Intact Aminoacyl-tRNA Is Required To Trigger GTP Hydrolysis by Elongation Factor Tu on the Ribosome†

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ABSTRACT: GTP hydrolysis by elongation factor Tu (EF-Tu) on the ribosome is induced by codon recognition. The mechanism by which a signal is transmitted from the site of codon−anticodon interaction in the decoding center of the 30S ribosomal subunit to the site of EF-Tu binding on the 50S subunit is not known. Here we examine the role of the tRNA in this process. We have used two RNA fragments, one which contains the anticodon and D hairpin domains (ACD oligomer) derived from $tRNA^{Phe}$ and the second which comprises the acceptor stem and T hairpin domains derived from $tRNA^{Ala}$ (AST oligomer) that aminoacylates with alanine and forms a ternary complex with EF-Tu·GTP. While the ACD oligomer and the ternary complex containing the Ala-AST oligomer interact with the 30S and 50S A site, respectively, no rapid GTP hydrolysis was observed when both were bound simultaneously. The presence of paromomycin, an aminoglycoside antibiotic that binds to the decoding site and stabilizes codon−anticodon interaction in unfavorable coding situations, did not increase the rate of GTP hydrolysis. These results suggest that codon recognition as such is not sufficient for GTPase activation and that an intact tRNA molecule is required for transmitting the signal created by codon recognition to EF-Tu.

The binding of aminoacyl-tRNA (aa-tRNA)[1] to the A site of the ribosome is facilitated by elongation factor Tu. A stable ternary complex of EF-Tu, aa-tRNA, and GTP binds to the ribosome. Initial binding of the ternary complex to the ribosome (*1*) is followed by codon recognition. Provided aa-tRNA recognizes a correct codon, the ternary complex is stabilized (*2*, *3*) by interactions of the tRNA both with the mRNA and with the ribosome. The 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 (*3*−*5*) which is followed by rapid GTP hydrolysis. As a consequence, the conformation of EF-Tu switches from the GTP form to the GDP form (*6*, *7*) which has a greatly reduced affinity for aa-tRNA (*8*). Therefore, aa-tRNA is released from EF-Tu·GDP, accommodates in the A site, and takes part in peptidyltransferase reaction, while EF-Tu·GDP leaves the ribosome.

In the absence of the ribosomes, the rate of GTP hydrolysis in EF-Tu·GTP or in the ternary complex is very low, about 10^{-5}−10^{-4} s^{-1} (*4*, *9*). The intrinsic GTPase activity of the ternary complex is stimulated on the ribosome (*10*) in a manner that depends on codon recognition (*4*). In the absence of mRNA, or when there is a nonmatching codon in the A site, the rate of GTP hydrolysis in the ternary complex remains low, about 10^{-2}−10^{-3} s^{-1} (*1*), whereas correct codon−anticodon interaction accelerates the GTPase by up to 5 orders of magnitude, to 50−500 s^{-1}, depending on ionic conditions (*3*).

The mechanism by which codon recognition leads to GTPase stimulation is not known. One possibility is that the codon−anticodon interaction creates a signal which is transmitted to the G domain of EF-Tu through the aa-tRNA (*1*). In the crystal structure of the ternary complex, there are interactions of the acceptor end of aa-tRNA with interface residues of domains I and II, as well as of the acceptor and T stems with domain III of EF-Tu (*11*, *12*). These interactions 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. Another possibility is that codon recognition is sensed by the ribosome, thus creating a conformational change in the decoding region that is transmitted to EF-Tu through coupled conformational changes of the ribosome. Whether codon recognition alone is sufficient for the stimulation of rapid GTP hydrolysis is not known.

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[1] Abbreviations: EF-Tu, elongation factor Tu; aa-tRNA, aminoacyl-tRNA; AcPhe-$tRNA^{Phe}$, *N*-acetylated phenylalanyl-$tRNA^{Phe}$.

