Kinetic aspects of tetracycline action on the acceptor (A) site of *Escherichia coli* ribosomes

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

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

Received 14 May 1982

1. INTRODUCTION

In many investigations on mechanisms of in vitro protein biosynthesis, it is often necessary to know the real distribution of different forms of tRNA between ribosomal donor (D) and acceptor (A) sites. The best known test for this purpose is inhibition of the A-site binding of tRNA by tetracyclines (TC). It is generally accepted, that TC, at moderate concentrations ($\leq 10^{-4}$ M), quantitatively blocks tRNA binding to the A, but not to the D site. In particular, with peptidyl-tRNA, complementary information can be obtained by using the puromycin reaction (reviews [1–3]).

Here, we show that a more complex character of TC action can be detected, when the binding of aa-tRNA to the A site is measured in a strictly quantitative way. Moreover, 2 different modes of inhibition by the antibiotic can be distinguished. If the resulting complexes of aa-tRNA with the A site are characterized by high stability (i.e., formed in the presence of the elongation factor (EF) Tu and GTP, or alternatively without the factor, but peptide bond formation was allowed to occur), TC slows down the kinetics of binding, but does not affect its final level. When the complex of aa-tRNA with the A site is formed in the absence of EF-Tu and GTP, and peptide bond formation is blocked, this type of binding has been proven to be reversible and relatively weak $K_a^A \sim 2–3 \cdot 10^7$ M^{-1}, [7]. In this case the main effect of TC is the decrease of K_a^A-value by 1 order of magnitude.

2. MATERIALS AND METHODS

30 S and 50 S ribosomal subunits were isolated from *E.coli* MRE-600 as in [4]. Enriched preparations of [^{14}C]Phe-tRNA^{Phe} or [^{3}H]Phe-tRNA^{Phe} (1400 pmol/A_{260} unit) and Ac-[^{14}C]Phe-tRNA$^{Phe}_{ox\text{-}red}$ (1350 pmol/A_{260} unit), as well as fractionated poly(U) (30 000 M_r) were prepared as in [5–7]. In all experiments (with the exception of one in fig.1D) we used TC at low concentration ($2 \cdot 10^{-5}$ M) to exclude any unspecific action of the antibiotic on the D site. $\bar{\nu}^{\Sigma}$, $\bar{\nu}^{D}$ and $\bar{\nu}^{A}$ are numbers of tRNA molecules bound to one ribosome or to its D and A sites, respectively. K_a^A is association constant of tRNA with the A site. Tetracycline and kanamycin were from Serva (Heidelberg). [^{14}C]Phe (318 mCi/mmol) was from 'UVVVR' (CSSR) and [^{3}H]Phe (15.5 Ci/mmol) from 'Isotop' (USSR). Details of experiments are given in the figure legends. Amounts of ribosomes with ([^{14}C]Phe)$_2$–tRNA^{Phe} were determined as in [4].

3. RESULTS

We used, throughout this study, ribosomes with D and A sites both fully active in binding experiments. This was advantageous for the correct interpretation of the data described below.

To characterize comparatively both thermodynamic and kinetic aspects of aa-tRNA interaction with mRNA-programmed ribosomes, and TC inhi-

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

bitory efficiency in both cases, we reproduced, with more details, the experiment on titration of vacant 70 S . poly(U) complexes with Phe–tRNA^{Phe} (as

