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Effector region of the translation elongation factor EF-Tu·GTP complex stabilizes an orthoester acid intermediate structure of aminoacyl-tRNA in a ternary complex

(^{13}C NMR)

CHARLOTTE FÖRSTER, STEFAN LIMMER, WALTRAUD ZEIDLER, AND MATHIAS SPRINZL*

Laboratorium für Biochemie, Universität Bayreuth, D-95440 Bayreuth, Federal Republic of Germany

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ABSTRACT **tRNA^{Val} from *Escherichia coli* was aminoacylated with [1-^{13}C]valine and its complex with *Thermus thermophilus* elongation factor EF-Tu·GTP was analyzed by ^{13}C NMR spectroscopy. The results suggest that the aminoacyl residue of the valyl-tRNA in ternary complex with bacterial EF-Tu and GTP is not attached to tRNA by a regular ester bond to either a 2′- or 3′-hydroxyl group; instead, an intermediate orthoester acid structure with covalent linkage to both vicinal hydroxyls of the terminal adenosine-76 is formed. Mutation of arginine-59 located in the effector region of EF-Tu, a conserved residue in protein elongation factors and the α subunits of heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins), abolishes the stabilization of the orthoester acid structure of aminoacyl-tRNA.**

tRNAs are enzymatically aminoacylated at either the 2′- or the 3′-hydroxyl group of the 3′-terminal adenosine (1). After aminoacylation, the aminoacyl residue of free aminoacyl-tRNA can migrate between the 2′- and 3′-hydroxyls of the 3′-terminal adenosine. The rate of this aminoacyl migration, however, is slower under physiological conditions than the rate of bacterial polypeptide synthesis (2). Since the 3′-aminoacyl-tRNA is the isomer active as an acceptor of the peptidyl residue during the peptidyltransferase reaction (3), its formation must be enzymatically catalyzed.

Both the 2′ and 3′ isomers of aminoacyl-tRNA are potential substrates for the ribosomal aminoacyl (A) and peptidyl (P) binding sites. Nonisomerizable aminoacyl-tRNA having a 2′- or 3′-deoxyadenosine instead of adenosine on the 3′-CCA terminus was used to elucidate the substrate requirements in the ribosomal A and P sites (for a review, see ref. 4). Only the 3′-aminoacyl and 3′-peptidyl isomers are active as an acceptor and donor, respectively (3), and the free 2′-hydroxyl or the vicinal 3′-hydroxyl is not required for the peptidyl transfer. A nonisomerizable 3′-peptidyl-tRNA (2′-deoxy) was, however, inactive in the elongation factor EF-G-dependent translocation from the A to the P site (5). This explains why the nonisomerizable 3′-aminoacyl-tRNA (2′-deoxy) is inactive in template-dependent polypeptide synthesis (6). Thus the vicinal 2′-hydroxyl, although not required for the peptide transfer, is necessary for some unidentified step of the elongation cycle.

Elongation factor EF-Tu, a protein which carries the aminoacyl-tRNA to the ribosomal A site, has to recognize both 2′ and 3′ isomers of aminoacyl-tRNA formed by aminoacylation. By using the nonisomerizable 2′- and 3′-deoxy aminoacyl-tRNAs, respectively, it was demonstrated that both 2′-aminoacyl- and 3′-aminoacyl-tRNA interacted with the EF-Tu·GTP binary complex, although their affinity relative to native aminoacyl-tRNA was reduced (7, 8). From fast kinetic experiments using native aminoacyl-tRNA, it was later confirmed that EF-Tu·GTP bound both isomers and acted as an isomerase to produce the 3′ isomer, which is competent as a donor in the peptidyl transfer reaction (9, 10).

Based on an experiment performed with aminoacyl-tRNA analogues, the existence of a stable intermediate 2′,3′-orthoester structure of the aminoacyl residue in the complex with EF-Tu was suggested (4). In this communication we report an NMR study of [1-^{13}C]valyl-tRNA·EF-Tu·GTP complex which proves the existence of the orthoester acid intermediate.