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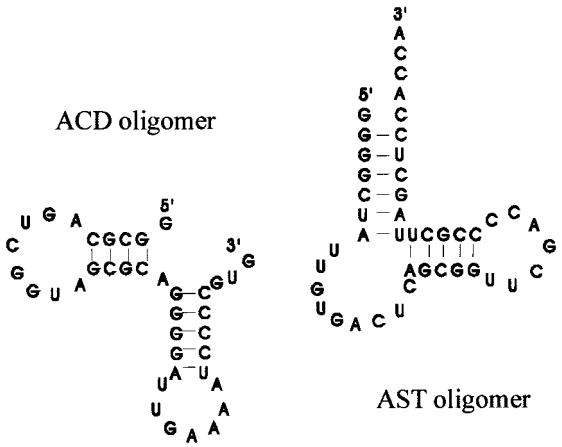

FIGURE 1: RNA oligomers comprising the anticodon and D hairpins derived from *E. coli* $tRNA^{Phe}$ (ACD oligomer) and the acceptor stem and T stem−loop of $tRNA^{Ala}$ (AST oligomer).

The present paper addresses this question by a split tRNA approach. Two RNA fragments are used that comprise different functional parts of the tRNA molecule (Figure 1). One 39 nt fragment, the ACD oligomer, consists of the anticodon and D loop hairpins and is expected to bind the 30S A site in a codon-dependent manner. The other is a 44 nt fragment (the AST oligomer) that contains the acceptor stem and T stem−loop sequences derived from $tRNA^{Ala}$ connected through a linker that was previously obtained by in vitro selection (*13*); the AST oligomer can be alanylated and in this form binds to EF-Tu·GTP to form a ternary complex. The goals of this work were first to verify that both tRNA fragments simultaneously interact with the ribosomes, and then to measure the rate of GTP hydrolysis in the EF-Tu·GTP·Ala-AST oligomer complex bound to the 50S A site in the absence or presence of the ACD oligomer in the 30S decoding region. The latter experiment was also performed in the presence of paromomycin, to promote the presumed structural transition of 16S rRNA toward the codon recognition conformation (*14*).

MATERIALS AND METHODS

Buffer and Reagents. Buffer A consisted of 50 mM Tris-HCl, pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTE, and 0.5 mM EDTA. The experiments were performed at 20 °C, if not stated otherwise. Poly(U), poly(A), and other chemicals were purchased from Boehringer-Mannheim and Merck. Radioactive compounds were from Amersham or ICN.

tRNAs. $tRNA^{Phe}$ from *E. coli* (1.7 nmol/A_{260} unit) was a gift of Drs. Yu. Semenkov and V. Katunin. AcPhe-$tRNA^{Phe}$ was prepared and purified to homogeneity (1.75 nmol/A_{260} unit) by HPLC on C-4 as described (*15*).

tRNA Fragments. RNA oligonucleotides comprising the anticodon D stem−loop (ACD oligomer) or acceptor stem T stem−loop (AST oligomer) domains equivalent to those of the respective tRNAs from *E. coli* were prepared by in vitro transcription using T7 RNA polymerase as described (*16*). Aminoacylation of the AST oligomer with ^{14}C-labeled alanine was performed as previously described (*13*). AST oligomer was renatured by heating at 70 °C for 5 min in 30 mM HEPES, pH 7.5, and 30 mM KCl, followed by addition of 10 mM $MgCl_2$ and cooling the sample to 37 °C at 1 °C/min. Aminoacylation was carried out in 30 mM HEPES, pH 7.5, 30 mM KCl, and 10 mM $MgCl_2$ using 20 μM AST oligomer, 50 μM [^{14}C]alanine, 10% of S100 extract, 2.5 mM ATP, and 7 mM 2-mercaptoethanol for 15 min at 37 °C. The reaction was stopped by addition of cold potassium acetate to 0.3 M. After phenol treatment and ethanol precipitation, [^{14}C]Ala-AST was dissolved in water and stored in aliquots at −80 °C. To form the ternary complex, EF-Tu was preincubated 15 min at 37 °C with 2 μM [γ-^{32}P]-GTP, 3 mM phosphoenolpyruvate, and 20 μg/mL pyruvate kinase. After addition of [^{14}C]Ala-AST, the incubation was continued for another 5 min at 37 °C. To obtain labeled ACD, [^{3}H]ATP was added to the in vitro transcription reaction (2.5×10^5 dpm/μL). The specific activity of [^{3}H]-ACD was about 250 dpm/pmol.

