Influence of systematic error on the shape of the Scatchard plot of tRNA^{Phe} binding to eukaryotic ribosomes

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Scatchard plots of tRNA^{Phe} binding to poly(U)-programmed human 80 S ribosomes can be curved, either concave upwards or concave downwards, depending on the experimental conditions. The influence of a systematic error on the shape of the Scatchard plots has been analysed in a model experiment where the binding proceeds at two independent sites. The Scatchard plot for this binding model has a concave-upwards shape. When the concentration of the ribosomes is kept constant, a small systematic

error in tRNA concentration changes this Scatchard plot markedly to a concave-downwards plot as though a co-operative interaction occurred. In contrast, when the tRNA concentration exceeds the ribosomal concentration and their concentration ratio is constant, the Scatchard plot is stable with respect to the systematic error. We suggest the latter type of experiment to be more appropriate. The results also imply a non-co-operative interaction of tRNA^{Phe} with the 80 S ribosome.

INTRODUCTION

Different forms of tRNA, i.e. deacylated, aminoacyl- or peptidyl-tRNA, interact non-enzymically with the P and A sites of poly(U)-programmed *Escherichia coli* 70 S ribosomes [1,2]. Similarly, tRNA interacts with the P and A sites on eukaryotic 40 S and 80 S ribosomes from rat and rabbit livers [3–5]. A third site or E site, specific for deacylated tRNA, has been found on both prokaryotic and eukaryotic ribosomes [5–9]. The binding of tRNA to eukaryotic ribosomes has been reported to proceed with positive co-operativity between the P and A sites [4,5]. In contrast, there seems to be no positive co-operativity in the tRNA-ribosome interaction in the prokaryotic system [1,2]. Moreover, the binding of deacylated tRNA to the E site and of aminoacyl-tRNA to the A-site of 70 S ribosomes has been reported to occur with negative co-operativity [6]. In our earlier study, the binding of different tRNA^{Phe} forms to the P and A sites of human 80 S ribosomes was shown to proceed without co-operative effects [10]. The affinity of deacylated tRNA^{Phe} for the P site of 80 S ribosomes was found to be no less than 10^{10} M^{-1} at 20 °C and 13 mM Mg²⁺ [10]. This greater affinity of the P site on human 80 S ribosomes for the deacylated tRNA was in contrast with the affinity of approx. $3 \times 10^7 \text{ M}^{-1}$ at 20 °C and 20 mM MgCl₂ observed for rabbit liver ribosomes [5]. The affinity for the human 80 S ribosomal P site was even higher than that reported for *E. coli* 70 S ribosomes, approx. $2 \times 10^9 \text{ M}^{-1}$ [11].

Here we show that this high affinity of tRNA for the P site necessitates special precautions in the preparation of binding experiments. Both ‘co-operative’ and ‘non-co-operative’ Scatchard plots of deacylated tRNA and AcPhe-tRNA^{Phe} binding to human 80 S ribosomes can be obtained, depending on the experimental conditions. We analyse the effect of systematic error on the shape of the Scatchard plot for both types of experiment by modelling data. When the ribosomal concentration is kept constant the Scatchard plot is sensitive even to a small systematic error. As a result the binding curve adopts a concave-

downwards shape characteristic of a co-operative interaction. However, when the ratio of concentrations of tRNA to ribosomes is kept constant, the Scatchard plot is stable with respect to this systematic error. We suggest the latter type of experiment to be more appropriate. Our data also imply a non-co-operative interaction of tRNA^{Phe} with the 80 S ribosome.