MATERIALS AND METHODS

Four hundred fifty nanomoles of tRNA^{Val} was quantitatively aminoacylated with a mixture of [1-^{13}C]valine/[^{14}C]valine (specific activity, 0.7 cpm/pmol), with valyl-tRNA synthetase from *Escherichia coli* in 50 mM Hepes, pH 7.6/150 mM KCl/12.5 mM $MgCl_2$/2.5 mM 2-mercaptoethanol/10 mM ATP/1 mM valine for 1 hr at 37°C. The aminoacylated tRNA was applied to a Sephadex A-25 (Pharmacia) column equilibrated in 20 mM potassium phosphate, pH 4.8/10 mM $MgCl_2$/200 mM NaCl. Isocratic elution was carried out with 450 mM NaCl, and then the aminoacyl-tRNA was eluted with a linear gradient from 450 to 1000 mM NaCl. Desalting of tRNA was achieved by Bio-Gel P-6 (Bio-Rad) gel filtration. Nucleotide-free EF-Tu from *Thermus thermophilus* cells was prepared (11). Its GTP-binding capacity was 22,000 pmol/mg of protein. Overexpression and purification of the Arg^{59} → Thr (R59T) mutant EF-Tu were performed as described (12) after site-specific mutagenesis (13). Proton-decoupled ^{13}C NMR spectra were recorded at 125.7 MHz on a Bruker AM 500 spectrometer. [1-^{13}C]valyl-tRNA was measured in 50 mM potassium phosphate, pH 4.8/100 mM NaCl/15 mM $MgCl_2$/10% (vol/vol) ^2H_2O. The [1-^{13}C]valyl-tRNA^{Val}·EF-Tu complex was measured with 0.9 mM [1-^{13}C]valyl-tRNA^{Val} after complex formation with 1.08 mM EF-Tu and 1.3 mM GTP or guanosine 5′-[β,γ-imido]triphosphate (GTP[β,γ-NH]) in 50 mM potassium borate, pH 7.5/50 mM KCl/10 mM $MgCl_2$/10% ^2H_2O. [1-C-^{13}C]valyl-tRNA^{Val} with the R59T mutant EF-Tu was measured under the same conditions.

In all samples, [methyl-^{13}C]methionine was used as a reference and its methyl resonance was set at 15 ppm. The rate of GTP hydrolysis in the ternary complex was determined by HPLC analysis of an NMR sample aliquot (11), and deacylation of aminoacyl-tRNA was determined by precipitation of the trichloroacetic acid-insoluble radioactivity.

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Abbreviations: GTP[β,γ-NH], guanosine 5′-[β,γ-imido]triphosphate; A site, aminoacyl site; P site, peptidyl site.

*To whom reprint requests should be addressed.

RESULTS AND DISCUSSION

The ^{13}C NMR spectra of [1-^{13}C]valyl-tRNA^{Val} and [1-^{13}C]valyl-tRNA^{Val}·EF-Tu·GTP ternary complex are shown in Fig. 1. The resonance of the [^{13}C]carbonyl of valine is expected at 175.3 ppm (14, 15). Esterification of tRNA^{Val} by [1-^{13}C]valine gives rise to two resonances at 169.8 and 169.5 ppm corresponding to slowly exchanging structures of 3′-valyl-tRNA^{Val} and 2′-valyl-tRNA^{Val}, respectively. The ratio of the isomers is approximately 7:3 in favor of the 3′ isomer (2). The broad resonance signals between 60 and 90 ppm are due to the natural ^{13}C abundance of the ribose carbon atoms in tRNA^{Val} (16). Upon binding of valyl-tRNA^{Val} to EF-Tu·GTP the resonances around 170 ppm belonging to the sp^2-hybridized [^{13}C]carbon-1 are no longer detectable in the spectrum, and two new resonances around 63 ppm arise (Fig. 1*b*). This large upfield shift can be explained by a structure in which carbon-1 of valine is sp^3-hybridized in the form of an orthoester acid intermediate as shown in Fig. 2. As compared with a true orthoester, the carbon-1 signals of the aminoacyl residue in the 2′,3′ intermediate of the aminoacyl-tRNA are shifted significantly further upfield (14, 15). The electron density at carbon-1 of the aminoacyl-tRNA in the protein-complexed form has to be increased considerably relative to carbon-1 of a regular orthoester in order to explain the strong enhancement of shielding and the resulting upfield shift. The extraordinary stability of the orthoester acid intermediate and the observed NMR spectra can be understood by assuming an interaction between the former carbonyl oxygen of the aminoacyl residue and EF-Tu. To our knowledge, this is the first example of a ^{13}C NMR spectrum of a protein-stabilized orthoester acid intermediate with an sp^3-hybridized carbon-1 atom.

