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Initial Binding of the Elongation Factor Tu·GTP·Aminoacyl-tRNA Complex Preceding Codon Recognition on the Ribosome*

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The first step in the sequence of interactions between the ribosome and the complex of elongation factor Tu (EF-Tu), GTP, and aminoacyl-tRNA, which eventually leads to A site-bound aminoacyl-tRNA, is the codon-independent formation of an initial complex. We have characterized the initial binding and the resulting complex by time-resolved (stopped-flow) and steady-state fluorescence measurements using several fluorescent tRNA derivatives. The complex is labile, with rate constants of 6×10^7 M^{-1} s^{-1} and 24 s^{-1} (20 °C, 10 mM Mg^{2+}) for binding and dissociation, respectively. Both thermodynamic and activation parameters of initial binding were determined, and five Mg^{2+} ions were estimated to participate in the interaction. While a cognate ternary complex proceeds from initial binding through codon recognition to rapid GTP hydrolysis, the rate constant of GTP hydrolysis in the non-cognate complex is 4 orders of magnitude lower, despite the rapid formation of the initial complex in both cases. Hence, the ribosome-induced GTP hydrolysis by EF-Tu is strongly affected by the presence of the tRNA. This suggests that codon-anticodon recognition, which takes place after the formation of the initial binding complex, provides a specific signal that triggers fast GTP hydrolysis by EF-Tu on the ribosome.

Binding of aminoacyl-tRNA (aa-tRNA)[1] to the ribosomal A site takes place in a complex with elongation factor Tu (EF-Tu) and GTP ("ternary complex") and proceeds in several steps. After the initial contact of the ternary complex with the ribosome, codon recognition takes place, which is followed by the hydrolysis of GTP, the dissociation of the factor, and the accommodation of aa-tRNA in the A site. EF-Tu-binding sites were localized at the base of the L7/L12 stalk on the 50 S subunit and on the 30 S subunit in the upper part of the body (1, 2). Domain VI of 23 S RNA was identified to participate in the function of EF-Tu (3–5), and also the 530 loop of 16 S RNA seems to be in direct contact with EF-Tu (6). Additionally, a number of proteins of both large and small subunits have been localized In the vicinity of A site-bound EF-Tu or aminoacyl-tRNA.

We have shown recently that the first step in the interaction between the ribosome and the EF-Tu·aa-tRNA complex is a codon-independent step, referred to as initial binding (7). After subsequent codon-anticodon recognition, a cognate ternary complex is found in the pre-A site (8), or recognition site (9), or T/A state (10), in which the anticodon of the aa-tRNA is bound to the mRNA, while the acceptor domain is still bound to EF-Tu. In the absence of cognate codon recognition, the EF-Tu·aa-tRNA complex does not enter this state and remains in the initial binding state, from which it is rejected with high probability. Initial binding and the initial complex have not been studied in detail up to now.

In this paper, we characterize the initial binding and the resulting complex with respect to kinetic and thermodynamic characteristics. Our results show that the initial binding of EF-Tu·GTP·aa-tRNA to the ribosome leads to a labile complex with properties fitting to the initial screening of ternary complexes by the ribosome. Most significantly, the rate constants of formation and dissociation of the initial complex are such that they will not limit appreciably the rate of cognate aa-tRNA binding under physiologically relevant conditions. Furthermore, in the absence of codon recognition, ribosome-mediated GTP hydrolysis in the EF-Tu·GTP·aa-tRNA complex is extremely slow, in accordance with a model where the GTPase of EF-Tu is triggered by cognate codon-anticodon interaction (11).

MATERIALS AND METHODS

Buffer and Reagents—Buffer A contained 25 mM Tris-HCl, pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM dithioerythritol, and 0.5 mM EDTA. In some experiments, the concentration of $MgCl_2$ was different, as indicated. The experiments were performed at 20 °C, if not stated otherwise. Poly(U), poly(A), and other chemicals were purchased from Boehringer Mannheim. Radioactive compounds were from Amersham Corp. or Institute for Research, Production, and Application of Radioisotopes (Prague, Czech Republic).

tRNAs and Proflavin-labeled tRNA Derivatives—$tRNA^{Phe}$ from brewers' yeast (charging capacity of 1.7 nmol/A_{260} unit) was purchased from Boehringer Mannheim. *Escherichia coli* $tRNA^{Phe}$ (1.7 nmol/A_{260} unit), $tRNA_2^{Leu}$ (1.5 nmol/A_{260} unit), and $tRNA^{Lys}$ (1.6 nmol/A_{260} unit) were from Subriden RNA. AcPhe-$tRNA^{Phe}$ was prepared and purified to homogeneity (1.75 nmol/A_{260} unit) by HPLC on a C4 column as described (12). Acetylation of Phe-$tRNA^{Phe}$ was performed according to Rappoport and Lapidot (13) and was quantitative, as verified by hydrolysis and subsequent HPLC analysis on an RP-8 column. The fluorescent $tRNA^{Phe}$ and $tRNA^{Leu}$ derivatives, $tRNA^{Phe}$(Prf16/17) and $tRNA_2^{Leu}$(Prf16,17,20), were prepared as described previously (14) and purified by gel filtration on Superdex 75. The fluorescent $tRNA^{Phe}$ contained one dye/molecule and was chargeable to 1.5 nmol/A_{260} unit. The fluorescent $tRNA^{Leu}$ contained three dyes/molecule and was chargeable to 1.1 nmol/A_{260} unit. Phe-$tRNA^{Phe}$(Flu8) from *E. coli* (1.3 nmol/A_{260} unit) containing fluorescein attached to thiouridine 8 was a gift from A. Johnson.

Ribosomes and Elongation Factor Tu—70 S ribosomes from *E. coli* MRE600 were prepared as described (15). Ribosome concentrations were calculated from absorption measurements on the basis of 23 pmol/A_{260} unit. The activity of the ribosomes was 85–90% in binding AcPhe-$tRNA^{Phe}$ to both A and P sites and >80% in peptide bond formation. Ribosome concentrations given below are based on tRNA binding activity. EF-Tu was prepared from *E. coli* K12 using a procedure combined from the protocols of Leberman *et al.* (16) and Ehrenberg *et al.* (17) with some modifications (15).

