

Complete kinetic mechanism of elongation factor Tu-dependent binding of aminoacyl-tRNA to the A site of the *E.coli* ribosome

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The kinetic mechanism of elongation factor Tu (EF-Tu)-dependent binding of Phe-tRNA^{Phe} to the A site of poly(U)-programed *Escherichia coli* ribosomes has been established by pre-steady-state kinetic experiments. Six steps were distinguished kinetically, and their elemental rate constants were determined either by global fitting, or directly by dissociation experiments. Initial binding to the ribosome of the ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} is rapid ($k_1 = 110$ and $60/\mu\text{M/s}$ at 10 and 5 mM Mg^{2+} , 20°C) and readily reversible ($k_{-1} = 25$ and $30/\text{s}$). Subsequent codon recognition ($k_2 = 100$ and $80/\text{s}$) stabilizes the complex in an Mg^{2+} -dependent manner ($k_{-2} = 0.2$ and $2/\text{s}$). It induces the GTPase conformation of EF-Tu ($k_3 = 500$ and $55/\text{s}$), instantaneously followed by GTP hydrolysis. Subsequent steps are independent of Mg^{2+} . The EF-Tu conformation switches from the GTP- to the GDP-bound form ($k_4 = 60/\text{s}$), and Phe-tRNA^{Phe} is released from EF-Tu·GDP. The accommodation of Phe-tRNA^{Phe} in the A site ($k_5 = 8/\text{s}$) takes place independently of EF-Tu and is followed instantaneously by peptide bond formation. The slowest step is dissociation of EF-Tu·GDP from the ribosome ($k_6 = 4/\text{s}$). A characteristic feature of the mechanism is the existence of two conformational rearrangements which limit the rates of the subsequent chemical steps of A-site binding.

Keywords: codon recognition/conformational change/EF-Tu function/GTP hydrolysis/protein synthesis

Introduction

The binding of aminoacyl-tRNA (aa-tRNA) to the A site of the ribosome is catalyzed by elongation factor Tu (EF-Tu). EF-Tu, aa-tRNA and GTP form a stable ternary complex that binds to the ribosome. The process comprises several steps (Figure 1), as has been established by experiments with either ternary complexes containing non-hydrolyzable and slowly hydrolyzable analogs of GTP (Kaziro, 1978, and references cited therein; Karim and Thompson, 1986; Rodnina *et al.*, 1994) or ribosomes in different functional states (Rodnina *et al.*, 1994, 1995, 1996). Initial binding of the ternary complex to the ribosome (Rodnina *et al.*, 1996) is followed by codon recognition. Provided aa-tRNA recognizes a correct codon, the complex of EF-Tu·GTP·aa-tRNA is greatly stabilized

(Eccleston *et al.*, 1985) by interactions of the tRNA with the mRNA and, possibly, the ribosome. Codon-anticodon interaction provides an activation signal that is transmitted to the G domain of EF-Tu and leads to the formation of the activated GTPase state of the ribosome·EF-Tu·aa-tRNA complex (Rodnina *et al.*, 1995) which is followed by GTP hydrolysis. As a consequence, the conformation of EF-Tu switches from the GTP- to the GDP-form (Abel *et al.*, 1996; Polekhina *et al.*, 1996) which has a greatly reduced affinity for aa-tRNA (Dell *et al.*, 1990). Subsequently, aa-tRNA is released from EF-Tu·GDP, accommodates in the A site and takes part in the peptidyl-transferase reaction, while EF-Tu·GDP leaves the ribosome.

The rate constants of some elementary steps of A-site binding were measured first by Thompson and colleagues (Thompson, 1988, and references therein). Using a combination of single- and multiple-turnover approaches, they were able to measure the rate constants of codon-anticodon dissociation (k_{-2} in Figure 1, $0.002/\text{s}$; Thompson and Karim, 1982), of GTP hydrolysis ($k_{\text{GTP}} = 20/\text{s}$; Eccleston *et al.*, 1985) and of peptide bond formation ($k_{\text{pep}} = 0.8/\text{s}$, Eccleston *et al.*, 1985); the rate constant of EF-Tu·GDP dissociation was deduced from the data ($k_6 = 0.2/\text{s}$). Most of those experiments were performed at 5°C in the presence of 5 mM MgCl_2 ; a few measurements were made at higher temperatures (up to 25°C ; Eccleston *et al.*, 1985) and in different buffer conditions (Thomas *et al.*, 1988).

Later, rate constants of GTP hydrolysis and dipeptide formation were determined by Ehrenberg and colleagues (Bilgin *et al.*, 1992). Working in a polyamine-containing, low- Mg^{2+} buffer ('polymix', Jelenc and Kurland, 1979), they obtained rate constants for GTP hydrolysis of $100/\text{s}$ and for peptide bond formation of $50/\text{s}$ (37°C). From the temperature dependence of the data of Thompson and colleagues (Eccleston *et al.*, 1985), a rate constant of GTP hydrolysis at 37°C of $80/\text{s}$ is estimated, in good agreement with the value of Bilgin *et al.* (1992). On the other hand, Thompson's rate constant of peptide bond formation extrapolated to 37°C , $7/\text{s}$, is significantly less than that measured by Bilgin *et al.* (1992). Most probably, the difference is due to differences in buffer conditions, because an ~ 5 -fold increase in the rate of dipeptide formation was observed upon varying the concentrations of Mg^{2+} and polyamines (Thomas *et al.*, 1988).

In the earlier studies mentioned above, only the rates of GTP hydrolysis and peptide bond formation were measured directly. Therefore, the rate constants of transient steps of A-site binding, such as A-site accommodation of aa-tRNA, had to be deduced. Similarly, because no observable factor was available to determine the rate constants of initial binding and codon recognition, only the overall rates of these two reactions were estimated.