Investigations with nonisomerizable aminoacyl-tRNA analogues support the interpretation of the NMR results. Modifications which prevent aminoacyl migration between the 2′ and 3′ vicinal hydroxyls and prevent formation of the intermediate orthoester acid also lower the affinity of aminoacyl-

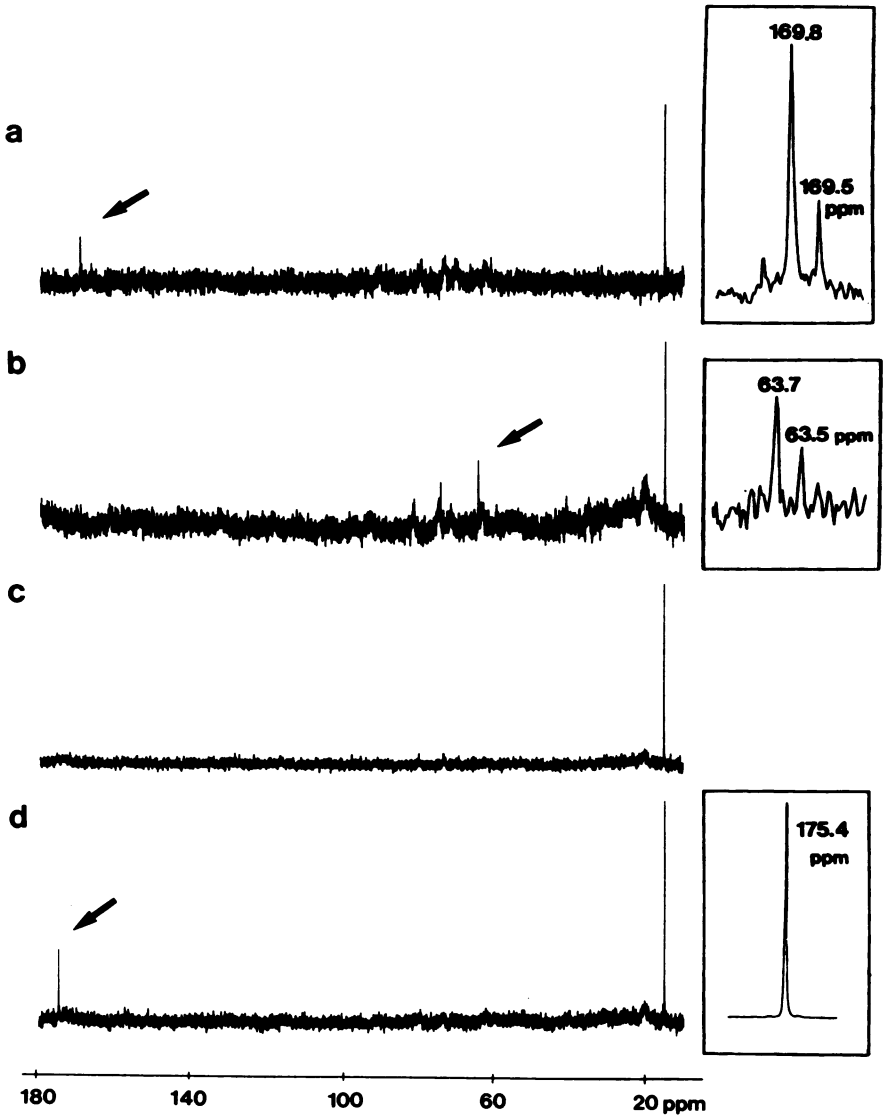

FIG. 1. Part of the proton-decoupled 125.7-MHz ^{13}C NMR spectra. (*a*) *E. coli* [1-^{13}C]valyl-tRNA^{Val}. The resonances at 169.8 and 169.5 ppm represent the 3′ and 2′ isomers of valyl-tRNA^{Val}, respectively [22,000 free induction decays (FIDs) accumulated at room temperature; measuring time, 15.5 hr]. (*b*) [1-^{13}C]valyl-tRNA^{Val}·EF-Tu·GDP/GTP. The signals at 63.7 ppm and 63.5 ppm are due to aminoacyl carbon-1 of aminoacyl-tRNA (17,000 FIDs accumulated at 10°C for ≈12 hr after complex formation). (*c*) [1-^{13}C]valyl-tRNA^{Val}-R59T EF-Tu·GDP/GTP (17,000 FIDs accumulated between ≈5.5 hr and ≈17.5 hr after complex formation; the spectrum taken between 0 and 5.5 hr after complex formation likewise did not contain resonances except that of the reference). (*d*) The same complex as in *c* after deacylation of aminoacyl-tRNA. The resonance at 175.4 ppm belongs to the carboxyl group of free valine. The spectrum was obtained after storage of the sample for 8 days at 4°C (27,000 FIDs accumulated during ≈17 hr). The ^{13}C-resonances of the carbon-1 atom are shown in expanded scale at right.

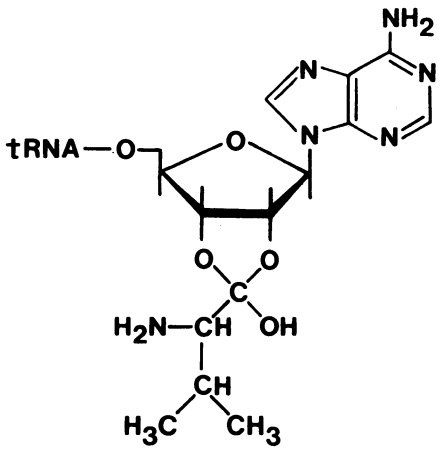

FIG. 2. Orthoester acid intermediate structure of the aminoacyl residue in the valyl-tRNA^{Val}·EF-Tu·GTP ternary complex. Stereoisomers are not indicated.

