Kinetic Parameters for tmRNA Binding to Alanyl-tRNA Synthetase and Elongation Factor Tu from *Escherichia coli*†

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ABSTRACT: Aminoacylation and transportation of tmRNA to stalled ribosomes constitute prerequisite steps for *trans*-translation, a process facilitating the release of stalled ribosomes from 3′ ends of truncated mRNAs and the degradation of incompletely synthesized proteins. Kinetic analysis of the aminoacylation of tmRNA indicates that tmRNA has both a lower affinity and a lower turnover number than cognate $tRNA^{Ala}$ for alanyl-tRNA synthetase, resulting in a 75-fold lower k_{cat}/K_M value. The association rate constant of Ala-tmRNA for elongation factor Tu in complex with GTP is about 150-fold lower than that of Ala-$tRNA^{Ala}$, whereas its dissocation rate constant is about 5-fold lower. These observations can be interpreted to suggest that additional factors facilitate tmRNA binding to ribosomes.

During the past two decades, in-depth analysis of the ribosomal translation mechanism has enhanced understanding of the gene expression pathway. More recent discoveries such as the incorporation of selenocysteine in response to a UGA stop codon in a special context indicate that totally unexpected features of the translation mechanism may still be discovered any time (*1*). Another new and exciting mechanism acting at the level of ribosomal translation was quite recently found (*2*). Serendipitous discovery of protein tagging in *Escherichia coli* (*3*) identified a new component of the translational apparatus, a small and stable RNA molecule called tmRNA[1] (*4*). Recent experimental and computer-aided studies identified more than 100 tmRNA-like sequences. All of them are of either bacterial or plastid origin (*5*).

Considerable experimental evidence suggests that tmRNA binds to ribosomes stalled at the 3′ end of a truncated mRNA without a stop codon. This event marks the beginning of a process called *trans*-translation. Although many molecular details of the *trans*-translation process are not yet known, the current model assumes that tmRNA acts as both tRNA and mRNA. This assumption is supported not only by functional analyses but also by the presence of tRNA- and mRNA-like modules in the tmRNA structure (*2*, *6*−*9*).

E. coli tmRNA is a highly structured molecule with a complex organization of secondary and tertiary interactions (*10*−*13*). The tRNA-like module of tmRNA is formed by its very 3′ and 5′ ends, and in most homologues, it mimics $tRNA^{Ala}$. Komine and colleagues (*14*) demonstrated that the *E. coli* tmRNA can be aminoacylated both in vivo and in vitro. Likewise, tmRNAs isolated from *Bacillus subtilis* and *Mycoplasma capricolum* accept alanine in vitro (*15*).

Aminoacylation of tmRNA constitutes an essential step in *trans*-translation, since tmRNA mutants that cannot accept alanine do not bind to 70S ribosomes in vivo (*14*, *16*). Since all aminoacylated tRNAs (with the exception of the initiator Met-$tRNA^{Met}$ and selenocysteinyl-$tRNA^{Sec}$) form tight ternary complexes with elongation factor Tu and GTP (EF-Tu·GTP) for their delivery to the ribosomal A site, it is reasonable to suppose that Ala-tmRNA may form a similar complex.

In this study, we present kinetic parameters of the alanylation of tmRNA and $tRNA^{Ala}$ transcripts by highly purified AlaRS. Subsequently, the alanylated transcripts were used to assess rate constants of ternary complex formation with highly purified EF-Tu.

EXPERIMENTAL PROCEDURES

Isolation of Proteins. C-Terminally $(His)_6$-tagged EF-Tu from *E. coli* was obtained from the pKECAHis expression vector (*17*). After induction, cells were collected by centrifugation (4000*g* for 10 min) at 4 °C and resuspended in 50 mM Tris-HCl (pH 7.6), 60 mM NH_4Cl, 7 mM $MgCl_2$, 0.1 mM PMSF, and 10 μM GDP. Cells were disrupted by sonication, and an S30 extract was prepared by centrifugation at 30000*g* for 15 min at 4 °C. EF-Tu·GDP was eluted from a Ni^{2+}-NTA−agarose (Qiagen) column by a gradient of 20 to 100 mM imidazolium chloride in the same buffer. EF-Tu·GDP was activated to EF-Tu·GTP by incubation at 37 °C for 15 min in 50 mM Tris-HCl (pH 7.6), 60 mM NH_4Cl, 7 mM $MgCl_2$, 2 mM GTP, 6 mM PEP, and 10 μg/mL PK immediately before use, and cooled to 4 °C.

