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Codon-dependent conformational change of elongation factor Tu preceding GTP hydrolysis on the ribosome

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The mechanisms by which elongation factor Tu (EF-Tu) promotes the binding of aminoacyl-tRNA to the A site of the ribosome and, in particular, how GTP hydrolysis by EF-Tu is triggered on the ribosome, are not understood. We report steady-state and time-resolved fluorescence measurements, performed in the *Escherichia coli* system, in which the interaction of the complex EF-Tu·GTP·Phe-tRNA^{Phe} with the ribosomal A site is monitored by the fluorescence changes of either mant-dGTP [3′-*O*-(*N*-methylanthraniloyl)-2-deoxyguanosine triphosphate], replacing GTP in the complex, or of wybutine in the anticodon loop of the tRNA. Additionally, GTP hydrolysis is measured by the quench–flow technique. We find that codon–anticodon interaction induces a rapid rearrangement within the G domain of EF-Tu around the bound nucleotide, which is followed by GTP hydrolysis at an ~1.5-fold lower rate. In the presence of kirromycin, the activated conformation of EF-Tu appears to be frozen. The steps following GTP hydrolysis—the switch of EF-Tu to the GDP-bound conformation, the release of aminoacyl-tRNA from EF-Tu to the A site, and the dissociation of EF-Tu·GDP from the ribosome—which are altogether suppressed by kirromycin, are not distinguished kinetically. The results suggest that codon recognition by the ternary complex on the ribosome initiates a series of structural rearrangements resulting in a conformational change of EF-Tu, possibly involving the effector region, which, in turn, triggers GTP hydrolysis.

Key words: effector region/GAP/kirromycin/mant-dGTP/rapid kinetics

Introduction

The binding of aminoacyl-tRNA to the A site of the ribosome is catalysed by elongation factor Tu (EF-Tu), which belongs to the family of GTP binding proteins. EF-Tu·GTP binds aminoacyl-tRNA with high affinity to form a complex which enters the ribosomal A site. During the binding reaction, EF-Tu hydrolyses GTP, and both GTP hydrolysis and subsequent dissociation from the ribosome of EF-Tu·GDP are necessary for aminoacyl-tRNA to reach the reactive state in the A site (Kaziro, 1978). Conformational changes of EF-Tu are known to play an important role in the mechanism of the reaction. The binding of GTP to EF-Tu brings about the conformation of the enzyme necessary for the interaction with aminoacyl-tRNA and, subsequently, the ribosome. The transition of the factor from the GTP-bound form to the GDP-bound form, which follows the hydrolysis of GTP, involves an extensive conformational change of EF-Tu (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993) and leads to the release of the aminoacyl-tRNA and to the dissociation of the binary complex, EF-Tu·GDP, from the ribosome. The steps following GTP hydrolysis are inhibited by the antibiotic kirromycin which appears to block the transition of EF-Tu to the GDP-bound conformation and to freeze the enzyme in a conformation close to the GTP-bound one (Parmeggiani and Swart, 1985; Mesters *et al.*, 1994).

The various ligands of EF-Tu (GTP/GDP, aminoacyl-tRNA, ribosome, EF-Ts, kirromycin) occupy separate binding sites which are distributed among the three structural domains of the protein. The binding of GDP/GTP has been described in great detail including the structure of the binding pocket which is located in domain I (G domain, residues 1–200) of EF-Tu (Clark *et al.*, 1990; Kjeldgaard and Nyborg, 1992; Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993), as well as the differences between the GDP and the GTP form (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993). The interaction with the ribosome has been shown to involve domain II of the protein (Swart *et al.*, 1987; Tubulekas and Hughes, 1993). Based on the structural similarities between EF-Tu and p21 (Pai *et al.*, 1990), the region encompassing amino acids 40–60 in domain I (effector region) was discussed as an additional site for ribosome interaction (La Cour *et al.*, 1985; Clark *et al.*, 1990). The binding site for kirromycin is formed by residues of domains I and III (Abdulkarim *et al.*, 1994; Mesters *et al.*, 1994).

It is not known how the interaction with elongating ribosomes is activating the GTPase of EF-Tu, although interactions of the factor with the 530 loop of 16S RNA [Powers and Noller (1993) and references cited therein] and with domain VI of 23S rRNA (Hausner *et al.*, 1987; Moazed *et al.*, 1988; Tapprich and Dahlberg, 1990) have been implied. On the side of the factor, evidence from proteolysis experiments performed with EF-Tu from *Thermus thermophilus* supports the view that the region formed by residues 52–60 serves the function of an effector binding region (Peter *et al.*, 1990), i.e. responds to an external interaction by a conformational change which, in turn, triggers GTP hydrolysis. However, neither the external effector nor the structural changes of EF-Tu that accompany the binding of the ternary complex to the ribosome and induce GTP hydrolysis have been characterized in detail.

Here we report a study in which a fluorescent GTP derivative, mant-dGTP [3′-*O*-(*N*-methylanthraniloyl)-2-deoxyguanosine triphosphate], which is fully active in

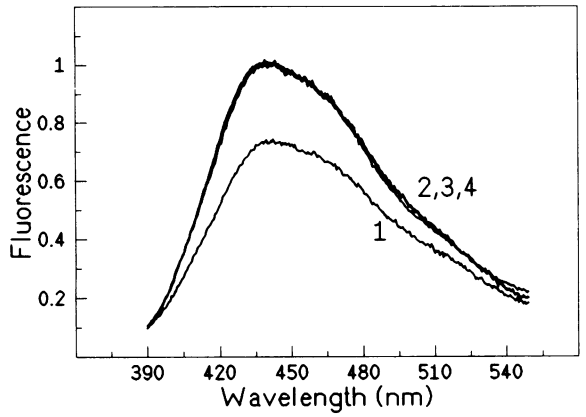

Fig. 1. Fluorescence spectra of mant-dGTP. Free in solution (1), EF-Tu·mant-dGTP (2), EF-Tu·mant-dGTP·Phe-tRNA^{Phe} (3) and EF-Tu·mant-dGDP (4; obtained from sample 3 by adding kirromycin). EF-Tu (10 μM) was incubated with mant-dGTP (2 μM), ATP (3 mM), phosphoenol pyruvate (6 mM) and pyruvate kinase (0.1 μg/ml) in buffer A for 60 min at 37°C; Phe-tRNA^{Phe} (10 μM) and kirromycin (50 μM) were added subsequently.

ternary complex formation and A-site binding, is used to follow the binding of the ternary complex to the A site in rapid kinetic experiments employing the stopped-flow technique. In parallel, GTP hydrolysis is followed in quench–flow experiments. The fluorescent GTP derivative reports conformational changes of EF-Tu which are due to the interaction of EF-Tu with the ribosome and depend on cognate codon–anticodon interaction. The kinetic experiments reveal the rapid formation of a transient conformational state of EF-Tu which is induced by codon recognition and precedes the hydrolysis of GTP. The transient activated state of EF-Tu can be trapped by kirromycin and, therefore, studied spectroscopically.

