Quantitative study of interaction of deacylated tRNA with *Escherichia coli* ribosomes

Role of 50 S subunits in formation of the E site

S.V. Kirillov, E.M. Makarov and Yu.P. Semenkov

B.P. Konstantinov Nuclear Physics Institute, USSR Academy of Sciences, Gatchina, Leningrad district 188350, USSR

Received 1 April 1983

The 30 S subunit contains 2 sites for tRNA binding (Phe-tRNA, AcPhe-tRNA, $tRNA_{OH}^{Phe}$) with the functional properties of D and A sites of the 70 S ribosome after attachment of 50 S subunit. The third (E) site specific for deacylated tRNA is introduced into 70 S ribosome by its 50 S subunit. The E-site binding of $tRNA_{OH}^{Phe}$ is not sensitive to either tetracycline and edeine, and practically codon-independent. The affinity constant of $tRNA_{OH}^{Phe}$ for the E site is 2–3 orders of magnitude lower than that for the D site.

Deacylated tRNA–ribosome interaction *Ribosome, exit site* *Antibiotic, tetracycline, edeine* *Site distribution, between subunits*

1. INTRODUCTION

Deacylated tRNA appears in a translating ribosome as a result of peptide bond formation between peptidyl-tRNA, located at the donor (D) site, and a newly selected aminoacyl-tRNA, located at the acceptor (A) site. At the next step of the elongation cycle, translocation, removal of deacylated tRNA from the D site is a key event in this complicated process. For this reason, a detailed study of the interaction of deacylated tRNA with ribosomes (thermodynamics and kinetics of binding; number of active sites) is important for better understanding of protein biosynthesis.

In 1965, Wettstein and Noll [1] showed, using polysomes from rat liver, that for each active ribosome bound, on the average, at least 2 and at most 3 tRNA molecules: aminoacyl-tRNA, at the 'decoding' (A) site; peptidyl-tRNA, at the 'condensing' (D) site; and deacylated tRNA, at a third site. They found that the latter is specific exclusively for deacylated tRNA and characterized by relatively weak and reversible binding. Thus, it was regarded as an 'exit' site.

Nonetheless, during the last 2 decades, Watson's 2-site model of the ribosome [2], based on functional properties of peptidyl- and aminoacyl-tRNA, has been generally accepted. As a consequence, in all studies of the interaction of deacylated tRNA with ribosomes, it was admitted, a priori, that only the D and A sites exist for binding.

However, it was demonstrated using different techniques, that ribosomes contain, besides the usual D and A sites, a third site, specific for deacylated tRNA only [3,4].

Here, we confirm this result and show additionally that:

(i) The third, so-called E site on the 70 S ribosome is formed by its 50 S subunit. The 30 S subunit contains only 2 sites able to bind deacylated tRNA ($tRNA_{OH}^{Phe}$), as well as aminoacyl-tRNA ($Phe\text{-}tRNA^{Phe}$) or peptidyl-tRNA ($Ac\text{-}Phe\text{-}tRNA^{Phe}$) [5];

(ii) Edeine and tetracycline block almost quantitatively the binding of 2 $tRNA_{OH}^{Phe}$ molecules/70 S ribosome, obviously, at the D and A sites. The E-site binding is not sensitive to either antibiotic;

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

(iii) Contrary to [3] and in accordance with [4], the binding of $tRNA^{Phe}_{OH}$ at the E site is practically codon-independent;
(iv) The affinity constant of $tRNA^{Phe}_{OH}$ for the E site is 2–3 orders of magnitude lower than that for the D site.

