MECHANISM OF CODON–ANTICODON INTERACTION IN RIBOSOMES

Codon–anticodon interaction of aminoacyl-tRNA at the ribosomal donor site

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1. Introduction

According to current concepts in protein biosynthesis, aminoacyl-tRNA is never placed at the donor (D) site of the ribosome during elongation, because this site, at that time, is always occupied by nascent peptidyl-tRNA. But in model systems, containing free ribosomes charged with mRNA, aminoacyl-tRNA can bind both at D and A (acceptor) sites.

It was shown earlier that at low [Mg^{2+}] (5–6 mM) the D site was preferred, but at higher [Mg^{2+}] most of the bound aminoacyl-tRNA was found at the A site [1,2]. This was true also for peptidyl-tRNA [3,4]. However in [5], enriched Phe-tRNA^{Phe} bound initially at the D site both at low (10 mM) and high (30 mM) [Mg^{2+}]. Moderate concentrations of cognate tRNA^{Phe}, but not non-cognate tRNAs, were found to be strong competitors for D-site binding [5]. The data mentioned are somewhat contradictory. Thus, we re-examined the problem of Mg^{2+}-dependent aminoacyl-tRNA distribution between the A and D sites of 70 S · poly(U) complex.

Using ribosomes with fully active sites, we were able to show that purified Phe-tRNA^{Phe} has a higher affinity towards the D site at all [Mg^{2+}] studied (5–20 mM); at excess of ribosomes Phe-tRNA^{Phe} binds exclusively at the D site. However, if non-enriched preparations of Phe-tRNA were used, the latter was found preferentially in the A site (up to 80%, at 20 mM Mg^{2+}). Obviously, a high excess of natural mixture of non-cognate deacylated tRNAs is also a strong competitor for D-site binding, at least at high [Mg^{2+}].

This finding allowed us to measure the association constants of Phe-tRNA^{Phe} with the D site in the presence and absence of poly(U). It was found that the presence of only the appropriate codon in the D site increased the K_a by 2–3 orders of magnitude. This is a strong argument for the contribution of codon–anticodon interaction when Phe-tRNA^{Phe} is bound at the D site of 70 S · poly(U) complex.

2. Materials and methods

Isolated 30 S and 50 S subunits, enriched [^{14}C]-Phe-tRNA^{Phe} (1440 pmol/A_{260} unit) and fractionated poly(U) (with av. chain-length 90 nucleotides) were prepared as in [6–8]. A natural mixture of non-cognate deacylated tRNAs (tRNA^{-Phe}) (fractions of tRNA from a salt gradient) revealed no detectable aminoacylation with [^{14}C]phenylalanine in the course of purification of [^{14}C]Phe-tRNA^{Phe} by BD-cellulose chromatography [7]. [^{14}C]Phe-tRNA^{Phe} was preincubated 5 min at 40°C in buffer I (0.02 M bis-Tris–HCl (pH 6.5); 0.02 M $MgCl_2$; 0.2 M NH_4Cl; 0.001 M EDTA) before addition to ribosomes to obtain the pure high-affinity (native) conformer [9]. Association constants of Phe-tRNA^{Phe} with the 70 S · poly(U) complex were determined by the method of variable volume [9] using the equation:

$$1/\beta = 1/\gamma \,.\, K_a \,.\, r + 1/\gamma$$

where: β = the relative amount of aminoacyl-tRNA bound to ribosomes; γ = the active fraction of Phe-tRNA^{Phe}; r = the concentration of free 70 S · poly(U) complex; and K_a = the association constant. In fig.4A

we used the method of constant volume [8]. In this case the amount of ribosomes in the incubation mixtures was fixed, but the amount of [^{14}C]Phe-tRNA^{Phe} was variable. The binding isotherm is expressed by the equation:

$$1/\bar{\nu} = 1/M + 1/M \,.\, K_a \,.\, C,$$

where: $\bar{\nu}$ = the average number of tRNA molecules bound per ribosome; C = the concentration of free tRNA; and M = the fraction of active ribosomal sites. Designations K_a^D and K_a^A will be used to distinguish association constants of aminoacyl-tRNA with the D and A sites, respectively.