Results

Binding of mant-dGTP and mant-dGDP to EF-Tu

The binding of mant-dGTP to EF-Tu leads to an increase in the fluorescence by ~30% without a significant spectral shift (Figure 1); the same effect we observe with mant-dGDP (not shown), in accord with earlier results obtained with mant-GDP (Eccleston *et al.*, 1989). The lack of any fluorescence difference between EF-Tu·mant-dGTP and EF-Tu·mant-dGDP was verified in an experiment with the antibiotic kirromycin which induces GTP hydrolysis on EF-Tu·GTP in the absence of ribosomes (Chinali *et al.*, 1977). When the EF-Tu·mant-dGTP complex is incubated with kirromycin, the fluorescence does not change (Figure 1) while, as revealed by HPLC analysis, all mant-dGTP bound to EF-Tu is hydrolysed under these conditions (data not shown). The fluorescence of the EF-Tu·mant-dGTP complex does not change either when Phe-tRNA^{Phe} is bound to the complex (Figure 1). Thus, the environment of the mant group of mant-dGTP bound to EF-Tu does not change extensively upon either hydrolysis of mant-dGTP or binding of aminoacyl-tRNA.

To determine the cause of the increase in mant-dGTP fluorescence upon binding to EF-Tu, we have monitored the solvent accessibility of the fluorophore in the complex with EF-Tu by the technique of fluorescence quenching. As a quencher, acrylamide was used, which has been shown to quench efficiently the fluorescence of mant-GDP (Eccleston *et al.*, 1989). Stern–Volmer quenching constants, calculated from the dependence of the relative fluorescence on the concentration of acrylamide (Materials and methods), are 3/M and 1.5/M for free and EF-Tu-bound mant-dGTP, respectively. Essentially the same numbers were reported for free and EF-Tu-bound mant-GDP (Eccleston *et al.*, 1989). A 2-fold difference in the quenching constants of free and EF-Tu-bound nucleotides is of the order expected from the lowering of the diffusion constant of mant-dGTP upon binding to EF-Tu. Hence, the mant group is probably not shielded appreciably in the complex; some shielding by nearby residues of the protein cannot be excluded, since the quenching rate constant has not been determined.

Activity of the ternary complex containing mant-dGTP

The complex EF-Tu·mant-dGTP·Phe-tRNA^{Phe} is as stable as the corresponding complex with GTP and can also be purified by gel chromatography (Materials and methods). For the stopped-flow experiments, purified complexes were used. With the isolated complexes, both the efficiency of the complex binding to the A site and the extent of peptide bond formation under the conditions of the kinetic experiments were checked routinely by the nitrocellulose filter binding assay and by HPLC analysis of the peptides, respectively.

According to these assays, >90% of the complex EF-Tu·mant-dGTP·[^{14}C]Phe-tRNA^{Phe} was bound by an excess of poly(U)-programmed ribosomes carrying AcPhe-tRNA^{Phe} in the P site, and >80% of the ribosome-bound radioactivity was found in the dipeptide AcPhe[^{14}C]Phe. HPLC analyses run in parallel showed that, following the binding of the EF-Tu complex to the ribosome, all mant-dGTP in the complex is hydrolysed to mant-dGDP. Thus, the results of the biochemical assays demonstrate that mant-dGTP is as active as GTP in all steps of A-site binding and subsequent peptide bond formation.

Also kinetically, mant-dGTP is equivalent to GTP. This has been verified by fluorescence stopped-flow experiments using EF-Tu complexes containing a fluorescent tRNA derivative (Rodnina *et al.*, 1994) and either GTP or mant-dGTP (data not shown).

Conformational changes of EF-Tu during ternary complex binding to the A site indicated by mant-dGTP fluorescence

In the stopped-flow experiment, the binding of purified EF-Tu·mant-dGTP·Phe-tRNA^{Phe}(yeast) to poly(U)-programmed ribosomes is accompanied by a biphasic change in the fluorescence of mant-dGTP (Figure 2): a rapid (k_{app1} = 18/s) fluorescence increase by ~60% is followed by a slower (k_{app2} = 5/s) fluorescence decrease of the same amplitude. No signal change is observed with poly(A)-programmed ribosomes (Figure 2), which means that codon–anticodon interaction is essential for the observed reactions to occur. Qualitatively the same results are obtained with ternary complex containing Phe-tRNA^{Phe} from *Escherichia coli*, except that both steps are slightly slower at 16 and 3/s, respectively. The nature of the P site-bound tRNA, AcPhe-tRNA^{Phe} or deacyated tRNA^{Phe}

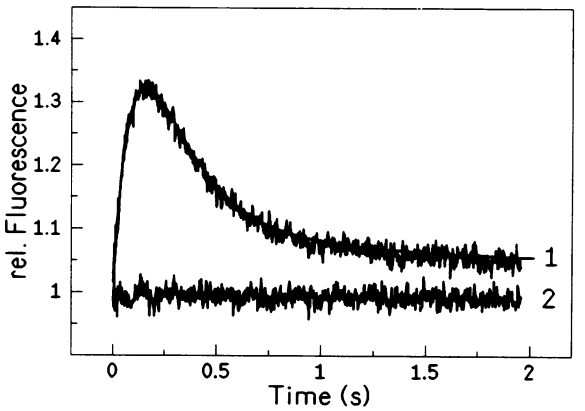

Fig. 2. Fluorescence changes of mant-dGTP during A-site binding of the ternary complex. Purified EF-Tu·mant-dGTP·Phe-tRNA^{Phe} was mixed with ribosome complexes as indicated: (1) poly(U)-programmed ribosomes with AcPhe-tRNA^{Phe} in the P site; (2) poly(A)-programmed ribosomes with tRNA^{Lys} in the P site. The curves were evaluated by two-exponential fitting assuming pseudo first-order kinetics (smooth line): (1) k_{app1} = 18/s, A_1 = 61%, k_{app2} = 5/s, A_2 = –59%.