MATERIALS AND METHODS

[¹⁴C]Phe-tRNA^{Phe} from *E. coli* (1600 pmol/*A*₂₆₀ unit) was isolated as described [12]. N-Ac[¹⁴C]Phe-tRNA^{Phe} was prepared as described in [13]. Enriched yeast tRNA^{Phe} (1600 pmol/*A*₂₆₀ unit) was kindly provided by Dr. V. I. Katunin and was 3'-end ³²P-labelled as described [14]. Poly(U) was purchased from Reanal (Hungary). Ribosomal subunits (40 S and 60 S) were isolated from normal full-term human placenta as described in [15]; 80 S ribosomes were prepared from reactivated subunits as described in [10]. The binding reaction was performed at 20 °C for 90 min in buffer A [13 mM MgCl₂/0.6 mM EDTA/120 mM KCl/20 mM Tris/HCl (pH 7.5)] and assayed by nitrocellulose filtration. The concentration of poly(U) was 5 *A*₂₆₀ units/ml. The fraction of 80 S ribosomes active in tRNA binding was found to be approx. 40% [10]. In the present paper the quantity and concentration of 80 S ribosomes were considered to be the quantity and concentration of the active fraction of ribosomes. On this scale the plateau for AcPhe-tRNA^{Phe} binding is 2 mol per 1 mol of 80 S ribosomes. tRNA-binding curves were analysed in Scatchard coordinates, *v*/*C* against *v*, where *v* is the extent of tRNA binding, on a molar basis, and *C* is the concentration of free tRNA.

RESULTS AND DISCUSSION

To demonstrate the effect of experimental conditions on the shape of the Scatchard plot of tRNA^{Phe} or AcPhe-tRNA^{Phe} binding to 80 S ribosomes, the following two types of binding experiment were performed.

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First, to obtain a 'co-operative' Scatchard plot, i.e. concave-downwards, the concentration of the ribosomes was kept constant while the tRNA concentration was varied. Figure 1 demonstrates Scatchard plots obtained under these experimental conditions. The character of the binding curves is clearly a concave-downwards; they have the appearance of co-operative ones. These conditions are close to those used previously [4,5]. The Scatchard plots were analysed with the assumption of two-site binding and of infinite co-operativity, i.e. 'all-or-none' reaction [16]. The Hill coefficient for both experiments was greater than 1, suggesting a co-operative interaction. The association constants were estimated to be approx. 10^8 M^{-1} and 10^7 M^{-1} for the binding of deacylated tRNA^{Phe} and AcPhe-tRNA^{Phe} respectively. These values are close to the association constant of $3 \times 10^7 \text{ M}^{-1}$ reported for the binding of tRNA^{Phe} to rat liver ribosomes [4,5]. However, the concave-downwards Scatchard

Figure 1 Scatchard plot of binding of Ac[¹⁴C]Phe-tRNA^{Phe} (A) or deacylated [³²P]tRNA^{Phe} (B) to 80 S ribosomes in the presence of poly(U)

80 S ribosomes (2.5 pmol) were incubated in a volume of 25 μl with 0.5–50 pmol of Ac[¹⁴C]Phe-tRNA^{Phe} (A) or deacylated [³²P]tRNA^{Phe} (B). Symbols: ν , level of binding, mol of Ac[¹⁴C]Phe-tRNA^{Phe} or deacylated [³²P]tRNA^{Phe} per mol of active fraction of ribosomes; C , concentration of free tRNA.

plots can be approximated in a different way, depending on the number of binding sites and on the physical model for this co-operative interaction. Therefore our approximation might not be accurate.

Secondly to obtain a 'non-co-operative' curve, 80 S ribosomes and [³²P]tRNA^{Phe} or Ac[¹⁴C]Phe-tRNA^{Phe} were mixed so that the ratio of [ligand]₀ to [80 S]₀ was constant and more than 2 for the part of the curve where $\nu \leq 1$. Points referring to the initial part of the binding curves were obtained by dilution of the mixtures containing 2 pmol of 80 S ribosomes with buffer A. Ligand was added after dilution. For the rest of the curve the ratio of [ligand]₀ to [80 S]₀ was varied to provide an excess of the ligand so as to saturate the ribosomes. As shown in Figure 2, no co-operativity was observed. The affinity of deacylated tRNA^{Phe} for the P site of the 80 S ribosome evaluated from the data shown in Figure 2 is higher than 10^{10} M^{-1} , in accordance with earlier data [10]. Also, the affinity of Ac[¹⁴C]Phe-tRNA^{Phe} is strong, approx. 10^9 M^{-1} . This result shows that the high affinity of tRNA species for the human ribosomal P site might be a general feature of tRNA-ribosome interaction in this system.

