QUANTITATIVE STUDY OF THE INTERACTION OF AMINOACYL-tRNA WITH THE A SITE OF *ESCHERICHIA COLI* RIBOSOMES

Equilibrium and kinetic parameters of binding in the absence of EF-Tu factor and GTP

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1. Introduction

Phe-tRNA^{Phe} has a higher affinity to the D site of 70 S ribosomes over wide concentrations of Mg^{2+} and in the absence of elongation factor Tu (EF-Tu) and GTP [1]. If ribosomes were in excess, Phe-tRNA^{Phe} was found exclusively at the D site. This result allowed us to derive the thermodynamic parameters of the D-site binding of aminoacyl-tRNA [2].

On the other hand, if high concentrations of Phe-tRNA^{Phe} were used, the A site began to fill after D-site saturation, and quantitative formation of (Phe)$_2$-tRNA^{Phe} occurred [3]. It made impossible the determination of thermodynamic parameters of A-site binding.

Here, to avoid this difficulty, we used 70 S ribosomes, where the D sites were initially preoccupied by an analogue of peptidyl-tRNA, *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$, exempt from donor activity [4–6]. In this system we measured both association and dissociation rate constants, and the equilibrium binding constants of Phe-tRNA^{Phe} with the vacant A site of 70 S · poly(U) · peptidyl-tRNA$^{Phe}_{ox.-red.}$ complex. The latter appeared by 2–3 orders of magnitude lower, than the binding constant of Phe-tRNA^{Phe} with the D site of 70 S · poly(U) complex.

2. Materials and methods

30 S and 50 S ribosomal subunits were isolated from *Escherichia coli* MRE-600 as in [3]. Enriched preparations of [^{14}C]Phe-tRNA^{Phe} (1440 pmol/A_{260} unit) and [^{3}H]Phe-tRNA^{Phe} (1440 pmol/A_{260} unit), as well as fractionated poly(U) (30 000 M_r) were prepared as in [8,9]. Enriched deacylated tRNA^{Phe} was obtained by deacylation of a labeled preparation Phe-tRNA^{Phe}. Its oxidation–reduction was performed according to [4,10]. To remove the traces of Na-borohydride, the final preparation of tRNA$^{Phe}_{ox.-red.}$ was passed additionally through a Sephadex G-15 column in 0.05 M Na-acetate buffer (pH 5.5). Aminoacylation of tRNA$^{Phe}_{ox.-red.}$ was made in 1 ml buffer: 0.1 M Tris–HCl (pH 7.6); 0.02 M $MgCl_2$; 0.075 M NH_4Cl; 0.006 M 2-mercaptoethanol, containing 20 A_{260} units tRNA$^{Phe}_{ox.-red.}$, 100 nmol [^{14}C]phenylalanine, 4 μmol ATP and 120 μg S-100 supernatant free of tRNAs. After 60 min incubation at 37°C aminoacylation yield attained 620 pmol [^{14}C]Phe/A_{260} unit. Acetylation of [^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$ was performed according to [11]; the yield of acetyl-Phe-tRNA$_{ox.-red.}$ was usually 97–98%. Then *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$ was additionally purified on a BD-cellulose column to a final enrichment 1350 pmol/A_{260} unit.

To obtain S-100 supernatant free of tRNAs, the supernatant fraction (after the first pelleting of ribosomes from the crude extract [3]) was dialysed against buffer A: 0.02 M Tris–HCl (pH 7.3); 0.01 M $MgCl_2$; 0.3 M NaCl; 0.5 mM EDTA; 6 mM 2-mercaptoethanol. Then the protein was applied to a DEAE-cellulose column (3000 A_{280} units per 40 × 3.6 cm column) in buffer A, and eluted with the same buffer. Under these conditions tRNAs were adsorbed on DEAE-cellulose, and A_{280}/A_{260} ratio of the final S-100 supernatant was 1.5 instead of initial 0.7.

The binding assays were performed in the buffer I: 0.02 M Tris–HCl (pH 7.3); 0.2 M NH_4Cl, containing different concentrations of Mg^{2+} (as indicated in

figure legends). Reaction with puromycin was as in [12]. For double-label counting ($^{14}C/^{3}H$) nitrocellulose filters were dissolved in 1 ml acetone followed by addition of 15 ml scintillation liquid (3 g PPO + 0.1 g POPOP per liter toluene).