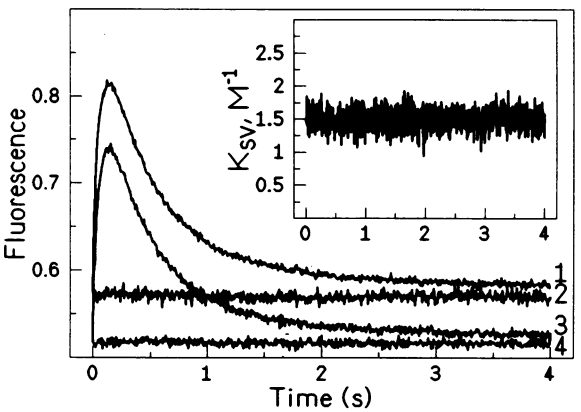

Fig. 3. Fluorescence quenching of mant-dGTP during A-site binding. EF-Tu·mant-dGTP·Phe-tRNA^{Phe} was mixed with poly(U)-programmed ribosomes with AcPhe-tRNA^{Phe} in the P site as in Figure 2 without added quencher (1) and with acrylamide (0.05 M) present in both solutions (3); curves (2) and (4) show the signals of control experiments with buffer alone instead of ribosomes in the absence and presence of acrylamide, respectively. Fluorescence is given in arbitrary units. The inset shows the quenching constant, $K_{SV}(t)$, calculated from curves (1) and (3) according to the Stern–Volmer equation: $I_1(t)/I_3(t) = 1 + K_{SV}(t)\cdot 0.05$.

does not influence the results, i.e. none of the steps is due to peptide bond formation.

The changes in the fluorescence signal are not due to the hydrolysis of mant-dGTP, since the fluorescent group is not sensitive to the conformational change of EF-Tu which accompanies the hydrolysis (see above). To explain the fluorescence change, two possibilities may be considered. First, the fluorescence may be increased by shielding from solvent access of the fluorophor brought about by binding to the ribosome and decreased again by subsequent deshielding due to dissociation of the EF-Tu following the hydrolysis of mant-dGTP. Alternatively, the observed fluorescence changes may reflect a conformational change of EF-Tu at the GTP binding site, which takes place after codon–anticodon recognition in the A site, and another conformational change, probably the reversal of the first, which follows the hydrolysis of mant-dGTP.

To test the shielding hypothesis, the stopped-flow experiment was performed in the presence of acrylamide (50 mM) which efficiently quenches the fluorescence of mant-dGTP in the EF-Tu complex (see above) and thereby lowers the initial signal observed in the binding experiment. A shielding brought about by the ribosome should then lead to a larger fluorescence increase upon formation of the A-site complex, compared to the signal change observed in the absence of the quencher. In the stopped-flow experiment, the addition of acrylamide has no detectable influence on the amplitudes of the observed signal changes (Figure 3). This observation excludes the possibility that mant-dGTP experiences an additional shielding against solvent access by the binding of the ternary complex to the ribosome. Rather, it strongly supports the alternative interpretation, i.e. that the observed fluorescence effects are caused by conformational changes of EF-Tu induced by codon–anticodon interaction on the ribosome.

Kirromycin freezes an intermediate state of EF-Tu during A-site binding

The antibiotic kirromycin, apart from stimulating the GTPase activity of EF-Tu, inhibits the dissociation of EF-Tu·GDP from the ribosome (Wolf *et al.*, 1977), presumably by trapping the factor in a conformation like the GTP-bound one (Parmeggiani and Swart, 1985; Mesters *et al.*, 1994). To see which of the steps reported by mant-dGTP is/are influenced by kirromycin, the stopped-flow experiment was performed in the presence of the antibiotic. To avoid the premature hydrolysis of mant-dGTP in the ternary complex (which kirromycin bound to the complex will induce) as much as possible, the antibiotic was added only to the ribosome solution to be mixed with the ternary complex. Figure 4 shows that, in the presence of kirromycin, the first step is affected only slightly in that the amplitude is lowered somewhat, presumably by some premature kirromycin-induced hydrolysis of mant-dGTP in the ternary complex, while the second step, i.e. the signal decrease, is suppressed completely. The biochemical analysis reveals that the binding of the ternary complex is ~30% lower, again attributable to premature hydrolysis

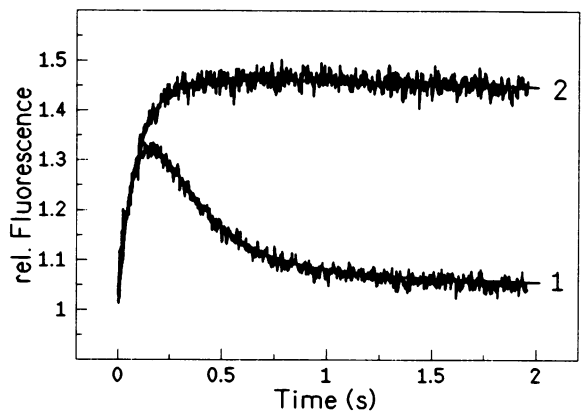

Fig. 4. Effect of kirromycin on A-site binding of EF-Tu·mant-dGTP·Phe-tRNA^{Phe}. Experiment (1) was performed as in Figure 2, while in (2) kirromycin (40 μM) was present in the ribosome solution. Parameters of the two-exponential fits: (1) without kirromycin k_{app1} = 18/s, A_1 = 61%, k_{app2} = 5/s, A_2 = –59%; (2) with kirromycin k_{app1} = 18/s, A_1 = 48%, k_{app2} = 3/s, A_2 = –1%.