Preparation of EF-Tu·GTP·aa-tRNA Complexes—About 7 nmol of

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[1] The abbreviations used are: aa-tRNA, aminoacyl-tRNA; EF-Tu, elongation factor Tu; HPLC, high pressure liquid chromatography; mant-dGTP, 3′-*O*-(*N*-methylanthranilyl)-2-deoxyguanosine triphosphate.

EF-Tu were incubated for 10 min at 37 °C with 1 mM GTP, 3 mM ATP, 6 mM phosphoenolpyruvate, and 0.1 μg/ml pyruvate kinase in 200 μl of buffer A; then, 3 nmol of [^{14}C]Phe-tRNA^{Phe}(Prf16/17) (800 dpm/pmol) were added (final volume of 230 μl), and incubation was continued for 5 min. The complex was purified by gel filtration on Superdex 75 (2 × HR 10/30, Pharmacia Biotech Inc.) (18). Under these conditions, a quinternary complex ((EF-Tu·GTP)$_2$·aa-tRNA) is eluted from the column (18); whether the quinternary complex or the ternary complex (EF-Tu·GTP·aa-tRNA) is the one binding to the ribosome at the lower working concentration is not known. Since we did not observe different kinetics with complexes prepared as quinternary or ternary complex (data not shown), for the present purpose, the complexes are not distinguished and are referred to as ternary complex.

The complex eluted from the gel filtration column (~1 μM) was stored on ice and diluted to the usual working concentration (0.2 μM) immediately before the stopped-flow experiment. The same procedure was applied to prepare the EF-Tu·GTP complexes of Leu-tRNA^{Leu} (Prf16,17,20) and Phe-tRNA^{Phe}(Flu8). The complexes containing [γ-^{32}P]GTP were prepared in the same way, except that nucleotide-free EF-Tu was used (18).

Preparation of Ribosome Complexes—To block the P site, the ribosomes were incubated in buffer A for 15 min at 37 °C either with a 1.1-fold excess (relative to ribosomes active in binding) of AcPhe-tRNA^{Phe} (or tRNA^{Phe}) and 2 A_{260} units/ml poly(U) or with a 1.1-fold excess of tRNA^{Lys} and 1 A_{260} unit/ml poly(A). The final concentration of the complexes was 0.6 μM. When both P and A sites were to be blocked, the same procedure was applied with the amounts of AcPhe-tRNA^{Phe} indicated.

Biochemical Assays—The amount of [^{14}C]Phe-tRNA^{Phe} bound to ribosomes was determined by nitrocellulose filtration by directly applying aliquots of the reaction mixture to the filters (Sartorius Corporation) and subsequently washing with buffer A. [γ-^{32}P]GTP hydrolysis was measured (18) under the conditions of the stopped-flow experiments. The reaction was stopped by adding an equal volume of 1 M $HClO_4$, 3 mM potassium phosphate; sodium molybdate was added; and the complexed $^{32}P_i$ was extracted into isopropyl acetate and determined by liquid-liquid scintillation counting.

Stopped-flow Experiments—Fluorescence stopped-flow measurements were performed, and the data were evaluated as described previously (15). The fluorescence of proflavin or fluorescein was excited at 436 nm and measured with two photomultipliers after passing through KV 500 filters (Schott). The data were evaluated by fitting an expression that contained the sum of up to three exponential terms (characterized by variable time constants (k_{app}) and amplitudes (A)) and another variable for the final signal. With the apparatus used, time constants up to 200 s^{-1} could be measured.

The experiments were performed by rapidly mixing equal volumes (60 μl each) of the ternary complex, purified by gel filtration, and the ribosome complex to give final concentrations of 0.1 and 0.3 μM, respectively. If not stated otherwise, the temperature was 20 °C. From experiment to experiment, the reproducibility of the rate constants given is about ±10% and that of the amplitudes about ±15%; within one experiment, the reproducibility from shot to shot was ~5% for both parameters.

To determine rate constants of initial binding, ribosome titrations were performed, *i.e.* rates were measured at a fixed concentration of EF-Tu·GTP·aa-tRNA (0.1 μM), and increasing concentrations of ribosome complex (from 0.3 up to 2.5 μM). To measure rates at ribosome concentrations lower than 0.3 μM, experiments were performed at a 0.02 μM concentration of the ternary complex. Rate constants were determined from the concentration dependence of the apparent rate constants on the basis of a two-step model (19) (Scheme 1).

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} D$$

SCHEME 1.

For states C and D, the relative fluorescence quantum yields (ξ_C and ξ_D) were calculated from the time course of the reaction at 20 °C and 10 mM Mg^{2+}, setting the initial fluorescence (ξ_A) of the ternary complex to 1 and taking into account the rate constants k_1, k_{-1}, k_2, k_{-2} determined from the titration. The fitting was performed according to Equations 1–4, which were derived adopting previously described procedures (19),[2] assuming pseudo first-order conditions with respect to the ribosome concentration:

$$F(t) = F_\infty + \Delta F_1 e^{-\lambda_1 t} + \Delta F_2 e^{-\lambda_2 t} \quad \text{(Eq. 1)}$$

$$F_\infty = \xi_C a_0 + (\xi_A - \xi_C)\bar{A} + (\xi_D - \xi_C)\bar{D} \quad \text{(Eq. 2)}$$

$$\Delta F_1 = \alpha_1((\xi_A - \xi_C)k_{-1} + (\xi_D - \xi_C)(\lambda_1 - k_1 b_0 - k_{-1})) \quad \text{(Eq. 3)}$$

$$\Delta F_2 = \alpha_2((\xi_A - \xi_C)k_{-1} + (\xi_D - \xi_C)(\lambda_2 - k_1 b_0 - k_{-1})) \quad \text{(Eq. 4)}$$

