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\textsuperscript{Phe} to the A site of poly(U)-programed \textit{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·GDP from the ribosome is rapid (\(k_{1} = 100 \text{ and } 60/\mu\text{M/s at 10 and 5 mM Mg}^{2+}, 20^\circ\text{C}\)) and readily reversible (\(k_{-1} = 25 \text{ and } 30/s\)). Subsequent codon recognition (\(k_{2} = 100 \text{ and } 80/s\)) stabilizes the complex in an Mg\(^{2+}\)-dependent manner (\(k_{-2} = 0.2 \text{ and } 2/s\)). It induces the GTPase conformation of EF-Tu (\(k_{5} = 500 \text{ and } 55/s\), 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 (\(k_{4} = 60/s\)) and Phe-tRNA\textsuperscript{Phe} is released from EF-Tu·GDP. The accommodation of Phe-tRNA\textsuperscript{Phe} in the A site (\(k_{5} = 8/s\)) 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 (\(k_{6} = 4/s\)). 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 peptidyltransferase 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/s; Thompson and Karim, 1982), of GTP hydrolysis (\(k_{GTP} = 20/s\); Eccleston et al., 1985) and of peptide bond formation (\(k_{\text{pep}} = 0.8/s\); Eccleston et al., 1985); the rate constant of EF-Tu·GDP dissociation was deduced from the data (\(k_{6} = 0.2/s\)). 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/s and for peptide bond formation of 50/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/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/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.
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 MgCl$_2$ 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 Mg$^{2+}$ concentration (Rodnina et al., 1996). Upon codon recognition, the fluorescence rises further (Rodnina et al., 1994) and reaches saturation at ~60/s at 10 mM Mg$^{2+}$; at 5 mM Mg$^{2+}$, $k_{app1}$ saturates at ~80/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 Mg$^{2+}$. 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\textsuperscript{Phe\textsubscript{Prf16/17}} to the A site at 10 and 5 mM Mg\textsuperscript{2+}, 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: \(k_{\text{app1}} = 63/s\), \(A_1 = 52\%\), \(k_{\text{app2}} = 8/s\), \(A_2 = -22\%\) at 10 mM Mg\textsuperscript{2+}; \(k_{\text{app1}} = 40/s\), \(A_1 = 44\%\), \(k_{\text{app2}} = 11/s\), \(A_2 = -27\%\) at 5 mM Mg\textsuperscript{2+}. (B) Concentration dependence of \(k_{\text{app1}}\) at 10 mM (●) and 5 mM (○) Mg\textsuperscript{2+}. (C) Concentration dependence of \(k_{\text{app2}}\) at 10 mM (●) and 5 mM (○) Mg\textsuperscript{2+}.

Fig. 3. Conformational changes of EF-Tu during A-site binding. (A) Time course of binding of EF-Tu·mant-dGTP·Phe-tRNA\textsuperscript{Phe} to the A site at 10 and 5 mM Mg\textsuperscript{2+}, 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: \(k_{\text{app1}} = 61/s\), \(A_1 = 49\%\), \(k_{\text{app2}} = 3.8/s\), \(A_2 = -45\%\) at 10 mM Mg\textsuperscript{2+}; \(k_{\text{app1}} = 28/s\), \(A_1 = 76\%\), \(k_{\text{app2}} = 5.5/s\), \(A_2 = -69\%\) at 5 mM Mg\textsuperscript{2+}. (B) Concentration dependence of \(k_{\text{app1}}\) at 10 mM (●) and 5 mM (○) Mg\textsuperscript{2+}. Dotted lines show data from Figure 2B, for comparison. (C) Concentration dependence of \(k_{\text{app2}}\) at 10 mM (●) and 5 mM (○) Mg\textsuperscript{2+}.