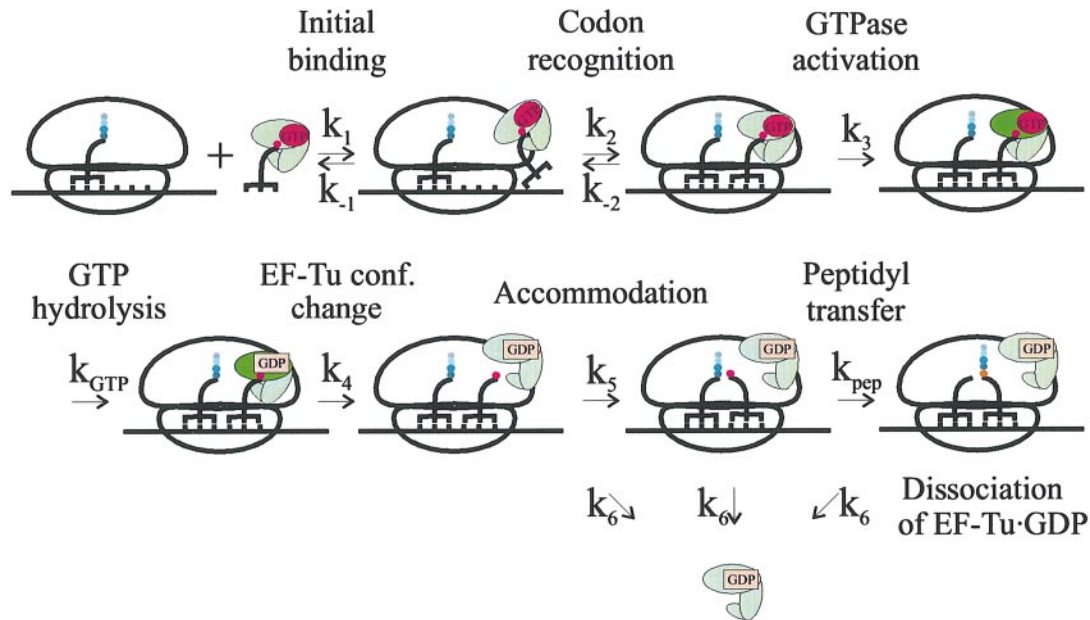


Fig. 1. Mechanism of EF-Tu-dependent binding of aa-tRNA to the ribosomal A site. EF-Tu (light green) is depicted in three conformations: the GTP-bound form, the transient GTP-activated form on the ribosome (G domain dark green) and the GDP-bound form which dissociates from the ribosome. See text for details.

The rate of the conformational change of EF-Tu from the GTP- to the GDP-form has not been determined.

In the present work, we have analyzed the complete kinetic mechanism of EF-Tu-dependent binding of Phe-tRNA^{Phe} to the A site of poly(U)-programed ribosomes from *Escherichia coli*. Several steps of A-site binding were monitored directly by stopped-flow fluorescence using proflavin-labeled tRNA, Phe-tRNA^{Phe}(Prf16/17) (Rodnina *et al.*, 1994) and GTP (mant-dGTP) (Rodnina *et al.*, 1995). The rates of GTP hydrolysis and peptide bond formation were measured by quench flow. Additionally, k_{-2} was measured directly in chase experiments monitored by fluorescence. Rate constants of all elementary steps are reported for 10 and 5 mM MgCl₂ at 20°C.

Results

Conformational changes in aa-tRNA during A-site binding

The interaction of the ternary complex, EF-Tu-GTP-Phe-tRNA^{Phe}(Prf16/17), with the ribosome leads to a characteristic biphasic fluorescence change (Figure 2A) that is due to conformational changes in the D loop of the tRNA where the proflavin label is bound (Rodnina *et al.*, 1995, 1996). According to our previous analysis, the formation of the initial binding complex (Figure 1) is accompanied by a 10% fluorescence increase, independently of the Mg²⁺ concentration (Rodnina *et al.*, 1996). Upon codon recognition, the fluorescence rises further (Rodnina *et al.*, 1994) and reaches an ~40% increase in the activated GTPase conformation (Rodnina *et al.*, 1996). At 5 mM Mg²⁺, the fluorescence increase is somewhat smaller (30%). The fluorescence does not change when GTP is hydrolyzed and when EF-Tu changes conformation from the GTP- to the GDP-bound form. The subsequent decay of the high fluorescence intermediate is related to the accommodation of the tRNA in the A site, while the

following step of peptide bond formation does not change the fluorescence further (Rodnina *et al.*, 1994).

To obtain data for the determination of rate constants of individual steps, stopped-flow experiments were performed at different ribosome concentrations (pseudo-first-order conditions). For an initial analysis and a qualitative description of the data, apparent rate constants (k_{app}) were determined by two-exponential fitting of the time curves (Materials and methods). (Note that the smooth curves in Figure 2A do not represent the results of the fits, but are calculated from the rate constants obtained by global fitting, as described below.)

The dependence of the two apparent rate constants, k_{app1} and k_{app2} , upon the ribosome concentration is shown in Figure 2B and C. The apparent rate constant measured by the fluorescence increase, k_{app1} , reaches saturation at ~60/s at 10 mM Mg²⁺; at 5 mM Mg²⁺, k_{app1} saturates at ~40/s. The step represented by k_{app1} is determined predominantly by codon recognition (Rodnina *et al.*, 1994; Vorstenbosch *et al.*, 1996). The rate of the fluorescence decrease (k_{app2}) saturates at ~8 (10)/s at 10 (5) mM Mg²⁺. Following codon recognition, Phe-tRNA^{Phe} is bound stably to the ribosome, and, after GTP hydrolysis, is accommodated in the A site and takes part in peptide bond formation. Since neither GTP hydrolysis nor peptide bond formation affect the fluorescence of Phe-tRNA^{Phe}(Prf16/17) (Rodnina *et al.*, 1994), the step represented by k_{app2} is assigned to the accommodation in the A site.