where

$$\lambda_1 = \frac{1}{2}(k_1 b_0 + k_{-1} + k_2 + k_{-2} + \sqrt{(k_1 b_0 + k_{-1} - k_2 - k_{-2})^2 + 4k_{-1}k_2}) \quad \text{(Eq. 5)}$$

$$\lambda_2 = \frac{1}{2}(k_1 b_0 + k_{-1} + k_2 + k_{-2} - \sqrt{(k_1 b_0 + k_{-1} - k_2 - k_{-2})^2 + 4k_{-1}k_2}) \quad \text{(Eq. 6)}$$

$$\alpha_1 = \frac{1}{k_{-1}}\left(\frac{K_1 a_0 b_0 (1 + K_2)}{1 + K_1 b_0 (1 + K_2)}\right) - \alpha_2 \quad \text{(Eq. 7)}$$

$$\alpha_2 = \frac{1}{\lambda_1 - \lambda_2}\left(\frac{a_0}{1 + (K_2)^{-1}(1 + K_1 b_0)^{-1}}\right) + \frac{\lambda_1 - k_1 b_0 - k_{-1}}{k_{-1}}\left(\frac{K_1 a_0 b_0 (1 + K_2)}{1 + K_1 b_0 (1 + K_2)}\right)) \quad \text{(Eq. 8)}$$

where a_0 and b_0 are the initial concentrations of the ternary complex and ribosomes, respectively; K_1 and K_2 are binding constants; and $\bar{A}$ and $\bar{D}$ are equilibrium concentrations of A and D, respectively.

The dependence of the fluorescence of A (free ternary complex) and of D (ribosome-bound ternary complex) on both Mg^{2+} concentration and temperature was measured at a saturating concentration of poly(A)-programed ribosomes. The fluorescence of Phe-tRNA^{Phe}(Prf16/17) both in the free ternary complex and in the ribosome-bound state was not affected by Mg^{2+} (3.5–10 mM Mg^{2+}) (data not shown); thus, the relative fluorescence quantum yields of states A, C, and D, measured at 10 mM Mg^{2+}, were used for fitting the time courses of the initial binding at lower Mg^{2+} concentrations for the determination of the individual rate constants of the reaction. The increase in temperature affected the fluorescence of both the free and ribosome-bound ternary complexes. The respective temperature coefficients, which were similar for both states, were taken into account for the determination of the quantum yields of states A, C, and D for further fitting the rate constants. The activation parameters of the initial binding reaction were calculated from the temperature dependence of the rate constants (19).

Fluorescence Titrations—Fluorescence measurements were made on a Schoeffel RRS 1000 spectrofluorometer. The excitation wavelength was 460 nm; emission was measured at 510 nm.

To determine the K_d of the initial binding complex, the fluorescence of 0.1–0.2 μM purified EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe}(Prf16/17) was measured alone and with the addition of increasing amounts (up to 2.5 μM) of poly(A)-programed ribosomes with tRNA^{Lys} in the P site. The resulting fluorescence was corrected for dilution (<15%) and for background fluorescence (~2%). The data were evaluated by fitting Equation 9:

$$y = \frac{x}{x + K_d} \quad \text{(Eq. 9)}$$

where

$$y = \frac{F(c) - F_0}{F_{max} - F_0} \quad \text{(Eq. 10)}$$

$$x = c_a - y \cdot c_t \quad \text{(Eq. 11)}$$

where F_0 is the initial fluorescence of the ternary complex; F_{max} is the maximum fluorescence after addition of saturating amounts of ribosomes; $F(c)$ is the fluorescence at a given concentration of free ribosomes, x; and c_a and c_t are the added concentrations of ribosomes and ternary complex, respectively. The fitting program was TableCurve (Jandel). The standard thermodynamic parameters of the interaction

[2] M. Ehrenberg, S. Kaijser, M. Pavlov, and A. Sverredal, *Kinetics and Probabilities*, lectures for the teaching course in Uppsala University.

TABLE I
Fluorescence characteristics of different EF-Tu·GTP·aa-tRNA complexes in the initial complex with the ribosome

Aa-tRNA in ternary complex	Ribosome complex	Fluorescence change[a]
		%
Phe-tRNA^{Phe}(Prf16/17)	70 S·poly(A)·tRNA^{Lys}(P site)	7
Phe-tRNA^{Phe}(Prf16/17)	70 S	7
Phe-tRNA^{Phe}(Prf16/17)	30 S·poly(U)	<1
Phe-tRNA^{Phe}(Prf16/17)	50 S	<1
Phe-tRNA^{Phe}(Flu8)	70 S·poly(A)·tRNA^{Lys}(P site)	9
Leu-tRNA^{Leu}(Prf16,17,20)	70 S·poly(A)·tRNA^{Lys}(P site)	6
Phe-tRNA^{Phe}(Wye37)	70 S·poly(A)·tRNA^{Lys}(P site)	0
Phe-tRNA^{Phe}(Prf37)	70 S·poly(A)·tRNA^{Lys}(P site)	0

[a] Relative to the fluorescence of the corresponding ternary complex in the absence of ribosomes. The fluorescence changes were measured in stopped-flow experiments as described under "Material and Methods": the sum of the amplitudes of the fast and slow steps (A_1 and A_2) is given.

were calculated from the temperature dependence of the K_d. The number of Mg^{2+} ions participating in the interaction was estimated as described (21).

Fluorescence quenching titrations were performed by measuring the fluorescence intensity in the absence (F_0) and presence (F) of increasing amounts of potassium iodide (up to 100 mM). To determine the quenching constant, K_{SV}, the data were analyzed according to the Stern-Volmer equation for collisional quenching as described (22).

Modeling the Time Course of A Site Binding—The modeling was performed by solving numerically the differential equations using *Mathematica* software (Wolfram Research) according to Schemes 2 and 3:

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} D \overset{k_3}{\longrightarrow} E \overset{k_4}{\longrightarrow} F$$

SCHEME 2.

$$G + B \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} H \underset{k_{-2}'}{\overset{k_2'}{\rightleftharpoons}} I$$

SCHEME 3.

where A and G denote cognate and non-cognate ternary complexes, respectively; B denotes ribosomes; and C, D, E, F, H, and I denote intermediates of the A site binding of cognate and non-cognate ternary complexes. The formation of the non-cognate initial complex, H, is followed by the slow formation of I (see "Results"); this step is not observed in the cognate case (15). Steps 2–4 represent codon-anticodon interaction, GTP hydrolysis, and peptide bond formation.

