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# Puromycin reaction for the A site-bound peptidyl-tRNA

Yu. Semenkov, T. Shapkina, V. Makhno and S. Kirillov

*B.P. Konstantinov Petersburg Nuclear Physics Institute, Academy of Sciences, 188350 Gatchina, Saint Petersburg district, USSR*

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AcPhe$_2$-tRNA$^{Phe}$ synthesized in 70S ribosomes after consecutive binding of AcPhe-tRNA$^{Phe}$ at the P sites and EF-Tu-directed binding of Phe-tRNA$^{Phe}$ at the A sites is able to react quantitatively with puromycin in the absence of EF-G. A detailed study of the kinetics of the puromycin reaction, its comparison with that of spontaneous translocation, the use of antibiotic viomycin as an effective inhibitor of spontaneous translocation revealed that, besides spontaneous translocation, this peptidyl-tRNA could react with puromycin being located at the A site. This leads to the conclusion that the transpeptidation reaction per se triggers conformational changes in the ribosomal complex bringing the 3'-end of a newly synthesized peptidyl-tRNA nearer to the peptidyl-site of the peptidyltransferase center. This is detected functionally as the ability of such an A site bound peptidyl-tRNA to react with puromycin. This reaction is highly pronounced at elevated (25°C) temperature but can be hardly detected at 0°C.

70S Ribosome; A site; Puromycin reaction; Viomycin; Spontaneous translocation

## 1. INTRODUCTION

The majority of the models of protein biosynthesis accept operational definition of the P and A sites based on the puromycin reaction of the bound peptidyl-tRNA, namely: a positive puromycin reaction indicates the P-site location of peptidyl-tRNA whereas a negative one is evidence for its A-site location. It is generally accepted that the A site-bound peptidyl-tRNA acquires the capability to react with puromycin only after translocation.

It should be noted, however, that the puromycin reaction per se is an exclusive test for a peptidyl residue in the peptidyl-tRNA molecule rather than for the tRNA moiety itself. This principal uncertainty of the puromycin test becomes important and should be taken into account in the case where the peptidyl-tRNA might occupy some intermediate (hybrid) states during its movement through the translating ribosome. The existence of such states has been proposed, indeed, many years ago by Bretscher [1] and Spirin [2] and was recently confirmed by Moazed and Noller [3]. Using the structural approach they have found that after or during peptide bond formation the 3'-ends of two tRNAs move with respect to the 50S subunit; the deacylated tRNA binds to the 50S E site (hybrid P/E state) whereas the newly formed peptidyl-tRNA binds to the 50S P site (hybrid A/P state).

In this work we show that the peptidyl-tRNA (AcPhe$_2$-tRNA$^{Phe}$) synthesized in ribosomes is able to react with puromycin when bound at the A site. This means that this peptidyl-tRNA can be detected in the hybrid state of the ribosomal A site (A/P state according to the nomenclature in [3]) in a functional way too. The essential feature of such an 'A-site puromycin reaction' is its rate constant which is at least two orders of magnitude lower when compared with that for the P site-bound AcPhe$_2$-tRNA$^{Phe}$. It follows from these results also that: (i) the puromycin reaction detects, indeed, localization of the peptidyl residue but not the tRNA moiety (see above); (ii) the operational definition of the P and A sites (i.e. more strictly, the P/P and A/P states [3] corresponding functionally to the post- and pretranslocative states) for the growing peptidyl-tRNA should be based on kinetic discrimination between puromycin reactions of the P site- and A site-bound peptidyl-tRNA rather than on a 'positive' or 'negative' puromycin reaction.

*Correspondence address:* S. Kirillov, B.P. Konstantinov Petersburg Nuclear Physics Institute, Academy of Sciences, 188350 Gatchina, Saint Petersburg district, USSR. Fax: (7)(812) 71-37196.