In the first experiment, points in the initial part of the curve ($\nu \leq 1$) demonstrate that most of the ligand was bound to the ribosome so that concentration of free ligand in solution was relatively low and was measured indirectly as difference between two relatively large values: the initial ligand concentration and that of the bound ligand. Hence the experimental error, which is the sum of the errors, increases greatly, especially taking into account the fact that the fraction of tRNA that is competent in binding might not reach 100% and lies in the range 80–100% [11,17]. To investigate the influence of systematic error on the shape of the Scatchard plots we modelled the experimental data for both types of experiment.

In modelling experiments, we assumed that under certain conditions tRNA binding would proceed at two ribosomal sites. This is obviously true for charged tRNA, because this ligand can occupy only two, P and A, sites of the human 80 S ribosome [10]. This is also true for equilibrium binding of deacylated tRNA, which fills the human 80 S ribosomal sites successively in accordance with their affinities [10]. Because the affinity of the third site is at most one-tenth that of the two strongest sites [10], under non-saturating conditions deacylated tRNA binds mostly to the two strongest sites, including the strongest P site. In the following model we set the dissociation constant for the strongest binding site at 1 nM and the ratio of the affinities of the sites at 0.01.

To model the data we solved the following algebraic equation for binding to two sites:

$$\nu/C = 1/(k + C) + 1/(\alpha k + C) \quad (1)$$

where $\nu/C = p/R_0C$, p is the concentration of the complex formed, k is the dissociation constant for the weaker binding site and α is the ratio of the dissociation constants for the two sites ($\alpha < 1$). Eqn. (1) leads to the following cubic equation for the concentration of the complex (p):

$$p^3 - [(\alpha + 1)k + 2C_0 + 2R_0]p^2 + \{(\alpha k + C_0)(k + C_0) + C_0[(\alpha + 1)k + 2C_0] + 2R_0C_0\}p - R_0C_0[(\alpha + 1)k + 2C_0] = 0 \quad (2)$$

where R_0 and C_0 are the initial concentrations of the ribosomes and tRNA respectively. The appropriate solution for eqn. (2) has been chosen according to the inequality $p \leq C_0$.

To model the first set of experimental conditions, i.e. constant ribosomal concentration, we assume that $R_0 = 0.1 \mu\text{M}$. The values of ν and ν/C were calculated from eqn. (2) by varying C_0 from 0.001 to 0.2 μM in steps of 0.002 μM .

Figure 2 Scatchard plot of binding of Ac[¹⁴C]Phe-tRNA^{Phe} (A) or deacylated [³²P]tRNA^{Phe} (B) to the 80 S-poly(U) complex

The ratio of [ligand]₀ to [80 S]₀ was not more than 3 for all of the reaction mixtures. The volume of mixtures was 25–400 μl. v and C are as defined in the legend to Figure 1.

To model the second set of experimental conditions, we assume that $C_0/R_0 = 2$. The values of v and v/C for various C_0 values were calculated as above. In modelling the data for both types of experiment, we calculated the concentration, C , of free tRNA with the following values of systematic error: $C = C_0 - p$ (for the first curve the systematic error $\epsilon = 0$) and $C = C_0(1 + \epsilon) - p$ (for the second, third and fourth curves $\epsilon = 0.05$, $\epsilon = 0.1$ and $\epsilon = 0.2$ respectively). The curves shown in Figure 3 reveal striking differences for these two experiments. In the first case ($R_0 = \text{constant}$) the Scatchard plot changes its shape to a concave-downwards plot even for a systematic error of 5%. Surprisingly,

Figure 3 Scatchard plot for the model data on the binding of tRNA to 80 S ribosomes

(A) The initial ribosome concentration is constant ($R_0 = 0.1 \mu\text{M}$). (B) The ratio of tRNA to ribosome concentration is constant ($C_0/R_0 = 2$). Plots for systematic errors of 0% (curve 1), 5% (curve 2), 10% (curve 3) and 20% (curve 4) are shown. Association constants for the binding sites are assumed to be 10^9 and 10^7 M^{-1} respectively. v and C are as defined in the legend to Figure 1.

for the second type of experimental condition ($C_0/R_0 = 2$), the shape of the Scatchard plot was stable with respect to these systematic errors.