The AlaRS enzyme was obtained as a $(His)_6$-tagged protein from the pQE-875 expression vector (*18*). The latter construct

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[1] Abbreviations: AlaRS, alanyl-tRNA synthetase; EF-Tu, elongation factor Tu; NTA, nitrilotriacetic acid; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; *ssrA*, small stable RNA A gene; TCA, trichloroacetic acid; tmRNA, transfer messenger RNA.

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was a kind gift from P. Schimmel. Induction and isolation of the AlaRS were performed as described for EF-Tu, however without GDP.

The protein purity was checked by SDS−PAGE with 8% (w/v) gels and subsequent Coomassie Brilliant Blue staining. Fractions containing purified protein were pooled and concentrated using Amicon Centriflo ultrafiltration cones. For storage, both proteins were kept in buffer with 10% (v/v) glycerol at −20 °C.

Transcription and Aminoacylation. The plasmid pALA119, carrying the gene for $tRNA^{Ala}$ under control of the T7 promoter, was kindly provided by J. Horowitz (*19*). Plasmid pssrA, containing the T7 promoter directly followed by the *ssrA* gene encoding tmRNA, was constructed as described in Wower et al. (manuscript in preparation). Plasmids were transformed into *E. coli* strain JM109.

Both $tRNA^{Ala}$ and tmRNA were synthesized by in vitro runoff transcription of the plasmids after *Mva*I (MBI Fermentas) digestion, by means of the T7 RiboMAX large-scale RNA production system (Promega) as described by the supplier. In vitro synthesis of ^{3}H-labeled ([^{3}H]GTP; 370 × 10^9 Bq/mmol) RNAs was carried out with the T7 Riboprobe system (Promega) as described by the manufacturer.

Aminoacylation kinetics of $tRNA^{Ala}$ transcripts with alanine were studied at 37 °C, in 100 μL reaction mixtures containing 50 mM Tris-HCl (pH 7.6), 60 mM NH_4Cl, 7 mM $MgCl_2$, 2 mM ATP, 6 mM PEP, 10 μg/mL PK, 15 μM [^{3}H]-Ala (3.63 × 10^{10} Bq/mmol), 0.25 μM AlaRS, and $tRNA^{Ala}$ at the indicated concentrations. Alanylation of tmRNA was carried out as described for $tRNA^{Ala}$, with the exception that 1 μM AlaRS was used. After the indicated incubation time, 15 μL aliquots were precipitated in 1 mL of 10% (w/v) TCA and filtered on GF/C (Whatmann) filters. The amount of incorporated radioactivity was determined by liquid scintillation counting. When RNA aminoacylation in the presence of EF-Tu·GTP was studied, a preparation of EF-Tu·GDP was converted into EF-Tu·GTP as described above, and subsequently added to the aminoacylation mixtures at the indicated concentrations.

For the isolation of alanylated transcripts, aminoacylation reactions were performed as described above, except that 2 μM [^{3}H]Ala (1.92 × 10^{12} Bq/mmol) was used. After incubation for 15 min, the reaction was quenched by addition of 0.1 volume of 2 M ice-cold potassium acetate (pH 5.0) and the mixture immediately treated with 1 volume of phenol saturated with 0.2 M potassium acetate (pH 5.0) and 1 volume of chloroform. After extraction, the aqueous layer was precipitated twice in 70% (v/v) EtOH and 0.2 M potassium acatetate (pH 5.0) and the precipitate was washed twice with the same solution. Washed RNA pellets were redissolved in 10 mM potassium acetate (pH 5.0) up to a final concentration of 0.2 μM.

Equilibrium Constants of Ternary Complexes. To determine rate constants and the equilibrium constant of ternary complex formation, a special RNase A protection assay was performed with a set of increasing EF-Tu·GTP concentrations (*20*). The system can be described as follows:

$$[^3\text{H}]\text{Ala} + \text{RNA} \underset{\text{RNase A}}{\overset{k_2}{\leftarrow}} [^3\text{H}]\text{Ala-RNA} + \text{EF-Tu·GTP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{EF-Tu·GTP·}[^3\text{H}]\text{Ala-RNA} \quad (1)$$

Ternary complex formation between EF-Tu·GTP and alanylated $tRNA^{Ala}$ or tmRNA is governed by the kinetic parameters k_1 and k_{-1}. The k_2 rate constant determines the cleavage rate of an aminoacylated tRNA, when not in complex with EF-Tu·GTP.