Conformational transitions of EF-Tu

Conformational changes of EF-Tu taking place upon binding of the ternary complex to the A site were monitored by the fluorescence of a GTP derivative, mant-dGTP, which behaves biochemically as GTP (Rodnina *et al.*, 1995). The interaction of EF-Tu-mant-dGTP-Phe-tRNA^{Phe} with the ribosome results in a biphasic fluorescence change (Figure 3A). The increase in mant-dGTP fluorescence

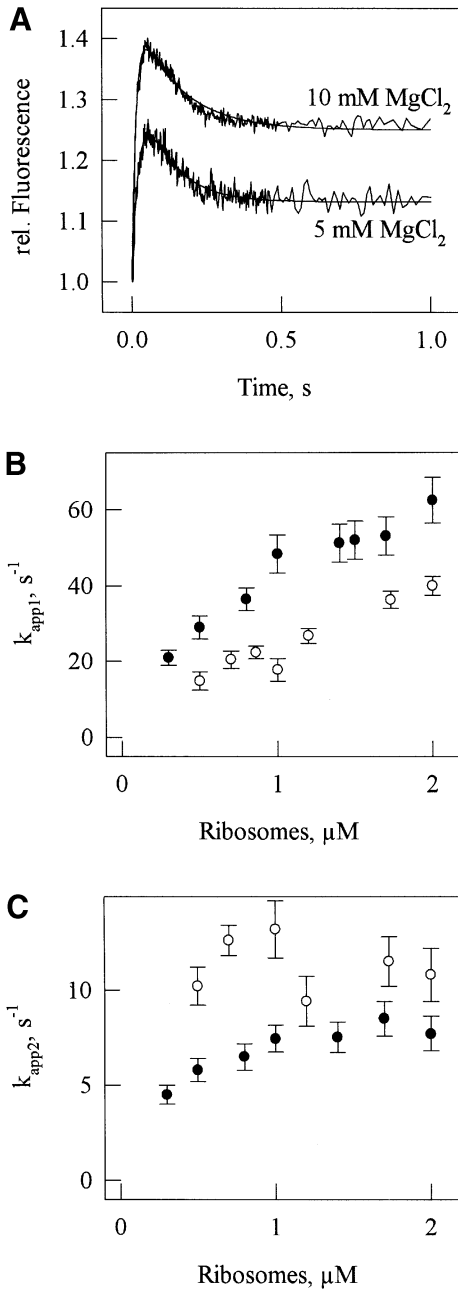


Fig. 2. Conformational changes of aa-tRNA during A-site binding. (A) Time courses of binding of EF-Tu-GTP-Phe-tRNA^{Phe}(Prf16/17) to the A site at 10 and 5 mM Mg²⁺, monitored by proflavin fluorescence. Concentrations after mixing were 0.1 μM ternary complex and 2 μM ribosomes. Smooth lines show the time curves as calculated from using the rate constants of elementary steps (Table I). Parameters of two-exponential fits: $k_{app1} = 63/s$, $A_1 = 52\%$, $k_{app2} = 8/s$, $A_2 = -22\%$ at 10 mM Mg²⁺; $k_{app1} = 40/s$, $A_1 = 44\%$, $k_{app2} = 11/s$, $A_2 = -27\%$ at 5 mM Mg²⁺. (B) Concentration dependence of k_{app1} at 10 mM (●) and 5 mM (○) Mg²⁺. (C) Concentration dependence of k_{app2} at 10 mM (●) and 5 mM (○) Mg²⁺.

reports a conformational rearrangement within the G domain of EF-Tu which precedes the hydrolysis step, i.e. the transition to the activated GTPase state, while the fluorescence decrease is due to the dissociation of EF-Tu-mant-dGDP from the ribosome (Rodnina *et al.*, 1995).

Apparent rate constants, k_{app1} (fluorescence increase) and k_{app2} (fluorescence decrease), and their dependence upon the ribosome concentration were determined by two-

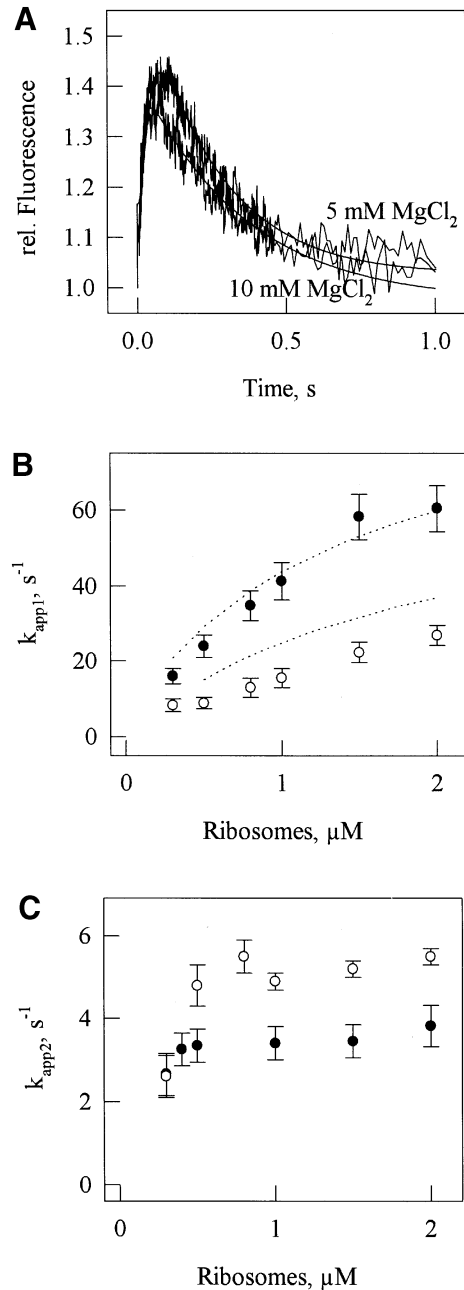


Fig. 3. Conformational changes of EF-Tu during A-site binding. (A) Time course of binding of EF-Tu-mant-dGTP-Phe-tRNA^{Phe} to the A site at 10 and 5 mM Mg²⁺, monitored by mant fluorescence. Concentrations after mixing were 0.1 μM ternary complex and 2 μM ribosomes. Smooth lines show the time curves as calculated from the rate constants of elementary steps (Table I). Parameters of two-exponential fits: $k_{app1} = 61/s$, $A_1 = 49\%$, $k_{app2} = 3.8/s$, $A_2 = -45\%$ at 10 mM Mg²⁺; $k_{app1} = 28/s$, $A_1 = 76\%$, $k_{app2} = 5.5/s$, $A_2 = -69\%$ at 5 mM Mg²⁺. (B) Concentration dependence of k_{app1} at 10 mM (●) and 5 mM (○) Mg²⁺. Dotted lines show data from Figure 2B, for comparison. (C) Concentration dependence of k_{app2} at 10 mM (●) and 5 mM (○) Mg²⁺.