3. Results and discussion

It was shown earlier that peptidyl-tRNA binds exclusively at the D site of 70 S · poly(U), if the latter is in excess; the ratio of association constants of peptidyl-tRNA with the D and A sites, K_a^D/K_a^A, being equal to 30–50 [7,13]. As can be seen from table 1, *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$, in these conditions, binds also exclusively at the D site, because the binding is not sensitive to tetracycline. The lack of puromycin reactivity proves the quantitative cleavage of the C2′–C3′ bond in 3′-terminal adenosine of acetyl-Phe-tRNA$^{Phe}_{ox.-red.}$ (compare lines 2 and 4, table 1). Fig.1A shows that modified acetyl-Phe-tRNA$^{Phe}_{ox.-red.}$ binds to 70 S ribosomes as fast as the unmodified one [7]; moreover, the association constants of both analogues of peptidyl-tRNA are indistinguishable ((2.8 ± 0.2) × 10^8 M^{-1} at 8 mM Mg^{2+}, fig.1B).

To saturate the D sites quantitatively at various conditions, we measured K_a^D values for *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$ at different Mg^{2+} concentrations and temperatures (table 2). From these data the enthalpy of interaction was calculated at 8 mM Mg^{2+} (13 ± 2 kcal/mol); it appeared close to the value found earlier for the system with unmodified *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} [7].

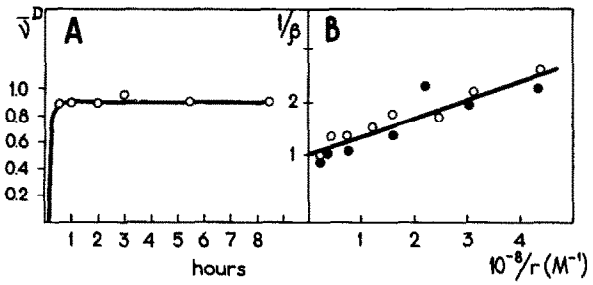

Fig.1. Binding of *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$ at the D site of 70 S · poly(U) complex at 0°C. (A) Kinetics of binding. Incubation mixtures contained in 100 μl buffer I (with 10 mM Mg^{2+}): 6 pmol 30 S subunits; 9 pmol 50 S subunits; 5 μg poly(U); 10 pmol *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$; $\bar{\nu}^D$, no. acetyl-Phe-tRNA molecules bound/ ribosome. (B) Adsorption isotherms of *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} (–•–) and *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$ (–○–) at the D site of 70 S · poly(U) complex, measured by the method of variable volume [1]. Incubation mixtures contained in 0.1–4.0 ml buffer I (with 10 mM Mg^{2+}): 9 pmol 30 S subunits; 12 pmol 50 S subunits; 15 μg poly(U); 5 pmol peptidyl-tRNA; incubation, 2 h at 0°C, β, portion of peptidyl-tRNA bound to ribosomes at equilibrium; *r*, concentration of free 70 S · poly(U) complex.

All following experiments were in two steps:

(i) The ternary 70 S · poly(U) · peptidyl-tRNA$^{Phe}_{ox.-red.}$ complex was formed with controlled $\bar{\nu}^D$ values = 1.0 ± 0.1 (as in fig.2A,3).

(ii) After attainment of equilibrium, [^{3}H]Phe-tRNA^{Phe} was added.

Fig.2A demonstrates that the non-enzymatic binding of aminoacyl-tRNA at the A site is very slow; several hours are needed to reach equilibrium at 0°C. This

Table 1
Reaction of prebound *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} and *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$ with puromycin

Expt. no.	tRNA	Tetracycline (2×10^{-5} M)	tRNA bound (pmol)	Portion tRNA bound (β)	*N*-Acetyl-Phe-puromycin synthesized	
					(pmol)	(%)
1	*N*-Acetyl-	+	4.47	0.99	0	0
2	Phe-tRNA$^{Phe}_{ox.-red.}$	–	4.56	1.01	0	0
3	*N*-acetyl-	+	3.42	0.76	2.85	84
4	Phe-tRNA^{Phe}	–	3.77	0.84	2.95	78

Incubation mixtures contained in 100 μl buffer I (with 20 mM Mg^{2+}): 10 pmol 30 S subunits; 15 pmol 50 S subunits; 5 μg poly(U); 4.5 pmol *N*-acetyl-[^{14}C]Phe-tRNA^{Phe} or *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$. After 20 min incubation at 20°C in the absence or presence of 2×10^{-5} M tetracycline, puromycin was added to final conc. 2×10^{-4} M, and the mixtures were incubated additionally 30 min at 30°C

Table 2
K_a^D values for *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$ at different Mg^{2+} concentrations and temperatures