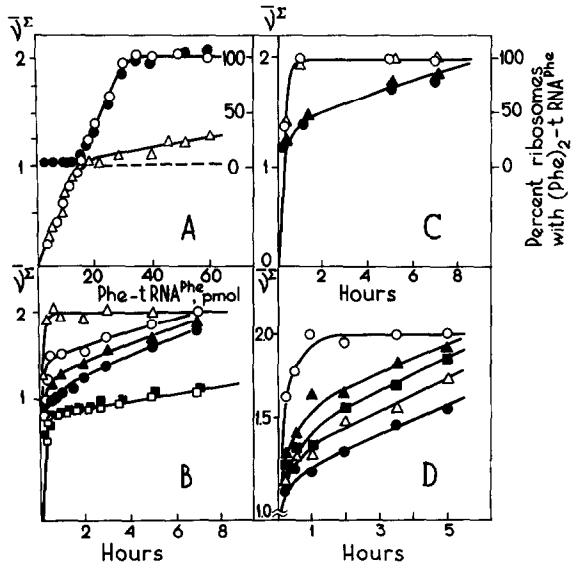

Fig.1. Binding of 2 molecules of Phe-tRNA^{Phe} to the vacant 70 S . poly(U) complex in the absence of EF-T$_u$ and GTP. (A). Titration of the 70 S . poly(U) complex with [^{14}C]Phe-tRNA^{Phe}. Mixtures contained in 200 μl buffer I (0.02 M Tris–HCl, (pH 7.4); 0.02 M $MgCl_2$; 0.2 M NH_4Cl; 0.001 M EDTA): 10 pmol 30 S subunits, 12 pmol 50 S subunits, 5 μg poly(U) and 2.5–70 pmol [^{14}C]Phe-tRNA^{Phe}. After 90 min incubation at 0°C $\bar{\nu}^{\Sigma}$-values were determined by the nitrocellulose filter technique (–○–). Parallel samples were used to measure the binding in the presence of 2 . 10^{-5} M TC (–△–) or to determine portions of ribosomes bearing ([^{14}C]-Phe)$_2$-tRNA^{Phe} (in the absence of TC; –●–). (B–D). Kinetics of the binding of Phe-tRNA^{Phe} to the 70 S . poly(U) complex. Incubation mixtures contained in 200 μl buffer I: 10 pmol 30 S subunits, 12 pmol 50 S subunits, 10 μg poly(U) and amounts indicated below [^{14}C]Phe-tRNA^{Phe}. (B). Mixtures contained 58 (–△–), 34 (–○–) and 20 (–□–) pmol [^{14}C]Phe-tRNA^{Phe}; closed symbols, analogous experiments in the presence of 2 . 10^{-5} M TC. (C). Mixtures contained 58 pmol [^{14}C]Phe-tRNA^{Phe} in the absence (–○–) or presence (–●–) of 2 . 10^{-5} M TC. Parallel samples were used to determine portions of ribosomes bearing ([^{14}C]Phe)$_2$-tRNA^{Phe} (–△–, without and –▲–, with TC). (D). Mixtures contained 62 pmol [^{14}C]Phe-tRNA^{Phe} without TC (–○–) and with 10^{-6} M (–▲–), 5 . 10^{-6} M (–■–), 10^{-5} M (–△–) and 10^{-4} M (–●–) TC.

in [8]). Up to $\bar{\nu}^{\Sigma}$-values close to unity (fig.1A), we find in ribosomes only Phe-tRNA, but not (Phe)$_2$-tRNA^{Phe} (–●–). As a second molecule of aa-tRNA per ribosome iis bound, synchronous formation of (Phe)$_2$-tRNA^{Phe} does readily occur. This means, that over 0–1 $\bar{\nu}^{\Sigma}$, the same sites are occupied in all ribosomes; these sites are TC-resistant, i.e., they are D sites (cf. –○– and –△– at low inputs of Phe-tRNA^{Phe}). Successive filling of the A sites is possible only at higher concentrations of aa-tRNA. These results, combined with [8,9], represent more strict evidence that the D site of vacant 70 S . poly(U) complex is thermodynamically preferable for Phe-tRNA^{Phe} when the binding is carried out non-enzymatically. We find, further, that TC does not quantitatively block the A-site binding. The extent of inhibition depends on both aa-tRNA concentration (–△–) and incubation times (as will be seen below). The latter circumstance induced us to study kinetic aspects of TC action on ribosomal A and D sites.

Fig. 1B shows the kinetics of binding of Phe-tRNA^{Phe} in the absence of EF-T$_u$ and GTP. At different excesses of aa-tRNA over ribosomes, one ribosomal site is filled very fast (within 1–2 min) and independent of TC; obviously, it is the D site. The binding to the second, i.e., A site is much slower and strongly depends on aa-tRNA concentration. TC only decelerates this process, but does not decrease the final level of binding. Interestingly, the most inhibitory effect of the antibiotic is observed at high concentrations of aa-tRNA; at lower concentrations of the latter the inhibition becomes negligible and tends to zero (cf. –□– and –■–).