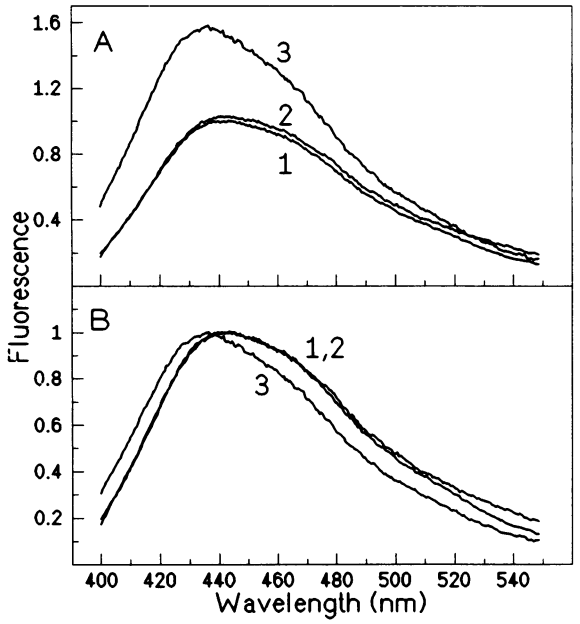

Fig. 5. Emission spectra of EF-Tu·mant-dGTP·Phe-tRNA^{Phe} (1), EF-Tu·mant-dGDP·kirromycin (2), and of ribosome-bound EF-Tu·mant-dGDP·Phe-tRNA^{Phe}·kirromycin (3). In (**A**), the spectra are shown as measured; in (**B**), spectra (2) and (3) are normalized to spectrum (1). (1) EF-Tu·mant-dGTP·Phe-tRNA^{Phe} (0.2 μM) was purified by gel filtration, λ_{max} 444 nm; (2) obtained from sample 1 by incubation with 20 μM kirromycin, λ_{max} 444 nm; (3) obtained from sample 1 by adding poly(U)-programmed ribosomes with AcPhe-tRNA^{Phe} in the P site (0.24 μM) containing kirromycin (20 μM after mixing), λ_{max} 437 nm. Blank spectra, measured with the respective samples without mant-dGTP, are subtracted.

of mant-dGTP, whereas peptide bond formation is inhibited completely. These results suggest that the fluorescence decrease observed during the interaction of EF-Tu·mant-dGTP·Phe-tRNA^{Phe} with the A site is in fact related to the release of EF-Tu·mant-dGDP from the ribosome or a conformational change of EF-Tu preceding it.

In the presence of kirromycin, not only the relative fluorescence of mant-dGDP in the ribosome complex is increased by ~50%, as also seen in the stopped-flow experiment (Figure 4), but also the emission spectrum is blue-shifted by 7 ± 2 nm (Figure 5). The latter observation may be taken as an indication that in the state frozen on the ribosome by kirromycin the environment of the fluorophor is more hydrophobic than in both the initial (EF-Tu·mant-dGTP·Phe-tRNA^{Phe}) or final (EF-Tu·mant-dGDP) states.

Codon recognition monitored by wybutine fluorescence

To monitor codon–anticodon interaction directly, we have utilized the fluorescence of wybutine in yeast tRNA^{Phe} which, due to its position 3′ to the anticodon, is sensitive to codon binding (Paulsen *et al.*, 1982). When Phe-tRNA^{Phe} in the complex with EF-Tu·GTP binds to the A site of poly(U)-programmed ribosomes, the fluorescence of wybutine increases rapidly, whereas there is no effect when the A site is misprogrammed with poly(A) (Figure 6). Most of the fluorescence change (>85%) is due to a rapid step (k_{app} = 23/s) which is followed by a slow further increase (k_{app} = 2/s) with small amplitude. The

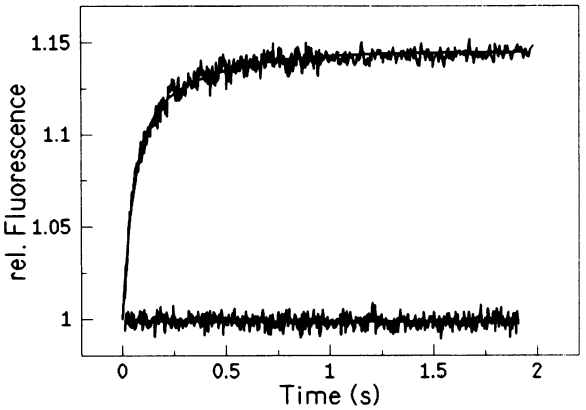

Fig. 6. Changes in wybutine fluorescence during A-site binding of EF-Tu·GTP·Phe-tRNA^{Phe}. Ribosomes were programmed with poly(U) (upper trace) or poly(A) (lower trace) and contained, respectively, AcPhe-tRNA^{Phe} or tRNA^{Lys} in the P site. The stopped-flow experiment was carried out as in Figure 2. Parameters of the two-exponential fit: k_{app1} = 23/s, A_1 = 12%; k_{app2} = 2/s, A_2 = 2%.

same result is obtained when GTP in the ternary complex is replaced by GTPγS, when tRNA^{Phe} rather than AcPhe-tRNA^{Phe} is in the P site, or when kirromycin is present (data not shown). Thus, the rapid increase in wybutine fluorescence exclusively reflects the codon–anticodon recognition step and none of the subsequent steps. (The origin of the slower small increase of wybutine fluorescence is not clear at present.) As shown earlier (Paulsen *et al.*, 1982), the emission spectrum of wybutine in tRNA^{Phe} is blue-shifted upon binding the codon triplet in the A site; this indicates that, by binding to the codon, the stacking interactions of wybutine with the neighbouring adenines are changed, i.e. that the conformation of the anticodon loop changes.