## 2. MATERIALS AND METHODS

The isolation of highly active 'tightly coupled' 70S ribosomes and enriched Ac[$^{14}$C]Phe-tRNA$^{Phe}$ and [$^3$H]Phe-tRNA$^{Phe}$ (1600–1800 pmol/$A_{260}$ unit) was described earlier [4,5]. Elongation factors Tu·Ts and G were purified as in [6]. In the experiments puromycin (Calbiochem), viomycin (Sigma), and labelled [$^3$H]- and [$^{14}$C]phenylalanine (UVVVR) with specific activities 800 dpm/pmol and 1900 dpm/pmol, respectively, were used.

The pretranslocative complexes with Ac[$^{14}$C]Phe[$^3$H]Phe-tRNA$^{Phe}$ at the A sites were prepared at 0°C as follows. In the first step mixtures contained in 50 μl of the buffer A: 10 pmol 70S ribosomes, 5 μg poly(U) and 12–14 pmol Ac[$^{14}$C]Phe-tRNA$^{Phe}$. After 100% occupation of the P sites [4] 10 μl of the ternary complex were added containing 10 pmol EF-Tu·Ts, 8–9 pmol active [$^3$H]Phe-tRNA$^{Phe}$ and GTP (final concentration $5 \cdot 10^{-4}$ M). All [$^3$H]Phe-tRNA$^{Phe}$ added was bound to ribosomes. According to HPLC analysis [7] 85% ribosomes bearing bound [$^3$H]Phe-tRNA$^{Phe}$ formed Ac[$^{14}$C]Phe[$^3$H]Phe-tRNA$^{Phe}$.

All experiments on the measuring of the kinetics of the puromycin reaction (except that shown in Fig. 2B) were carried out at 25°C. The components needed for each experiment were added to the complexes prior to raising the temperature. The amounts of Ac[$^{14}$C]Phe[$^{3}$H]Phe-puromycin formed were determined as in [8] and represented in this work as percentage $^{3}$H radioactivity extractable into ethylacetate with respect to the amount of Ac[$^{14}$C]Phe[$^{3}$H]Phe-tRNA$^{Phe}$ initially formed in ribosomes.

Final concentrations of the antibiotics were $10^{-3}$ M for puromycin and $10^{-4}$ M for viomycin. The low-magnesium buffer A contained, according to recommendations in [9], 20 mM Tris-HCl, pH 7.7, 25° C; 6 mM $MgCl_2$, 200 mM $NH_4Cl$; 0.6 mM spermine; 0.4 mM spermidine; 6 mM 2-mercaptoethanol.

## 3. RESULTS

If puromycin and EF-G are added to the pretranslocative complexes (see section 2) followed by raising the temperature from 0 to 25°C, all AcPhe$_2$-tRNA$^{Phe}$ rapidly reacts with puromycin, obviously as a result of the factor-dependent translocation (Fig. 1A,1). In the absence of EF-G, AcPhe$_2$-tRNA$^{Phe}$ also completely reacts with puromycin although much slower (Fig. 1A,2). The latter result could be readily explained by the high ability of this peptidyl-tRNA to translocate spontaneously [10]. If so, AcPhe$_2$-tRNA$^{Phe}$ must reveal both a fast and quantitative puromycin reaction after either several minutes preincubation of the complex with EF-G or 60 min preincubation in its absence as can be expected from the data in Fig. 1A,1 and 2, respectively. This is the case when EF-G is present (Fig. 1B,1). Surprisingly, the kinetics of the puromycin reaction in the system without EF-G appeared to be strikingly heterogeneous: approx. 50% AcPhe$_2$-tRNA$^{Phe}$ reacts rapidly (Fig. 1B,2, fast phase) whereas the other half of peptidyl-tRNA forms AcPhe$_2$-puromycin very slowly and the rate of this process (Fig. 1B,2, slow phase) is comparable with that in Fig. 1A,2.

The control result in Fig. 1B,1 indicates that the puromycin reaction for AcPhe$_2$-tRNA$^{Phe}$ already pretranslocated at the P sites requires 10–20 s for its completion. This means that after 60 min preincubation of the system without EF-G, spontaneous translocation occurred in only ~50% (compare Fig. 1B,1 and 2) but not in all pretranslocative complexes, as was expected initially. This makes us draw the conclusion that the rest of peptidyl-tRNA has been able to react with puromycin not being located at the P sites. The simplest, and at the same time, paradoxical explanation is that the A site-bound AcPhe$_2$-tRNA$^{Phe}$ is able to react with puromycin too.