Mg^{2+} (mM)	$K_a^D \times 10^{-8}$ (M^{-1})			
	0°C	10°C	19°C	32°C
4	0.74 ± 0.06			
6	2.1 ± 0.1			
8	2.8 ± 0.2	2.6 ± 0.1	0.72 ± 0.05	0.26 ± 0.06
10	~10			0.56 ± 0.15
15	>10			4.2 ± 0.5
20	>10			4.4 ± 0.4

Measurements were made as described in legend to fig.1B

binding is reversible, because after dilution of the incubation mixtures [^{3}H]Phe-tRNA^{Phe} dissociates from the complex, and $\bar{\nu}^A$ value tends to a new equilibrium value (cf. –□– and –△–, fig.2A). It is essential that $\bar{\nu}^D$ does not change upon dilution at 0°C (–■–, –▲–, fig.2A). These data provide us criteria for the correct determination of equilibrium K_a^A constants.

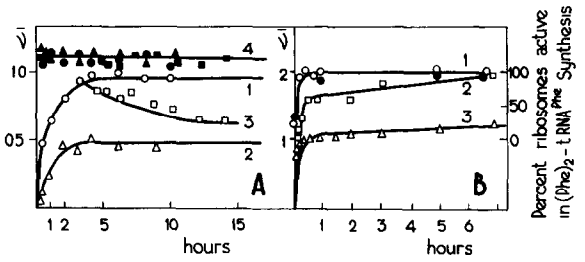

Fig.2. (A) Kinetics of association and dissociation of [^{3}H]Phe-tRNA^{Phe} at the A site of ribosomes at 0°C. Two sets of incubation mixtures contained in buffer I (with 10 mM Mg^{2+}): 5 pmol 30 S subunits; 7 pmol 50 S subunits; 5 μg poly(U); 11 pmol *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$, mixture vol. 100 μl; 40 pmol *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$, mixture vol. 600 μl. After 2 h incubation at 0°C 11 pmol [^{3}H]Phe-tRNA^{Phe} was added to each mixture, and kinetics of aminoacyl-tRNA binding was measured both in more (–○–) and less (–△–) concentrated mixtures. After 3 h 30 pmol *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$ was added to the part of more concentrated mixtures followed by dilution (with pure buffer I) to 600 μl (–□–). Curve 4, control. $\bar{\nu}^D$ values for *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$ in expt: 1 (–●–); 2 (–▲–); 3 (–■–). (B) Kinetics of binding of [^{14}C]Phe-tRNA^{Phe} and formation of ([^{14}C]Phe)$_2$-tRNA^{Phe}. Incubation mixtures contained in 200 μl buffer I (with 20 mM Mg^{2+}): 10 pmol 30 S subunits; 15 pmol 50 S subunits; 5 μg poly(U); 58 pmol (–○–, curve 1), 34 pmol (–□–, curve 2), 20 pmol (–△–, curve 3) [^{14}C]Phe-tRNA^{Phe}. In one of these experiments the fraction of ribosomes active in ([^{14}C]Phe)$_2$-tRNA^{Phe} formation was determined, as in [3].

Results of these measurements are documented in fig.3 and table 3. We see that association constants of codon-dependent, factor-free binding of Phe-tRNA^{Phe} with the A site of the ternary complex appear equal to 2.4×10^7 M^{-1} at 10 mM and 6×10^7 M^{-1} at 20 mM Mg^{2+} and 0°C. After comparison of these values with those for the D-site binding ($K_a^D = 2 \times 10^9$ M^{-1} at 7 mM and ~10^{10} M^{-1} at 10 mM Mg^{2+} and 0°C [1,2]), we draw the conclusion that the affinity of Phe-tRNA^{Phe} to the D site is by 2–3 orders of magnitude higher, than to the A site.

The very slow kinetics of Phe-tRNA^{Phe} binding at the A site is not the consequence of the presence of artificial peptidyl-tRNA at the D site. The same situation we observe when intact [^{14}C]Phe-tRNA^{Phe} fills both D and A sites (fig.2B). The first molecule of