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, \(k_{\text{app1}}\) (fluorescence increase) and \(k_{\text{app2}}\) (fluorescence decrease), and their dependence upon the ribosome concentration were determined by two-exponential fitting. \(k_{\text{app1}}\) saturates at ~55/s (10 mM Mg\textsuperscript{2+}) or 25/s (5 mM Mg\textsuperscript{2+}) (Figure 3B). At 10 mM Mg\textsuperscript{2+}, 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 Mg\textsuperscript{2+}, the rate of the fluorescence increase of mant-dGTP is slower than that observed with proflavin, indicating a delay between codon
recognition and GTPase activation at that Mg\(^{2+}\) concentration. \(k_{\text{app}}\) does not depend on the ribosome concentration and is 3 and 5/s at 10 and 5 mM Mg\(^{2+}\), 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 \(\mu\)M). Apparent rate constants of GTP hydrolysis of 55 and 25/s at 10 and 5 mM Mg\(^{2+}\), 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 Mg\(^{2+}\) (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 Mg\(^{2+}\) 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 Mg\(^{2+}\) 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 \(\mu\)M 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 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-[\(^{3}H\)Phe]-tRNA\(^{\text{Phe}}\) to the A site, with AcPhe-tRNA\(^{\text{Phe}}\) in the P site, was measured by quench flow at near-saturating concentration of ribosomes (2 \(\mu\)M). As shown in Figure 5, the time course is sigmoidal, at both 10 and 5 mM Mg\(^{2+}\). 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 Mg\(^{2+}\), 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 Mg\(^{2+}\), 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 Mg\(^{2+}\) \([k = 1/(35–18 ms)\) 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
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 \(^{14}\)C-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 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 (\( k_{d} \)) as well as the rate constant of accommodation in the A site (\( k_{a} \)) is 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 (\( k_{p} \)) of 3–4/s, is the slowest reaction of the sequence.

With the rate constants of Table I, time courses 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.

<table>
<thead>
<tr>
<th>Step</th>
<th>Rate constant (/s)</th>
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<tr>
<td></td>
<td>Mg(^{2+}) concentration</td>
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<td></td>
<td>10 mM</td>
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<tr>
<td>Initial binding</td>
<td>( k_{1} )</td>
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<tr>
<td>CoA dissociation</td>
<td>( k_{1} )</td>
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<tr>
<td>Codon recognition</td>
<td>( k_{2} )</td>
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<tr>
<td>GTPase activation and GTP hydrolysis</td>
<td>( k_{3} )</td>
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<tr>
<td>GTP-GDP conformation change of EF-Tu</td>
<td>( k_{4} )</td>
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<tr>
<td>Aa-tRNA accommodation and peptide bond formation</td>
<td>( k_{5} )</td>
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<tr>
<td>Dissociation of EF-Tu</td>
<td>( k_{6} )</td>
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\(^a\) \( \mu \)M/s.
\(^b\) Determined previously (Rodnina et al., 1996).
\(^c\) Determined independently (Figure 6).

\(^d\) Grouped for the analysis, because the former reaction is rate-limiting and the latter follows instantaneously.
The calculation gives molecules that are in the correct orientation for binding. The stabilization of the codon–anticodon complex in the ribosome contribute up to a factor of 10 (5–6 kJ/mol) to the present values (Thompson, 1986). While determined (Thompson and Karim, 1982; Karim and Thompson, 1986) for the first time, since in earlier publications only transported (Grosjean et al., 1976; 0.1–4.7/s at 9°C; Grosjean et al., 1976) result in reduced affinity for both GDP and GTP, as well as decreased intrinsic and GAP-stimulated GTPase activity of p21ras (John et al., 1993). With EF-Tu, only the affinity for GDP is decreased 300-fold in the absence of Mg2+ (John et al., 1993). Mutations of the three residues of p21ras that participate in Mg2+ coordination (Pai et al., 1990) result in reduced affinity for GDP and GTP, as well as decreased intrinsic and GAP-stimulated GTPase activity of p21ras (John et al., 1993). With EF-Tu, only the affinity for GDP is decreased when Mg2+ is removed by adding EDTA or when the mutation D80N is introduced; the affinity for GDP is not changed (Harmark et al., 1992). Interestingly, Mg2+ 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, k3. It is likely, therefore, that the effect of Mg2+ on k3 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 Mg2+ on this step is in line with this idea.