GTP hydrolysis takes place after codon–anticodon interaction

To determine the rate of GTP hydrolysis during A-site binding of the ternary complex under the conditions used for the stopped-flow experiments, quench–flow measurements were performed. [^{3}H]GTP is hydrolysed at a rate of (12 ± 3)/s when the EF-Tu complex containing Phe-tRNA^{Phe} binds to poly(U)-programmed ribosomes carrying AcPhe-tRNA^{Phe} in the P site (Figure 7). In contrast, when the ternary complex EF-Tu·[^{3}H]GTP·Phe-tRNA^{Phe} is bound to poly(A)-misprogrammed ribosomes, [^{3}H]GTP is hydrolysed extremely slowly under the present conditions (~10^{-3}/s; Rodnina *et al.*, in preparation), although a complex with the ribosomes is formed rapidly (Rodnina *et al.*, 1993). (The formation of this initial complex is not reported by the fluorophores used in the present study, but by a proflavin label in the D loop of tRNA.) Thus, codon–anticodon interaction is absolutely required in order to get rapid GTP hydrolysis.

Discussion

Binding of mant-dGTP to EF-Tu

According to the structure of the GMPPNP complex of EF-Tu from *T.thermophilus* (Berchtold *et al.*, 1993) and *Thermus aquaticus* (Kjeldgaard *et al.*, 1993), the ribose moiety of the nucleotide is positioned such that the 2′-

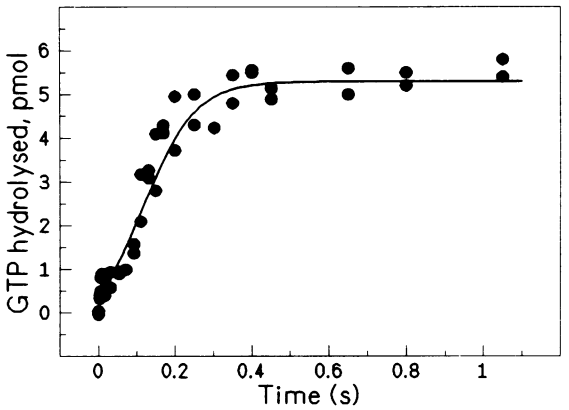

Fig. 7. Time course of GTP hydrolysis during A-site binding of ternary complex. Quench–flow experiments were performed with poly(U)-programmed ribosomes carrying AcPhe-tRNAPhe in the P site and purified ternary complex with [^{3}H]GTP under the conditions of Figure 2 as described in Materials and methods. The apparent rate constant of GTP hydrolysis, k_{app}(GTP) = (12 ± 3)/s, was estimated by the fitting procedure described previously (Bilgin *et al.*, 1992) to take into account the sigmoidal behaviour in the beginning.

and 3′-hydroxyl groups are pointing towards the solvent and should not be protected in the complex with the protein. Thus, the mant group at the 3′ position in mant-dGTP is also expected to be exposed in the complex with EF-Tu, in accordance with the fluorescence quenching data. On the other hand, the data do not exclude that the fluorescence increase without spectral shift observed upon binding the nucleotide to the factor may nevertheless be due to some shielding by residues of the protein located in the vicinity of the 3′ mant group.

The hydrolysis of mant-dGTP to mant-dGDP in the complex with EF-Tu, induced by kirromycin, has no effect on the fluorescence signal. Thus, the environment of the fluorophor in the G domain is not different in the GTP-bound and GDP-bound conformations.

GTP hydrolysis is initiated by a conformational change of EF-Tu

The large fluorescence increase that accompanies the binding of the ternary complex containing mant-dGTP to the ribosome shows that the environment of the fluorescent group changes extensively upon binding. As suggested by the quenching experiments, the effect does not originate from a protection of the fluorophor by the ribosome. Thus, the observed fluorescence increase is likely to reflect a structural rearrangement of EF-Tu that leads to a conformation around the nucleotide which differs from the one in the GTP- or GDP-bound state of the free factor. The observation that kirromycin is keeping EF-Tu in the high fluorescence state on the ribosome suggests that the antibiotic freezes the factor not only in a GTP-like conformation with respect to the arrangement of the structural domains of the protein (Parmeggiani and Swart, 1985; Mesters *et al.*, 1994), but in a conformation of the G domain which represents the activated state of the enzyme with respect to GTP hydrolysis.

In the crystal structure of EF-Tu·GMPPNP from thermophiles (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993), the ribose moiety of the nucleotide is located close to the effector region (amino acids 41–62; Peters *et al.*, 1990), the closest amino acid being Tyr 47 (or Phe 46 in the *E.coli* protein). Thus, it is conceivable, though at present speculative, that it is a movement of the effector region towards the GTP site in the G domain that causes the observed fluorescence change and constitutes the trigger of GTP hydrolysis.

The hydrolysis of GTP is followed by the conformational switch of EF-Tu from the GTP-bound to the GDP-bound form. Since we did not observe any fluorescence change due to these reactions in ternary complex not bound to the ribosome (Figure 1), the fluorescence decrease observed with mant-dGTP on the ribosome is attributed to the returning of the factor from the activated state to the ground state that either precedes or accompanies the transition to the GDP-bound conformation, i.e. the step blocked by kirromycin. In the latter conformation, due to a lowered binding affinity, EF-Tu releases the aminoacyl-tRNA, thereby allowing the aminoacyl end of the tRNA to accommodate in the A site, and EF-Tu·GDP dissociates from the ribosome. A fluorescence change in the same time range was also seen with a fluorescent label in the tRNA (Rodnina *et al.*, 1994), suggesting that the steps following GTP hydrolysis are coupled, probably through the conformational switch of EF-Tu.

Coupling of codon-dependent conformational changes of aminoacyl-tRNA and EF-Tu

The question remains how codon–anticodon interaction, conformational change of EF-Tu and GTP hydrolysis are related to each other. The simplest model assumes that these events occur independently and are not causally related. Although this model is not strictly excluded, and never can be on the basis of kinetic data alone, it is unlikely in that the signal change exhibited by mant-dGTP on EF-Tu as well as rapid GTP hydrolysis do not take place at all in the absence of codon–anticodon interaction. An alternative model would be that the conformational change of the anticodon loop, and possibly also of the mRNA, that is induced by codon–anticodon interaction creates a conformational signal which is transmitted to EF-Tu. In fact, preliminary data suggest that, at high ribosome concentration, the rate of codon recognition is significantly larger than the rate of the conformational change of EF-Tu. This finding is consistent with, though not proving, the sequential model.