Ribosomes and Elongation Factor Tu. 70S ribosomes from *E. coli* MRE 600 were prepared as described (*17*). Ribosome concentrations were calculated from absorption measurements on the basis of 23 pmol/A_{260} unit. The activity of the ribosomes was 85−90% in tRNA binding and peptide bond formation. EF-Tu was prepared from *E. coli* K12 using the previously described procedure (*17*). To prepare ribosome complexes, 70S ribosomes were incubated in buffer A for 15 min at 37 °C with a 1.4-fold excess of AcPhe-$tRNA^{Phe}$ and 1 mg/mL poly(U), or with a 1.2-fold excess of $tRNA^{Lys}$ and 0.1 mg/mL poly(A).

GTP Hydrolysis. To measure [γ-^{32}P]GTP hydrolysis, EF-Tu complexes were prepared in Buffer A. EF-Tu (0.5 μM) was incubated with 2 μM [γ-^{32}P]GTP (about 5000 dpm/pmol), 3 mM phosphoenolpyruvate, and 10 μg/mL pyruvate kinase for 20 min at 37 °C. To form ternary complexes, 0.5 μM [^{14}C]Ala-AST or [^{14}C]Phe-$tRNA^{Phe}$ was added to EF-Tu·[γ-^{32}P]GTP and incubated further for 2 min at 37 °C. Before mixing, both EF-Tu and ribosome complexes were precooled to 20 °C. If not state otherwise, the concentrations of both complexes after mixing were 0.3 μM. Aliquots were withdrawn after 5−120 s of incubation at 20 °C and quenched with 1 M $HClO_4$ and 3 mM potassium phosphate, and [^{32}P]P_i was extracted and measured as described (*3*).

RESULTS

Binding of the ACD Oligomer to the Ribosomal A Site. To demonstrate that the RNA fragment containing the anticodon of $tRNA^{Phe}$ is a suitable substrate for the A site, the binding of ^{3}H-labeled ACD to the ribosomes was studied by nitrocellulose filtration. After 1 h of incubation, a stoichiometric amount of ACD was bound to poly(U)-programmed ribosomes with AcPhe-$tRNA^{Phe}$ in the P site (Figure 2). The interaction of ACD with the ribosome was codon-dependent, since very little binding was found with poly(A)-programmed ribosomes. When the P site was unoccupied, ACD was bound to both A and P sites (not shown), indicating that the oligomer may compete with AcPhe-$tRNA^{Phe}$ for the P site. However, less than 10% of prebound AcPhe-$tRNA^{Phe}$ was released from the ribosome during the incubation under conditions of Figure 2 (not shown), allowing only a small fraction of the P sites to be occupied by ACD at high concentrations of the oligomer. From Figure 2, the apparent K_d of the interaction is estimated to 1 ± 0.2 μM. The true value is probably somewhat smaller, since the incubation was not extended to full saturation in

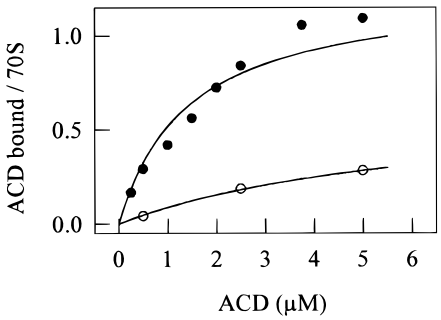

FIGURE 2: Binding of ACD to the ribosomal A site. Titration in the presence of poly(U)- (●) or poly(A)-programmed (○) ribosome complexes (P site occupied). 10 pmol (0.25 μM) of ribosome complexes was incubated with the indicated concentrations of [^{3}H]-ACD for 1 h at 20 °C in the presence of 20 mM $MgCl_2$.