exponential fitting. k_{app1} saturates at ~55/s (10 mM Mg²⁺) or 25/s (5 mM Mg²⁺) (Figure 3B). At 10 mM Mg²⁺, the rate of the mant-dGTP fluorescence increase is very similar to that seen with proflavin fluorescence (shown as a dashed line in Figure 3B). At 5 mM Mg²⁺, the rate of the fluorescence increase of mant-dGTP is slower than that observed with proflavin, indicating a delay between codon

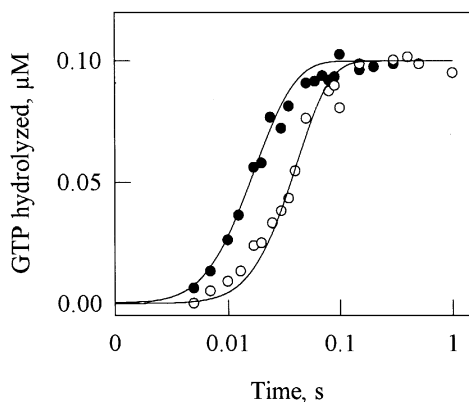


Fig. 4. GTP hydrolysis by the EF-Tu-GTP-Phe-tRNA^{Phe} complex during A-site binding at 10 mM (●) and 5 mM (○) Mg²⁺. Smooth lines show the time curves as calculated from the rate constants of elementary steps (Table I). Parameters of single-exponential fits: $k_{app} = 55/s$ at 10 mM Mg²⁺; and $k_{app} = 23/s$ at 5 mM Mg²⁺.

recognition and GTPase activation at that Mg²⁺ concentration. k_{app2} does not depend on the ribosome concentration and is 3 and 5/s at 10 and 5 mM Mg²⁺, respectively (Figure 3C).

GTP hydrolysis

The rate of GTP hydrolysis was measured by quench flow at increasing concentrations of ribosomes. Figure 4 shows the time courses of GTP hydrolysis at near-saturating ribosome concentration (2 μM). Apparent rate constants of GTP hydrolysis of 55 and 25/s at 10 and 5 mM Mg²⁺, respectively, were obtained by single-exponential fitting. The extent of GTP hydrolysis in both cases was close to 100%.

The concentration dependence of the apparent rate constant of GTP hydrolysis at 10 mM Mg²⁺ (not shown) was similar to that observed for codon recognition and GTPase activation, and reached the same saturation level (~60/s). This indicates that, at saturation, the rates of both GTPase activation and GTP hydrolysis are limited by the rate of codon recognition. The time delay between codon recognition and GTPase activation is only ~2 ms, suggesting that the latter reaction is very fast.

At a lower Mg²⁺ concentration, the apparent rate constants of GTPase activation and GTP hydrolysis were smaller, 25/s, while the apparent rate constant of codon recognition was ~40/s. This suggests that, at lower Mg²⁺ concentration, GTPase activation, rather than codon recognition, is rate limiting. The time delay between codon recognition and GTPase activation is 15–20 ms, hence the rate constant of the latter reaction is ~60/s.

While the rates of GTPase activation (monitored by mant-dGTP fluorescence) and GTP hydrolysis (measured by quench flow) coincide under the present experimental conditions, they were found to be significantly different (44 and 32/s, respectively, at 2 μM ribosomes) when EF-Tu carrying a mutation, G222D, was studied (Vorstenbosch *et al.*, 1996). Moreover, as the mant fluorescence is the same in the GTP- and GDP-form of EF-Tu, GTP hydrolysis does not affect the fluorescence (Rodnina *et al.*, 1995). Thus, the step observed by mant fluorescence is physically different from and precedes GTP hydrolysis, and is therefore assigned to GTPase activation. In the wild-type

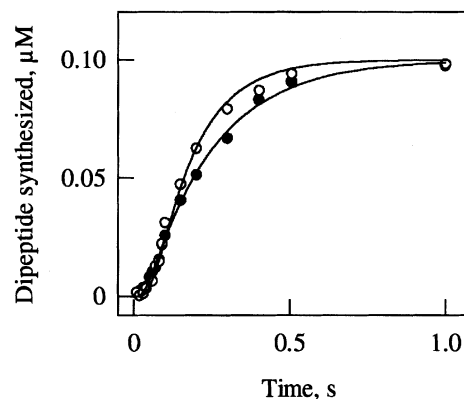


Fig. 5. Peptide bond formation at 10 mM (●) and 5 mM (○) Mg²⁺. Smooth lines show the time curves as calculated from the rate constants of elementary steps (Table I).

situation, GTP hydrolysis apparently takes place instantaneously after GTPase activation. Therefore, for global fitting, as described below, the two steps were grouped, and the rate constant determined from either mant-dGTP fluorescence or GTP hydrolysis is referred to as the GTPase rate constant.

Peptide bond formation

The time course of AcPhePhe dipeptide formation after binding of EF-Tu-GTP-[³H]Phe-tRNA^{Phe} to the A site, with AcPhe-tRNA^{Phe} in the P site, was measured by quench flow at near-saturating concentration of ribosomes (2 μM). As shown in Figure 5, the time course is sigmoidal, at both 10 and 5 mM Mg²⁺. The length of the lag phase is determined by the time required for all steps that take place before the dipeptide is formed. The rates of peptide bond formation were estimated to be 7 and 8/s at 10 and 5 mM Mg²⁺, respectively, the same as the rates of aa-tRNA accommodation in the A site determined by fluorescence. This suggests that the rate of peptide bond formation is limited by the preceding accommodation step and that, therefore, the rate constant determined from the time course of dipeptide formation reflects the accommodation of aa-tRNA in the A site, rather than the chemistry step which appears to follow instantaneously.