Analysis for (Phe)$_2$-tRNA^{Phe} synthesis showed that the portion of ribosomes with dipeptides formed is always equal to that with the A site occupied, irrespective of whether TC is present or not (fig.1C). This means that TC, per se, does not prevent correct positioning of aa-tRNA at the functional A site, but only decelerates some stage(s) of this complicated process. The binding of resulting depeptidyl-tRNA to the A site is very stable: successive raising of temperature till 30°C accompanied by 3-fold dilution of the mixtures does not lead to any dissociation of (Phe)$_2$-tRNA^{Phe} from ribosomes after 4 h additional incubation (not shown). The situation described above is observed in a wide range of TC concentrations (fig.1D).

The next experiment was performed in two steps

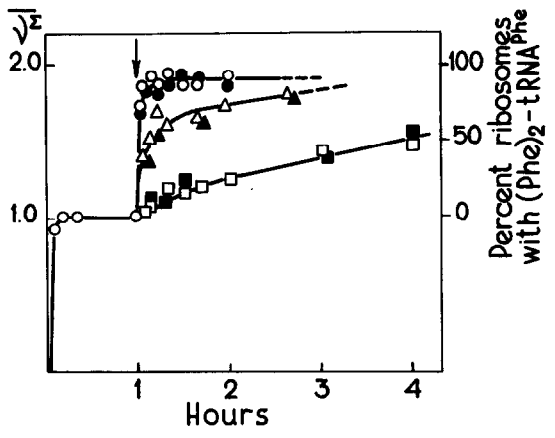

Fig.2. Comparative kinetics of Phe-tRNA^{Phe} binding to the A site and (Phe)$_2$-tRNA^{Phe} synthesis in the presence and absence of EF-Tu and GTP. Incubation mixtures contained in 200 μl buffer I (with 10 mM [Mg^{2+}]): 10 pmol 30 S subunits, 12 pmol 50 S subunits, 10 μg poly(U) and 12 pmol [^{14}C]Phe-tRNA^{Phe}. After 60 min incubation at 0°C the second portion of aa-tRNA (12 pmol) was added in the presence of 2 pmol EF-Tu and GTP (final conc. $3 \cdot 10^{-4}$ M), and kinetics of the binding was measured (—○—). Analogous experiments were made without the factor (—△—) and with EF-Tu and GTP, but in the presence of $2 \cdot 10^{-5}$ M TC (—□—); closed symbols, corresponding experiments on kinetics of ([^{14}C]Phe)$_2$-tRNA^{Phe} synthesis.

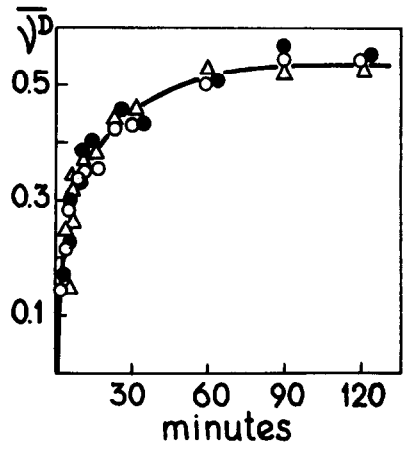

Fig.3. Kinetics of Phe-tRNA^{Phe} binding to the D site of 70 S · poly(U) complex. Incubation mixtures contained in 1 ml buffer I (6 mM Mg^{2+}): 10 pmol 30 S subunits, 12 pmol 50 S subunits, 10 μg poly(U) and 8.2 pmol [^{14}C]Phe-tRNA^{Phe}. Incubation was performed at 0°C without antibiotics (—○—) and in the presence of $2 \cdot 10^{-5}$ M TC (—●—) or 10^{-4} M kanamycin (—△—).