2. MATERIALS AND METHODS

Active 30 S and 50 S subunits, as well as fractionated poly(U) (M_r 30000) and enriched [^{14}C]Phe-$tRNA^{Phe}$ (1500 pmol/A_{260} unit) and Ac-[^{14}C]Phe-$tRNA^{Phe}$ (1400 pmol/A_{260} unit), were obtained as in [5,6]. Deacylated [^{14}C]$tRNA^{Phe}_{OH}$ (1290 pmol/A_{260} unit) was isolated according to [4]. In the binding experiments, except that in fig.4C, incubation mixtures contained in 100–120 μl buffer TAM: 10 pmol 30 S subunits, 12 pmol 50 S subunits, 10 μg poly(U); and variable amounts of tRNAs. After 3 h incubation at 0°C, $\bar{\nu}^{\epsilon}$ values (total number of tRNA molecules bound/ribosome) were determined by the nitrocellulose filter technique. Antibiotics edeine (Calbiochem, USA) and tetracycline (Serva, FRG) were used, where indicated, at final concentrations 10^{-6} M and 10^{-4} M, respectively. Corrections for the actual fraction of active [^{14}C]$tRNA^{Phe}_{OH}$ (55–60%) were made in all experiments. Buffer TAM: 0.02 M Tris–HCl (pH 7.4); 0.02 M $MgCl_2$; 0.2 M NH_4Cl; 0.001 M EDTA.

3. RESULTS

We showed that active poly(U)-programmed 30 S subunits are able to bind simultaneously 2 molecules of aminoacyl-tRNA (Phe-$tRNA^{Phe}$) or peptidyl-tRNA (Ac-Phe-$tRNA^{Phe}$) [5,7]. We observe a similar situation for deacylated $tRNA^{Phe}_{OH}$ (fig.1, —○—)). Tetracyline, a specific inhibitor of the A site, blocks almost quantitatively the binding of one $tRNA^{Phe}_{OH}$ molecule (—●—). In the presence of both tetracycline and edeine (an inhibitor of the D-site binding; [8,9]), $\bar{\nu}^{\epsilon}$ value is close to 0 (—△—). This means that $tRNA^{Phe}_{OH}$ binds specifically to the parts of the D and A sites belonging to the 30 S subunit.

Unlike the 30 S subunits, every 70 S ribosome binds at saturation up to 3 molecules of $tRNA^{Phe}_{OH}$ (fig.2A, —○—), but only 2 molecules of Phe-$tRNA^{Phe}$ (—●—) or Ac-Phe-$tRNA^{Phe}$ [10,11].

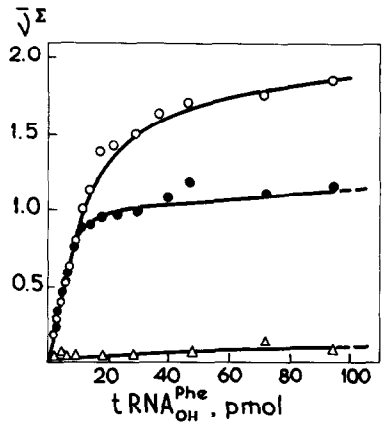

Fig.1. Titration of the 30 S·poly(U) complex with deacylated $tRNA^{Phe}_{OH}$: in the absence of antibiotics (—○—); in the presence of tetracycline (—●—); in the presence of edeine and tetracycline (—△—).

Comparing this result with that in fig.1, we can suggest that the third, E site on the 70 S ribosome is formed by its 50 S subunit. A direct experiment confirms this suggestion: addition of 50 S subunits to the complexes [30 S·poly(U)·$(tRNA^{Phe}_{OH})_2$] and [30 S·poly(U)·$(Phe\text{-}tRNA^{Phe})_2$] results in additional binding of a third $tRNA^{Phe}_{OH}$ molecule, but not Phe-$tRNA^{Phe}$ (cf. —○— and —●—, fig.2B).