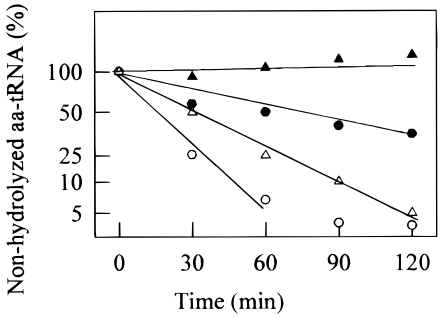

FIGURE 3: Hydrolysis protection of [^{14}C]Ala-AST (●) and [^{14}C]-Phe-tRNA^{Phe} (▲) (both 0.5 μM) by EF-Tu·GTP. EF-Tu·GTP was prepared as described under Materials and Methods, except that 1 mM GTP was used. Closed symbols, in the presence of EF-Tu·GTP; open symbols, in the absence of the factor. Incubation at 37 °C.

order to minimize the loss of AcPhe-tRNA^{Phe} from the P site.

ACD binding to the A site is very sensitive to the Mg^{2+} concentration. At 10 mM Mg^{2+}, only about 50% of the A sites were occupied even at 5 μM ACD (not shown). Therefore, ribosomal complexes containing ACD in the A site were routinely prepared at 20 mM Mg^{2+} and diluted to the lower Mg^{2+} concentration (10 mM) immediately before adding ternary complex.

Stability of the EF-Tu·GTP Complex with Ala-AST. Since the AST fragment was selected under conditions somewhat different from those used for the present experiments (*13*), Ala-AST binding to *E. coli* EF-Tu and the stability of the complex at the present assay conditions were verified using an assay that measures the protection of the aminoacyl ester bond from spontaneous hydrolysis (Figure 3); control experiments were performed with Phe-tRNA^{Phe}. At 37 °C, free Phe-tRNA^{Phe} was hydrolyzed with a half-lifetime of about 30 min. The addition of EF-Tu·GTP strongly protected Phe-tRNA^{Phe} from hydrolysis, such that no significant hydrolysis was observed after 2 h of incubation. Free Ala-AST was hydrolyzed about twice as fast as Phe-tRNA^{Phe}, and the addition of EF-Tu·GTP increased the half-life to about 60 min. This demonstrates that Ala-AST formed a complex with EF-Tu·GTP which was sufficiently stable for the experiments reported in the following.

Ribosome-Stimulated GTP Hydrolysis of EF-Tu·GTP·Ala-AST. To study the binding of the ternary complex EF-Tu·GTP·Ala-AST to the ribosome, the stimulation of GTP