Here we define [EF-Tu·GTP] as *T*, [EF-Tu·GTP·[^{3}H]Ala-RNA] as *C*, [[^{3}H]Ala-RNA] as *A*, and [RNA] as *D*. T_0 (=$T + C$) is the total amount of EF-Tu·GTP used, whereas *S* (=$A + C$) is the total amount of precipitable counts present in [^{3}H]Ala-RNA. For a steady-state approximation, the following equation is valid:

$$k_2A = k_{-1}C - Tk_1A \quad (2)$$

With the assumption that $T_0 \gg C$, so that $T_0 - C \approx T_0$, the time dependence of *S* can be derived for a range of T_0 concentrations, with the parameter *p* having the dimensions of a decay rate constant:

$$S_t = S_0e^{-pt} \quad (3)$$

$$p = \frac{k_2k_{-1}}{k_2 + k_{-1} + k_1T_0} \quad (4)$$

All three rate constants can be derived from one set of experiments. First, the natural logarithms taken from the ratios of precipitable ^{3}H counts at time *t* over those at time zero are plotted versus time for a set of experiments with various EF-Tu concentrations. Thereafter, the reciprocal values of the obtained slopes from these linear fits can be plotted as a function of the total EF-Tu·GTP concentrations (T_0) that were used. The slope obtained in the absence of EF-Tu directly yields k_2. Rate constants and the equilibrium constant can then be calculated from this slope according to eqs 3 and 4. In brief, the assay measures the steady-state kinetics of a reaction in which the initial dynamic equilibrium between association and dissociation is drawn toward dissociation by a continuous elimination of noncomplexed Ala-RNA.

Experimentally, the RNase A protection assay was performed as follows. Each assay was carried out in 200 μL of the mixture described above, supplemented with 5 nM [^{3}H]-Ala-RNA (1.92 × 10^{12} Bq/mmol) and EF-Tu•GTP concentrations in the 0.1−20 μM range. After equilibration for 15 min at 4 °C, the decay measurement of precipitable [^{3}H]Ala started after the addition of RNase A from bovine pancreas, at a final concentration of 6 nM. At regular time intervals, 30 μL aliquots were taken, mixed with 100 mM bulk tRNA (Sigma) as a carrier, and immediately precipitated in 10% (w/v) TCA for filtration and liquid scintillation counting.

Binding assays with ^{3}H-labeled non-aminoacylated RNAs with $(His)_6$-tagged EF-Tu·GDP or EF-Tu·GTP carried out according to ref *20* were as follows. The reaction mixtures were the same as in the RNase A protection assay, with [^{3}H]-Ala-RNA or deacylated ^{3}H-labeled RNA, and without PEP and PK when binding to EF-Tu·GDP was studied. Samples of 20 μL of a 60% (v/v) Ni^{2+}-NTA−agarose suspension were added to the reaction mixtures in 0.45 μm Ultrafree-MC centrifugal filter units (Millipore). The units were shaken at 4 °C for 30 min. The nonbound components were spun through the filter unit at 14 000 rpm for 1 min (4 °C), and precipitated with 10% (w/v) TCA in the presence of 100

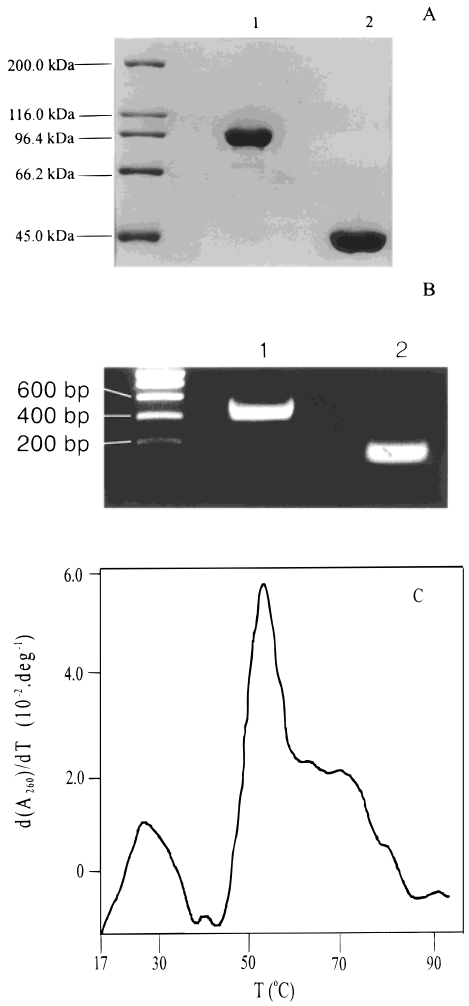

FIGURE 1: Analysis of isolated proteins and transcripts. (A) SDS−PAGE with protein markers, showing a single band for the His-tagged AlaRS (lane 1) around 96 kDa and the His-tagged EF-Tu (lane 2) around 45 kDa. (B) Nondenaturing agarose gel with DNA markers, showing the T7 transcripts of tmRNA (lane 1) and $tRNA^{Ala}$ (lane 2) as single bands. (C) UV-absorbance melting curve of the tmRNA transcript.

mM bulk tRNA. The retained Ni^{2+}-NTA-associated components were eluted by treatment at 4 °C with 200 μL of 100 mM imidazolium chloride in assay buffer for 5 min. After centrifugation, the eluted fraction was also precipitated in 10% (w/v) TCA with 100 mM bulk tRNA. TCA precipitates were filtered on GF/C filters. Radioactivity was quantified by liquid scintillation counting.

