MECHANISM OF CODON–ANTICODON INTERACTION IN RIBOSOMES

Comparative study of interaction of Phe-tRNA^{Phe} and *N*-acetyl-Phe-tRNA^{Phe} with the donor site of *Escherichia coli* ribosomes

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1. Introduction

Conformational changes of tRNA in solution, depending e.g., on medium conditions, aminoacylation and interaction of oligonucleotides with anticodon, have been intensively studied. Substantial flexibility was detected in TψC and D loops, anticodon stem and loop and the 3′-end of the tRNA molecule (review [1]).

However, the biological relevance of conformational transitions in tRNA has been questionable. Obviously, it is characterization of stable conformations of tRNA in ribosomes, which are fixed in different functional states, that may be the initial step in investigation of structural dynamics of tRNA during protein biosynthesis.

Here we have made a comparative study of interactions of aminoacyl-tRNA (Phe-tRNA^{Phe}) and peptidyl-tRNA (*N*-acetyl-Phe-tRNA^{Phe}) with the donor (D) site of vacant 70 S ribosomes both in the presence and absence of messenger (poly(U)). The K_a-value of peptidyl-tRNA with the D-site, in the absence of poly(U), was double that of aminoacyl-tRNA. But in the presence of poly(U) aminoacyl-tRNA has a higher affinity to the D-site than peptidyl-tRNA. A possible difference in the anticodon loop conformations of both forms of tRNA, bound to the D-site, is suggested.

2. Materials and methods

Isolated 30 S and 50 S subunits, enriched in [^{14}C] Phe-tRNA^{Phe} (1440 pmol/A_{260} unit) and fractionated poly(U) with an average chain length 90 nucleotides were prepared as in [2–4]. An analogue of peptidyl-tRNA, *N*-acetyl-[^{14}C]Phe-tRNA^{Phe}, was synthesized according to [5] starting from [^{14}C]Phe-tRNA^{Phe}. Details concerning each experiment are indicated in the figure legends. K_a-values of tRNA with 70 S ribosomes were measured using two equivalent methods:

Method 1: The amounts of ribosomes and tRNA were kept constant in incubation mixtures, but their volumes were varied; binding isotherms are described by the equation:

$$1/\beta = 1/\gamma + 1/\gamma \times K_a \times r$$

where, β is the portion of tRNA bound, γ its active fraction, r the concentration of free ribosomes and K_a the association constant.

Method 2: The amount of ribosomes and the volume of the mixtures were fixed, but the amounts of tRNA added were variable. In this case the binding isotherm is described by the equation:

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

where, $\bar{\nu}$ is the average number of tRNA molecules bound/ribosome, M the fraction of active ribosomal sites and C the concentration of free tRNA. For the determination of the yield of [^{14}C]Phe-puromycin 2 ml portions of 0.1 M Na-phosphate buffer (pH 8.3) were added to incubation mixtures, and the reaction product was extracted into ethylacetate according to [6]. All experiments were performed in buffer 1 (0.02 M Bis-Tris (pH 6.5)) containing 0.2 M NH_4Cl and $MgCl_2$ at concentrations indicated in the figure legends.

Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris-(hydroxymethyl)methane; TET, tetracycline; K_a^D and K_a^A, association constants of tRNA with the D (donor) and A (acceptor) sites of ribosomes, respectively

3. Results

Purified peptidyl- and aminoacyl-tRNA have a higher affinity to the D-, than to the A-site of 70 S · poly(U) complex. K_a^D/K_a^A ratios were estimated to be equal to 30–50 for *N*-acetyl-Phe-tRNA^{Phe} and Phe-Lac-tRNA^{Phe} [7,8], and up to 2–3 orders of magnitude for Phe-tRNA^{Phe} [9]. If ribosomes were taken in excess, both forms of tRNA were found exclusively in the D-site [7,9,10]. For this reason we have chosen, for the measurements of K_a^D-values, method 1, when the ratio ribosomes/tRNA >1 was kept constant in all mixtures, but their volumes were varied ($1/\beta$ *vs* $1/r$ plot, see section 2).