tRNA for EF-Tu·GTP by about 2 orders of magnitude (B. Blechschmidt, personal communication). Inhibition of aminoacyl migration can be prevented by (*i*) oxidation of the 2′-3′ carbon-carbon bond by periodate (17) or (*ii*) the replacement of one hydroxyl by hydrogen or by an amino group (6).

Due to the GTPase activity of EF-Tu in the ternary complex (18) it was not possible to prevent EF-Tu·GDP formation in the course of the NMR experiment. There are two signals in the high-field region of the [1-^{13}C]valyl-tRNA^{Val}·EF-Tu ^{13}C NMR spectrum around 63 ppm (Fig. 3*a*). The intensities of the peaks reflect the gradually changing proportions of valyl-tRNA·EF-Tu·GTP and valyl-tRNA·EF-Tu·GDP complexes during the accumulation of the free induction decays over a period of 5 hr. The correlation between the appearance of the 63.7-ppm resonance and the formation of GDP was confirmed by HPLC analysis of an aliquot of an NMR sample. The amount of GTP hydrolyzed to GDP after 5 hr was ≈60%. One of these signals, at 63.5 ppm, which we ascribe to the GTP complex, decreases as the hydrolysis of GTP proceeds and finally disappears when the GTP hydrolysis is complete (Fig. 3*b*). The second resonance, at 63.7 ppm, increasing with the progress of the GTP hydrolysis, belongs to the [1-^{13}C]valyl-tRNA^{Val}·EF-Tu·GDP complex. This complex is remarkably stable at 0.9 mM concentration. Finally, after several days, as valine is produced by hydrolysis of valyl-tRNA^{Val}, the resonance of the free [1-^{13}C]valine at 175.3 ppm becomes detectable in the same sample (Fig. 3*b*).