Structural Analysis. UV-absorbance melting curves were obtained on a Beckmann spectrophotometer equipped with a temperature regulator. Conditions were adopted from Nameki et al. (*21*). Measurements were taken at RNA transcript concentrations of 12 μg/mL in 10 mM cacodylate buffer (pH 7.3), 1 mM $MgCl_2$, and 50 mM NaCl and were started at 17 °C. The temperature was increased at a rate of 0.4 °C/min until a final temperature of 95 °C was reached.

RESULTS

Kinetics of Alanylation. His-tagged AlaRS and EF-Tu for kinetic measurements were isolated by Ni^{2+}-NTA affinity chromatography. SDS−PAGE analysis showed that both proteins were highly pure (Figure 1A). The His-tagged

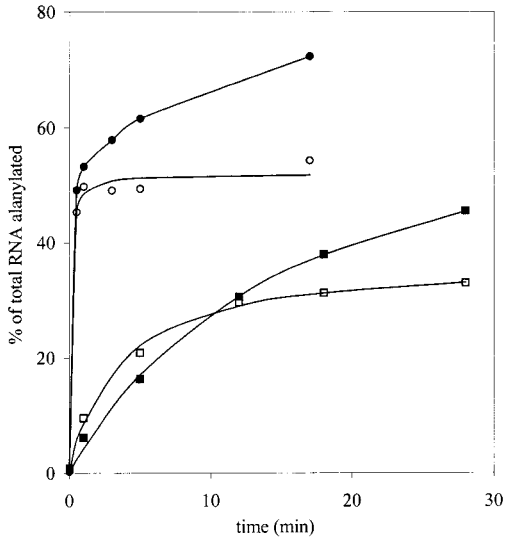

FIGURE 2: Aminoacylation kinetics at 37 °C of $tRNA^{Ala}$ (○ and ●) and tmRNA transcripts (□ and ■) in the absence (white symbols) or presence (black symbols) of His-tagged EF-Tu·GTP. Charging of 1.5 μM $tRNA^{Ala}$ or tmRNA was carried out with His-tagged AlaRS at 0.25 or 1.0 μM, respectively, while EF-Tu·GTP, if present, was used at 5 or 10 μM, respectively.

AlaRS migrates as a single band around 96 kDa, corresponding to the monomeric subunit (*22*), and EF-Tu has its single band around 45 kDa.

Transcripts of tmRNA and tRNA were purified by ammonium acetate/2-propanol precipitation. Subsequently, they were checked by PAGE analysis under denaturing conditions; the transcripts appeared to comigrate with the native RNAs (not illustrated). Analysis by nondenaturing agarose gel electrophoresis revealed only one band (Figure 1B), indicating that our procedure of synthesis and purification yields monomeric transcripts in one structural conformation. This was confirmed by UV-absorbance melting experiments (Figure 1C). The melting profile exhibited two transitions, at 26.3 °C and around 55 °C, in good agreement with the UV-absorbance melting profile reported for native *E. coli* tmRNA with two transitions around 25 and 57.5 °C, respectively (*21*). In addition, aminoacylation experiments showed that typically up to 30% of all tmRNAs are alanylated by AlaRS after prolonged incubation. Similarly prepared $tRNA^{Ala}$ transcripts could be alanylated up to 50% of the total amount of transcript (Figure 2, white symbols).

Assuming that plateau levels in RNA aminoacylation kinetics represent steady states for their aminoacylation reaction and the spontaneous hydrolysis of their aminoacyl moieties, we analyzed whether EF-Tu·GTP would raise plateau levels by protection against deacylation upon ternary complex formation. Indeed, in the presence of 5 μM EF-Tu·GTP, more than 70% of the total $tRNA^{Ala}$ could be alanylated, whereas in the presence of 10 μM EF-Tu·GTP, more than 40% of tmRNA transcripts were charged with an alanine residue (Figure 2, black symbols). These results indicate that the tmRNA and tRNA transcripts obtained with our procedure are functionally active to at least these levels. Control experiments demonstrated that EF-Tu·GDP has no stimulating effect on the aminoacylation of both RNAs (not illustrated). These results also indicate that in the presence of EF-Tu·GTP not only Ala-$tRNA^{Ala}$ but also Ala-tmRNA is protected against hydrolysis of the alanyl ester bond.