3. Results

Fig.1A illustrates the ability of 70 S ribosomes to bind simultaneously two molecules of Phe-tRNA^{Phe} at high (20 mM) [Mg^{2+}]. Obviously, the site with higher affinity for Phe-tRNA^{Phe} is filled first, and the second site is occupied at higher aminoacyl-tRNA concentrations. To discriminate between the sites we used tetracycline (TET) at an extremely low concentration (2×10^{-5} M), which inhibits the binding of Phe-tRNA^{Phe} at the A site of 30 S subunits but does not affect the D-site binding [6]. As can be seen from fig.1A (–●–), the inhibitory action of TET is developed only when $\bar{\nu} \geqslant 1$. This result together with the fact that both D and A sites are really filled ($\bar{\nu} \to 2$, when $C \to \infty$) allows us to conclude

(i) The site with higher affinity for Phe-tRNA^{Phe} is the D site;

(ii) The A site is occupied only after saturation of the D site.

A test with puromycin supports this conclusion. In fig.2 we used an excess of ribosomes over aminoacyl-tRNA to effect TET-resistant binding only. After the equilibrium was reached (not shown) the temperature was raised and puromycin added. In a control experiment (without puromycin) the level of binding decreases at elevated temperature (–○–) due to partial dissociation of the ternary complex, but the bound Phe-tRNA^{Phe} is entirely reactive with puromycin (–●–). TET, as expected, changed neither the level of initial binding, nor the kinetics and the extent of Phe–puromycin formation (–△–,–▲–).

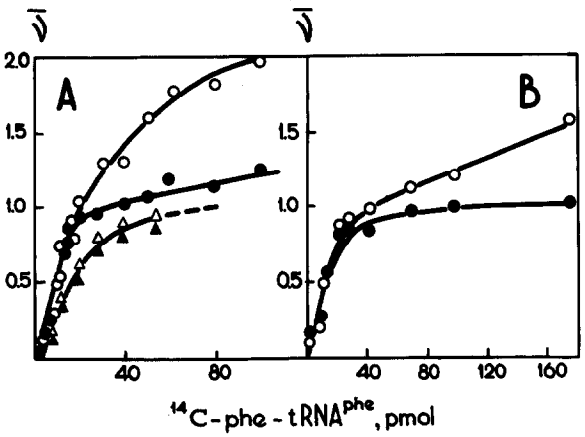

Fig.1. Titration of the 70 S · poly(U) complex with [^{14}C]Phe-tRNA^{Phe}. Mixtures contained in 1 ml buffer 1: 10 pmol 30 S subunits, 15 pmol 50 S subunits, 10 μg poly(U) and variable amounts of [^{14}C]Phe-tRNA^{Phe}. Incubation was performed for 90 min at 0°C in the absence (–○–) or in the presence (–●–) of 2×10^{-5} M TET. (A) Binding at 20 mM; (B) at 5 mM Mg^{2+}. In experiments without poly(U) volumes of samples were 0.2 ml ((–△–) –TET; (–▲–) +TET).

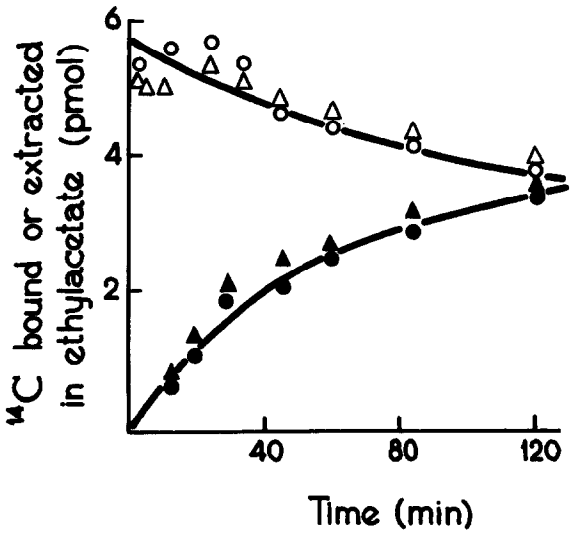

Fig.2. Kinetics of the reaction of prebound [^{14}C]Phe-tRNA^{Phe} with puromycin. Mixtures contained in 0.25 ml buffer 1: 10 pmol 30 S subunits, 15 pmol 50 S subunits, 5 μg poly(U), 6.8 pmol [^{14}C]Phe-tRNA^{Phe} and TET (where indicated). After 60 min incubation at 0°C (not shown) the temperature was raised to 30°C and the kinetics of binding was measured in the absence (–○–) and in the presence (–△–) of TET. To another set of mixtures puromycin (2×10^{-4} M) was added simultaneously with the increase of temperature, and the kinetics of [^{14}C]Phe–puromycin formation was measured in the absence (–●–) and in the presence (–▲–) of TET.