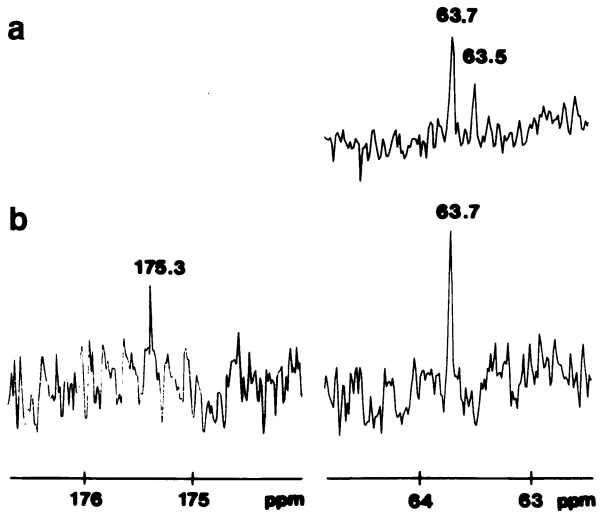

FIG. 3. GTPase activity of EF-Tu and deacylation of aminoacyl-tRNA in the [1-^{13}C]valyl-tRNA^{Val}·EF-Tu·GTP complex as monitored by ^{13}C NMR spectroscopy. 15 min–5 hr after complex formation (*a*) and after storage of the sample for 8 days at 4°C, measuring time 12.5 hr (*b*). The resonance at 63.5 ppm corresponds to [1-^{13}C]valyl-tRNA^{Val}·EF-Tu·GTP, and that at 63.7 ppm to [1-^{13}C]valyl-tRNA^{Val}·EF-Tu·GDP. The signal at 175.3 ppm represents the carboxyl group of [1-^{13}C]valine.

The formation of the aminoacyl-tRNA·EF-Tu·GDP complex was reported previously (19). Experiments presented in Figs. 1 and 3 demonstrate that EF-Tu·GDP does not dissociate from the aminoacyl-tRNA spontaneously in the absence of ribosomes and that the orthoester acid structure is still preserved in this complex. The T state, biochemically defined as a complex of aminoacyl-tRNA·EF-Tu·GTP with the programmed ribosomes that is incompetent to participate in the peptidyltransferase reaction (20), may correspond to the intermediate orthoester acid structure. Since the EF-Tu GTPase is not sufficient to induce the release of the aminoacyl-tRNA (Fig. 3), it is likely that the ribosomal A site plays an active role in directing the relaxation of this high-energy intermediate to the reactive 3′-aminoacyl-tRNA, which is then competent to function as a peptidyl acceptor (3). The rate of this accommodation of the aminoacyl residue might be controlled, thus providing a way to regulate the stringency of the proofreading mechanism (21). The orthoester acid intermediate structure (Fig. 2) is consistent with the observation that the aminoacyl-tRNA is protected from spontaneous hydrolysis by binding to EF-Tu.

GTP hydrolysis during the NMR experiment can be inhibited by using the slowly hydrolyzable GTP analogue GTP[β,γ-NH]. Indeed, only one signal appears for the [1-^{13}C]valyl-tRNA·EF-Tu·GTP[β,γ-NH] ternary complex in the 63.7-ppm region (Fig. 4). This signal is stable for several hours and disappears only after several days, due to hydrolysis of the aminoacyl ester bond. At the same time, the resonance of the free [1-^{13}C]valine at 175.3 ppm becomes detectable. The positions of the orthoester ^{13}C resonances are slightly different in the ternary complexes containing GTP or GTP[β,γ-NH], reflecting some structural alterations induced by the analogue.

In contrast to the results obtained with wild-type EF-Tu, the ^{13}C NMR spectra of the complex of [1-^{13}C]valyl-tRNA^{Val} with the R59T mutant EF-Tu·GTP does not display resonances in the whole region from 170 ppm to 15 ppm after formation of the complex (Fig. 1*c*). Neither a signal around 64 ppm for the intermediate orthoester acid structure nor a signal due to the free valyl-tRNA^{Val} around 170 ppm is observed within 24 hr. However, after the sample is kept for

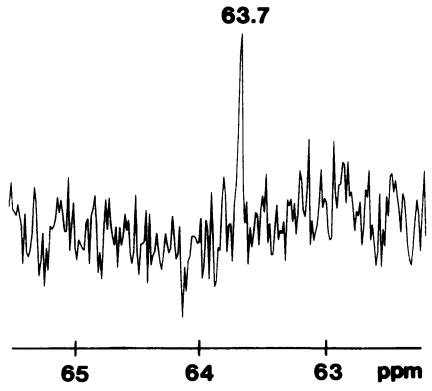

FIG. 4. Part of the 125.7-MHz ^{13}C NMR spectrum of [1-^{13}C]valyl-tRNA^{Val}·EF-Tu·GTP[β,γ-NH], measured between 0.5 and 16.5 hr after complex formation. The resonance at 63.7 ppm represents carbon-1 of the orthoester acid intermediate of aminoacyl-tRNA in complex with EF-Tu·GTP[β,N-NH].