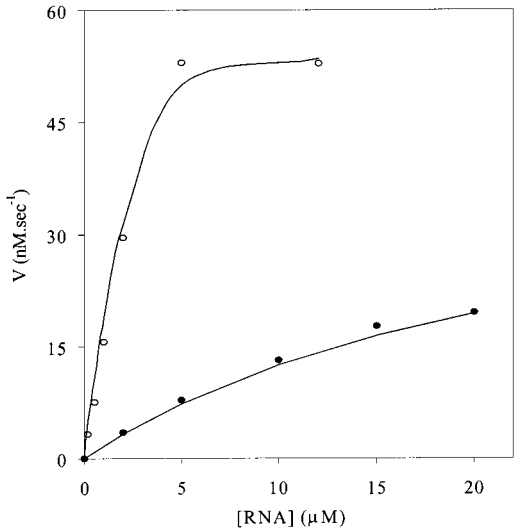

FIGURE 3: Michaelis−Menten plot of the alanylation rate catalyzed by His-tagged AlaRS at 37 °C as a function of the tRNA^{Ala} (○) or tmRNA (●) concentration. The two sets of data were obtained with 0.25 μM AlaRS and a 20 s reaction time or with 1.0 μM AlaRS and a 60 s reaction time, respectively.

Table 1: Kinetic Parameters for the Alanine Charging by His-Tagged AlaRS of tRNA^{Ala} and tmRNA Transcripts[a]

	tRNA^{Ala} with 0.25 μM AlaRS	tmRNA with 1.0 μM AlaRS
V_{max} (nM s^{-1})	50 ± 5	43 ± 4
K_M (μM)	1.5 ± 0.3	24 ± 3
k_{cat} ($\times 10^{-3}$ s^{-1})	200 ± 40	43 ± 4
k_{cat}/K_M ($\times 10^4$ M^{-1} s^{-1})	13 ± 4	0.18 ± 0.03

[a] Values were determined from Michealis−Menten plots such as in Figure 3.

Since tmRNA transcripts can be used by AlaRS as a substrate, we wanted to characterize the kinetic parameters of this reaction and compare them with those of the alanylation reaction with tRNA^{Ala}. Preliminary experiments demonstrated that tmRNA could only be aminoacylated to significant levels in the presence of a relatively high concentration of AlaRS. We therefore used 1.0 μM instead of 0.25 μM AlaRS, as was used for tRNA^{Ala}. In the experiments for the determination of k_{cat} and K_M values, the amounts of Ala-tRNA^{Ala} that formed were measured after the initial 20 s of each reaction; for tmRNA, the amounts of Ala-tmRNA were determined after the initial 60 s of each reaction. Both reaction times were in the linear part of the alanylation reaction, and for these periods, the extent of spontaneous hydrolysis of the alanine ester bond was negligible (Figure 2, white symbols). From results such as those depicted in Figure 3 (the calculated parameters are listed in Table 1), one can see that AlaRS aminoacylates tmRNA at a much lower rate and with a lower affinity than tRNA^{Ala}. In reaction with tmRNA, it exhibits an almost 5-fold lower k_{cat} and a 16-fold higher K_M than with tRNA^{Ala}. Consequently, the k_{cat}/K_M values show a difference of about 75-fold. We conclude that the *E. coli* tmRNA transcript can be alanylated by the AlaRS enzyme, albeit at a considerably lower efficiency than the tRNA^{Ala} transcript.

Dynamic Equilibrium Parameters for Ternary Complex Formation. In the preceding subsection, first indications were already obtained for a protective binding of EF-Tu·GTP to