The length of the lag phase in Figure 5 (35 and 60 ms at 10 and 5 mM Mg²⁺, respectively) is significantly longer than the time delay due to all reactions up to and including GTP hydrolysis (1/55 s = 18 ms and 1/25 s = 40 ms, respectively). This suggests the existence of an additional kinetic step between GTP hydrolysis and accommodation. From the delay difference, the rate constant of this step is estimated to be ~60/s, independently of Mg²⁺ [$k = 1/(35-18)$ ms) and $k = 1/(60-40)$ ms, respectively], the same value as determined by global analysis (see below). The step which is known to follow GTP hydrolysis and precede the accommodation of aa-tRNA in the A site and peptide bond formation is the conformational rearrangement of EF-Tu from the GTP- to the GDP-form, which leads to the release of aa-tRNA (Kaziro, 1978). Thus, we attribute the step with rate constant 60/s to that conformational rearrangement of EF-Tu.

Determination of dissociation constants k_{-1} and k_{-2}

A unique solution of the reaction mechanism of Figure 1 cannot be achieved unless independent information is

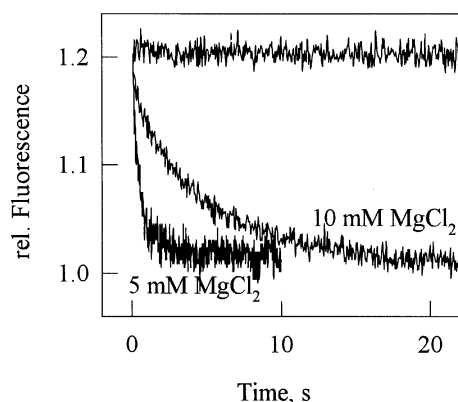


Fig. 6. Determination of the dissociation rate constant of the codon-anticodon complex. Parameters of the single exponential fits: $k_{-2} = 0.2/s$ at 10 mM Mg^{2+} and $2/s$ at 5 mM Mg^{2+} .

available in addition to the time curves presented. Therefore, we have determined the values of two dissociation rate constants independently.

k_{-1} , the dissociation rate constant of the initial binding complex, has been estimated previously to be 25 and 30/s at 10 and 5 mM Mg^{2+} (Rodnina *et al.*, 1996). k_{-2} , the dissociation rate constant of the codon recognition complex, was determined by chase experiments using a non-hydrolyzable GTP analog, GDPNP, as follows. It is known that, in the presence of GDPNP, A-site binding is blocked at the codon recognition step (Kaziro, 1978), and we have shown that the rates of codon recognition are unaffected when GTP is replaced with GDPNP (Rodnina *et al.*, 1994). Thus, to determine k_{-2} , the pre-formed complex of EF-Tu·GDPNP·Phe-tRNA^{Phe}(Prf16/17) with poly(U)-programed ribosomes was mixed rapidly with a 10-fold excess of unlabeled EF-Tu·GDPNP·Phe-tRNA^{Phe} in the stopped-flow apparatus, and proflavin fluorescence was monitored (Figure 6). The time courses could be fitted with single-exponential functions. Since rebinding of the fluorescent ternary complex is inhibited by the presence of an excess of the unlabeled one, the resulting time constants represent the rate constants of ternary complex dissociation from the codon recognition complex, $k_{-2} = 0.2$ and $2/s$ at 10 and 5 mM Mg^{2+} , respectively.

Ribosome binding and dissociation of EF-Tu·GDPNP·Phe-tRNA^{Phe} was also followed by nitrocellulose filtration. Upon mixing the ternary complex containing [¹⁴C]Phe-tRNA^{Phe} with ribosomes, >85% of radioactivity was bound to the ribosome. Addition of excess non-labeled EF-Tu·GDPNP·Phe-tRNA^{Phe} led to a fast chase of radioactively labeled ternary complex (not shown). The dissociation was complete within 10 s, indicating a dissociation rate constant >0.1/s (10 mM Mg^{2+}), consistent with the kinetically determined value.

Determination of elemental rate constants by global fitting

To calculate rate constants of all elemental steps of A-site binding, the kinetic data were combined into data sets which contained time courses of proflavin and mant-dGTP fluorescence changes, GTP hydrolysis and dipeptide formation at four different ribosome concentrations. Additionally, independently determined values of k_{-1} and k_{-2} (see above) were used as fixed parameters. The combined

Table I. Elemental rate constants of EF-Tu-dependent aa-tRNA binding to the A site.

Step	Rate constant (/s)	Mg^{2+} concentration	
		10 mM	5 mM
Initial binding	k_1	110 ± 10^a	60 ± 10^a
	k_{-1}	25 ± 5^b	30 ± 5^b
Codon recognition	k_2	100 ± 15	80 ± 10
	k_{-2}	0.2 ± 0.1^c	2 ± 0.5^c
GTPase activation and GTP hydrolysis ^d	k_3	500 ± 100	55 ± 15
GTP-GDP conformation change of EF-Tu	k_4	60 ± 20	60 ± 20
Aa-tRNA accommodation and peptide bond formation ^d	k_5	7 ± 2	8 ± 1
Dissociation of EF-Tu	k_6	3 ± 1	4 ± 1

^a $\mu M/s$.

^bDetermined previously (Rodnina *et al.*, 1996).

^cDetermined independently (Figure 6).

^dGrouped for the analysis, because the former reaction is rate-limiting and the latter follows instantaneously.

data sets were globally fitted by numerical integration, using the model of Figure 1.

