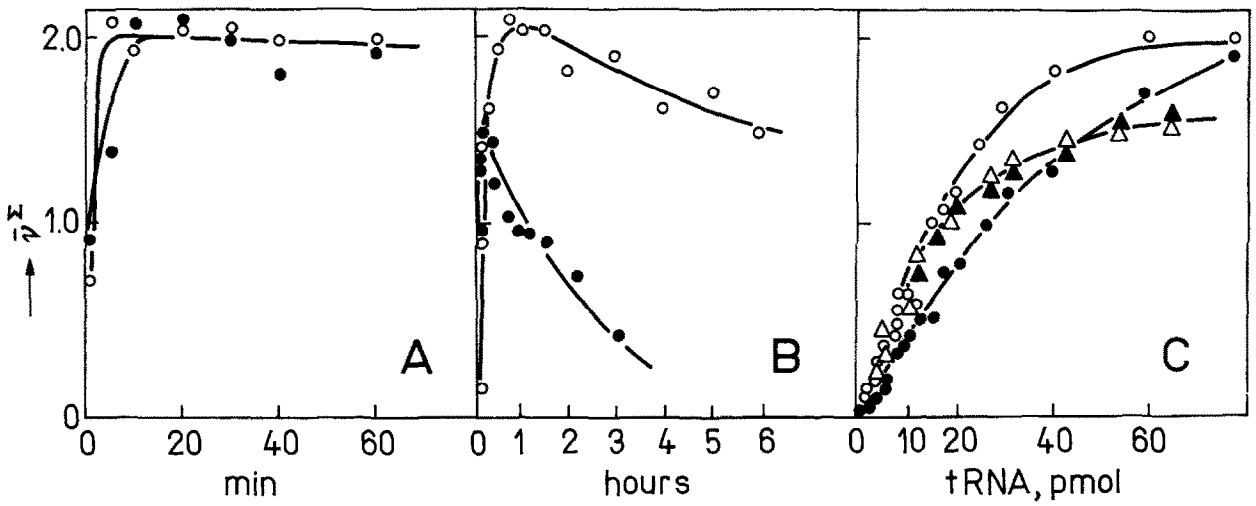

Fig.2. (A) Comparative kinetics of heat activation of 40 S (○) or 30 S subunits (●). Subunits were heated in TAM buffer at 40°C for the indicated periods and chilled to 0°C. Then 10 μg poly(U) and 80 pmol [^{14}C]Phe-tRNA^{Phe} were added to 10 pmol portions of activated subunits followed by 45 min incubation at 0°C. The numbers of aminoacyl-tRNA molecules bound per subunit in the mixtures (i.e. $\bar{\nu}^{\Sigma}$ values) were determined after filtration through nitrocellulose filters. (B) Kinetics of [^{14}C]Phe-tRNA^{Phe} binding to activated 40 S subunits at 0°C (○) and 30°C (●). (C) Titration of activated 40 S · poly(U) complex with *E. coli* [^{14}C]Phe-tRNA^{Phe} (△), yeast [^{14}C]Phe-tRNA^{Phe} (▲), *E. coli* Ac-[^{14}C]Phe-tRNA^{Phe} (○) and deacylated *E. coli* [^{14}C]tRNA^{Phe} (●). Incubations were for 3 h at 0°C.

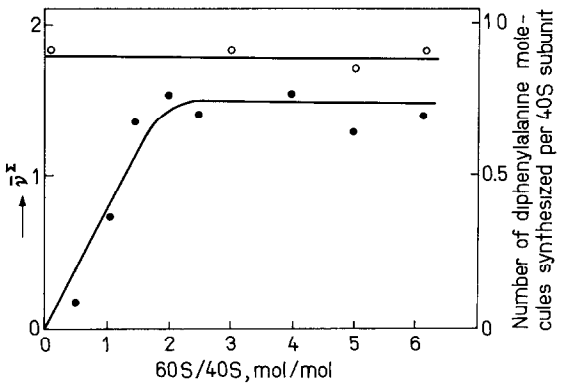

Fig.3. Involvement of 2 Phe-tRNA^{Phe} molecules prebound to activated 40 S subunits in dipeptide synthesis after addition of activated 60 S subunits. Two series of mixtures, each containing 10 pmol 40 S subunits, 10 μg poly(U) and 80 pmol [^{14}C]Phe-tRNA^{Phe} in 150 μl TAM buffer, were incubated for 20 min at 0°C (final $\bar{\nu}^{\Sigma}$ = 1.8). Then 0–70 pmol 60 S subunits were added to each mixture, and the incubation continued for an additional 15 min. Mixtures from the first series were used to measure the dependence of $\bar{\nu}^{\Sigma}$ on the 60 S/40 S ratio (○). Analogous mixtures from the second series were treated as in [15] to determine the number of diphenylalanine molecules synthesized per 40 S subunit as a function of the 60 S/40 S (●).

molecules prebound to 40 S subunits appear to be involved in dipeptide synthesis, one can draw the conclusion that the 2 sites on the 40 S subunits are of functional relevance, i.e., by definition, they are parts of the P and A sites of 80 S ribosomes.

Thus, in spite of considerable differences in structure, 40 S ribosomal subunits from eukaryotes display important functional similarities when compared with 30 S subunits from prokaryotes: they contain one mRNA-binding center and two tRNA binding centers.

Double-reciprocal or Scatchard plots derived from the data in fig.2C (not shown) indicated that the interaction of tRNA with the 2 sites on 40 S subunits was most likely a cooperative interaction. Hence, additional experiments are required to determine the thermodynamic quantities of the 40 S-tRNA interaction; these are presently in progress.

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