To demonstrate the possibility of such an 'A-site puromycin reaction' more clearly, it is necessary to construct a system where AcPhe$_2$-tRNA$^{Phe}$ could be both synthesized and stopped at the A site. We used for this purpose the antibiotic viomycin which is known as an effective inhibitor of spontaneous translocation [10].

Fig. 2A,1 shows the kinetics of spontaneous translocation measured by a routine procedure: the pretranslocative complexes incubated at 25°C for the indicated times were chilled to 0°C (to stop spontaneous translocation [10]; see below), and the puromycin reaction was carried out at this temperature for 20 min to determine the portions of AcPhe$_2$-tRNA$^{Phe}$ at the P sites. We observed, first of all, that the rate of real spontaneous translocation is noticeably slower than that of the process shown in Fig. 1A,2. Then, the spontaneous translocation of approx. 50% AcPhe$_2$-tRNA$^{Phe}$ takes, indeed, 60 min as had been deduced above after comparison of the curves 1 and 2, Fig. 1B. Viomycin, as can be seen in Fig. 2A,2, apparently stops

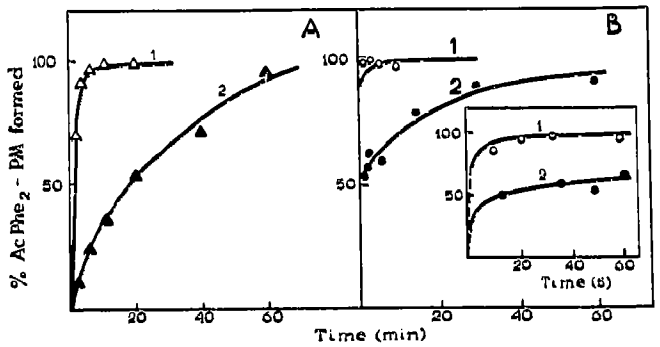


Fig. 1. Kinetics of AcPhe$_2$-puromycin formation in the presence and absence of EF-G. A. The pretranslocative complex was formed at 0° C as described in section 2. Then it was subdivided into four portions, puromycin was added, with or without EF-G, to two of them, and after raising temperature to 25°C, the kinetics of Ac[$^{14}$C]Phe[$^{3}$H]Phe-puromycin formation was measured (1 and 2 respectively). B. Two other portions were preincubated either for 5 min in the presence of stoichiometric amounts of EF-G or 60 min in its absence, then puromycin was added followed by measurement of the kinetics of AcPhe$_2$-puromycin formation (1, +EF-G; 2, −EF-G). Insert: the same but within 0–1 min.

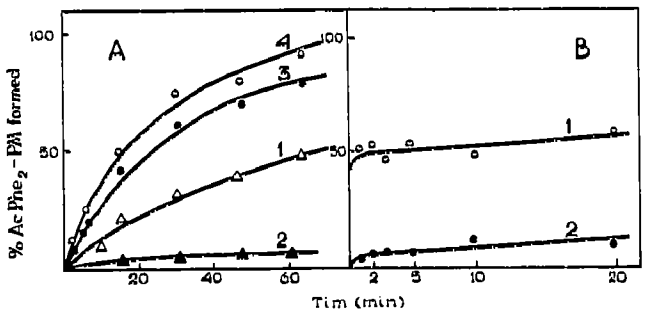


Fig. 2. The use of viomycin to discriminate between spontaneous translocation and 'the A-site puromycin reaction'. A Kinetics of spontaneous translocation at 25°C measured in the absence and presence of viomycin; 1 and 2, respectively (for details, see the text); 3, kinetics of the puromycin reaction in the presence of viomycin; 4, kinetics of the puromycin reaction without viomycin (taken from Fig. 1A,2). B. The pretranslocative complexes were preincubated at 25°C for 60 min without or with viomycin. Then they were chilled to 0°C followed by measurement of the kinetics of the puromycin reaction at this temperature (1, −viomycin; 2, +viomycin).

the spontaneous translocation, leaving $AcPhe_2$-$tRNA^{Phe}$ at the A sites. Therefore, the kinetics of $AcPhe_2$-puromycin formation obtained in the presence of viomycin (Fig. 2A,3) reflects the kinetics of the puromycin reaction for the A site-bound $AcPhe_2$-$tRNA^{Phe}$ in a pure form.