The rate constants obtained are shown in Table I. The rate constants of initial binding determined here are similar to the values determined in the poly(A) system (60 and $10/\mu M/s$; at 10 and 5 mM Mg^{2+} ; Vorstenbosch *et al.*, 1996). The forward rate constant of codon recognition, k_2 , is not significantly influenced by Mg^{2+} , whereas the dissociation rate constants, k_{-2} , determined at 10 and 5 mM Mg^{2+} differ by a factor of 10. A 10-fold difference is also observed for the GTPase rate constants, k_3 , determined at the two Mg^{2+} concentrations. k_3 represents the rate of GTPase activation which limits GTP hydrolysis; thus, the rate of GTP hydrolysis itself could not be measured. The reaction is probably very fast ($k_{GTP} > 1000/s$). The rate constant of the conformational change of EF-Tu from the GTP-bound to the GDP-bound form (k_4) as well as the rate constant of accommodation in the A site (k_5) are practically independent of Mg^{2+} . Accommodation is followed instantaneously by peptide bond formation, and only the lower limit for the rate constant of the latter reaction can be estimated ($k_{pep} > 100/s$). The dissociation of EF-Tu·GDP from the ribosome, with a rate constant (k_6) of 3–4/s, is the slowest reaction of the sequence.

With the rate constants of Table I, time curves were calculated for given concentrations of ribosomes. As shown in Figures 2A, 3A, 4 and 5, the calculated curves describe the experimentally obtained time curves quite well, thus supporting the model and the rate constants derived from it.

Discussion

Initial binding

The first step in the sequence of interactions between the ribosome and the ternary complex of EF-Tu, GTP and aa-tRNA is the codon-independent formation of an initial complex (Rodnina *et al.*, 1996). At this stage, the ternary complex is bound to the ribosome in a labile fashion

independently of the codon in the A site. The association rate constant (k_1) is $110/\mu\text{M/s}$ at 10 mM Mg^{2+} , and somewhat lower, $60/\mu\text{M/s}$, at 5 mM Mg^{2+} . These values are unexpected. The association rate constant for a bimolecular reaction is given by the product of the encounter frequency, Z , a steric factor, p , and an activation energy term $[\exp(-E_a/RT)]$. For the encounter frequency, a value of $10^4/\mu\text{M/s}$ is expected, assuming radii of the ribosome and EF-Tu-GTP-aa-tRNA complex of 110 and 35 \AA , respectively. The activation energy, E_a , of the reaction has been determined to be 10 kJ/mol (Rodnina *et al.*, 1996). This allows us to estimate the value for the steric factor ($0 < p < 1$), which gives an estimate of the fraction of molecules that are in the correct orientation for binding. The calculation gives $p = 0.1\text{--}0.6$ (20°C), depending on the Mg^{2+} concentration.

Compared with other systems, this value for p is unusually high. It indicates that the encounter of the ternary complex with the ribosome is non-random. One possible explanation is pre-orientation, for instance by electrostatic forces, of the ternary complex upon approaching the ribosome, such as to hit the A-site region more frequently than other regions of the ribosome. Another possible explanation is suggested by the observation that EF-Tu early in the sequence of A-site binding interacts with proteins L7/L12 (Stark *et al.*, 1997) which are present in four copies on the 50S ribosomal subunit. A possible scenario is that the formation of the initial binding complex starts with binding of EF-Tu to one of the four copies of L7/L12, most likely to the highly mobile C-terminal domain (Traut *et al.*, 1995), thus introducing a favorable statistical factor. According to this model, the role of EF-Tu in the ternary complex is to promote the binding to the ribosome and to position aa-tRNA such that the anticodon is pre-oriented for codon recognition.

Codon recognition

The rate constant of codon recognition (k_2) is measured here for the first time, since in earlier publications only the overall rate of binding and codon recognition were determined (Thompson and Karim, 1982; Karim and Thompson, 1986). While k_2 is largely independent of the Mg^{2+} concentration, the stability of the codon-anticodon complex, as indicated by the dissociation rate constant, k_{-2} , decreases significantly with decreasing Mg^{2+} concentration. While k_{-2} is $0.2/\text{s}$ at 10 mM Mg^{2+} , it is $\sim 2/\text{s}$ at 5 mM Mg^{2+} . Nevertheless, the affinities of the codon-anticodon complexes, with K_d values of $4 \times 10^{-10}\text{ M}$ and $1 \times 10^{-8}\text{ M}$ at 10 and 5 mM Mg^{2+} , respectively, are remarkably high. Previous values for the dissociation rate constant were $0.002/\text{s}$ (5°C) and $0.2/\text{s}$ (25°C), both at 5 mM Mg^{2+} (Karim and Thompson, 1986); the difference from the present values may, at least in part, be due to the use of different experimental approaches and the use of different non-hydrolyzable GTP analogs.

The present values for the dissociation rate constants of the codon recognition complex are of the order of 10 times lower than those of the complexes formed from tRNAs with complementary anticodons ($3\text{--}14/\text{s}$ at 20°C ; Grosjean *et al.*, 1976; $0.1\text{--}4.7/\text{s}$ at 9°C ; Grosjean *et al.*, 1978). This suggests that binding interactions with the ribosome contribute up to a factor of 10 ($5\text{--}6\text{ kJ/mol}$) to the stabilization of the codon-anticodon complex in the

A site. Footprinting data suggest that the stabilization involves interactions of the anticodon arm of the tRNA with the decoding center of 16S rRNA (Moazed and Noller, 1990; Purohit and Stern, 1992). It is possible that the formation of the codon-anticodon complex induces or stabilizes a particular conformation of the decoding region which is crucial for the further steps of A-site binding, especially for the GTPase activation and accommodation of the aa-tRNA. Direct evidence suggesting conformational mobility of the decoding region has been obtained by NMR (Fourmy *et al.*, 1998).

GTPase activation and GTP hydrolysis

Triggering of fast GTP hydrolysis in the EF-Tu-GTP-aa-tRNA complex on the ribosome requires correct codon-anticodon interaction, because in the presence of a non-cognate codon (no base pair possible) the rate of GTP hydrolysis remains very low ($10^{-3}\text{--}10^{-2}/\text{s}$) (Rodnina *et al.*, 1996). In the presence of a fully complementary cognate codon, the GTPase rate constant (k_3) increases to $500/\text{s}$, i.e. $\sim 10^5$ times. Decreasing the Mg^{2+} concentration to 5 mM decreases the GTPase rate constant to $55/\text{s}$. The latter value is in the range of previous values determined by other groups, i.e. $25/\text{s}$ under comparable conditions (Thompson and Dix, 1982; Eccleston *et al.*, 1985) and $100/\text{s}$ in polyamine-containing buffer ('polymix') at 37°C (Bilgin *et al.*, 1992).