Previously, the ribosome has been implicated in signalling the event of cognate codon–anticodon interaction to EF-Tu and activating the GTPase, e.g. through structural rearrangements of 23S rRNA (Moazed *et al.*, 1988; Tapprich and Dahlberg, 1990) and/or 16S rRNA (Powers and Noller, 1994). Our results suggest the tRNA may be involved as well. In the crystal structure of the ternary complex, interactions of the acceptor end of the tRNA with interface residues of domains I and II, as well as of the acceptor and T stems with domain III of EF-Tu, are seen (J.Nyborg, personal communication). The interactions between the acceptor domain of the tRNA and the factor may function in the direct communication between the two molecules, thereby providing a potential link between codon–anticodon interaction and GTP hydrolysis by EF-Tu. It has been shown that binding of the codon, or a tRNA with a complementary anticodon, induces a conformation of the tRNA in which tertiary

structure interactions between D and T loops are opened (Rigler and Wintermeyer, 1983; Moras *et al.*, 1985). Thereby, the acceptor domain of the tRNA, upon codon–anticodon interaction, may become free to move relative to the anticodon domain and change the interactions with the factor. In this model, the tRNA that has recognized the cognate codon in the A site is acting as an effector of EF-Tu. Presumably, the ribosome is also involved, possibly by providing binding interactions that promote the formation of the activated structure of EF-Tu.

GTP hydrolysis by EF-Tu needs to be triggered by codon–anticodon interaction

It has been reported that the binary complex EF-Tu·GTP binds to the ribosome slowly (5×10^4/M/s) and hydrolyses GTP at a significant rate ($\geq$1/s), when bound to the ribosome (Thompson *et al.*, 1986). It was suggested, therefore, that the rate constant for GTP hydrolysis is determined exclusively by the interactions of EF-Tu with the ribosome and is independent of the aminoacyl-tRNA (Thompson, 1988). Our GTPase data show that the interaction of the non-cognate ternary complex with the ribosome is radically different from that of the binary complex, EF-Tu·GTP. In contrast to the latter, the ternary complex binds to the ribosome quite rapidly (~5×10^7/M/s), independent of the codon in the A site (Rodnina *et al.*, 1993). Thus, the low efficiency of GTP hydrolysis upon interaction of the ternary complex with misprogrammed ribosomes, which was repeatedly reported, is not due to the low rate of binding to the ribosome. Moreover, the rate constant of GTP hydrolysis in EF-Tu·GTP·Phe-tRNA^{Phe} on ribosomes misprogrammed with poly(A) has been determined to be ~10^{-3}/s (Rodnina *et al.*, manuscript in preparation), which is 10 000 times lower than the rate of GTP hydrolysis in the cognate complex. The fact that the GTPase is practically completely suppressed when a non-cognate EF-Tu·GTP·aminoacyl-tRNA complex is bound to the ribosome also suggests, in line with the conclusions from the fluorescence data, that fast GTP hydrolysis is induced by codon–anticodon interaction.

Materials and methods

Buffers and reagents

The following buffer was used (buffer A): 25 mM Tris–HCl (pH 7.5), 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTE, 0.5 mM EDTA. The experiments were performed at 20°C, if not stated otherwise.

Poly(U), poly(A), ATP, GTP, DTE, spermidine, putrescine, pyruvate kinase and phosphoenol pyruvate were from Boehringer-Mannheim. Radioactive compounds were from Amersham or UVVVR, Prague. Mant-dGTP and mant-dGDP were gifts of R.Goody; kirromycin was donated by A.Parmeggiani.

tRNA, EF-Tu and ribosomes

Tightly coupled 70S ribosomes from *E.coli* MRE 600 (Rodnina *et al.*, 1994; 90% active in tRNA binding to both P and A sites, assuming 23 pmol/A_{260} unit), EF-Tu from *E.coli* K12 (Ehrenberg *et al.*, 1990; fully active in binding Phe-tRNA^{Phe} or GDP), [^{14}C]Phe-tRNA^{Phe} and Ac[^{14}C]Phe-tRNA^{Phe} (purified by HPLC on RP-4 to 1.75 nmol/A_{260} unit) from *E.coli* and [^{14}C]Phe-tRNA^{Phe} from yeast (1.5 nmol/A_{260} unit) were prepared as described previously (Rodnina and Wintermeyer, 1992). tRNA^{Lys} (charging capacity 1.5 nmol/A_{260} unit) from *E.coli* was purchased from Subriden RNA.

EF-Tu·GTP·Phe-tRNA^{Phe} complexes

For preparative purposes, ~7 nmol of nucleotide-free EF-Tu (Rodnina and Wintermeyer, 1995) were incubated for 10 min at 37°C with 0.5 mM mant-dGTP, 3 mM ATP, 6 mM phosphoenol pyruvate and 0.1 μg/ml pyruvate kinase in buffer A, followed by the addition of 3 nmol of [^{14}C]Phe-tRNA^{Phe} (final volume 230 μl) and incubation for 5 min. The EF-Tu·Phe-tRNA^{Phe} complex was purified by gel filtration on Superdex 75 (two Pharmacia HR 10/30 columns in tandem) using FPLC. Fractions of 0.16 ml were collected and 10 μl aliquots were taken to determine the elution position of the complex by counting radioactivity. In this chromatography, the EF-Tu·GTP·Phe-tRNA^{Phe} complex was completely separated from unbound Phe-tRNA^{Phe}, tRNA^{Phe}, free EF-Tu and smaller molecules. The final concentration of the complex was determined according to the radioactivity of [^{14}C]Phe-tRNA^{Phe} in the sample; usually it was ~0.7–0.9 μM. The complexes were stored on ice and diluted to the desired concentration (0.2 μM) immediately before the stopped-flow experiment.

As described elsewhere, the composition of the EF-Tu·GTP complex with aminoacyl-tRNA depends on the relative amounts of factor and tRNA (Rodnina and Wintermeyer, 1995). Under the present conditions, a quinternary complex (EF-Tu·GTP)$_2$·Phe-tRNA^{Phe} is formed, as described previously (Ehrenberg *et al.*, 1990). Control experiments, using the true ternary complex, have revealed the same kinetic picture as reported here (data not shown). Thus, for the present purpose, the two types of EF-Tu complexes are not distinguished.

