Interaction of puromycin with acceptor site of human placenta 80 S ribosomes

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The complex *N*-AcPhe-tRNA^{Phe}·poly(U)·80 S ribosome from human placenta was treated with puromycin taken in various concentrations. Based on the kinetic data of *N*-acetylphenylalanyl-puromycin formation, the association constant of puromycin with the acceptor site of the ribosome was estimated to be $(3.96 \pm 0.84) \times 10^4$ M^{-1} at 37°C.

Puromycin; Ribosome; Human placenta; Rate constant; Affinity

1. INTRODUCTION

One of the essential functions of ribosomes in the course of translation is peptidyl transfer. All types of ribosomes perform this reaction in the same way – without using protein factors or nucleoside triphosphates; peptidyltransferase center is located on large ribosomal subunit [1]. Antibiotic puromycin imitating the 3′-end of aminoacyl-tRNA is widely used for investigation of tRNA-ribosome interactions and peptidyltransferase center as it accepts polypeptide chain or aminoacyl residue attached to tRNA being located in peptidyl (P) site of ribosome (for review see [2]). Puromycin reaction is common for P-site-bound peptidyl-tRNA in all classes of ribosomes, so affinity of puromycin as minimal acceptor substrate to the ribosomal peptidyltransferase center reflects properties of this center.

Puromycin affinity to *E. coli* ribosomes was studied earlier [3–5] whereas parameters of puromycin interaction with eucaryotic ones were not measured. Here we have calculated the association constant of puromycin with human placenta 80 S ribosomes using the kinetic data on *N*-AcPhe-puromycin formation at 37°C at various puromycin concentrations.

2. MATERIALS AND METHODS

40 S and 60 S ribosomal subunits were isolated from normal full-term human placenta according to [6]. Enriched preparation of [^{14}C]Phe-tRNA^{Phe} from *E. coli* (1700 pmol/A_{260} unit) was prepared as in [7]. *N*-Ac[^{14}C]Phe-tRNA^{Phe} (1500 pmol/A_{260} unit) was synthesized according to [8]. Poly(U) was purchased from Reanal (Hungary), puromycin from Fluka.

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To obtain 80 S ribosomes, 40 S and 60 S subunits were re-activated by incubation in buffer A (0.013 M $MgCl_2$, 0.12 M KCl, 0.006 M EDTA, 0.02 M Tris-HCl, pH 7.5) at 37°C for 10 min and mixed in a molar ratio 40 S/60 S = 1:1.3 (assuming that 1 A_{260} unit corresponds to 50 pmol of 40 S, or 25 pmol of 60 S subunits [9]). Binding of *N*-AcPhe-tRNA^{Phe} and poly(U) to 80 S ribosomes was performed by incubation of the components in buffer A at 20°C for 15 min. The concentrations of 80 S ribosomes, *N*-Ac[^{14}C]Phe-tRNA^{Phe} and poly(U) were 4.2×10^{-7} M; 1.6×10^{-7} M and 2.3 A_{260} units/ml, respectively. After the incubation, puromycin was added to the reaction mixtures and temperature was raised up to 37°C. Aliquots were removed from the mixtures, diluted with 15 vols of 0.1 M NaAc, pH 5.0, and the amount of *N*-Ac[^{14}C]Phe-puromycin formed was determined by extraction into ethylacetate according to [10]. The kinetic data were processed on an Apple II personal computer with the help of non-linear regression program [11].

3. RESULTS AND DISCUSSION

Activity of 80 S ribosomes was tested by non-enzymatic poly(U)-dependent binding of [^{14}C]Phe-tRNA^{Phe} and accompanying diphenylalanine formation as in [12]. Under conditions of saturation of the ribosomes with Phe-tRNA^{Phe} (at a more than 5-fold excess of tRNA with respect to ribosomes), the binding level was about 0.7 mol of the Phe-tRNA^{Phe} per mol of ribosomes; the level of $(Phe)_2$ formation was about 0.3 mol per mol of ribosomes. Thus under saturating conditions, both A and P sites of the active fraction of ribosomes are occupied by Phe-tRNA^{Phe} and about 35% of the ribosomes are active in this binding. Also, it is evident that almost all ribosomes that are active in Phe-tRNA^{PHe} binding are competent in peptidyltransferase reaction.