8 days at 4°C, a signal at 175.4 ppm shows up that originates from free [1-^{13}C]valine which is formed by deacylation of tRNA (Fig. 1*d*). This mutant EF-Tu forms a ternary complex with GTP and aminoacyl-tRNA as determined by fluorescence measurements (22) and does not differ significantly from wild-type EF-Tu with regard to intrinsic GTPase activity and translation. The apparent equilibrium dissociation constants (K_d values) of wild-type EF-Tu and R59T mutant EF-Tu for complex formation with aminoacyl tRNA and GTP are 1.7×10^{-9} M and 3.6×10^{-9} M, respectively. We explain the lack of resonance signals in the case of the mutant protein (Fig. 1*c*) by assuming an intermediate rate of exchange between the 2′ and 3′ isomers and the orthoester acid structure of aminoacyl-tRNA. Thus the mutation of arginine-59 to threonine results in acceleration by several orders of magnitude of the exchange between the aminoacyl and orthoester acid tRNAs in the mutant ternary complex.

The above-described results point to a possible role for arginine-59 in the binding of aminoacyl-tRNA to EF-Tu·GTP. Remarkably, a corresponding arginine residue is conserved in almost all GTP/GDP-binding proteins (23), such as EF-Tu, EF-G, SelB, and the α subunits of eukaryotic heterotrimeric G proteins. In the case of growth hormone-secreting human pituitary tumor cells, it was demonstrated that overstimulation of adenylyl cyclase was caused by mutations in the α_s subunit of the adenylyl cyclase-stimulatory regulator G_s. These natural mutations occurred either at glutamine 227 or at the conserved residue arginine-201 (which corresponds to arginine-59 in EF-Tu) and prevented GTPase activity (24). Also, amino acid replacements in the same conserved arginine were reported to occur in the α chain of G_{i2} derived from endocrine tumor cells of the ovary and of the adrenal cortex (25).

From the presented data it follows that arginine-59 in *T. thermophilus* EF-Tu stabilizes the orthoester acid intermediate of aminoacyl-tRNA either by direct interaction or indirectly by affecting the EF-Tu conformation in the vicinity of the tRNA binding site. Arginine-59 could represent a key element of the molecular switch mechanism and stimulation of the effector-dependent GTPase. Indeed the switch from p21^{ras}·GDP to p21^{ras}·GTP is accompanied by a large movement of tyrosine-32 (26). This residue corresponds to arginine-59 in *T. thermophilus* EF-Tu (27).

A transition-state analogue of serine proteases, *N*-tosyl-L-phenylalanylchloromethane, in which the hydrated carbonyl carbon atom is sp^3-hybridized, binds selectively to *E. coli* EF-Tu, becomes covalently attached to cysteine-81, and inhibits aminoacyl-tRNA binding (28). The specific effect of this inhibition on EF-Tu becomes obvious if one considers it as an analogue of the aminoacyl-tRNA-orthoester. The fact that cysteine-82 and arginine-59 in *T. thermophilus* EF-Tu (corresponding to cysteine-81 and arginine-58 in *E. coli* EF-Tu) are close in the *T. thermophilus* EF-Tu·GTP[β,γ-NH] tertiary structure (29) supports this interpretation.

In summary, our experiments demonstrate the stabilization of an intermediate state of a molecule (aminoacyl-tRNA) by interaction with a protein (EF-Tu). In free aminoacyl-tRNA, the orthoester acid structure of the aminoacyl ester linkage is the intermediate form which has to be passed through during transesterification from 2′-aminoacyl-tRNA to 3′-aminoacyl-tRNA and vice versa. A stereoselective binding of the orthoester acid tRNA by the protein may prepare the controlled release of only the 3′-aminoacyl-tRNA isomer, which is the substrate required in the ribosomal A site.

We still cannot explain why the nonisomerizable 3′-peptidyl-tRNA cannot be translocated from the A site to the P site (5). There could be only two solutions for the problem: either EF-G, like EF-Tu, requires the orthoester acid structure of the peptidyl-tRNA or (a suggestion more heretical) a second EF-Tu·GTP promotes the binding of peptidyl-tRNA to the P site. Although the ternary complexes of peptidyl-tRNA and EF-Tu·GTP are not formed in solution, recent evidence suggests a participation of two EF-Tu·GTP complexes in one elongation cycle (30).

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