Ribosome complexes

To form a ribosome complex with blocked P site, ribosomes (0.6 μM) were incubated for 15 min at 20°C with poly(U) (2 A_{260} units/ml) and with a 1.1-fold excess relative to active ribosomes of AcPhe-tRNA^{Phe} or tRNA^{Phe}. Alternatively, the ribosomes were programmed with poly(A) (1 A_{260} unit/ml), and the P site was blocked with a 1.1-fold excess of tRNA^{Lys}.

Biochemical assays

Biochemical assays were performed as previously described (Rodnina *et al.*, 1994).The extent of hydrolysis of mant-dGTP to mant-dGDP was analysed by HPLC on RP 8. The sample was applied in 65 mM potassium phosphate (pH 6.0), 2 mM tetrabutylammonium hydrogen sulphate, 15% acetonitrile, and the column was developed with 22% acetonitrile in the same buffer at a flow rate of 1 ml/min; the fluorescence in the eluate was measured by a flow-through monitor (Merck-Hitachi F-1100). A clear separation was achieved, mant-dGDP eluting ~1 min after mant-dGTP.

Fluorescence measurements

Fluorescence measurements were performed on a Schoeffel RRS 1000 spectrofluorimeter which was interfaced to a PC/AT computer equipped with a mathematical co-processor. The excitation wavelength was 370 nm; emission spectra (scanned at 0.2 nm intervals) were corrected for the wavelength dependence of the detection system (Paulsen *et al.*, 1983). To eliminate polarization effects, the emitted light was passed through a depolarizer placed in front of the emission monochromator.

For measuring the emission spectrum of EF-Tu-bound mant-dGDP, nucleotide-free EF-Tu (10 μM) was incubated with mant-dGDP (2 μM) in buffer A for up to 60 min at 37°C in the dark. Alternatively, the spectrum of EF-Tu·mant-dGDP was obtained after incubating EF-Tu·mant-dGTP (see below) with kirromycin (20 μM, 15 min, 20°C) to induce mant-dGTP hydrolysis (Figure 1). In the experiments with mant-dGTP, additionally 3 mM ATP, 6 mM phosphoenol pyruvate and 0.1 μg/ml pyruvate kinase were added. To obtain the EF-Tu·mant-dGTP·Phe-tRNA^{Phe} complex, 10 μM Phe-tRNA^{Phe} was added.

Fluorescence quenching titrations were performed by measuring the fluorescence intensity in the absence (I_o) and in the presence (I) of increasing amounts of acrylamide (up to 100 mM). To determine the quenching constant, K_{SV}, the data were analysed according to the Stern–Volmer equation for collisional quenching, as described previously (Robertson and Wintermeyer, 1981).

Kinetic experiments

The stopped-flow experiments were performed at 20°C by rapidly (dead time 2 ms) mixing 60 μl each of the solutions of the ternary complex (0.2 μM) and the ribosome complex (0.6 μM) in a stopped-flow apparatus (SFM 3, Biologic, France), as described previously (Rodnina *et al.*, 1994). Excitation wavelengths were 363 nm for mant-dGTP and 312 nm for wybutine; the fluorescence of both mant and wybutine was measured after passing cut-off filters (KV 408, Schott). The data (4000 data points per shot; several shots averaged) were evaluated by fitting an expression containing the sum of two exponential terms, characterized by variable time constants, k_{app}, and amplitudes, A (given relative to the starting

signal of ternary complex alone), by a least-squares procedure (Rodnina *et al.*, 1994). The reproducibility of rate constants and amplitudes in independent experiments was better than ±10% and ±20%, respectively; within one experiment, the reproducibility from shot to shot was ±5% for both parameters.

Quench–flow experiments were performed as described previously (Bilgin *et al.*, 1992) under conditions identical to those employed in the fluorescence stopped-flow experiments. Equal volumes of ribosome complex and purified ternary complex containing [^{3}H]GTP were rapidly mixed, and after the desired incubation time the reaction was terminated by quenching with formic acid. After centrifugation, an aliquot from the supernatant was analysed by TLC chromatography on PEI–cellulose to determine the relative amounts of [^{3}H]GTP and [^{3}H]GDP.

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References

Abdulkarim,F., Liljas,L. and Hughes,D. (1994) Mutations to kirromycin resistance occur in the interface of domains I and III of EF-Tu·GTP. *FEBS Lett.*, **352**, 118–122.

Berchtold,H., Reshetnikova,L., Reiser,C.O.A., Schirmer,K., Sprinzl,M. and Hilgenfeld,R. (1993) Crystal structure of active elongation factor Tu reveals major domain rearrangements. *Nature*, **365**, 126–132.

Bilgin,N., Claesens,F., Pahwerk,H. and Ehrenberg,M. (1992) Kinetic properties of *Escherichia coli* ribosomes with altered forms of S12. *J. Mol. Biol.*, **224**, 1011–1027.

Chinali,G., Wolf,H. and Parmeggiani,A. (1977) Effect of kirromycin on elongation factor Tu. *Eur. J. Biochem.*, **75**, 55–65.

Clark,B.F.C., Kjeldgaard,M., La Cour,T.F.M., Thirup,S. and Nyborg,J. (1990) Structural determination of the functional sites of *E. coli* elongation factor Tu. *Biochim. Biophys. Acta*, **1050**, 203–208.

Eccleston,J.F., Kanagasabai,T.F., Molloy,D.P., Neal,S.E. and Webb,M.R. (1989) The application of fluorescent and photosensitive analogues of guanine nucleotides to the function and structure of G-binding proteins. In Bosch,L., Kraal,B. and Parmeggiani,A. (eds), *The Guanine-Nucleotide Binding Proteins*. Plenum Publishing Corp., New York, pp. 87–99.

Ehrenberg,M., Bilgin,N. and Kurland,C.G. (1990) Design and use of a fast and accurate *in vitro* translation system. In Spedding,G. (ed.), *Ribosomes and Protein Synthesis. A Practical Approach*. Oxford University Press, New York, pp. 101–130.