For *N*-AcPhe-puromycin synthesis to be performed, *N*-AcPhe-tRNA^{Phe} should be bond at a ribosomal donor (peptidyl, P) site. Upon non-enzymatic binding the affinity of *N*-AcPhe-tRNA^{Phe} to the P site is higher than to the A site on *E. coli* 70 S ribosomes [13].

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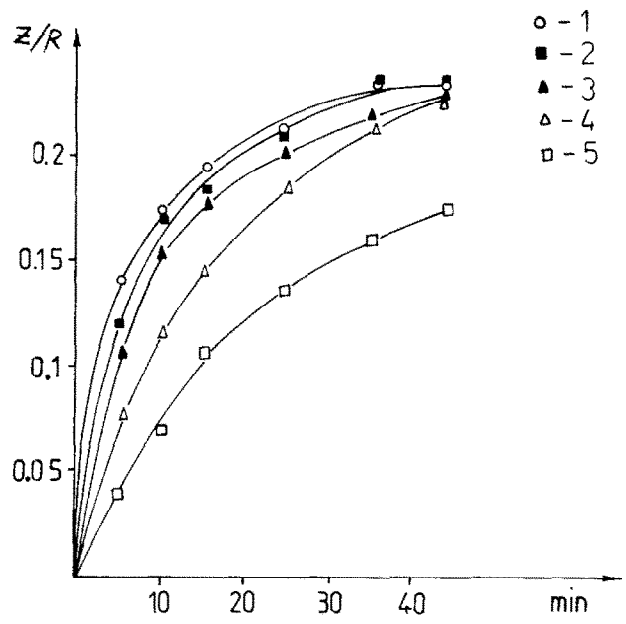

Fig. 1. Kinetic curves of *N*-Ac[^{14}C]Phe-puromycin formation upon treatment of the complex *N*-Ac[^{14}C]Phe-tRNA^{Phe}·poly(U)·80 S ribosome with different concentrations of puromycin at 37°C. (1) 130 μM puromycin (for 400 μM puromycin the same curve was obtained, not shown in this figure); (2) 67 μM; (3) 40 μM; (4) 23 μM; (5) 7.5 μM. The extent of *N*-Ac[^{14}C]Phe-tRNA^{Phe} binding was 0.24 (without template below 0.01) mol per mol of ribosomes.

Therefore the tRNA derivative should preferentially bind at the P site under conditions of the absence of an excess of tRNA to ribosomes (see section 2). As seen from Fig. 1, all *N*-AcPhe-tRNA^{Phe} bound to the ribosomes is puromycin-reactive, i.e. is bound at the P site. To estimate association constant of puromycin with the acceptor site of the peptidyltransferase center (K_p) the rates of *N*-AcPhe-puromycin formation were measured under treatment of the complex *N*-AcPhe-tRNA^{Phe}·poly(U)·80 S ribosome with different concentrations of puromycin.

The mechanism of *N*-AcPhe-puromycin formation is described by the kinetic scheme:

$$RT + P \rightleftarrows RTP \qquad K_p$$
$$RTP \rightarrow RT' + Z \qquad k$$

where RT is the complex *N*-Ac[^{14}C]Phe-tRNA^{Phe}·poly(U)·80 S ribosome; P, puromycin; RTP, complex of RT with puromycin; RT′ the complex 80 S ribosome·tRNA^{Phe}·poly(U); Z, *N*-AcPhe-puromycin; k, rate constant of *N*-AcPhe-puromycin formation. Under quasi-equilibrium conditions and in the case when the initial concentration of puromycin (p_0) is much higher than the concentration of RT (r_0), the time course of the product formation can be described by the expression:

$$[Z] = r_0 \cdot [1 - \exp(-k_{eff} \cdot t)], \quad (1)$$

where

$$k_{eff} = k \cdot K_p \cdot p_0/(1 + K_p \cdot p_0) \quad (2)$$

According to these equations the association constant K_p may be determined from the dependence of k_{eff} on puromycin concentration. The kinetics of the product formation at various puromycin concentrations was investigated for this purpose (see Fig. 1). The values of k and K_p calculated from these data according to Eqn 2 are equal to (0.152 ± 0.009) min^{-1} and (3.96 ± 0.84) × 10^4 M^{-1}, respectively.