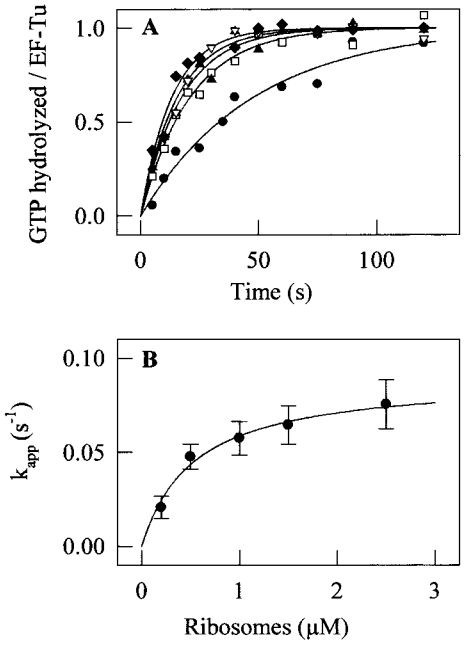

FIGURE 4: GTP hydrolysis in EF-Tu·[γ-^{32}P]GTP·[^{14}C]Ala-AST (0.3 μM, prepared as described under Materials and Methods) on poly-(A)-programmed ribosomes. (A) Time courses of GTP hydrolysis at 20 °C in the presence of 0.3 μM (●), 0.5 μM (□), 1.0 μM (▲), 1.5 (▽), and 2.5 μM (◆) ribosomes. (B) Dependence of k_{app} on ribosome concentration. k_{app} values were estimated by exponential fitting of the time courses shown in (A). $K_d = 0.5 \pm 0.1$ μM; $k_{GTP} = 0.07 \pm 0.01$ s^{-1}.

hydrolysis was measured in the presence of increasing concentrations of ribosomes. Time courses of GTP hydrolysis at 0.3−2.5 μM ribosomes are shown in Figure 4A. Intrinsic, ribosome-independent GTP hydrolysis was negligible, similarly to that observed for the ternary complexes containing native Phe-tRNA^{Phe} [about 2×10^{-5} s^{-1}; (*4*)]. To determine the rate constant of ribosome-stimulated GTP hydrolysis, and the affinity of EF-Tu·GTP·Ala-AST for the ribosome, the time courses presented in Figure 4A were evaluated, and the apparent rate constants were plotted as a function of ribosome concentration (Figure 4B). The saturation level gives estimates for the rate constant of GTP hydrolysis in the complex, 0.07 s^{-1}, and for the K_d, 0.5 ± 0.1 μM.

Effect of Codon−Anticodon Interaction by ACD on GTP Hydrolysis in EF-Tu. The effect of codon recognition was studied at conditions where the GTPase rate was linearly dependent on the ribosome concentration (Figure 4B), to achieve the maximum sensitivity of the assay. At this ribosome concentration, 0.3 μM, ribosome-bound EF-Tu·GTP·Ala-AST hydrolyzed GTP at a rate of 0.022 s^{-1}, independent of the mRNA in the ribosome complex (Figure 5A). In the presence of 3 μM ACD, i.e., at about 80% occupancy of the A sites throughout the incubation time, the GTPase of EF-Tu·GTP·Ala-AST increased to 0.037 s^{-1}, a 1.7-fold increase. This is a negligible effect, compared to the 10^5-fold stimulation of GTP hydrolysis observed with intact cognate aa-tRNA (*3*).

To increase the stability of codon binding of ACD, we have used the antibiotic paromomycin which binds to the ribosomal decoding region and induces a conformational change of 16S rRNA (*18*), thereby stabilizing the binding of near-cognate aa-tRNA (*14*, *18*). In this experiment, the GTPase activation of the binary complex EF-Tu·GTP was