These results allow us to decipher the evens 'hidden' in Fig. 1A,2 and 1B,2. When the pretranslocative complex is incubated without puromycin, $AcPhe_2$-$tRNA^{Phe}$ is involved in a slow process, i.e. spontaneous translocation (Fig. 2A,1). If the latter is blocked but puromycin is present in the system, $AcPhe_2$-$tRNA^{Phe}$ participates in another slow process, i.e. 'the A-site puromycin reaction' (Fig. 2A,3). So incubation of the system with puromycin when spontaneous translocation is allowed to proceed (–viomycin) leads to the situation where $AcPhe_2$-$tRNA^{Phe}$ is involved simultaneously in these two parallel slow processes (Fig. 2A,4 identical to Fig. 1A,2). Preincubation of the pretranslocative complex without antibiotics results in the accumulation of $AcPhe_2$-$tRNA^{Phe}$ at the P sites due to spontaneous translocation (~50% after 60 min preincubation, see Fig. 2A,1). That is why we observe in this case, after the addition of puromycin, the two-phase kinetics of the puromycin reaction: the P site-bound $AcPhe_2$-$tRNA^{Phe}$ reacts extremely rapidly (see Fig. 1B,2, insert), whereas the behaviour of the second portion of peptidyl-tRNA still bound at the A sites is explained, obviously, by the processes just described above.

In conclusion, we feel the necessity to answer the questions: 'why has the A-site puromycin reaction not been noticed earlier?' The main reason is that the puromycin reaction is usually carried out at 0°C to avoid both spontaneous translocation and the problem of 'puromycin overreaction' [11,12]. In the experiment shown in Fig. 2B,2 the pretranslocative complexes were incubated at 25°C for 60 min without and with viomycin. Then the mixtures were chilled to 0°C and the kinetics of the puromycin reaction were measured at this temperature (curves 1 and 2, respectively). In the presence of viomycin when spontaneous translocation is negligible, a very small slope of curve 2 obviously reflects extremely slow rate of the A-site puromycin reaction. It is clear, therefore, that it could hardly be detected at 0°C. On the other hand, the P site-bound $AcPhe_2$-$tRNA^{Phe}$ reacts with puromycin rapidly at 0°C (–viomycin; the first point in curve 1). The further course of this curve shows that the rate of spontaneous translocation at 0°C is negligible too, and its extent (after incubation at 25°C) can be measured correctly enough, independent of whether the puromycin reaction is carried out at 0°C for 1 or 20 min. It is not so if the value at elevated (25°C) temperature is measured. In this case it is necessary to perform 'the kinetic discrimination' between the P site- and the A site-bound $AcPhe_2$-$tRNA^{Phe}$, i.e. to carry out the puromycin reaction within 1 min (see Fig. 1B,2 and insert) to avoid some sort of the 'puromycin overreaction' brought about by both the A-site puromycin reaction and spontaneous translocation (Fig. 1B,2, slow phase).

## 4. DISCUSSION

The ability of peptidyl-tRNA synthesized in the ribosomal A site to react with puromycin without translocation has led us to the conclusion that the transpeptidation reaction per se induces conformational changes in the pretranspeptidative complex (i.e. in tRNAs and/or ribosomes). Just before the peptide bond formation, peptidyl- and aminoacyl-tRNAs (after GTP hydrolysis and dissociation of the EF-Tu·GDP complex) occupy their 'canonical' P and A sites (Fig. 3, state A). In the course of transpeptidation the 3'-end of a newly synthesized peptidyl-tRNA appears in close vicinity to the P site of the peptidyltransferase center (PTC) whereas the rest of peptidyl-tRNA molecule remains to be bound at the A site (Fig. 3, state B). The A site of PTC is now accessible for the puromycin molecule, but the hybrid position of peptidyl-tRNA results in a very slow formation of peptidyl-puromycin (the rate constants of the puromycin reactions for the P site- and A site- (i.e. B-state) bound $AcPhe_2$-$tRNA^{Phe}$ differ by at least 2–3 orders of magnitude as can be roughly evaluated after comparison of Figs. 1B,1 and 2A,3, respectively). The state B seems to be stable (see below) and can be considered as the real pretranslocative one. The the EF-G·GTP complex comes to the ribosome thus promoting subsequent movement of peptidyl-tRNA at the P site (Fig. 3, state B···N).