To demonstrate further the instability in the first type of experiment ($R_0 = \text{constant}$) we assume a systematic error to be proportional to the concentration of ribosomes. This can occur when a contaminating nuclease is brought to the binding reaction with the ribosomes and if the concentration of tRNA is saturated for such an enzyme. Earlier, we observed degradation of the isolated tRNA^{Phe} anticodon hairpin incubated with ribosomes when the radioactively labelled 3'-end nucleotide was not in a double-helical structure [11]. Also, we observed a more pro-

Figure 4 Scatchard plot for model data for binding of tRNA to 80 S ribosomes at a constant initial ribosome concentration ($R_0 = 0.1 \mu\text{M}$)

The systematic error is assumed to be proportional to the initial ribosome concentration. Plots of systematic errors of 0.1% (curve 1), 1% (curve 2) and 5% (curve 3) are shown. v and C are as defined in the legend to Figure 1.

nounced degradation of ^{32}P -labelled tRNA^{Phe} in the presence of eukaryotic ribosomes than in incubation with *E. coli* ribosomes (S. Nekhai, unpublished work). This degradation did not allow us to perform footprinting experiments on tRNA^{Phe} binding to eukaryotic ribosomes, as done with *E. coli* ribosomes [18]. Therefore this degradation of tRNA in the presence of eukaryotic ribosomes might lead to the systematic error described above.

We set the systematic error to a value proportional to the concentration of the ribosomes ($\epsilon = \gamma R_0$, $\gamma = 0.001$, $\gamma = 0.01$, $\gamma = 0.05$). The results in Figure 4 show a marked change in the shape of the binding curve, which takes a concave-downwards shape. In the second type of experiment ($C_0/R_0 = 2$) the situation is obviously the same as in the previous modelling, i.e. the binding curve remains concave upwards. The Scatchard plot for the systematic error of 5% is close to that for tRNA^{Phe} binding to 80 S ribosomes (compare curve 3 in Figures 4 and 1B). This shows that our model describes relatively accurately the first type of experimental condition.

The results reported can be summarized as follows: co-operative-type Scatchard plots can be obtained if non-optimal conditions for binding reaction are used, i.e. the concentration of ribosomes is constant, when $v \leq 1$. If this is combined with a very high affinity of tRNA for the P site, which is true for eukaryotic ribosomes, most of the ligand is bound to the ribosome. Hence determination of the free ligand concentration is not accurate. As a result, a systematic error in tRNA concentration might change

the shape of the Scatchard plot of binding. This can also lead to underestimation of the association constants. Therefore the association constants for the binding of tRNA^{Phe} and AcPhe-tRNA^{Phe} to 80 S ribosomes were almost two orders of magnitude lower when determined from Figure 1 (10^8 and 10^7 M^{-1} respectively) than those from Figure 2 (10^{10} and 10^9 M^{-1} respectively). More importantly, the results from Figure 2 allow us to propose a less sophisticated model for the binding, that is the binding to two independent sites on 80 S ribosomes.

The analysis of the points in the first half of the curve ($v \leq 1$) taken from [4], of Ac[^{14}C]Phe-tRNA^{Phe} binding to rabbit liver 80 S ribosomes, shows that 80–90% of the ligand added was bound to the ribosome. As shown above, this part of the curve is mainly competent for the co-operative character of the curve. This implies that the co-operativity observed previously [4,5] might have been based on non-optimal experimental conditions. Our observations give a simple explanation for the increase in the co-operative effect with decreasing incubation temperature and increasing Mg^{2+} concentration described earlier [4]. Because both of these conditions lead to the increase in the affinity of tRNA for the ribosomal P site [1], the binding might be even more sensitive to the systematic error described in this paper. As a result, the pseudo-co-operative effect might be more pronounced.

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