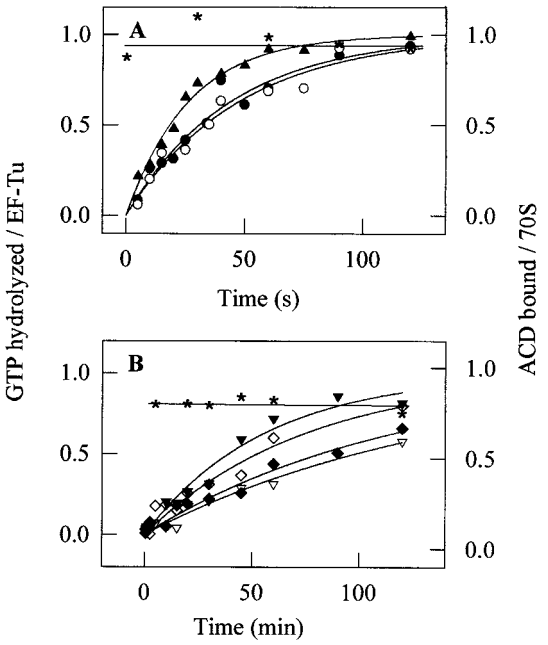

FIGURE 5: Effect of A site-bound ACD fragment on GTP hydrolysis. (A) Time courses of GTP hydrolysis in EF-Tu·[γ-^{32}P]-GTP·[^{14}C]Ala-AST (0.3 μM) induced by 0.3 μM ribosomes in the presence of poly(A) (○), poly(U) (●), or poly(U) and ACD in the A site (▲) at 10 mM Mg^{2+}, 20 °C. The apparent rate constants are 0.021 ± 0.006, 0.022 ± 0.006, and 0.037 ± 0.005 s^{-1}, respectively. (B) Time courses of GTP hydrolysis in EF-Tu·[γ-^{32}P]GTP with 0.3 μM poly(U)-programmed 70S ribosomes in the absence (◆,◇) or presence (▼,▽) of 2 μM ACD, with (▼,◆) or without (▽,◇) the addition of 5 μM paromomycin (20 mM Mg^{2+}, 20 °C). Apparent rate constants range between 0.009 ± 0.003 and 0.018 ± 0.003 min^{-1}, respectively. (*) Ribosome-bound [^{3}H]ACD.

monitored, and 20 mM Mg^{2+} was used in order to stabilize the ACD fragment in the A site sufficiently to allow longer incubation (Figure 5B). The rate of GTP hydrolysis was about 0.009 min^{-1} with EF-Tu·GTP alone, and was 2 times higher, 0.018 min^{-1}, when both ACD and paromomycin were present. Thus, the stimulatory effect of ACD remained small, also in the presence of antibiotic.

DISCUSSION

It is well established that the anticodon stem−loop of tRNA binds to both A and P sites of the ribosome (*19*, *20*). In the P site, the anticodon stem−loop interacts exclusively with the small subunit of the ribosome (*19*−*22*). Binding of the anticodon stem−loop of tRNA to the A site was shown to be functionally relevant, since it is competent for translocation (*20*). In the present paper, we show that also the tRNA fragment that comprises the anticodon arm−D arm domain of $tRNA^{Phe}$ (ACD oligomer) binds to the A site of the ribosome in a codon-dependent manner. The affinity of ACD binding for the A site ($K_d \approx 1$ μM, 20 mM Mg^{2+}) is comparable to that of deacylated $tRNA^{Phe}$ (*23*). Taken together, these data suggest that the ACD oligomer interacts with the 30S A site in a similar manner as the pertinent part of intact tRNA.

The ternary complex of aa-tRNA with EF-Tu·GTP rapidly binds to the ribosome ($k_1 \approx 10^8$ M^{-1} s^{-1}) and forms a labile ($k_{-1} = 25$ s^{-1}) initial binding complex (*1*, *3*). The formation of the initial binding complex is independent of mRNA, and the occupancy of the A site by tRNA does not inhibit the initial binding of the ternary complex containing a full-size aa-tRNA (*1*). Subsequent codon recognition causes a conformational change in the complex that results in a 10^5-fold acceleration of GTP hydrolysis by EF-Tu (*3*, *4*, *15*). When the codon does not match, the GTPase activity of EF-Tu remains low, about 0.002 s^{-1} (*4*). The ternary complex EF-Tu·GTP·Ala-AST on the ribosome hydrolyzes GTP at a similar low rate, 0.07 s^{-1}, thus demonstrating that in the complex lacking the anticodon and D stem−loop domain GTP hydrolysis is also tightly controlled and is not stimulated in the initial binding complex. The K_d values of the initial ribosome complexes of the ternary complexes containing Ala-AST and $Phe\text{-}tRNA^{Phe}$ are about the same, 0.5 and 0.4 μM, respectively, which is in keeping with the notion that the binding is dominated by EF-Tu−ribosome interactions (*1*, *3*).