RESULTS

Initial Binding Affects Fluorophores in the Elbow Region of the tRNA—To study the initial binding of the ternary complex to the A site, the interaction of EF-Tu·GTP·aa-tRNA complexes with poly(A)-programed ribosomes containing tRNA^{Lys} in the P site was monitored by various fluorescent labels in Phe-tRNA^{Phe} bound to EF-Tu·GTP (Table I). For all experiments, the respective ternary complex (or quinternary complex; see "Materials and Methods") was purified by gel filtration from deacylated tRNA and excess EF-Tu. The isolated ternary complex was rapidly mixed with the preformed ribosome complex in a stopped-flow apparatus, and the fluorescence signal was monitored.

The interaction of the ternary complex with poly(A)-misprogramed ribosomes leads to a rapid increase in the fluorescence of proflavin in the D-loop of either Phe-tRNA^{Phe}(Prf16/17) or Leu-tRNA^{Leu}(Prf16,17,20) (Fig. 1). With both tRNA derivatives, the fluorescence increase is biphasic, with apparent rate constants of about 40 and 3 s^{-1}, respectively. The presence or absence of the mRNA does not influence the initial binding since EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) interacting with vacant or with poly(A)-programed ribosomes exhibits the same effects (Table I). A fluorescence change is also observed with fluorescein at position 8 (Table I).

Neither wybutin in the anticodon loop of tRNA nor mant-dGTP replacing GTP in the ternary complex shows any significant fluorescence change upon binding of the respective ternary complex of Phe-tRNA^{Phe} to poly(A)-programed ribosomes (Fig. 1 and Table I), in contrast to the extensive signal changes observed with both labels upon binding to ribosomes programed with poly(U) (11, 15). Replacement of wybutin at position 37 with proflavin also did not result in the appearance of any fluorescence change (Table I).

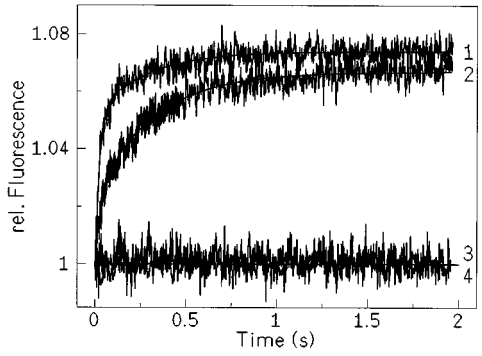

FIG. 1. **Time course of initial binding of EF-Tu·GTP·1Phe-tRNA^{Phe}(Prf16/17) (*trace 1*), EF-Tu·GTP·Leu-tRNA^{Leu}-(Prf16,17,20) (*trace 2*), and EF-Tu·mant-dGTP·Phe-tRNA^{Phe} (*trace 3*) to 70 S ribosomes programed with poly(A) and carrying tRNA^{Lys} in the P site and control without ribosomes (*trace 4*).** Stopped-flow experiments were performed as described under "Materials and Methods." Parameters of the two-exponential fits are as follows: 1) k_{app1} = 43 s^{-1} and A_1 = 6%, k_{app2} = 3 s^{-1} and A_2 = 2%; 2) k_{app1} = 41 s^{-1} and A_1 = 2%, k_{app2} = 3 s^{-1} and A_2 = 4%.

In analogous stopped-flow experiments with EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) and ribosomal subunits, a slight fluorescence increase with k_{app} around 15 s^{-1} is detectable (<1%) (Table I). This shows that, although there seems to be an interaction of the ternary complex with either subunit alone, the proper formation of the initial complex requires 70 S ribosomes. The biochemical analysis shows that, upon rapid filtration without prior dilution, a small and variable fraction (10–15%) of the non-cognate complex is retained on Millipore filters; however, the complex is quite unstable and readily dissociates upon dilution shortly before filtration.

The accessibility of the dye in Phe-tRNA^{Phe}(Prf16/17) in the initial complex to solvent access was measured under equilibrium conditions upon adding KI as a quencher. The interaction with poly(A)-programed ribosomes does not change the quenching constant (K_{SV} = 7.5 M^{-1}), indicating that there is no direct contact of the ribosome with the dye, but rather that the fluorescence change is due to a conformational change around the fluorophore. This conclusion is supported by preliminary results of fluorescence lifetimes measurements that show that, upon initial binding, the three lifetimes remain the same, but their relative contribution is changed.[3]

Effect of Blocking the A Site on Initial Binding—To charac-

[3] L. Kuhn and T. Kulinski, unpublished data.

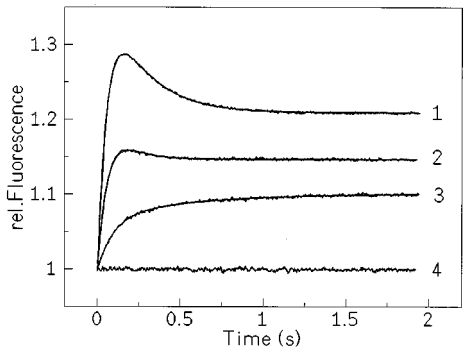

FIG. 2. **Effect of preoccupancy of the A site with AcPhe-tRNA^{Phe} on the binding of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17).** The relative amount of Ac[^{14}C]Phe-tRNA^{Phe} bound (added) per ribosome was as follows: *trace 1*, 0.93 (1.1), P site blocked, A site free; *trace 2*, 1.32 (1.5), P site blocked, A site partially blocked (~40%); *trace 3*, 1.97 (5.0), both P and A sites fully blocked; *trace 4*, control without ribosomes. The amount of ribosome-bound Ac[^{14}C]Phe-tRNA^{Phe} was the same before and after binding the ternary complex. Parameters of the two-exponential fit are as follows: k_{app1} = 9 s^{-1} and A_1 = 7%, k_{app2} = 2 s^{-1} and A_2 = 3%.