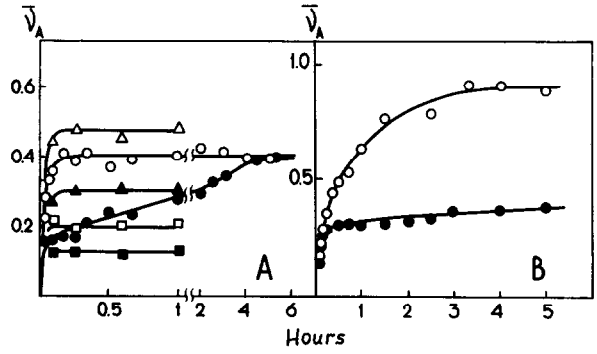

Fig.4. Kinetics of Phe-tRNA^{Phe} binding to the A site of 70 S · poly(U) complex. Incubation mixtures contained in 100 μl buffer I (with 10 mM [Mg^{2+}]): 10 pmol 30 S subunits, 12 pmol 50 S subunits, 10 μg poly(U) and 20 pmol Ac-[^{14}C]Phe-tRNA$^{Phe}_{ox\text{-}red}$, and were incubated for 2 h at 0°C (the first stage). At the second stage: (A): 1.1 (—■—); 2.0 (—□—); 3.2 (—▲—); 4.1 (—○—) and 4.7 (—△—) pmol [^{3}H]Phe-tRNA^{Phe} was added to each mixture in the presence of 2 pmol EF-T$_u$ and GTP (final conc. $3 \cdot 10^{-4}$ M); (—●—) the experiment analogous to (—○—), but in the presence of $2 \cdot 10^{-5}$ M TC (B) 20 pmol [^{3}H]Phe-tRNA^{Phe} was added to the mixtures without EF-Tu and GTP in the absence (—○—) or presence (—●—) of $2 \cdot 10^{-5}$ M TC. The level of Ac-[^{14}C]Phe-tRNA$^{Phe}_{ox\text{-}red}$ bound was constant ($\bar{\nu}^D \sim 1.0 \pm 0.1$) during the second stage of the experiments.

(fig.2). Initially, the D site was filled by Phe-tRNA^{Phe}, when nearly stoichiometric amounts of the latter were added to the 70 S · poly(U) complex. Then a second portion of aa-tRNA was added with or without EF-T$_u$ and GTP. We see that the factor drastically accelerates A-site binding and successive peptide bond formation. TC, again, only decelerates both processes, as in case of factor-free system (see fig.1).

Further, we checked, in more detail, the action of TC separately on the D and A sites. The first is easily realized, if ribosomes are in excess over aa-tRNA ([8,9] and fig.1A). To visualize the kinetics of binding, we used simultaneously both large volumes of incubation mixtures and low [Mg^{2+}] (6 mM). TC affects neither kinetics nor the final level of D-site binding (fig.3). The antibiotic kanamycin, an inhibitor of translocation [10,11], reveals no effect either. This result suggests that aa-tRNA binds directly to the D site, not via a transient binding to

Table 1

Characteristics of Phe-tRNA^{Phe} binding to the A site of 70 S ribosomes and TC action on different types of the A-site binding

Expt. no.	EF-T_u and GTP	Peptide bond formation	Kinetics of the binding	Relative stability	TC affects		Fig. no.
					Kinetics of the binding	Final level	
1	–	+	Slow	High	+	–	1
2	+	+	Fast	High	+	–	2
3	+	–	Fast	High	+	–	4A
4	–	–	Slow	Moderate ($K_a^A \sim 3 \times 10^7$ M^{-1})	?	+	4B

the functional A site. Similar results were reported with respect to peptidyl-tRNA and deacylated tRNA [12–14]. So, we can assume, that such behaviour of the system is common for all forms of tRNA^{Phe} (peptidyl-, aminoacyl- and deacylated) and governed rather by the structure of tRNA^{Phe} itself, than by the nature of the residue attached (or not) to its 3′-terminus. From the experiment in fig.3, we can also estimate the association rate constant of aa-tRNA to the D site. It appeared $\sim 10^7$ l . mol^{-1} . min^{-1}, 50-fold higher than that for factor-free A-site binding [7].

To bind Phe-tRNA^{Phe} to the A site only, we used the system in [7]. At first the D site was saturated with an analogue of peptidyl-tRNA (Ac-Phe-tRNA$^{Phe}_{ox\text{-}red}$) exempt from donor activity [15,16]; then [^{3}H]Phe-tRNA^{Phe} was added. In the presence of EF-Tu and GTP the kinetics of binding is fast (fig.4A) and the resulting complex of aa-tRNA with the A site is characterized by high stability, because:

(i) All [^{3}H]Phe-tRNA^{Phe} added binds to the A site even if a catalytic amount of EF-Tu is present (see, for details, legend to fig.4A);

(ii) No exchange of bound [^{3}H]Phe-tRNA^{Phe} with a 10-fold excess of unlabeled one in solution was detected during 8 h incubation at 0°C (not shown).