Fig.3 shows the effect of antibiotics on the $tRNA^{Phe}_{OH}$–70 S ribosome interaction. In the

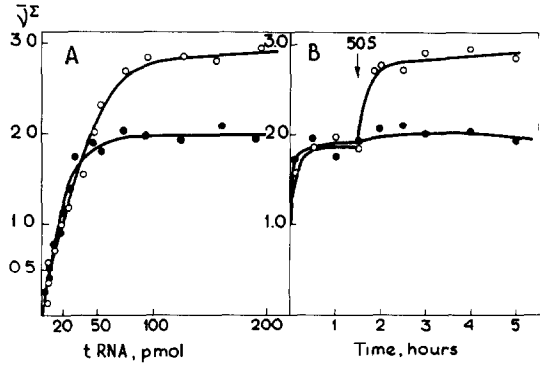

Fig.2. (A) Titration of the 70 S·poly(U) complex with [^{14}C]$tRNA^{Phe}_{OH}$ (—○—) and [^{14}C]Phe-$tRNA^{Phe}$ (—●—). (B) Kinetics of [^{14}C]$tRNA^{Phe}_{OH}$ (—○—) and [^{14}C]Phe-$tRNA^{Phe}$ (—●—) binding to the 30 S·poly(U) complex. After 90 min incubation at 0°C 50 S subunits were added, and the kinetic measurement continued. Each incubation mixture contained a saturating amount of labeled tRNA (120 pmol).

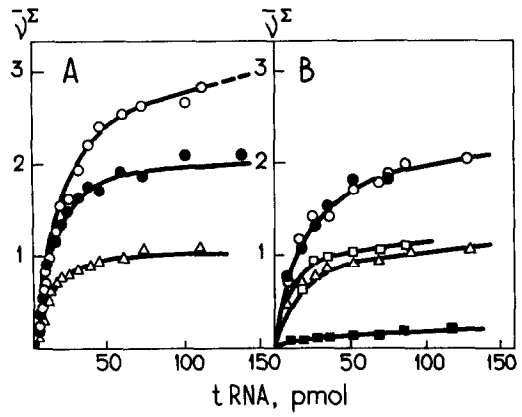

Fig.3. (A) Titration of the 70 S·poly(U) complex with [^{14}C]tRNA$^{Phe}_{OH}$: in the absence of antibiotics (—○—); in the presence of tetracycline (—●—) and tetracycline + edeine (—△—). (B) The same, but in the absence of poly(U). A control experiment with Ac-[^{14}C]Phe-tRNA^{Phe} was done in the absence (—□—) and in the presence (—■—) of edeine. (Without poly(U) Ac-Phe-tRNA^{Phe} binds exclusively at the D site [3,10]).

presence of poly(U) (fig.3A), tetracycline inhibits the binding of about 1 tRNA$^{Phe}_{OH}$ molecule (—●—), and tetracycline + edeine – about 2 tRNA$^{Phe}_{OH}$ molecules (—△—), obviously, at the D and A sites. Taking into account the results in fig.1 and 2, we can conclude, that the E site is insensitive to both antibiotics. In the absence of messenger, each ribosome binds readily 2 tRNA$^{Phe}_{OH}$ molecules (fig.3B, —○—). Tetracycline has no effect in this case (cf. —○— and —●—). This is not surprising, because the codon-less binding at the A site is not realized for all 3 biological forms of tRNA [10,12,13]. When tetracycline and edeine are both present, we find, again, binding of tRNA$^{Phe}_{OH}$ at the E site only (—△—). (A control experiment with Ac-Phe-tRNA^{Phe} shows clearly, that edeine is an effective inhibitor of D-site binding in the absence of mRNA; cf. —□— and —■—, fig.3B.)

It follows from these experiments, that we can measure selectively the binding properties of the E site in the presence of mRNA, using tetracycline and edeine, and in the absence of mRNA, using edeine alone. Titration curves, corresponding to the E-site binding in both cases (—△—, fig.3A,B), are represented in fig.4A in inverse coordinates.