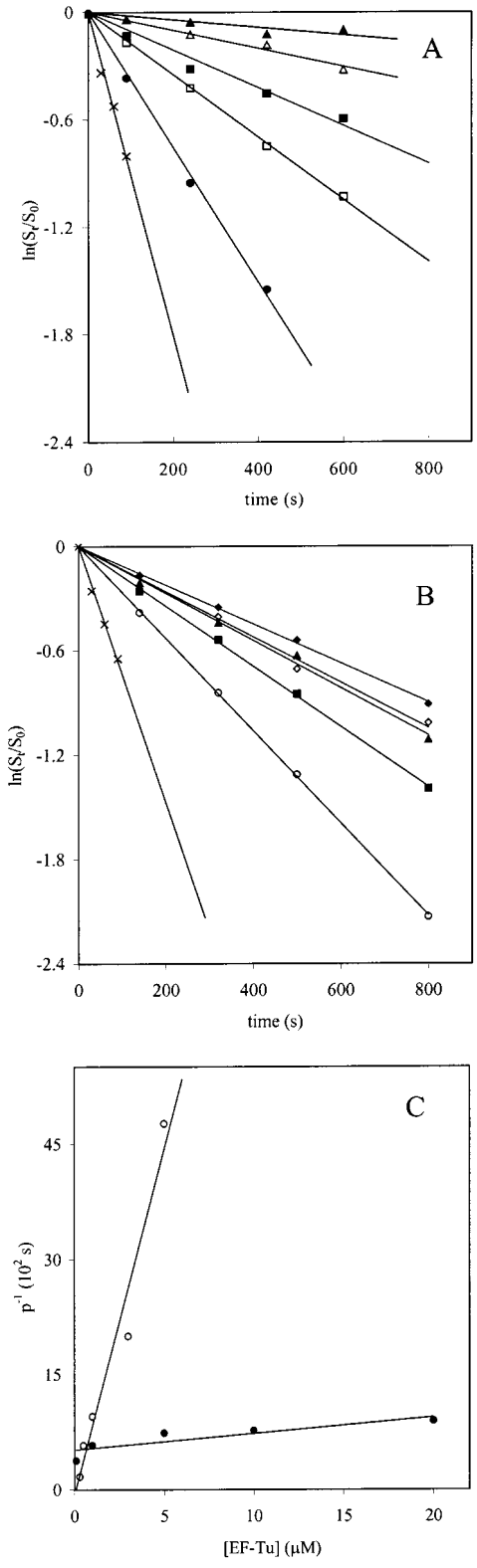

FIGURE 4: Parameters of ternary complex formation determined by protection against RNase A cleavage of the alanyl−adenosine phosphodiester bond. [^{3}H]Ala-tRNA^{Ala} (A) and [^{3}H]Ala-tmRNA (B) were incubated with various concentrations of His-tagged EF-Tu·GTP: 0 (×), 0.1 (○), 0.3 (●), 0.5 (□), 1 (■), 3 (△), 5 (▲), 10 (◇), and 20 μM (◆). The data are plotted as the natural logarithm of the ratios of precipitable ^{3}H counts at time t over those at time zero as a function of time. In panel C, the reciprocal slopes (p^{-1}) of the linear fits were plotted vs the EF-Tu·GTP concentrations for both Ala-tRNA^{Ala} (○) and Ala-tmRNA (●), to calculate the kinetic parameters (for details, see Experimental Procedures).

Table 2: Kinetic Parameters for Ternary Complex Formation of Ala-tRNA^{Ala} and Ala-tmRNA with His-Tagged EF-Tu·GTP at 4 °C[a]

	tRNA^{Ala}	tmRNA
k_2 ($\times 10^{-3}$ s^{-1})	10 ± 1	7.4 ± 0.5
k_{-1} ($\times 10^{-3}$ s^{-1})	14 ± 3	3.2 ± 0.9
k_1 ($\times 10^{3}$ M^{-1} s^{-1})	93 ± 1	0.6 ± 0.1
K_d (μM)	0.15 ± 0.03	5.0 ± 1.6

[a] Values were calculated from protection assays against RNase A cleavage as illustrated in Figure 4. The parameters are explained in eq 1.

tmRNA after alanylation. To determine association and dissociation rate constants together with the equilibrium constant of ternary complex formation for either RNA molecule with EF-Tu·GTP, a special RNase A protection assay was carried out with a set of increasing concentrations of His-tagged EF-Tu (for a description of the system, see eq 1 in Experimental Procedures). Representative results with such sets are shown in panels A and B of Figure 4 for the protection against RNase A cleavage of [^{3}H]Ala-tRNA^{Ala} and [^{3}H]Ala-tmRNA, respectively. The cleavage rate in the absence of EF-Tu is not much different for both RNA species, as reflected in their k_2 values (Table 2). From plots of the reciprocal values of the slopes (p^{-1}) of the lines in panels A and B of Figure 4 as a function of the EF-Tu concentration (Figure 4C), parameters k_{-1}, k_1, and K_d' were determined, as listed in Table 2. The dissociation rate k_{-1} appears to be on the same order of magnitude for both RNAs, although 4−5-fold higher for Ala-tRNA^{Ala}. In contrast, the K_d' values for both ternary complexes are very different, 0.15 μM for Ala-tRNA^{Ala} as compared to 5 μM for Ala-tmRNA. This is almost entirely caused by the very low association rate (k_1) for Ala-tmRNA with EF-Tu·GTP, about 150-fold lower than that for Ala-tRNA^{Ala}.