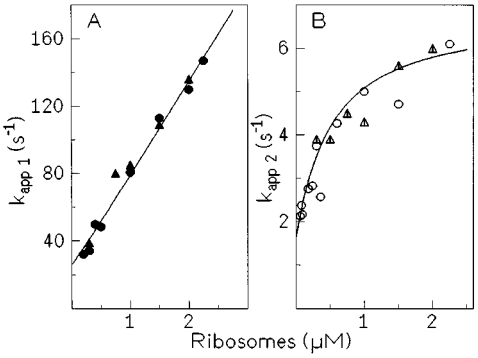

FIG. 3. **Concentration dependence of k_{app1} (*A*) and k_{app2} (*B*) of the binding of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) (● and ○) or EF-Tu·GTP·Leu-tRNA^{Leu}(Prf16,17,20) (▲ and △) to poly(A)-programed ribosomes.** The *lines* represent the fits according to the equations for λ_1 and λ_2 (see "Material and Methods") with the following parameters: $k_1 = 5.6 \times 10^7$ M^{-1} s^{-1}, k_{-1} = 24 s^{-1}, k_2 = 4 s^{-1}, and k_{-2} = 1.5 s^{-1}.

terize the site of initial binding of the ternary complex, the effect of progressively blocking the A site with AcPhe-tRNA^{Phe} was studied (Fig. 2). The biphasic increase and decrease in the fluorescence, which is typical for cognate A site binding (7, 15), is gradually lost until (when all A sites are filled) the biphasic fluorescence increase that signifies non-cognate binding remains, although the first step in this case is slower than the corresponding step in the misprogramed situation of Fig. 1. The qualitative similarity of the results obtained with A site-blocked ribosomes to those seen with misprogramed ribosomes (Fig. 1) indicates that, in fact, initial binding takes place on A site-blocked ribosomes, while the following rearrangement of the initial complex, *i.e.* codon recognition (15), does not. Accordingly, a fraction of Phe-tRNA^{Phe} (10–15%) added in the ternary complex can be found by nitrocellulose filtration to be labily bound to ribosomes with fully occupied A and P sites.

Rate Constants of Initial Binding of EF-Tu·GTP·aa-tRNA—Upon increasing ribosome concentration, k_{app1} increases linearly, while k_{app2} saturates (Fig. 3). This suggests a two-step mechanism where the second-order binding step is followed by a first-order rearrangement (19). On the basis of this model, $k_1 = (5.6 \pm 0.5) \times 10^7$ M^{-1} s^{-1} and $k_{-1} = 24 \pm 2$ s^{-1} are obtained from the titration curves (Fig. 3).

The second step is characterized by the rate constants $k_2 = 4 \pm 1$ s^{-1} and $k_{-2} = 1.5 \pm 0.5$ s^{-1}. The origin of this step is not clear at present. It was observed with several preparations of ribosomes that all were highly active. Also, neither k_{app1} nor k_{app2} was affected by the addition of EF-Tu·GTP up to 5 μM (data not shown), thus excluding any influence of a reaction involving free EF-Tu·GTP, such as the rearrangement from the quinternary to the ternary complex (see "Materials and Methods"). The relative amplitude of the second step is larger for Leu-tRNA^{Leu}(Prf16,17,20) than for Phe-tRNA^{Phe}(Prf16/17). This may indicate that the slow step reflects a local movement of the fluorophore that is particular for each labeled tRNAs species and is not of immediate relevance for the mechanism of A site binding.

Thermodynamic Characteristics of the Initial Binding Complex—The K_d of the initial complex of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) with the ribosome was determined from fluorescence titrations measured at varying Mg^{2+} concentrations and temperatures. Under all conditions, saturating titration curves were obtained, which were then fitted assuming one binding site for the ternary complex. Examples of titration curves obtained at different Mg^{2+} concentrations are shown in Fig. 4*A*.

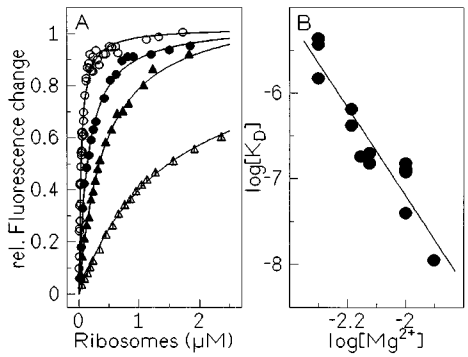

FIG. 4. **Mg^{2+} dependence of the affinity of the initial complex of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) with poly(A)-programed ribosomes.** *A*, titration of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) with increasing concentrations of ribosomes at 10 mM (○), 7.5 mM (●), 6.5 mM (▲), and 5 mM (△) $MgCl_2$; *B*, determination of the number of Mg^{2+} ions participating in the formation of the initial complex. K_d values were determined from titrations, examples of which are shown in *A*.

The affinity of the ternary complex for misprogramed ribosomes increases by >100-fold when the Mg^{2+} concentration is increased from 5 to 12.5 mM (Fig. 4). The slope of the linear plot of log(K_d) *versus* log(Mg^{2+}) (Fig. 4*B*) reveals that five Mg^{2+} ions are involved in initial binding (21). Extrapolation to a physiologically relevant Mg^{2+} concentration (<3 mM) yields a K_d of the initial binding complex of 30 μM. At the Mg^{2+} concentration used in the stopped-flow experiments (10 mM), the titration gives K_d = 0.15 μM, in agreement with the value calculated from the rate constants (0.16 μM).

To estimate the thermodynamic parameters of the initial complex (Table II), the K_d values obtained at different temperatures were plotted according to van't Hoff's equation. Extrapolation of the linear plots to 37 °C gives K_d = 0.24 μM at 10 mM Mg^{2+}. The negative values of the standard (298.15 K) enthalpy change (ΔH^0) and the positive values of the standard entropy change (ΔS^0) (Table II) indicate that the initial complex forms spontaneously at any temperature.