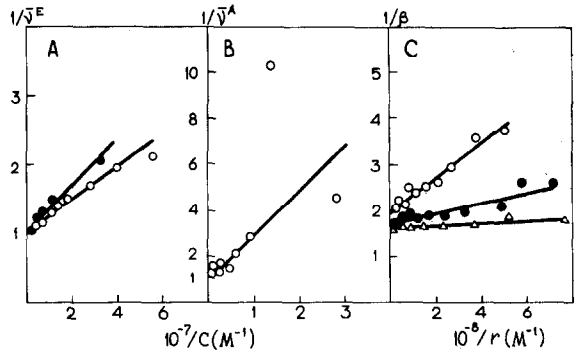

Fig.4. (A) The plot of $I/\bar{\nu}E$ vs I/C in the presence (—○—) and in the absence (—●—) of poly(U). (B) The plot of $I/\bar{\nu}A$ vs I/C (see, for details, the text). (C) The plot of I/β vs I/r, measured by the method of variable volume (details in [17]). Association constants of [^{14}C]tRNA$^{Phe}_{OH}$ with the D site of the 70 S·poly(U) complex (K^D_a) were determined in this case using the equation:

$$I/\beta = I/\gamma \cdot K^D_a \cdot r + I/\gamma$$

where:

β = the relative amount of [^{14}C]tRNA$^{Phe}_{OH}$ bound to ribosomes;

γ = the active fraction of [^{14}C]tRNA$^{Phe}_{OH}$;

r = the concentration of free 70 S·poly(U) complex

Incubation mixtures contained in 0.1–5.0 ml buffer TAM: 10 pmol 30 S subunits; 12 pmol 50 S subunits; 15 μg poly(U); and 8 pmol [^{14}C]tRNA$^{Phe}_{OH}$. Experiments were performed at 0°C and 6 mM (—○—), 10 mM (—●—) and 15 mM (—△—) [Mg^{2+}].

We see that:

(i) Both in the presence and absence of poly(U) all E sites are active in tRNA$^{Phe}_{OH}$ binding ($\bar{\nu}^E$ tends to I, when $C \longrightarrow \infty$);

(ii) The affinity constants for E site, K^E_a, are equal to 5×10^7 $M^{-1}$and 3×10^7 M^{-1}, respectively.

The difference between corresponding experimental points in curves —○— and —●— (fig.3A) can be treated, obviously, as the A-site binding; i.e., $\bar{\nu}^{\epsilon}$ (– tetracycline) $\bar{\nu}^{\epsilon}$ (+ tetracycline) $\simeq \bar{\nu}^A$. These data are plotted in fig.4B and allow one to estimate roughly the affinity constant of tRNA$^{Phe}_{OH}$ for the A site: $K^A_a \simeq 5 \times 10^6$ M^{-1}.

The affinity constants for the D site (K^D_a) were measured in a separate experiment under conditions when the ribosomes were in excess over tRNA$^{Phe}_{OH}$ and, therefore, only the D-site binding occurred (fig.4C). The K^D_a values appeared equal

to 5.5×10^8 M^{-1} at 6 mM, 1.5×10^9 M^{-1} at 10 mM and $\sim 4 \times 10^9$ M^{-1} at 15 mM [Mg^{2+}] and 0°C. At 20 mM [Mg^{2+}] virtually all active $tRNA_{OH}^{Phe}$ was bound (not shown), and we can only estimate the K_a^D value in this case; at least 10^{10} M^{-1}.

4. DISCUSSION

Here, we confirm data in [3,4] that 70 S ribosomes do contain a third, E site, physically distinct from the D and A sites and specific for deacylated tRNA. The main properties of this site can be summarized as follows:

(1) The affinity of deacylated tRNA for the E site is 2–3 orders of magnitude lower than that for the D site. On the contrary, in [3] the K_a^E/K_a^D ratio was claimed equal to 1. This discrepancy can be explained by their Scatchard plot containing few experimental points. Under conditions of excess ribosomes over tRNA, when the most stable binding can be detected, these points are absent.