The very low intrinsic GTPase activity of EF-Tu is stimulated on the ribosome by an as yet unknown mechanism. The stimulation is tightly controlled, in that the GTPase activity of EF-Tu, or the ternary complex, on the ribosome remains very low unless there is codon recognition. The extent of GTPase stimulation is determined by the nature of the codon−anticodon complex by the induced fit mechanism, as recognition of a cognate codon results in much faster GTP hydrolysis than recognition of a near-cognate codon [500 vs 50 s^{-1}; (*24*)]. Interestingly, the near-cognate GTPase rate is increased up to the cognate level when paromomycin, an aminoglycoside antibiotic, is present (*14*). The observation that paromomycin binds to the decoding region of 16S rRNA and induces a conformational change there (*18*, *25*) suggested that the formation of the cognate codon−anticodon duplex also induces a conformational change of the decoding center, and that this conformational change constitutes the signal that ultimately leads to GTPase activation.

There are two ways that a conformational change in the decoding center of the 30S ribosomal subunit could be transmitted to EF-Tu. One consists of coupled conformational changes of the ribosome that through direct ribosome−EF-Tu interactions may induce the GTPase of EF-Tu. In fact, structural rearrangements of 23S rRNA (*26*, *27*) and/or 16S rRNA (*28*) have been implicated in transmission of the GTPase activating signal to EF-Tu, and it has been suggested that conformational changes in 16S rRNA may be transmitted to the 50S subunit through the several interface contacts between the two subunits (*29*). Another way of signal transmission involves the tRNA and entails a conformational change or movement of the tRNA molecule that may affect the interactions of the acceptor end of aa-tRNA with EF-Tu in the ternary complex. The present work shows that when the tRNA in the codon recognition complex is present in the form of two fragments, one containing the anticodon and the other the aminoacyl end bound to EF-Tu, the rate of GTP hydrolysis is very low. This indicates that the signal transmission mechanism through the tRNA is important for GTPase activation, although it does not exclude a contribution of signal transmission through the ribosome.

There is direct evidence showing that codon recognition by the ternary complex on the ribosome leads to a coupled conformational change of the tRNA (*30*) and of the G domain of EF-Tu (*15*) which probably represents the transition to the GTPase state of the ternary complex which is followed by instantaneous GTP hydrolysis (*3*). The GTPase state is

stabilized by an antibiotic, kirromycin, that binds to EF-Tu and prevents the rearrangement of EF-Tu to the GDP-bound conformation and the release of the aa-tRNA. The three-dimensional reconstruction by electron cryomicroscopy at 18 Å resolution of the kirromycin-stalled codon recognition complex revealed a distortion of the ternary complex on the ribosome (*31*), compared to the structure of the free ternary complex (*11*). The anticodon domain of the tRNA appears to be displaced to reach into the decoding center, while EF-Tu is fixed by interactions of the G domain, and possibly of domain II, with structural elements of the 50S subunit, presumably including the sarcin−ricin stem−loop (*32*). Assuming that the overall L-shaped conformation of the tRNA is more or less rigid, the movement of the anticodon domain would lead to a movement of the whole tRNA in a lever-like fashion, and this would impose strain on the interactions of the acceptor stem−T stem loop domain with EF-Tu. Fragmenting the tRNA, as was done in the present work, obviously disrupts the lever and, thereby, the conformational coupling.

While the work presented here supports an important role for the tRNA in GTPase activation, it remains unclear whether the precise interactions responsible for this activity are provided by the acceptor arm of the tRNA, the ribosome, or both. The acceptor end of aa-tRNA in the ternary complex interacts with interface residues of all three domains of EF-Tu, including the switch I and switch II regions in the G domain (*11*, *12*). Conformational changes of these regions are thought to be involved in the GTPase reaction. It is conceivable, therefore, that changes at the interface between the tRNA and EF-Tu brought about by codon recognition on the ribosome directly induce the GTPase state of the G domain. Additionally, interactions of the G domain with the ribosome, such as those with the sarcin−ricin stem−loop and/or with protein L7/12 (*31*), may be involved. In such a case, transmission of the codon recognition signal through the tRNA would constitute a necessary condition that allows those interactions to take place in a way that leads to the stimulation of GTP hydrolysis.

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