Temperature and Mg^{2+} Dependence of Initial Binding Kinetics—Rate constants were determined for different temperatures and Mg^{2+} concentrations to allow extrapolations to physiologically relevant conditions. The apparent rate constants of the initial binding were measured at different temperatures, and the rate constants of the individual reactions were calculated as described under "Materials and Methods." Fig. 5 shows the temperature dependence of the rate constants of the two

TABLE II
Thermodynamic characteristics of the initial binding complex
The binding of EF-Tu·GTP·Phe-tRNA^{Phe} to poly(A)-programed, P site-blocked ribosomes was measured at two Mg^{2+} concentrations and four temperatures between 8 and 32 °C. Error margins are standard deviations.

Mg^{2+}	$\Delta G°$	$\Delta H°$	$\Delta S°$
mM	*kJ/mol*	*kJ/mol*	*J/mol/K*
7	−38.05 ± 0.05	−25.8 ± 2.2	41 ± 7
10	−38.90 ± 0.08	−23.7 ± 1.8	51 ± 6

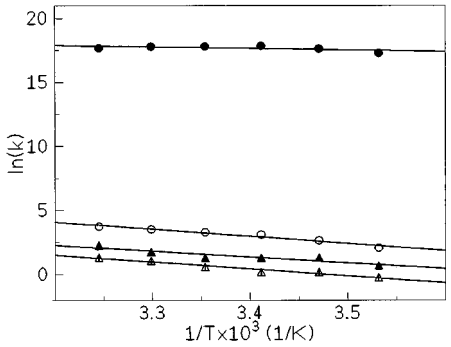

FIG. 5. **Temperature dependence of the rate constants of initial binding of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) to poly(A)-programed ribosomes.** ●, k_1; ○, k_{-1}; ▲, k_2; △, k_{-2}.

TABLE III
Activation parameters of the initial binding of EF-Tu·GTP·Phe-tRNA^{Phe} to poly(A)-programed, P site-blocked ribosomes
The rate constants at five temperatures between 10 and 35 °C have been determined. Error margins are standard deviations.

k	E_a	$\Delta G‡$	$\Delta H‡$	$\Delta S‡$
	kJ/mol	*kJ/mol*	*kJ/mol*	*J/mol/K*
k_1	10 ± 6	29 ± 9	8 ± 6	−71 ± 21
k_{-1}	46 ± 5	65 ± 7	44 ± 5	−71 ± 17
k_2	38 ± 8	69 ± 11	35 ± 8	−113 ± 27
k_2	44 ± 5	71 ± 7	42 ± 5	−99 ± 16

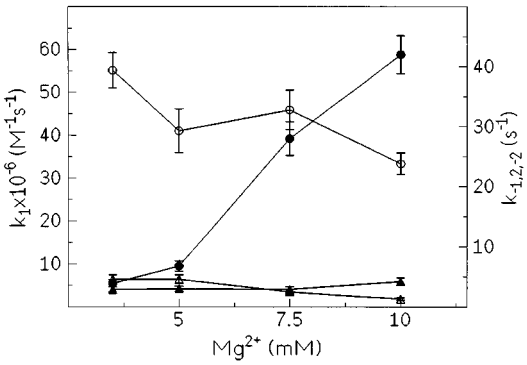

FIG. 6. **Mg^{2+} dependence of the rate constants of initial binding of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) to poly(A)-programed ribosomes containing tRNA^{Lys} in the P site.** ●, k_1; ○, k_{-1}; ▲, k_2; △, k_{-2}.

kinetic steps. The strongest effect is observed for k_{-1}. From the linear dependence of ln(k) on 1/T, the activation parameters of the initial binding were obtained (Table III).

A decrease in the Mg^{2+} concentration strongly affects the initial binding and results in a dramatic decrease in the amplitudes of both steps, while the values of k_{app1} and k_{app2} remain constant (data not shown). Analysis of the time course of the reaction at different Mg^{2+} concentrations (see "Materials and Methods") yields the Mg^{2+} dependence of all four individual rate constants (Fig. 6). The forward rate constant of the first step (k_1) is affected most. Extrapolation to 37 °C and 3.5 mM Mg^{2+} yields forward and backward rate constants for the second-order step of $k_1 = 1.2 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 65$ s^{-1}, respectively.

Effect of Non-cognate Ternary Complexes on the Kinetics of the Cognate Ternary Complex Binding to the A Site—Since initial binding is a step that is common for cognate and non-cognate ternary complexes, the question arises how the presence of an excess of non-cognate ternary complexes affects the rate of cognate ternary complex binding to the A site and subsequent amino acid incorporation into the peptide. This question is relevant for the *in vivo* situation where the reactants' concentrations are high and the ribosomes may be saturated with non-cognate ternary complexes. We modeled this situation on the basis of the rate constants of initial binding extrapolated to near-physiological conditions. The concentration of ribosomes was assumed to be 0.25 mM (23). The concentration of cognate ternary complexes was set to either 0.01 or 0.1 mM, and the concentration of non-cognate complexes was varied from 0 to 2.5 mM. The following rate constants for initial binding at 3.5 mM Mg^{2+} (Fig. 6) were used: $k_1 = k_1' = 1.2 \times 10^6$ M^{-1} s^{-1}, $k_{-1} = k_{-1}' = 65$ s^{-1}, $k_2' = 7.5$ s^{-1}, and $k_{-2}' = 7.6$ s^{-1}, where k and k' denote rate constants for cognate and non-cognate initial binding, respectively. In the cognate situation, initial binding is followed by codon recognition, GTP hydrolysis, and peptide bond formation. The rates of these reactions were set to 100, 60, and 20 s^{-1}, respectively, which is a compromise between our own data (15)[4] and the data of others (24).

The results of the modeling are shown in Fig. 7. In the presence of an excess of non-cognate ternary complexes, the rate of cognate amino acid incorporation into the polypeptide is decreased ~5 times in the presence of a 240-fold excess of non-cognate complexes, independent of the concentration of the cognate complex. For the situation *in vivo*, this excess is probably an overestimation (25). Thus, the modeling suggests that the rate of amino acid incorporation is only moderately affected by the presence of non-cognate ternary complexes at physiological concentrations.