(2) The E-site binding is rather insensitive to the template, as was revealed by 2 different experimental techniques: K_a^E-values are very close, both in the presence and in the absence of poly(U) (nitrocellulose filter technique, this paper); total non-specific deacylated tRNA ($tRNA^{-Phe}$) binds, in the presence of poly(U), as effectively as specific $tRNA_{OH}^{Phe}$ does (equilibrium sedimentation, [4]). These results differ strongly from those in [3], where an absolute dependence of the E-site binding on messenger was observed; i.e., the binding of only one $tRNA_{OH}^{Phe}$ molecule/70 S ribosome in the absence of poly(U). This contradiction can be related rather to the difference in ribosomal preparations than in medium conditions used, because at 15 mM [Mg^{2+}], 160 mM [NH_4^+] and 37°C (as in [3]) we also observed the binding of 2 $tRNA_{OH}^{Phe}$ molecules/1 non-programmed 70 S ribosome (not shown).

(3) The binding to the E site is insensitive to tetracycline and edeine, specific inhibitors of the D and A sites. This property is useful for discrimination between all 3 ribosomal sites, as shown here.

(4) The E site is introduced into a 70 S ribosome by its 50 S subunit. It is possible that only the E-site binding was observed [14,15], when studying the interaction between deacylated tRNA and 50 S subunits (discussed in [4]).

The role of the E site in protein biosynthesis is not yet clear. Its functional significance as an 'exit' site, as postulated in [1,16], remains to be proven.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge T.N. Makhno, N.P. Kleopina and A.A. Zverev for excellent technical assistance.

REFERENCES

[1] Wettstein, F.O. and Noll, H. (1965) J. Mol. Biol. 11, 35–53.
[2] Watson, J.D. (1964) Bull. Soc. Chim. Biol. 46, 1399–1425.
[3] Rheinberger, H.-J., Sternbach, H. and Nierhaus, K.H. (1981) Proc. Natl. Acad. Sci. USA 78, 5310–5314.
[4] Grajevskaja, R.A., Ivanov, Yu.V. and Saminsky, E.M. (1982) Eur. J. Biochem. 128, 47–52.
[5] Kirillov, S.V., Makhno, V.I. and Semenkov, Yu.P. (1980) Nucleic Acids Res. 8, 183–196.
[6] Rappaport, H. and Lapidot, Y. (1974) Methods Enzymol. 29E, 685–693.
[7] Semenkov, Yu.P., Katunin, V.I., Makarov, E.M. and Kirillov, S.V. (1982) FEBS Lett. 144, 121–124.
[8] Obrig, T., Irvin, J., Culp, W. and Hardesty, B. (1971) Eur. J. Biochem. 21, 32–41.
[9] Szer, W. and Kurilo-Borowska, Z. (1972) Biochim. Biophys. Acta 259, 357–368.
[10] Odinzov, V.B. and Kirillov, S.V. (1978) Nucleic Acids Res. 5, 3871–3879.
[11] Kirillov, S.V. and Semenkov, Yu.P. (1982) FEBS Lett. 148, 235–238.
[12] Peters, M. and Yarus, M. (1979) J. Mol. Biol. 134, 471–491.
[13] Kirillov, S.V., Katunin, V.I. and Semenkov, Yu.P. (1981) FEBS Lett. 125, 15–19.
[14] Cannon, M., Krug, R. and Gilbert, W. (1963) J. Mol. Biol. 7, 360–378.
[15] Grajevskaja, R.A., Saminsky, E.M. and Bresler, S.E. (1972) Biochem. Biophys. Res. Commun. 49, 635–640.
[16] Nierhaus, K.M. (1982) Curr. Topics Microbiol. Immunol. 97, 81–155.
[17] Kirillov, S.V., Kemkhadze, K.Sh., Makarov, E.M., Makhno, V.I., Odintsov, V.B. and Semenkov, Yu.P. (1980) FEBS Lett. 120, 221–224.