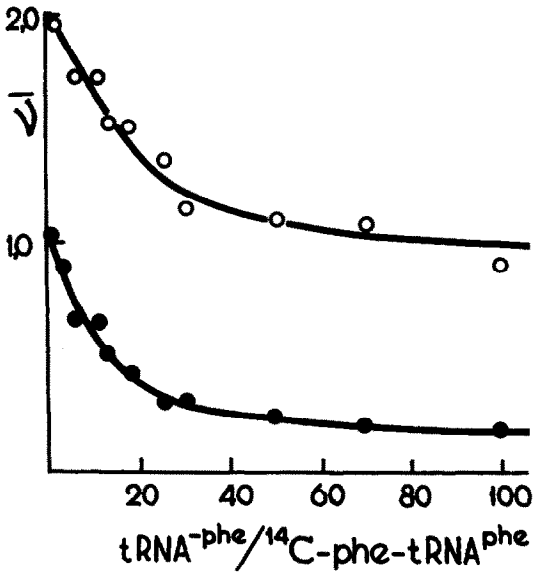

Fig.3. Inhibition of [^{14}C]Phe-tRNA^{Phe} binding by deacylated non-specific tRNA^{-Phe}. Mixtures contained in 0.4 ml buffer 1: 10 pmol 30 S subunits, 15 pmol 50 S subunits, 5 μg poly(U), 50 pmol [^{14}C]Phe-tRNA^{Phe} and 0–5000 pmol tRNA^{-Phe} in the absence (–○–) or in the presence (–●–) of 2×10^{-5} M TET.

In the absence of messenger RNA Phe-tRNA^{Phe} fills only the TET-resistant, i.e., D site (fig.1A (–△–, –▲–)). The acceptor site is not functional now (due to the very low K_a^A value).

A mixture of deacylated non-cognate tRNAs (tRNA^{-Phe}), which is a constituent of non-enriched Phe-tRNA, strongly inhibits the binding of Phe-tRNA^{Phe} to the D site (fig.3). Comparing the curves in the presence and absence of TET, we see that in case of a 40–50-fold excess of tRNA^{-Phe} (this equivalent to a usual non-enriched preparation of Phe-tRNA) the labeled aminoacyl-tRNA binds preferentially (up to 80%) to the A site. Similar results were obtained earlier when specific deacylated tRNA^{Phe} (but at much lower concentrations) was used as competitor for D-site binding [5,10,11].

All experiments shown above were performed at 20 mM Mg^{2+}. Fig.1B demonstrates that the situation does not change at a low, nearly 'physiological' [Mg^{2+}] (5 mM). It follows from these results that it is possible to study the interaction of Phe-tRNA^{Phe} with ribosomal D sites at all [Mg^{2+}] provided that an excess of ribosomes and highly purified aminoacyl-tRNA are used. Experiments made in such conditions are shown in fig.4B. At 20 mM Mg^{2+} (–●–) and in the presence of poly(U) virtually all Phe-tRNA^{Phe} added binds to

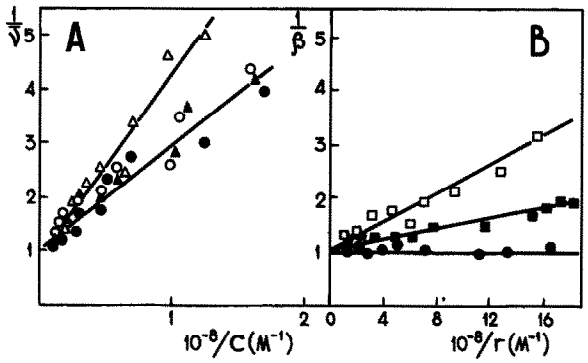

Fig.4. Measurements of K_a^D values in the absence and presence of messenger. (A) Mixtures contained in 0.25 ml buffer 1 (with 20 mM Mg^{2+}): 10 pmol 30 S subunits, 15 pmol 50 S subunits and 2–80 pmol [^{14}C]Phe-tRNA^{Phe}. (–●–) Without messenger; (–△–) the same in the presence of 2×10^{-5} M TET; (–○–, –▲–) in the presence of 10 μg poly(A) or poly(C), respectively. (B) Mixtures contained in 0.1–14 ml of buffer 1: 10 pmol 30 S subunits, 15 pmol 50 S subunits, 15 μg poly(U) and 5 pmol [^{14}C]Phe-tRNA^{Phe}. [Mg^{2+}], 20 mM (–●–); 7 mM (–■–) and 6 mM (–□–). Incubation, 90 min at 0°C.