Affinity assays with non-aminoacylated ^{3}H-labeled transcripts were carried out with increasing amounts of His-tagged EF-Tu·GTP immobilized on Ni^{2+}-NTA−agarose. The retained Ni^{2+}-NTA-associated components contained no TCA-precipitable counts from noncharged ^{3}H-labeled transcripts in contrast to positive controls with [^{3}H]Ala-RNAs (data not shown). These results show that EF-Tu·GTP exhibits measurable affinity only for Ala-tRNA^{Ala} and Ala-tmRNA. In addition, the same set of experiments conducted with EF-Tu·GDP revealed that EF-Tu·GDP exhibited no apparent affinity for both alanylated and noncharged tRNA^{Ala} or tmRNA (not shown).

DISCUSSION

We have presented kinetic parameters for the alanylation reaction of tmRNA with AlaRS and for the formation of a ternary complex of Ala-tmRNA with EF-Tu·GTP. Apparent rate constants were compared with those for tRNA^{Ala}. In this study, we used highly purified components. His-tagged AlaRS and EF-Tu were isolated by Ni^{2+}-NTA affinity chromatography, and both RNAs were obtained by in vitro T7 transcription. In contrast, previous studies by others were carried out with tmRNA obtained by in vivo overexpression from a high-copy number plasmid (*14*, *16*, *21*, *23*−*25*). Since *E. coli* tmRNA undergoes post-transcriptional base modifications (*23*), this method may yield a mixed population of partially modified and nonmodified tmRNA molecules. It has nevertheless been reported that in vivo overproduced tmRNA and tmRNA obtained by means of in vitro transcription exhibit a similar affinity for EF-Tu·GTP (*25*).

We therefore chose to do comparative affinity studies on in vitro transcripts that are homogeneous in this respect. UV-absorbance melting experiments revealed two conformational transitions, resembling the profile found for native *E. coli* tmRNA (*21*), and suggested that the tmRNA transcript assumes a structure similar to that of native modified tmRNA. Up to 30% of the total tmRNA transcripts could be alanylated in the reactions with purified components described in this study. This is much better than reported previously by other groups (*16*, *21*) and in the same range as found by Komine et al. (*14*). Additionally, in the presence of an excess of EF-Tu·GTP, nearly 50% of the total tmRNA transcript was alanylated. Alanylation of the tRNA^{Ala} transcript yielded 50% Ala-tRNA^{Ala}, and in the presence of EF-Tu·GTP, more than 70% of the total tRNA^{Ala} was alanylated. For isolation of aminoacylated tRNA (-like) species at increased yield, the introduction of EF-Tu·GTP with its protection of the 3′ aminoacyl acceptor end can thus be useful. In vivo, the high intracellular EF-Tu concentration may have a similar effect. The fact that not all RNA becomes aminoacylated may be due to 3′ or 5′ heterogeneities of the transcripts, which commonly occur with T7 polymerase, or due to a subsaturating amino acid concentration (*26*−*29*). In any event, data show that *E. coli* tmRNA does not require the recently found modifications for folding into a functional structure. This agrees with what is known for tRNA^{Ala} (*23*, *30*, and references therein).

The kinetics of alanylation were determined for both tmRNA and tRNA^{Ala} transcripts. Under the reaction conditions that were used, tmRNA alanylation by AlaRS exhibited a V_{max} comparable to that for charging of tRNA^{Ala} with 4-fold less AlaRS. However, a considerable difference in K_M values was observed. The determined K_M value of 1.5 μM for tRNA^{Ala} alanylation was also reported for AlaRS without His tag by other groups (*29*, *31*, *32*). Our calculated k_{cat} for tRNA^{Ala} is around 0.2 s^{-1}, while reported k_{cat} values are 0.9 (*29*, *32*) and 1.7 s^{-1} (*31*). Besides slightly different reaction conditions and the presence of His-tagged AlaRS, our use of T7 transcripts may also account for the different value. tmRNA appeared to have a K_M value of approximately 24 μM, 16-fold higher than for tRNA^{Ala}. Hence, the k_{cat}/K_M values of AlaRS for both substrates, as measures for the catalytic efficiency of the enzyme under subsaturating substrate conditions, differ 75-fold. Apparently, this does not prevent tmRNA from becoming alanylated at a sufficient level in vivo. A comparable situation occurs for a mutant tRNA^{Ala} with G3·U70 replaced with G3·A70. Although the k_{cat}/K_M was reported to be 90-fold lower than that for wild-type tRNA^{Ala} (*33*), the steady-state level of alanylation in vivo is about 78% of that of wild-type tRNA^{Ala} (*34*). The high intracellular EF-Tu concentration may contribute positively to the steady-state level of Ala-tmRNA (see above). The presence of a special protein factor for specific stimulation of the mutant tRNA^{Ala} alanylation can practically be excluded.