The data show that the rate constant of fast GTPase of EF-Tu on the ribosome strongly depends on the Mg^{2+} concentration. Many GTP-binding proteins have an absolute requirement for a divalent ion, usually Mg^{2+} , as a cofactor in the enzymatic reaction. Mg^{2+} ions are essential for either the binding of the nucleotide, GTP hydrolysis or the structural integrity of the protein. With p21^{ras}, the affinity for GDP is decreased 300-fold in the absence of Mg^{2+} (John *et al.*, 1993). Mutations of the three residues of p21^{ras} that participate in Mg^{2+} coordination (Pai *et al.*, 1990) result in reduced affinity for both GTP and GDP, as well as decreased intrinsic and GAP-stimulated GTPase activity of p21^{ras} (John *et al.*, 1993). With EF-Tu, only the affinity for GTP is affected when Mg^{2+} is removed by adding EDTA or when the mutation D80N is introduced; the affinity for GDP is not changed (Harmark *et al.*, 1992). Interestingly, Mg^{2+} ions are not required for the intrinsic or kirromycin-stimulated GTPase activity of EF-Tu (Ivell *et al.*, 1981). The rate of GTP hydrolysis on the ribosome is limited by the preceding step of the GTPase activation, represented by the present value of the GTPase rate constant, k_3 . It is likely, therefore, that the effect of Mg^{2+} on k_3 reflects the requirement for divalent ions in the interaction of the EF-Tu-GTP-aa-tRNA complex with the ribosome which leads to the formation of the GTPase activated state, rather than in the GTP cleavage reaction. The molecular mechanism of triggering the GTPase of EF-Tu by codon recognition on the ribosome has not been resolved yet. It is unclear how the codon recognition signal is transmitted to EF-Tu to induce the transition to the GTPase state and subsequent GTP hydrolysis. Most probably, conformational changes of 16S rRNA and tRNA are involved, and the strong effect of Mg^{2+} on this step is in line with this idea.

Conformational change of EF-Tu to the GDP-bound form and release of EF-Tu-GDP

Subsequent to GTP hydrolysis, aa-tRNA is released from EF-Tu, due to the conformational transition of EF-Tu into the GDP-bound form (Dell *et al.*, 1990; Abel *et al.*, 1996; Polekhina *et al.*, 1996). The rate constant of this extensive conformational change of EF-Tu (k_4) is determined here for the first time. At $\sim 60/s$ (20°C), the rearrangement is not rate-limiting for the further steps of A-site binding, and the rate constant is independent of the Mg^{2+} concentration. Also the dissociation of EF-Tu-GDP from the ribosome is practically independent of the Mg^{2+} concentration ($k_6 = 3\text{--}4/s$). Previously, the rate constant of EF-Tu-GDP dissociation from the ribosome was estimated to be $\sim 0.3/s$ (4°C) (Thompson and Dix, 1982; Thompson *et al.*, 1986). Taking into account the difference in the reaction temperature, these values are probably comparable.

A-site accommodation of aa-tRNA and peptide bond formation

Following the release from EF-Tu, the 3' end of aa-tRNA moves into the 50S A site (accommodation); the rate constant of accommodation (k_5) is $7\text{--}8/s$. The accommodation of aa-tRNA is probably independent of EF-Tu, as in the electron microscopic reconstruction of the complex there is a free space underneath the tRNA, and EF-Tu is located on the outside of the ribosome-tRNA complex (Stark *et al.*, 1997), i.e. it does not hinder the movement of the aminoacyl end of the tRNA into the peptidyl transferase center.

When aa-tRNA is accommodated in the A site, peptide bond formation takes place instantaneously ($k_{\text{pep}} > 100$), completing the sequence of A-site binding. The rate constants of peptide bond formation were determined previously to be $0.3\text{--}1.1/s$ for cognate Phe-tRNA^{Phe} at 4°C (Thompson and Dix, 1982; Eccleston *et al.*, 1985) and $50/s$ in polymix buffer at 37°C (Bilgin *et al.*, 1992). Our results show that the rate of dipeptide formation is limited by the accommodation in the A site. Since the accommodation step was not distinguished from peptide bond formation in the earlier experiments, the previous values probably also reflect the rate of accommodation. Here again, the differences between the previous and present rate constants may be attributed, at least partly, to the differences in incubation temperature (4 , 20 and 37°C , respectively) and ionic conditions.

The rate of protein elongation in *E.coli* is of the order of $10/s$, with variations depending on codon sequence and context (Sorensen and Pedersen, 1991). Poly(U) translation *in vitro* proceeds at similar rates (Wagner *et al.*, 1982). The rate constants of the individual steps of A-site binding which are reported here for poly(U)-programed ribosomes at 20°C and 5 and 10 mM Mg^{2+} are also in that range. The slowest step is the dissociation of EF-Tu-GDP; the rate constant is $4/s$ at 20°C , and it may be extrapolated to $\sim 15/s$ at 37°C . Thus, the present set of rate constants appears to be consistent with overall rates of translation both *in vitro* and *in vivo*.

Materials and methods

Biochemical assays

Ribosomes from *E.coli*, EF-Tu and tRNAs were prepared according to Rodnina and Wintermeyer (1995). Ternary complexes, EF-Tu-GTP-Phe-

tRNA, were purified by gel filtration on Superdex 75 in buffer A (50 mM Tris-HCl pH 7.5, 50 mM NH_4Cl , 10 mM MgCl_2 , 1 mM dithioerythritol) as described (Rodnina *et al.*, 1994). To fill the P site, ribosomes were incubated in buffer A for 15 min at 37°C with a 1.3-fold excess of AcPhe-tRNA^{Phe} and 1 mg/ml of poly(U).