TC, as in the experiment in fig.2, (where peptide bond synthesis was allowed to occur), only decelerates the binding, but does not affect its final level. Non-enzymatic binding of aa-tRNA to the A site in such a system is less stable, and its reversibility under these conditions was demonstrated in [7]. In this case, TC reveals a different mode of action (fig.4B): its main effect consists in a reduction of equilibrium level of binding due to the decrease of K_a^A to $\sim 3 \cdot 10^6$ M^{-1}, as compared with $\sim 3 \cdot 10^7$ M^{-1} in the absence of the antibiotic (calculated from the data in fig.4B).

4. CONCLUSION

We have considered 4 types of interaction of aa-tRNA with the ribosomal A site: factor-dependent and factor-free, with or without peptide bond synthesis; experimental data are summarized in table 1. Tetracycline never blocks the A-site binding completely; moreover, it reveals two different modes of action. Obviously, this facts reflects the complexity of the binding process. Many data lead to the suggestion that positioning of aa-tRNA at the functional A site involves, at least, two steps [7,17–21]. The overall reaction can be described by some equation(s), including intrinsic rate constants, which characterize each of the steps. It is likely that TC affects one (or more) of the rate constants. As regards inhibition, this must lead either to a deceleration of the binding, if the latter is irreversible under experimental conditions used (as in experiments 1–3, table 1), or to a decrease of the equi-

librium association constant, if the binding is reversible (as in expt.4, table 1).

Thus, for the correct interpretation of experiments on TC action, it is necessary to take into account not only the concentration of the antibiotic, but the concentration of aa-tRNA, the type of binding and the kinetics of binding as well.

REFERENCES

[1] Gale, E.F., Cundliffe, E., Reynolds P.E. and Richmond, M.H. (1972) in: The Molecular Basis of Antibiotic Action, J. Wiley London, New York.

[2] Vazquez, D. (1979) in: Inhibitors of Protein Synthesis, Springer-Verlag, Berlin, New York.

[3] Nierhaus, K.H. and Wittmann, H.G. (1980) Naturwissenschaften 67, 234–250.

[4] Kirillov, S.V., Machno, V.I. and Semenkov, Yu.P. (1980) Nucleic Acids Res. 8, 183–196.

[5] Kirillov, S.V., Makhno, V.I. and Semenkov, Yu.P. (1976) Dokl. Acad. Nauk. SSSR 229, 488–491.

[6] Semenkov, Yu.P., Makhno, V.I. and Kirillov, S.V. (1976) Molekul. Biol. 10, 754–763.

[7] Kemkhadze, K.Sh., Odintsov, V.B., Semenkov, Yu.P. and Kirillov. S.V. (1981) FEBS Lett. 125, 10–14.

[8] 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.

[9] Kemkhadze, K.Sh., Odintsov V.B., Makhno V.I., Semenkov, Yu.P. and Kirillov, S.V. (1981) Molekul. Biol. 15, 779–789.

[10] Modolell, J. and Vazquez, D. (1977) Eur. J. Biochem. 81, 491–497.

[11] Misumi, M., Nishimura, T., Komai, T. and Tanaka, N. (1978) Biochem. Biophys. Res. Commun. 84, 358–365.

[12] Watanabe, S. (1972) J. Mol.Biol. 67, 443–457.

[13] Wurmbach, P. and Nierhaus, K.H. (1979) Proc. Natl. Acad. Sci. USA 76, 2143–2147.

[14] Nierhaus, K.H., Rheinberger, H., Wurmbach, P. and Bergemann, K. (1980) in: Biological Implications of Protein–Nucleic Acids Interactions (Augustyniak ed) pp. 121–131, Poznan.

[15] Ofengand, J. and Chen, C.-M. (1972) J.Biol.Chem. 247, 2049–2058.

[16] Chinali, M., Sprinzl, M., Parmeggiani, A. and Cramer, F. (1974) Biochemistry 13, 3001–3010.

[17] Haenni, A.-L. and Lucas-Lenard, J. (1968) Proc. Natl. Acad. Sci. USA 61, 1363–1369.

[18] Thach, S. and Thach, R. (1971) Proc. Natl. Acad. Sci. USA 68, 1791–1795.

[19] Lake, J. (1977) Proc. Natl. Acad. Sci. USA 74, 1903–1907.

[20] Johnson, A., Fairclough, R. and Cantor, C. (1977) in: Nucleic Acids–Protein Recognition, pp. 469–490, Academic Press, New York.

[21] Thompson, R., Dix, D. and Eccleston, J. (1980) J.Biol.Chem. 255, 11088–11090.