GTP Hydrolysis upon Interaction of the Ribosomes with the Non-cognate Ternary Complex—The observation that the initial binding of the non-cognate ternary complex EF-Tu·GTP·aa-tRNA is fast raises the question of how fast is GTP hydrolysis in the complex. The addition of 0.3 μM poly(A)-programed ribosomes to 0.1 μM EF-Tu·[γ-^{32}P]GTP·Phe-tRNA^{Phe} results in a very slow GTP hydrolysis, with $k_{app} = 5 \times 10^{-4}$ s^{-1} (Fig. 8*A*). With cognate poly(U)-programed ribosomes, the rate of GTP hydrolysis is 12 s^{-1} under the same conditions (11). With increasing ribosome concentration, the rate of GTP hydrolysis in the non-cognate complex increases and reaches saturation (Fig. 8*B*). Thus, although all of the non-cognate EF-Tu·GTP·Phe-tRNA^{Phe} complex is rapidly bound to the ribosomes, the hydrolysis of GTP proceeds very slowly.

DISCUSSION

Initial Binding: A Step Common for Cognate and Non-cognate Ternary Complexes—Initial binding is the first step in the sequence of reactions leading to the binding of aa-tRNA to the A site. It is not influenced by the presence or absence of mRNA, either non-cognate or cognate. In the latter case, initial binding is followed by rapid codon-anticodon interaction. The rate of codon recognition increases with increasing ribosome concentration until saturation is reached at $k_{app} = 60$ s^{-1} at 20 °C (15).

[4] M. V. Rodnina, T. Pape, R. Fricke, L. Kuhn, and W. Wintermeyer, unpublished data.

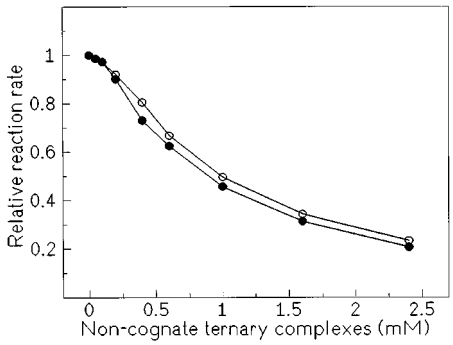

FIG. 7. **Modeling of the effect of non-cognate ternary complexes on cognate amino acid incorporation.** The concentrations used for the modeling were 0.25 mM ribosomes and 0.01 (○) or 0.1 mM (●) cognate ternary complex. The time of half-completion of the peptidyl transfer reaction ($\tau_{1/2}$) was calculated (see "Materials and Methods"), divided by the value in the absence of competitor, and plotted against the concentration of non-cognate complex.

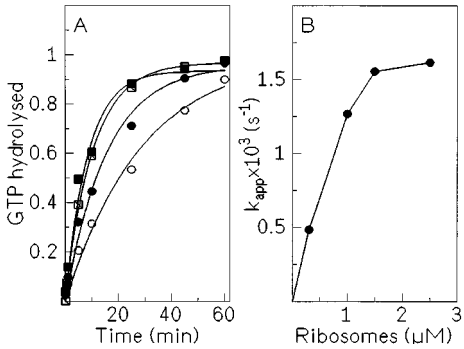

FIG. 8. **GTP hydrolysis in EF-Tu·[γ-^{32}P]GTP·Phe-tRNA^{Phe} on misprogramed ribosomes.** *A*, k_{app}^{GTP} in the presence of 0.3 μM (○), 1 μM (●), 1.5 μM (▨), or 2.5 μM (■) poly(A)-programed, P site-blocked ribosomes; *B*, dependence of k_{app}^{GTP} on the ribosome concentration. The value at saturation equals the first-order rate constant of GTP hydrolysis, $k^{GTP} = 1.7 \times 10^{-3}$ s^{-1}.

The saturating behavior shows that codon recognition is preceded by a second-order binding step, that is, the initial binding step. Analysis of previous titration data (15) leads to an estimate for the bimolecular rate constant of initial binding in the cognate system of $k_1 = 8 \times 10^7$ M^{-1} s^{-1}, which is close to the presently determined binding rate constant for the non-cognate system, 6×10^7 M^{-1} s^{-1}. Thus, the formation of the initial complex takes place in the same way in the cognate or non-cognate situation and precedes codon reading and recognition.

The Initial Binding Site of the Ternary Complex Is Distinct from the A Site—Interestingly, blocking the A site with pre-bound AcPhe-tRNA^{Phe} does not abolish the binding of the ternary complex; rather, a qualitatively similar biphasic fluorescence change is observed with the proflavin label in the D-loop, except that the first step is slower. The similarity of the signal suggests that a complex similar or identical to the initial binding complex is formed, although the A site is blocked. The slower rate of binding may be explained by electrostatic repulsion caused by the AcPhe-tRNA^{Phe} molecule occupying the A site, or else by some steric hindrance. This suggests that the ternary complex in the initial ribosome-binding complex occupies a binding site distinct from the A site, which probably means that neither the acceptor domain nor the anticodon region of the aa-tRNA is in the position of the final A site-bound state. Thus, the initial complex is clearly distinguished from the previously described recognition state (9) or T/A state (10), in which the anticodon region of the incoming (cognate) ternary complex is positioned in the decoding region of the A site.

Conformational Change of the tRNA during Initial Binding—The formation of the initial binding complex changes the fluorescence of reporter groups attached at positions 8, 16, 17, and 20 of the tRNA. These positions are clustered in the central part of the tRNA molecule, position 8 in the inner corner and positions 16, 17, and 20 in the D-loop on the outer side of the elbow. 4-Thiouridine at position 8 is oriented toward EF-Tu, but does not interact directly with it (26). Similarly, nucleotides 16, 17, and 20 in the D-loop do not contact the factor (27). Upon interaction with the ribosome, these positions are not shielded against solvent access, *i.e.* remain as exposed as in the free tRNA or ternary complex throughout the A site binding process. Thus, the fluorescence changes that take place upon initial binding of the ternary complex to the ribosome are most likely due to a conformational change of the aa-tRNA.