In our studies of Ala-tmRNA binding to EF-Tu·GTP, we took advantage of an RNase A protection assay to determine the rate constants of the dynamic binding equilibrium in a single set of experiments. For the Ala-tRNA^{Ala} transcript as

a reference molecule, a K_d' of 0.15 μM was measured. This value is higher than those reported by other groups for fully modified tRNA^{Ala} from cellular extracts (*35*, *36*). Further explanations for this systematic difference are the use of His-tagged EF-Tu, which causes a ≥3-fold increase in K_d' (*20*), and differences in reaction conditions [in particular, temperature and concentrations of buffer components which are very important for the tightness of the ternary complex (*36*, *37*)]. The functioning of the His-tagged EF-Tu is hardly affected because replacement of the wild-type *E. coli* gene, encoding EF-Tu, with His-tagged EF-Tu has been reported not to influence the growth rate (*38*). The K_d' value for Ala-tmRNA binding to EF-Tu·GTP in our system was about 30-fold higher in comparison with that for Ala-tRNA^{Ala} binding. While in the dynamic equilibrium reaction Ala-tmRNA exhibits a 4−5-fold smaller dissociation rate constant (k_{-1}) than Ala-tRNA^{Ala}, its association rate constant (k_1) is as much as 150-fold lower than that of Ala-tRNA^{Ala}.

This difference in association rate constants of Ala-tmRNA and Ala-tRNA^{Ala} for EF-Tu binding can at least partially be explained by their different relative masses of about 124 and 26 kDa, respectively, and the corresponding 1.7-fold difference in their Stokes radii (r). The association rate constant is, among other factors, dependent on the linear diffusion coefficient (proportional to r^{-1}), the rotational diffusion coefficient (proportional to r^{-3}), and the chance for a sterically productive encounter (proportional to r^{-2}). Altogether, these factors already make up for a 25-fold difference in k_1 values, without taking into account factors such as induced fit, or the presence of positive or negative determinants. The measured difference in dissociation rate constants of Ala-tmRNA and Ala-tRNA^{Ala} in their respective complexes with EF-Tu corresponds well with the assumption that mainly the 5-fold difference in their rotational diffusion coefficient may be responsible for the different outcome of k_{-1}. In this assumption, complex dissociation may start by the disruption of binding interactions between the constituting macromolecules as a result of their individual rotational movements. Notably, the 30-fold difference in apparent K_d' values of Ala-tmRNA and Ala-tRNA^{Ala} for EF-Tu binding is on the same order as the 15-fold difference in K_M values for their interaction as deacylated molecules with AlaRS. The same physicochemical reasoning may therefore explain both differences.

During the preparation of this paper, a related letter to the editor by Rudinger-Thirion et al. was published (*25*). They reported that the interactions of EF-Tu·GTP with Ala-tmRNA were at least as strong as that with Ala-tRNA^{Ala}, which therefore should rule out the requirement of a specialized factor for Ala-tmRNA delivery to the ribosome. Their measurements seem in conflict with our results, but a comparison is hardly possible. They only did a qualitative protection assay against spontaneous hydrolysis of the alanyl ester bond at a single EF-Tu concentration (2.5 μM) and based their conclusion on the similarity of the half-lives of both complexed Ala-RNA species (about 5 h at 37 °C). Furthermore, they used a heterologous EF-Tu from *Thermus thermophilus* in assay with tmRNA and tRNA^{Ala} from *E. coli*.

Besides Ala-tmRNA, another example of an aminoacylated elongator tRNA with a low affinity for EF-Tu·GTP is selenocysteinyl-tRNA^{Sec} (Sec-tRNA^{Sec}). The latter is an exceptional tRNA in the pathway for selenocysteine incorporation as programmed by the stop codon UGA in a special context. It turned out that EF-Tu binds to Sec-tRNA^{Sec} about 100-fold more weakly than to other aminoacyl-tRNAs in vitro (*39*). Sec-tRNA^{Sec}, however, makes use of a special EF-Tu homologue, called SELB (*1*). By analogy, we cannot completely rule out the possibility that Ala-tmRNA would also make use of a special protein factor not identified thus far. Quite recently, the group of Sauer reported on the function of a small protein called SmpB (*40*). It is encoded by the *smpB* gene located just upstream of the *ssrA* gene for tmRNA. They showed that deletion of *smpB* in *E. coli* results in the same phenotype as observed in *ssrA*-defective cells. From their in vitro experiments, they conclude that SmpB neither is required for nor significantly affects tmRNA alanylation. In addition, purified SmpB binds specifically and with high affinity to tmRNA. It seems that this interaction is needed for association of tmRNA with a stalled ribosome. The possible influence of SmpB on the stability of the ternary complex of EF-Tu·GTP with alanyl-tmRNA is therefore a next step to be studied.

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