In the absence of messenger *N*-acetyl-Phe-tRNA^{Phe} and Phe-Lac-tRNA^{Phe} bind to the D-site only [7,8]; we found that the same is true for Phe-tRNA^{Phe}. In the experiment shown on fig.1A, after the equilibrium binding at 0°C was reached, the temperature was raised to 30°C and puromycin was added. In a control experiment (without puromycin) the level of binding decrease (—○—), but the residual [^{14}C]Phe-tRNA^{Phe} reacts completely with puromycin (—●—). Further, in the absence of poly(U) [^{14}C]Phe-tRNA^{Phe} fills only one, TET-resistant site (—△—,—▲—, fig.1B). On the other hand, in the presence of poly(U) the ribosomes are able to bind simultaneously 2 molecules of aminoacyl-

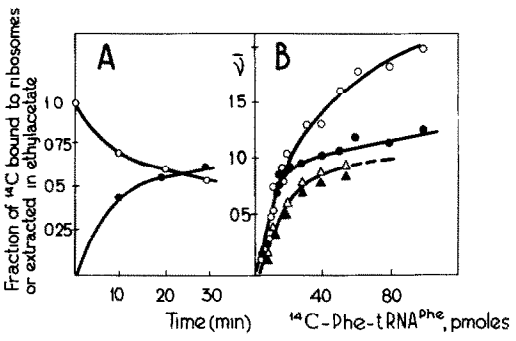

Fig.1. (A) Reaction of prebound [^{14}C]Phe-tRNA^{Phe} with puromycin in the absence of poly(U). Incubation mixtures contained in 0.2 ml buffer 1: 30 pmol 30 S subunits, 45 pmol 50 S subunits, and 10 pmol [^{14}C]Phe-tRNA^{Phe}. After 60 min incubation at 0°C the temperature was raised to 30°C and pH adjusted to 7.5. Then puromycin was added to half of the samples to a final concentration 2×10^{-4} M, and kinetics of further binding (—○—) as well as the yield of [^{14}C]Phe-puromycin (—●—) were measured in parallel. (B) Titration of 70 S ribosomes by [^{14}C]Phe-tRNA^{Phe}: (—○—) + poly(U), – TET; (—●—) + poly(U), + TET; (—△—) – poly(U), – TET; (—▲—) – poly(U), + TET. These data are taken from [9].

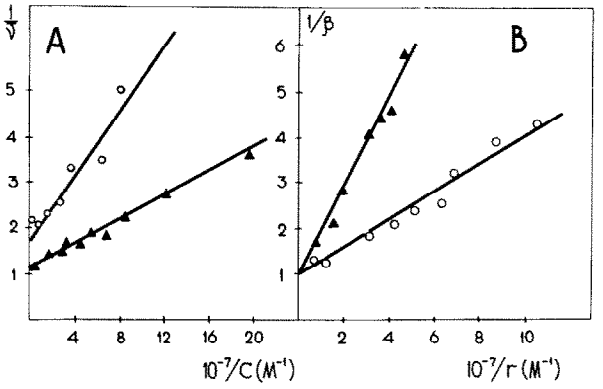

Fig.2. Adsorption isotherms of [^{14}C]Phe-tRNA^{Phe} and *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} at the D-site of 70 S ribosomes. (A) Binding in the absence of poly(U). Incubation mixtures contained in 0.2 ml buffer 1 (with 20 mM Mg^{2+}): 10 pmol 30 S subunits, 15 pmol 50 S subunits, and 4–80 pmol [^{14}C]-Phe-tRNA^{Phe} (—○—) or *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} (—▲—). Incubation time 60 min at 0°C. (B) Binding in the presence of poly(U). Incubation mixtures contained in 0.1–1.5 ml buffer 1 (with 10 mM Mg^{2+}: 10 pmol 30 S subunits, 15 pmol 50 S subunits, 15 μg poly(U) and 7 pmol [^{14}C]Phe-tRNA^{Phe} (—○—) or *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} (—▲—). Incubation time, 60 min at 30°C.

tRNA, one of them being TET-sensitive (compare —○— and —●—, fig.1B). It follows from these experiments, that aminoacyl-tRNA (as well as deacylated and peptidyl-tRNA [7,8]) can not bind to the acceptor site in the absence of mRNA, obviously, due to a too low K_a^A-value. Hence, we can use in this case any ribosome/tRNA ratios for K_a^D-values measurements, and method 2 is more convenient now ($1/\bar{\nu}$ *vs* $1/C$ plot, see section 2).

Fig.2A demonstrates the measurement of K_a^D-values in the absence of poly(U). From 60–90% of ribosomes contain active D-sites; the K_a for peptidyl-tRNA is the double of that for aminoacyl-tRNA. But in the presence of poly(U) we observe different results: all ribosomal D-sites are active, and the affinity of Phe-tRNA^{Phe} to the D-site of 70 S · poly(U) complex appears higher than that of *N*-acetyl-Phe-tRNA^{Phe}. The ratio of their K_a^D-values equals to 2–3. This value is reliable because the statistical error in K_a^D determinations does not exceed 15–20%; systematic errors were eliminated using the same preparations of ribosomes and tRNAs in all experiments.