Hausner,T.P., Atmadja,J. and Nierhaus,K.H. (1987) Evidence that 2661 region of 23S rRNA is located at the ribosome binding sites of both elongation factors. *Biochimie*, **69**, 911–923.

Kaziro,Y. (1978) The role of guanosine-5′-triphosphate in polypeptide chain elongation. *Biochim. Biophys. Acta*, **505**, 95–127.

Kjeldgaard,M. and Nyborg,J. (1992) Refined structure of elongation factor Tu from *Escherichia coli*. *J. Mol. Biol.*, **223**, 721–742.

Kjeldgaard,M., Nissen,P., Thirup,S. and Nyborg,J. (1993) The crystal structure of elongation factor EF-Tu from *Thermus aquaticus* in the GTP conformation. *Structure*, **1**, 35–50.

La Cour,T.F.M., Nyborg,J., Thirup.,S. and Clark,B.F.C. (1985) Structural details of the binding of guanosine diphosphate to elongation factor Tu from *E. coli* as studied by X-ray crystallography. *EMBO J.*, **4**, 2386–2388.

Mesters,J.R., Zeef,L.A.H., Hilgenfeld,R., Degraaf,J.M., Kraal,B. and Bosch,L. (1994) The structural and functional basis for the kirromycin resistance of mutant EF-Tu species in *E. coli*. *EMBO J.*, **13**, 4877–4885.

Moazed,D., Robertson,J.M. and Noller,H.F. (1988) Interaction of elongation factors EF-G and EF-Tu with a conserved loop in 23S rRNA. *Nature*, **334**, 362–364.

Moras,D., Dock,A.C., Dumas,P., Westhof,E., Romby,P., Ebel,J.P. and Giegé,R. (1985) The structure of yeast tRNA(Asp), a model for tRNA interacting with messenger RNA. *J. Biomol. Struct. Dyn.*, **3**, 479–492.

Pai,E.F., Krengel,U., Petsko,G.A., Goody,R.S., Kabsch,W. and Wittinghofer,A. (1990) Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 Å resolution: implications for the mechanism of GTP hydrolysis. *EMBO J.*, **8**, 2351–2359.

Parmeggiani,A. and Swart,G.W.M. (1985) Mechanism of action of kirromycin-like antibiotics. *Annu. Rev. Microbiol.*, **39**, 557–577.

Paulsen,H., Robertson,J.M. and Wintermeyer,W. (1982) Effect of ribosome binding and translocation on the anticodon of tRNA^{Phe} as studied by wybutine fluorescence. *Nucleic Acids Res.*, **10**, 2651–2663.

Paulsen,H., Robertson,J.M. and Wintermeyer,W. (1983) Topological arrangement of two transfer RNAs on the ribosome. Fluorescence energy transfer measurements between A and P site-bound tRNA^{Phe}. *J. Mol. Biol.*, **167**, 411–426.

Peter,M.E., Schirmer,N.K., Reiser,C.O.A. and Sprinzl,M. (1990) Mapping of the effector region in *Thermus thermophilus* elongation factor Tu. *Biochemistry*, **29**, 2876–2884.

Powers,T. and Noller,H.F. (1993) Evidence for functional interaction between elongation factor Tu and 16S RNA. *Biochemistry*, **90**, 1364–1368.

Powers,T. and Noller,H.F. (1994) The 530 loop of 16S rRNA: a signal to EF-Tu? *Trends Genet.*, **10**, 27–31.

Rigler,R. and Wintermeyer,W. (1983) Dynamics of tRNA. *Annu. Rev. Biophys. Bioeng.*, **12**, 475–505.

Robertson,J.M. and Wintermeyer,W. (1981) Effect of translocation on topology and conformation of anticodon and D loops of tRNA^{Phe}. *J. Mol. Biol.*, **151**, 57–79.

Rodnina,M.V. and Wintermeyer,W. (1992) Two tRNA-binding sites in addition to A and P sites on eukaryotic ribosomes. *J. Mol. Biol.*, **228**, 450–459.

Rodnina,M.V. and Wintermeyer,W. (1995) GTP consumption of elongation factor Tu during translation of heteropolymeric mRNAs. *Proc. Natl Acad. Sci. USA*, **92**, 1945–1949.

Rodnina,M.V., Fricke,R. and Wintermeyer,W. (1993) Kinetic fluorescence study on EF-Tu-dependent binding of Phe-tRNA^{Phe} to the ribosomal A site. In Nierhaus,K.H., Franceschi,F., Subramanian,A.R., Erdmann,V.A. and Wittmann-Liebold,B. (eds), *The Translational Apparatus. Structure, Function, Regulation, Evolution*. Plenum, New York and London, pp. 317–326.

Rodnina,M.V., Fricke,R., Kuhn,L. and Wintermeyer,W. (1994) Transient conformational states of aminoacyl-tRNA during ribosome binding catalyzed by elongation factor Tu. *Biochemistry*, **33**, 12267–12275.

Swart,G.W.M., Parmeggiani,A., Kraal,B. and Bosch,L. (1987) Effects of the mutation Gly-222→Asp on the functions of elongation factor Tu. *Biochemistry*, **26**, 2047–2054.

Tapprich,W.E. and Dahlberg,A.E. (1990) A single base substitution at position 2661 in *E. coli* 23S ribosomal RNA affects the binding of ternary complex to the ribosome. *EMBO J.*, **9**, 2649–2655.

Thompson,R.C. (1988) EF-Tu provides an internal kinetic standard for translational accuracy. *Trends Biochem. Sci.*, **13**, 91–93.

Thompson,R.C., Dix,D.D. and Karim,A. (1986) The reaction of ribosomes with elongation factor Tu·GTP complexes. *J. Biol. Chem.*, **261**, 4868–4874.

Tubulekas,I. and Hughes,D. (1993) A single amino acid substitution in elongation factor Tu disrupts the interaction between ternary complex and the ribosome. *J. Bacteriol.*, **175**, 240–250.

Wolf,H., Chinali,G. and Parmeggiani,A. (1977) Mechanism of the inhibition of protein synthesis by kirromycin. *Eur. J. Biochem.*, **75**, 67–75.

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