It should be noted that K_p value estimated indirectly may differ from the real value due to cooperative effects in the peptidyltransferase center [14]. But the effects of positive cooperativity upon binding of donor substrates and puromycin were observed on *E. coli* ribosomes under specific conditions (binding at 0°C in the presence of 40% ethanol) and were relatively weak [14] or not observed at all in another work from the same laboratory [15]. So we may conclude that K_p estimated here should not differ significantly from the real value. Moreover, data on 'fragment reactions' indicate that parameters of transpeptidation reaction do not depend on the nature of amino acid residue involved [16]. Hence, using of a single type of peptidyl-tRNA analogue (AcPhe-tRNA^{Phe}) is sufficient for the correct estimation of K_p value.

The obtained K_p value is higher than the reported earlier association constants of puromycin with *E. coli* ribosomes estimated to be in the range of 10^3–10^4 at 0°C [3–5,17]. Unfortunately, since there are no data on the parameters of puromycin interaction with *E. coli* ribosomes under physiological conditions (at 37°C and in the absence of organic solvents such as alcohols) we cannot directly compare the K_p values for *E. coli* and human ribosomes. The knowledge of the association constant of puromycin makes it possible to study affinities of different inhibitors of peptidyltransferase center (e.g. alkaloids [12]) to eucaryotic ribosomes and to investigate possible cooperative effects upon binding of acceptor and donor substrates at ribosomal peptidyltransferase center [14].

Table I

The values of k_{eff} at different puromycin concentrations

Puromycin (μM)	k_{eff} (min^{-1})
7.5	0.034 ± 0.001
23	0.067 ± 0.003
40	0.087 ± 0.017
67	0.122 ± 0.009
130	0.134 ± 0.024
400	0.134 ± 0.024

REFERENCES

[1] Wittman, H.G. (1986) in: Structure, Function and Genetics of Ribosomes (Hardesty, B. and Kramer, G. eds) pp. 1–27, Springer-Verlag, New York.

[2] Cooperman, B.S. (1979) in: Ribosomes - Structure, Function and Genetics (Chambliss, G, Craven, G.R., Davies, J., Kahan, L. and Nomura, M. eds) pp. 531-540, Baltimore, University Park Press.
[3] Fahnestock, S., Neumann, H., Shashoua, V. and Rich, A. (1970) Biochemistry 9, 2477-2483.
[4] Fernandez-Munoz, R. and Vazquez, D. (1973) Mol. Biol. Rep. 1, 27-32.
[5] Ivanov, Yu.V. and Saminsky, E.M. (1984) Biochim. Biophys. Acta 800, 203-206.
[6] Graifer, D.M., Zenkova, M.A., Karpova, G.G., Malygin, A.A. and Matasova, N.B. (1990) Mol. Biol. (Moscow) 24.
[7] Bulychev, N.V., Graifer, D.M., Karpova, G.G. and Lebedev, A.V. (1988) Bioorg. Khim. (Moscow) 1, 27-30.
[8] Rappaport, S. and Lapidot, Y. (1974) Methods Enzymol. 29E, 685-693.
[9] Semenkov, Yu.P., Kirillov, S.V. and Stahl, J. (1985) FEBS Lett. 193, 105-108.
[10] Monro, R.E. (1971) Methods Enzymol. 20, 472-481.
[11] Ebert, K. and Ederer, H. (1985) Computeranwendungen in der Chemie, VCH Verlagsgesellschaft mbh, Weinheim.
[12] Tujebajeva, R.M., Graifer, D.M., Karpova, G.G. and Ajtkhozhina, N.A. (1989) FEBS Lett. 257, 254-256.
[13] Kirillov, S.V. and Semenkov, Yu.P. (1982) FEBS Lett. 148, 235-238.
[14] Bourd, S., Kukhanova, M., Gottikh, B. and Krayevsky, A. (1983) Eur. J. Biochem. 135, 465-470.
[15] Kukhanova, M.K., Viktorova, L.S., Bourd, S.B., Gottikh, B.P., Krayevsky, A.A. and Sprinzl, M. (1980) FEBS Lett. 118, 176-179.
[16] Kukhanova, M.K. and Viktorova, L.S. (1985) Mol. Biol. (Moscow) 19, 371-376.
[17] Pestka, S. (1970) Arch. Biochim. Biophys. 136, 80-89.