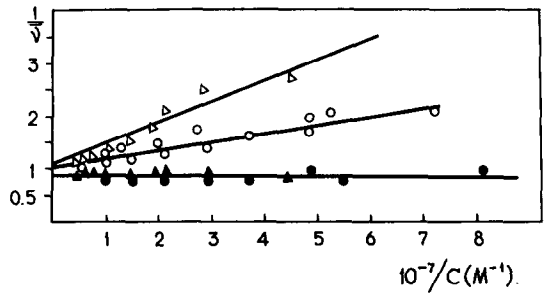

Fig.3. Adsorption isotherms of [^{3}H]Phe-tRNA^{Phe} at the A site of ribosomes at 0°C. Incubation mixtures contained in 150 μl buffer I with different Mg^{2+} concentrations: 7 pmol 30 S subunits; 10 pmol 50 S subunits; 5 μg poly(U); 13 pmol *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$. After 2 h incubation 4–35 pmol [^{3}H]Phe-tRNA^{Phe} was added to each mixture and incubation was continued for 3 h. Mg^{2+} was 10 mM (–△–) and 20 mM (–○–). Control: $\bar{\nu}^D$ values for *N*-acetyl-[^{14}C]Phe-tRNA$^{Phe}_{ox.-red.}$ in both experiments (–▲– and –●–, respectively).

Table 3
K_a^A values for [^{3}H]Phe-tRNA^{Phe} at 0°C and different Mg^{2+} concentrations

	8 mM Mg^{2+}	10 mM Mg^{2+}	15 mM Mg^{2+}	20 mM Mg^{2+}	30 mM Mg^{2+}
$K_a^A \times 10^{-7}$ (M^{-1})	1.87 ± 0.24	2.40 ± 0.30	5.0 ± 1.0	6.0 ± 1.0	8.3 ± 1.2

Measurements were made as described in legend to fig.3

aminoacyl-tRNA binds very fast, obviously, at the D site, because the binding is not inhibited by tetracycline (not shown). The A site is occupied much slower (–□–, –△–, at comparable concentrations of Phe-tRNA^{Phe}, fig.2A); this binding is not reversible, because (Phe)$_2$-tRNA^{Phe} is quantitatively formed while filling the A site (–●–, fig.2B).

Kinetic data in fig.2A allow us to compute the rate constants of association and dissociation of [^{3}H]Phe-tRNA^{Phe}, if the process is presumably described by the simple scheme:

$$T + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} TA \qquad (1)$$

where T is Phe-tRNA^{Phe}, A the vacant A site of the ternary 70 S · poly(U) · acetyl-Phe-tRNA$^{Phe}_{ox.-red.}$ complex, k_1 association and k_{-1} dissociation rate constants. The mean value of k_1 (determined from –○–, –△–, fig.2A), is equal to $(2 \pm 1) \times 10^5$ l/mol × min. As far as the equilibrium constant, K_a^A, is known (see table 3), it is easy to compute $k_{-1} = k_1/K_a^A \simeq 8 \times 10^{-3}$ min^{-1}. On the other hand, k_{-1} can be estimated independently from the experiment on dissociation of Phe-tRNA^{Phe} (–□–, fig.2A). Surprisingly, in this case k_{-1} appears equal to 1.2×10^{-3} min^{-1}, i.e., the dissociation of Phe-tRNA^{Phe} occurs much slower than expected.

Repeated experiments confirmed this discrepancy. This result led us to suggest, that the non-enzymatic interaction of aminoacyl-tRNA with the A site is more complicated and may be described, for instance, by the scheme:

$$T + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} TA^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} TA \qquad (2)$$

Here, TA* is an intermediate which is formed at first converting slowly into final TA complex, where Phe-tRNA^{Phe} becomes competent in its acceptor activity. At the first step of binding, quasi-equilibrium is attained, the admixture of TA complex is negligible and the binding is described by eq. (1). But in longer dissociation experiments (–□–, fig.2A) TA complex is appreciably accumulated. Such sequence of events (initial location of aminoacyl-tRNA at an intermediate site followed by its fastening at a functional A site) explains why the real rate constant of dissociation is lower than the expected one.

If so, we find some common features between the factor-free and factor-dependent binding of aminoacyl-tRNA at the A site. EF-Tu-dependent binding occurs, at least in two-steps. The first step, [GMP-P(CH$_2$)P], can be easily distinguished because peptidyl transfer cannot occur in the presence of non-hydrolyzable analogue of GTP [14]. However, after hydrolysis of GTP and removal of the EF-Tu · GDP complex from ribosome, aminoacyl-tRNA becomes competent in peptide bond formation. These findings have led to the assumption [15] of the 'accomodation' of aminoacyl-tRNA within the ribosome, and to the intermediate R (recognition) site, which is distinct from the A site [16]. At least two-step binding is needed for the selection process [17]. It is likely, that both factor-free and factor-promoted binding of aminoacyl-tRNA at the ribosomal A site occur in a similar way, the association rates of both steps being strongly increased by EF-Tu + GTP.

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