Other experimental procedures were as in Vorstenbosch *et al.* (1996), except for the following. To prepare the EF-Tu-GDPNP-Phe-tRNA^{Phe} complex, 10 μM EF-Tu was incubated with 2 μM EF-Ts, 4 mM GDPNP and 5 μM [^{14}C]Phe-tRNA^{Phe}(Prf16/17) in buffer A for 30 min at 37°C , and the ternary complex was purified by gel filtration. Before the stopped-flow experiment, 0.6 μM ribosomal complex was mixed with 0.2 μM ternary complex to form the codon recognition complex. The chase was initiated in the stopped-flow apparatus by the addition of an equal volume of 2 μM non-fluorescent ternary complex containing GDPNP.

Kinetic experiments

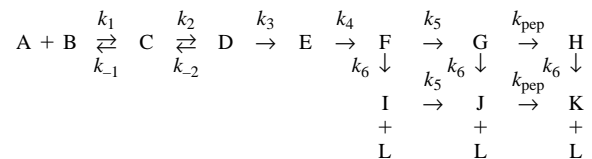
Fluorescence stopped-flow measurements were performed as described previously (Rodnina *et al.*, 1994). The fluorescence of proflavin was excited at 436 nm and measured with two photomultipliers after passing KV 500 filters (Schott). The fluorescence of mant-dGTP was excited at 363 nm and measured after passing KV 408 filters (Schott). With the apparatus used, time constants up to 500/s could be measured. The experiments were performed by rapidly mixing equal volumes (60 μl each) of the ternary complex (0.2 μM), purified by gel filtration, and the ribosome complex (0.6–4.0 μM). Unless stated otherwise, the temperature was 20°C .

Quench-flow experiments were performed using a KinTek quench-flow apparatus under conditions identical to those employed in the fluorescence stopped-flow experiments. To measure the rates of GTP hydrolysis, equal volumes (26 μl each) of ribosome complex and purified ternary complex containing [$\gamma\text{-}^{32}\text{P}$]GTP (1000 d.p.m./pmol) were mixed rapidly, and after the desired incubation time the reaction was terminated by quenching with 0.6 M $\text{HClO}_4/1.8$ mM potassium phosphate. To measure rates of dipeptide formation, ternary complexes containing [^3H]Phe-tRNA^{Phe} (8000 d.p.m./pmol) were used; reactions were stopped with 0.6 M KOH, incubated for 30 min at 37°C , neutralized, and analyzed by HPLC (Vorstenbosch *et al.*, 1996).

To obtain the values for apparent rate constants, the data were evaluated by fitting an expression which contained the sum of two exponential terms (characterized by variable time constants, k_{app} , and amplitudes, A) and another variable for the final signal using TableCurve software (Jandel Scientific).

Calculation of elemental rate constants

The rate constants were calculated from the combined sets of time courses for proflavin and mant-dGTP fluorescence changes, GTP hydrolysis and dipeptide formation at several different ribosome concentrations. The data were fitted by numerical integration using Scientist for Windows software (MicroMath Scientific Software). For this purpose, the kinetic scheme of Figure 1 was modified as follows.



EF-Tu and ribosome complexes are designated as A and B, respectively. The formation of the initial binding complex C is followed by codon recognition (D). The following steps of GTPase activation and GTP hydrolysis are not distinguished kinetically, and were therefore grouped, leading to formation of a transient complex E. The conformational change of EF-Tu from the GTP- to the GDP-bound form results in an intermediate F. aa-tRNA is accommodated in the A site (G) and can take part in peptide bond formation (H). The timing of EF-Tu-GDP dissociation from the ribosome is not known. Therefore, we assumed that it can dissociate at any time point after the formation of the GDP-bound form of EF-Tu, i.e. from intermediates F, G and H, resulting in complexes I, J and K, as well as free EF-Tu-GDP (L). With respect to the state of aa-tRNA on the ribosome, states F and I, G and J, as well as H and K are equivalent and represent the complexes after release from EF-Tu (F and I), accommodation (G and J) and peptide bond formation (H and K).

The overall fluorescence is determined by the concentration of the fluorescent species, and their respective relative fluorescence. For the

calculations, the fluorescence of proflavin in the free ternary complex (P_A) was set to 10 for 1 μM concentration, so that the fluorescence of a 0.1 μM ternary complex (standard concentration) is 1. The formation of the initial binding complex C leads to an increase of fluorescence to $P_C = 11$ independently of Mg^{2+} (Rodnina *et al.*, 1996). The fluorescence changes further in the codon-anticodon complex D to $P_D = 14.0$ and 12.7 at 10 and 5 mM Mg^{2+} , respectively (Rodnina *et al.*, 1994). The GTPase reaction and the conformational rearrangement of EF-Tu or dissociation of EF-Tu-GDP do not change the proflavin fluorescence; therefore, the same relative fluorescence P_D can be used for the intermediates E, F and I. The accommodation of aa-tRNA leads to a decrease in fluorescence which is not changed further upon peptide bond formation. The fluorescence of aa-tRNA in the accommodated state P_G is 12.3 and 11.5 at 10 and 5 mM Mg^{2+} and is the same for intermediates G, H, J and K. Thus, the overall proflavin fluorescence is $F_{\text{Prf}} = P_A \cdot A + P_C \cdot C + P_D \cdot (D + E + F + I) + P_G \cdot (G + H + J + K)$.

The fluorescence of the mant-group is set to 10 for the free ternary complex (M_A at 1 μM). It increases upon GTPase activation (intermediate E, relative fluorescence $M_E = 13.4$, and decreases to about the starting level upon dissociation of EF-Tu-GDP from the ribosome (complex L, relative fluorescence M_L) (Rodnina *et al.*, 1995). The overall mant fluorescence is $F_{\text{Mant}} = M_A \cdot (A + C + D) + M_E \cdot (E + F + G + H) + M_L \cdot L$.

For the calculations, the values of k_{-1} , P_A and P_C (determined previously; Rodnina *et al.*, 1996), as well as k_{-2} (determined independently in this study) were fixed. The fitting yields a unique solution for the rate constants k_{1-6} , as well as for the remaining fluorescence factors.

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