In the ternary complex, the conformation of aa-tRNA is slightly different from that in solution; the main differences were found in the association of the T- and D-loops and in the core region (26–32). Our data suggest that, upon initial interaction of the ternary complex with the ribosome, this region of the tRNA molecule is affected and changes the conformation. Subsequently, codon-anticodon recognition leads to yet another, and probably more extensive, rearrangement of this region of the tRNA. The latter rearrangement probably involves a transient unfolding of the D-loop that depends on cognate codon-anticodon interaction and hence may have an important role in the signal transduction from the site of codon-anticodon interaction to EF-Tu (11, 15).

Non-cognate Ternary Complexes Moderately Inhibit Translation in Vivo—It was shown experimentally that non-cognate ternary complexes at concentrations up to 40 μM do not inhibit the rate of polypeptide synthesis in the poly(U) system (33). This raises the question of whether the kinetic and thermodynamic parameters of initial binding determined here are consistent with this previous finding. We verified this by computer modeling, assuming equal binding of the cognate and non-cognate tRNAs to the ribosome in the initial binding step, followed by a sequence of steps leading to A site binding in the cognate case (15, 24). As modeling parameters, we took the rates of the initial binding extrapolated to low Mg^{2+} concentration ($k_1 = 1.2 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 65$ s^{-1}). When the concentrations of the ribosomes and ternary complexes for the modeling were set to those previously used experimentally (0.006 μM active ribosomes, 0.1 μM cognate aa-tRNA, and up to 40 μM non-cognate aa-tRNA) (33), practically no inhibition was observed (data not shown), in agreement with the experiment. However, concentrations *in vivo* are much higher than those that are feasible in the experiment, and the simulations performed under *in vivo* conditions (23) suggest that the rate of cognate amino acid incorporation is decreased up to 5 times at the highest concentrations.

At physiological concentrations of ternary complexes and ribosomes, the rate of the initial binding of the cognate ternary complex is not rate-limiting for A site binding anyway since the reaction is running at a rate of ~300 s^{-1} ($k_1 = 1.2 \times 10^6$ M^{-1} s^{-1}, ribosome concentration of 250 μM). It is to be noted that these conclusions may be restricted to conditions of exponential growth, *i.e.* high concentrations of ribosomes and ternary complexes. It is conceivable that a decrease in the concentrations of ribosomes and/or ternary complexes, *e.g.* in a situation of limited supply, may create a situation in the cell where the rate of initial binding may become rate-limiting for elongation.

The Ribosome Does Not Induce Fast GTP Hydrolysis in the Non-cognate Ternary Complex—The ribosomes appear to discriminate between cognate and non-cognate aa-tRNAs in two steps (34, 35). In the first step, referred to as initial selection, a ternary complex bound to the ribosome either dissociates (re-

jection) or undergoes GTP hydrolysis. After GTP hydrolysis, in a second selection step, the aa-tRNA either enters the A site and takes part in peptide bond formation or dissociates from the ribosome (proofreading). This model implies that the ribosome selects the ternary complexes according to the ratio of the forward rate constants of the following and the backward rate constant of the preceding steps. Therefore, Thompson *et al.* (36–38) argued that the ribosome must have a general criterion to distinguish between the cognate, near-cognate, and non-cognate aa-tRNAs and proposed that a tRNA-independent reaction, *i.e.* GTP hydrolysis, meets the requirements of an internal kinetic standard with respect to initial selection and thus determines the accuracy of protein synthesis. This conclusion was based on the comparison of the rate constants of ribosome-induced GTP hydrolysis in cognate (Phe-tRNA^{Phe}, 25 s^{-1}) and near-cognate (Leu-tRNA^{Leu}, 4 s^{-1}) ternary complexes with that of the binary complex EF-Tu·GTP (>1 s^{-1}), assuming that the latter mimics a ribosome-bound non-cognate ternary complex. However, this assumption is problematic since the binding of aa-tRNA substantially reduces the GTPase of EF-Tu·GTP, both in the free ternary complex (20) and, as shown here, in the non-cognate ribosome complex; therefore, data obtained with EF-Tu·GTP must not be taken for the ternary complex.

Our data clearly show that the ribosome interactions of the non-cognate ternary complex and the binary EF-Tu·GTP complex are grossly different, as evident from the comparison of the rate constants of binding and dissociation of the non-cognate ternary complex (6×10^7 M^{-1} s^{-1} and 24^{-1}, respectively) and of the binary complex (5×10^4 M^{-1} s^{-1} and 10^{-2} s^{-1}, respectively) determined under comparable conditions (37). The most important difference, however, comes from the measurements of the rate constants of GTP hydrolysis for the two types of complexes. For the binary complex, the rate of GTP hydrolysis is limited by the low rate of binding of EF-Tu·GTP to the ribosome. In contrast, for the ternary complex (cognate or non-cognate), the rate of the bimolecular reaction (6×10^7 M^{-1} s^{-1}) is not limiting, at least not at the concentrations we have used, and nevertheless, the rate constant of ribosome-induced GTPase in the non-cognate ternary complex is 2×10^{-3} s^{-1}, only 40 times faster than the rate constant of the intrinsic GTPase in the unbound complex (5×10^{-5} s^{-1}). In comparison, in the presence of correctly programed ribosomes, the rate of GTP hydrolysis is 12 s^{-1} (11), 4 orders of magnitude faster than the rate of intrinsic GTP hydrolysis. Thus, in the non-cognate situation, GTP hydrolysis does not occur in the physiologically relevant time range and therefore cannot be implied as a kinetic standard in the tRNA-independent rejection of EF-Tu·GTP·aa-tRNA complexes.

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Nucleic Acids, Protein Synthesis, and Molecular Genetics:

Initial Binding of the Elongation Factor Tu·GTP·Aminoacyl-tRNA Complex Preceding Codon Recognition on the Ribosome

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