This effect occurs at 10–20 mM Mg^{2+} (fig.3). Because of considerable differences in K_a^D-values in the absence and presence of poly(U), different tempera-

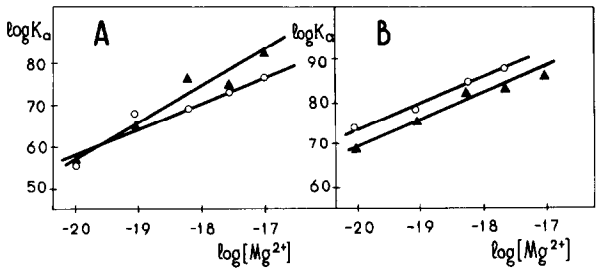

Fig.3. Log K_a^D as a function of log[Mg^{2+}] for [^{14}C]Phe-tRNA^{Phe} (–o–) and *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} (–▲–): (A) binding at 0°C and in the absence of poly(U); (B) binding at 30°C and in the presence of poly(U).

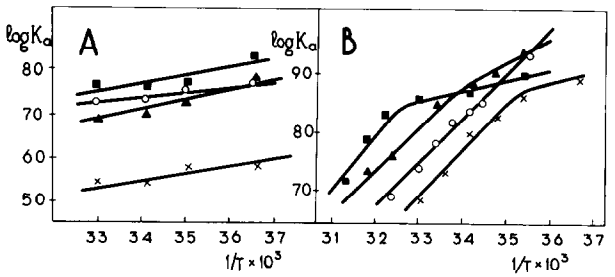

Fig.4. Van't Hoff plots for the binding of [^{14}C]Phe-tRNA^{Phe} and *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} at the D-site of ribosomes. (A) Experiments, in the absence of poly(U), were made with [^{14}C]Phe-tRNA^{Phe} at 20 mM Mg^{2+} (–o–) and *N*-acetyl-[^{14}C]-Phe-tRNA^{Phe} at 20 mM (–■–), 15 mM (–▲–) and 10 mM Mg^{2+} (–x–). (B) Experiments, in the presence of poly(U), were made with [^{14}C]Phe-tRNA^{Phe} at 10 mM Mg^{2+} (–o–) and *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} at 20 mM (–■–), 15 mM (–▲–) and 10 mM Mg^{2+} (–x–). The data at high temperature were corrected for spontaneous hydrolisis (for *N*-acetyl-Phe-tRNA^{Phe} $t_{1/2}$ = 8 h at 45°C).

tures were chosen for both sets of experiments (0°C, fig.3A and 30°C, fig.3B, respectively).

In fig.4 van't Hoff plots for peptidyl- and aminoacyl-tRNA, in the absence (fig.4A) and presence (fig.4B) of poly(U) are shown. Comparing the data (at the same Mg^{2+} levels) we conclude again, that the presence of the appropriate codon in the D-site causes a considerable increase of K_a^D-values for aminoacyl-tRNA as compared with peptidyl-tRNA at all temperatures examined. The enthalpy (ΔH^o) of the complex formation was determined from the data in fig.4 and the entropy (ΔS^o) of the complex was calculated from the Gibbs-Helmholtz equation: $\Delta S^o = (\Delta H^o - \Delta G^o)/T$. The free energy of binding, ΔG^o, was calculated according to the relationship: $\Delta G^o = -RT \ln K_a^D$. We see from table 1, the difference in thermodynamic parameters of the complexes with and without messenger. Without poly(U) the binding of Phe-tRNA^{Phe} as well as *N*-acetyl-Phe-tRNA^{Phe} is driven by both enthalpy and entropy whereas in the presence of poly(U) enthalpy is the driving force, ΔS^o being negative (except for the binding of *N*-acetyl-Phe-tRNA^{Phe} at 20 mM Mg^{2+}, over 10–34°C).

4. Discussion

In the absence of messenger *N*-acetyl-Phe-tRNA^{Phe} has higher affinity to the D-site of ribosome, than Phe-tRNA^{Phe}, obviously, due to higher affinity of the 3′-end of peptidyl-tRNA to the peptidyltransferase donor center [12]. In the presence of poly(U) codon–

Table 1
Thermodynamic characteristics of the complexes of Phe-tRNA^{Phe} and *N*-acetyl-Phe-tRNA^{Phe} with the D-site of 70 S ribosomes