This result obtained in a functional way is consistent with those on structural studies reported earlier. For example, in our work performed in collaboration with the Novosibirsk group [13], the photoreactive derivative of Phe-$tRNA^{Phe}$ has been used which forms crosslinks with the ribosomal proteins after irradiation of the ribosomal complexes with UV-light. Analysis of these

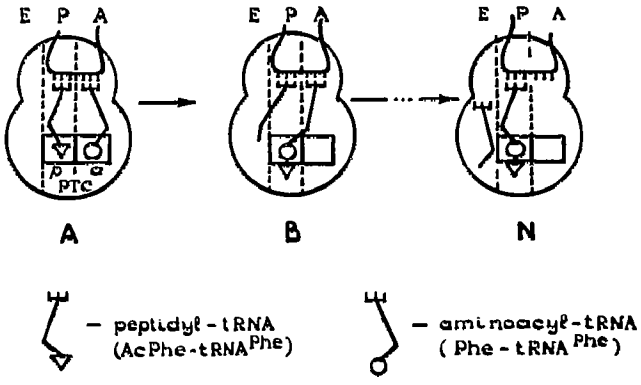


Fig. 3. The binding states of tRNA on the ribosome through a single cycle of elongation. (for details, see the text; due to a multipoint nature of tRNA interactions with the ribosomal sites, peptidyl-tRNA may undergo a number of intermediate states between the state B and P site too).

crosslinks revealed the protein arrangement of tRNA at the A site to differ strikingly prior to and after peptide bond formation. A more detailed information on the problem in question has been presented recently by Moazed and Noller [3]. By using the method of 'chemical footprinting' they have shown, in particular, that $AcPhe_2$-$tRNA^{Phe}$ synthesized in ribosomes occupies not the A site but the so-called A/P state, where the peptidyl-tRNA molecule itself is localized in the A site and its 3'-end is bound at the '50S P site'. This result is in excellent agreement with ours (compare Fig. 5 in [3] and Fig. 3, state B). On this basis, the authors predicted the potential capability of $AcPhe_2$-$tRNA^{Phe}$ in the A/P state (equivalent to state B in Fig. 3) to react with puromycin but failed to demonstrate that this carried out the puromycin reaction at 0°C. This discrepancy is readily overcome by the fact that 'the A-site puromycin reaction' is well expressed at higher temperatures only (compare Fig. 2B,2 and 2A,3). Moazed and Noller also suggested that immediately after peptidyl transfer, a newly formed peptidyl-tRNA occupies the A/A state (i.e. 'canonical' A site) followed by its transition to a more stable A/P state due to higher affinity of the peptidyl-3'-end of peptidyl-tRNA for the P site of PTC [3]. This suggests an oscillating movement of the nascent peptide during the elongation cycles. However, according to Hardesty et al. [14,15] the nascent peptide does not move appreciably and remains in a stationary position. It follows from these results and our findings that in the course of the transpeptidation reaction, the intermediate enzymatic complex decays in such a way that a newly formed peptidyl-tRNA binds at the A/P state directly, i.e. not via transient binding at the A/A state. This is accompanied by the concomitant movement of the nascent peptide for one amino acid relative to the P site of PTC in the direction from its C- to N-end.

If the situation described is close to the real one, aminoacyl, but not peptidyl, transfer takes place during the transpeptidation reaction, and, therefore, the terms 'peptidyltransferase reaction' and 'peptidyltransferase center' should be transformed into 'aminoacyltransferase reaction' and 'aminoacyltransferase center', respectively.

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