ribosomes. We can only estimate a lower limit of K_a^D values, 10^{10}–10^{11} M^{-1}. At low [Mg^{2+}] K_a^D is measurable and $=2 \times 10^9$ M^{-1} at 7 mM (–■–) and 7×10^8 M^{-1} at 6 mM Mg^{2+} (–□–). In the absence of poly(U) the K_a^D of 70 S ribosomes with Phe-tRNA^{Phe} $=5.5 \times 10^7$ M^{-1} at high (20 mM) [Mg^{2+}] (–●–, fig.4A), but practically no binding was detectable at 6–7 mM Mg^{2+}. TET has a slight effect on K_a^D (decreases to 3.5×10^7 M^{-1}). Addition of poly(A) or poly(C) does not change K_a^D value (fig.4A, –○–, –▲–). Comparing these data, we conclude that in the presence of the cognate codon at the D site K_a^D is increased by minimum 2–3 orders of magnitude. This is strong evidence for the contribution of codon–anticodon interaction at the ribosomal D site in aminoacyl-tRNA binding.

4. Discussion

We were able to show earlier that 30 S subunits contain two codon-dependent sites for Phe-tRNA^{Phe}. The K_a^D/K_a^A ratio was found to equal 10–30 at different [Mg^{2+}] [6]. It follows from the present study that the addition of 50 S subunits to the 30 S · poly(U) complex increases K_a^D values by 2–3 orders of magnitude, but does not change the general situation. At any [Mg^{2+}] pure Phe-tRNA^{Phe} fills at first the D site and only afterwards the A site. Two alternative explanations for this are possible:

(i) Both the A and D sites exist simultaneously and independently [12], but $K_a^D \gg K_a^A$;
(ii) The A site is 'generated' only after the filling of the D site ($K_a^A = 0$, when the D site is empty [5]), codon–anticodon interaction at the D site being necessary for this to occur [13,14].

We prefer the first explanation because: (a) the A site can be effectively filled after adsorption of non-specific deacylated $tRNA^{-Phe}$ in the D site (fig.3); and (b) after blocking the D site by the antibiotic edeine K_a^A for lactyl-Phe-$tRNA^{Phe}$ is the same as when the D site is specifically occupied [12].

We measured the K_a^D value for Phe-$tRNA^{Phe}$ at 7 mM Mg^{2+} and 0°C (2×10^9 M^{-1}), but the quantitative formation of $(Phe)_2$-$tRNA^{Phe}$, when $1 < \bar{\nu} \leqslant 2$ (not shown) made it impossible to determine the K_a^A value in such a system. However, when the D site is occupied by an analogue of peptidyl-tRNA exempt from donor activity (*N*-acetyl-Phe-$tRNA^{Phe}_{ox-red}$, [15]), the K_a^A value is easily measurable and $\approx 10^7$ M^{-1} at 0°C and 10 mM Mg^{2+} (unpublished).

Comparing the results of this study and data taken from the literature, we find some common features in the interaction with ribosomes of all forms of tRNA (aminoacyl-, peptidyl- and deacylated) participating in the elongation step:

(1) In the absence of messenger all forms of tRNA bind at the donor site only ([12,16]; fig.1A).
(2) In the presence of messenger they all have a higher affinity for the donor, than for the acceptor site ([12,16]; fig.1 and above).
(3) In all cases codon–anticodon interaction occurs at the donor site ([12–14,16]; fig.4).

This property of the tRNA molecule (its higher affinity to the D site) is the thermodynamic basis of the translocation process. We may assume also that the presence of the peptidyl- or aminoacyl- radical at the 3′-end of tRNA is not decisive for its preferential binding at the D site. This is not surprising, because there exist several regions common to all tRNA species which interact with the ribosome [11,17–20]. They can at most contribute in the free energy of this interaction [21]. However, in simpler, 'fragment' systems where these regions are absent, peptidyl- and aminoacyl- moieties do direct the binding of short 3′-end fragments of tRNA correspondingly at the donor and acceptor sites of peptidyltransferase center [22–24].

The detailed mechanism of aminoacyl-tRNA–ribosome interaction can be understood only when elongation factor EF-T_u plus GTP are present and external conditions are close to physiological. But we consider this study as an indispensable step in the investigation of more complex, factor-dependent systems.

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