Expt. no.	tRNA	Mg^{2+} (mM)	Poly(U)	Temperature	ΔH^o (kcal/mol)	ΔS^o (cal . mol^{-1} . deg^{-1})
1.	Phe-tRNA^{Phe}	20	–	0–30°C	–(6 ± 1)	14 ± 5
2.	Phe-tRNA^{Phe}	10	+	10–37°C	–(38 ± 3)	–(90 ± 10)
3.	*N*-Acetyl-Phe-tRNA^{Phe}	20	–	0–30°C	–(8 ± 3)	7 ± 11
4.	*N*-Acetyl-Phe-tRNA^{Phe}	15	–	0–30°C	–(10 ± 2)	3 ± 6
5.	*N*-Acetyl-Phe-tRNA^{Phe}	20	+	10–34°C	–(10 ± 1)	7 ± 4
				34–45°C	–(60 ± 12)	–(150 ± 40)
6.	*N*-Acetyl-Phe-tRNA^{Phe}	15	+	10–24°C	–(21 ± 1)	–(32 ± 4)
				24–41°C	–(34 ± 3)	–(75 ± 9)
7.	*N*-Acetyl-Phe-tRNA^{Phe}	10	+	10–30°C	–(36 ± 4)	–(86 ± 12)

anticodon interaction at the D-site is realized for all forms of $tRNA^{Phe}$ (deacylated, peptidyl- and aminoacyl-tRNA [7–11]) K_a^D-values being increased by 1–3 orders of magnitude [7–9]. Hence, from the comparison of two facts:

(i) Involvement of $tRNA^{Phe}$ anticodon in the interaction with 70 S · poly(U) complex;
(ii) Enormous increase of K_a^D for Phe-$tRNA^{Phe}$ as compared with *N*-acetyl-Phe-$tRNA^{Phe}$;

it is reasonable to speculate, that these forms of tRNA, being bound to the 70 S · poly(U) complex, have different stable conformations of anticodon loops.

It is known, indeed, that the conformation of anticodon loop is flexible. The 3′-stacked loop was observed in both crystal forms of $tRNA^{Phe}_{Yeast}$ [13,14]. Fluorescence study of Wybutine (Wye-base) and NMR spectroscopy as well as the investigation of complexed tRNAs with complementary anticodons confirmed a 3′-stacked conformation in solution [15–18]. However, sensitivity of Wye-base fluorescence towards variations in medium conditions shows that a 3′- to 5′-stacked conformational transition is possible [19], as proposed in [20]. The presence of two distinct conformations of $tRNA^{Phe}_{Yeast}$ anticodon loop was demonstrated by relaxation measurements [21,22]. Moreover, independent evidence for the presence of two conformations was obtained by lifetime and polarization measurements of the Wye-base fluorescence [23]. These data are interpreted in terms of a stacked and unstacked conformation of the Wye-base, which may correspond to the 3′- and 5′-stacked conformation of anticodon loop of $tRNA^{Phe}_{Yeast}$. According to [22], UUC-triplet binds preferentially to the 3′-stacked conformation of anticodon loop of $tRNA^{Phe}_{Yeast}$. Considering these data, we suggest that the anticodon loop of Phe-$tRNA^{Phe}$ is in 3′-stacked conformation during poly(U)-directed interaction with the D-site of ribosomes. Here, the short double-helix of the codon–anticodon complex is stabilized by the stacking interaction of hypermodified base ms^2i^6A at the 3′-side of the anticodon [18]. On the other hand, the anticodon loop of *N*-acetyl-Phe-$tRNA^{Phe}$ is presumably in the 5′-stacked conformation.

There is another peculiarity in the binding of peptidyl-tRNA with the D-site, non-linearity of van't Hoff plots (fig.4B). The reaction enthalpy differs strongly over the temperatures studied (table 1, no. 5,6). Such effect can be explained by many alternative models [24–26]; one of them seems us preferable. A temperature-dependent equilibrium must exist between two forms of peptidyl-tRNA, both of them able to interact with the D-site of 70 S · poly(U) complex. Enthalpy of transition is high enough and Mg^{2+}-dependent (fig. 4B, table 1). Obviously, the non-linearity observed is caused by variations in peptidyl-tRNA, not ribosomes, because:

(i) All experiments in the presence of poly(U) were performed at excess of ribosomes;
(ii) Non-linearity was not observed in the absence of poly(U) (fig.4A). This result is an additional evidence that just the anticodon region of peptidyl-tRNA is responsible for the non-linearity of van't Hoff plots;
(iii) Non-linearity was not observed either for Phe-$tRNA^{Phe}$ (fig.4B, –○–).

Taking into account estimations of K_a^D at 20 mM Mg^{2+} and 0°C ($>10^{11}M^{-1}$, [27]), we can suggest that van't Hoff plots for Phe-$tRNA^{Phe}$ is linear also at high (20 mM) Mg^{2+} levels. In these conditions the difference in K_a^D-values for aminoacyl- and peptidyl-tRNA reaches two orders of magnitude [7,27].

These experiments indicate that aminoacyl- and peptidyl-tRNA bound to the D-site are characterized by different conformations, presumably in their anticodon loops. Obviously, additional independent data are needed to support this assumption.

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