**Codon recognition**

The rate constant of codon recognition (k3) 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 k3 is largely independent of the Mg2+ concentration, the stability of the codon–anticodon complex, as indicated by the dissociation rate constant, k-2, decreases significantly with decreasing Mg2+ concentration. While k-2 is 0.2/s at 10 mM Mg2+, it is ~2/s at 5 mM Mg2+. Nevertheless, the affinities of the codon–anticodon complexes, with Kd values of 4×10-10 M and 1×10-8 M at 10 and 5 mM Mg2+, respectively, are remarkably high. Previous values for the dissociation rate constant were 0.002/s (5°C) and 0.2/s (25°C), both at 5 mM Mg2+ (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 (3–14/s at 20°C; Grosjean et al., 1976; 0.1–4.7/s at 9°C; Grosjean et al., 1978). This suggests that binding interactions with the ribosome contribute up to a factor of 10 (5–6 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 (10–3–10–2/s) (Rodrına et al., 1996). In the presence of a fully complementary cognate codon, the GTPase rate constant (k3) increases to 500/s, i.e. ~105 times. Decreasing the Mg2+ concentration to 5 mM decreases the GTPase rate constant to 55/s. The latter value is in the range of previous values determined by other groups, i.e. 25/s under comparable conditions (Thompson and Dix, 1982; Eccleston et al., 1985) and 100/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 Mg2+ concentration. Many GTP-binding proteins have an absolute requirement for a divalent ion, usually Mg2+, as a cofactor in the enzymatic reaction. Mg2+ ions are essential for either the binding of the nucleotide, GTP hydrolysis or the structural integrity of the protein. With p21ras, the affinity for GDP is decreased 300-fold in the absence of Mg2+ (John et al., 1993). 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, k3. It is likely, therefore, that the effect of Mg2+ on k3 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 Mg2+ 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 ($k_6$) 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 ($k_{6p} = 3-4$/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 7–8/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 ($k_{pp} > 100$), completing the sequence of A-site binding. The rate constants of peptide bond formation were determined previously to be 0.3–1.1/s for cognate Phe-tRNA$^{\text{Phe}}$ 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 results may 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$_4$Cl, 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$^{\text{Phe}}$ 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$^{\text{Phe}}$ complex, 10 $\mu$M EF-Tu was incubated with 2 $\mu$M EF-Ts, 4 $\mu$M GDPNP and 5 $\mu$M [3H]Phe-tRNA$^{\text{Phe}}$(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 $\mu$M ribosomal complex was mixed with 0.2 $\mu$m 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 $\mu$M 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 $\mu$l each) of the ternary complex (0.2 $\mu$m), purified by gel filtration, and the ribosome complex (0.6–4.0 $\mu$m). 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 $\mu$l each) of ribosome complex and purified ternary complex containing [γ-32P]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 HClO$_4$/1.8 mM potassium phosphate. To measure rates of dipeptide formation, ternary complexes containing [3H]Phe-tRNA$^{\text{Phe}}$ (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, $k_{pp}$ 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.

$$A + B \rightarrow \begin{array}{c} k_1 \downarrow \rightarrow C \rightarrow \begin{array}{c} k_2 \rightarrow D \rightarrow E \\ k_3 \rightarrow F \\ k_4 \rightarrow G \rightarrow H \end{array} \\ k_5 \downarrow \rightarrow k_{6p} \downarrow \rightarrow k_{6p} \downarrow \rightarrow k_{6p} \downarrow \rightarrow k_6 \downarrow \rightarrow k_{6p} \downarrow \rightarrow k_{6p} \downarrow \rightarrow k_6 \downarrow \rightarrow k_{6p} \downarrow \rightarrow k_6 \downarrow \rightarrow k_{6p} \downarrow \rightarrow k_6 \downarrow \rightarrow k_{6p} \downarrow \rightarrow k_6 \end{array}$$

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 J, 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 (P₃) 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 P₃ = 11 independently of Mg²⁺ (Rodnina et al., 1996). The fluorescence changes further in the codon-anticodon complex D to P₄ = 14.0 and 12.7 at 10 and 5 mM Mg²⁺, respectively (Rodnina et al., 1994). The GTase 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 P₄ 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 P₅ is 12.3 and 11.5 at 10 and 5 mM Mg²⁺ and is the same for intermediates G, H, J and K. Thus, the overall proflavin fluorescence is F₉ = P₅(A + P₅ + P₆)/2 + P₇(E + F + I) + P₈(G + H + I + K).

The fluorescence of the mant-group is set to 10 for the free ternary complex (M₃ at 1 μM). It increases upon GTase activation (intermediate E, relative fluorescence M₄ = 13.4, and decreases to about the starting level upon dissociation of EF-Tu·GDP from the ribosome (complex L, relative fluorescence M₅) (Rodnina et al., 1995). The overall mant fluorescence is F₉,mant = M₅(A + C + D) + M₆(ε + F + G + H) + M₇L.

For the calculations, the values of kₐ, P₅ and P₇ (determined previously; Rodnina et al., 1996), as well as k₋₅ (determined independently in this study) were fixed. The fitting yields a unique solution for the rate constants k₋₅, as well as for the remaining fluorescence factors.

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References


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