Energetic contribution of tRNA hybrid state formation to translocation catalysis on the ribosome

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Upon transpeptidylation, the 3′ end of aminoacyl-tRNA (aa-tRNA) in the ribosomal A site enters the A/P hybrid state. We report that transpeptidylation of Phe-tRNA to fMetPhe-tRNA on *Escherichia coli* ribosomes substantially lowers the kinetic stability of the ribosome–tRNA complex and decreases the affinity by 18.9 kJ mol^{-1}. At the same time, the free energy of activation of elongation factor G dependent translocation decreases by 12.5 kJ mol^{-1}, indicating that part of the free energy of transpeptidylation is used to drive translocation kinetically. Thus, the formation of the A/P hybrid state constitutes an important element of the translocation mechanism.

The elongation cycle of protein synthesis consists of three major steps. It begins with a ribosome that contains peptidyl-tRNA (or initiator tRNA) in the P site and a free codon in the A site. During the first step, a complex of elongation factor Tu (EF-Tu), GTP, and aminoacyl-tRNA (aa-tRNA) binds to the A site in a codon-dependent manner. Following codon recognition and GTP hydrolysis, aa-tRNA is released from EF-Tu and settles into the peptidyltransferase center. Once the aa-tRNA is accommodated, the peptide bond is formed instantaneously, yielding a deacylated tRNA in the P site and a peptidyl-tRNA in the A site in the P/E and A/P hybrid states[2], respectively. The elongation cycle is completed by translocation — that is, the movement of the mRNA–tRNA complex on the ribosome, which is catalyzed by elongation factor G (EF-G) with GTP. During translocation, the 3′ end of the peptidyl-tRNA moves into its puromycin-reactive position on the 50S subunit, and the anticodon end, along with the mRNA, moves along the 30S subunit. The peptidyl-tRNA is thereby displaced from the A site (A/P state) to the P site, while the P site-bound deacylated tRNA (P/E state) is moved to the E site and released from the ribosome[3,4]. Evidence for a stepwise movement of tRNAs on the ribosome and for the existence of hybrid states was first obtained by footprinting experiments using poly(U)-programmed ribosomes[2], and later also on ribosomes programmed with natural mRNA (S. Joseph, H.F. Noller & M.V.R., unpublished results).

To assess the energetic contribution to translocation of hybrid state formation, we have determined the kinetic stabilities and affinities of the A site complexes of Phe-tRNA^{Phe} and fMetPhe-tRNA^{Phe} and have related them to the rates of translocation from the two states.

Kinetic stability of tRNA–ribosome complexes

Two different A site complexes were studied (Fig. 1*a*). In complex 1, the A site contained fMetPhe-tRNA^{Phe} (in the A/P state). To form this complex, N-Formylmethionyl-tRNA^{fMet} (fMet-tRNA^{fMet}) was bound to the P site of ribosomes from *E. coli* programmed with a near-natural mRNA in the presence of all three initiation factors. Then, the ternary complex EF-Tu–GTP–Phe-tRNA^{Phe} was added in an amount slightly less than that of the initiation complex. Binding of Phe-tRNA^{Phe} to the A site and subsequent peptide bond formation resulted in a ribosome complex that had deacylated tRNA^{fMet} in the P site and fMetPhe-tRNA^{Phe} in the A site (in the A/P state[3]). In complex 2, the A site contained Phe-tRNA^{Phe}, most likely in the A/A state. In this case, initiation complexes were formed using deacylated tRNA^{fMet} instead of fMet-tRNA^{fMet}. Then, Phe-tRNA^{Phe} was bound to the A site with EF-Tu–GTP as above.

The stabilities of the two complexes were determined by monitoring binding levels during prolonged incubation (Fig. 1*b*). fMetPhe-tRNA^{Phe} dissociated readily with a half life of ~10 min. The dissociation was almost complete, and the small amount of fMetPhe-tRNA^{Phe} that was not released from the ribosome was bound to the P site, according to the puromycin reaction. In contrast, practically no dissociation of Phe-tRNA^{Phe} was observed within one hour, indicating a very stable complex. The presence or absence of initiation factors had no influence on the stability

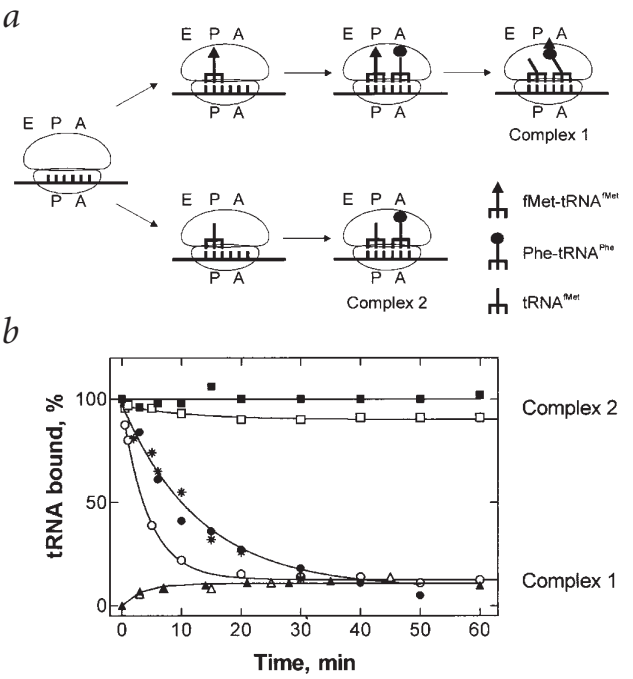

Fig. 1 Kinetic stability of A site tRNA complexes. ***a***, Preparation of complexes. Complex 1 (top row) was prepared by successively binding fMet-tRNA^{fMet} to the P site and Phe-tRNA^{Phe} to the A site of mRNA-programmed ribosomes; transpeptidylation resulted in the quantitative formation of fMetPhe-tRNA^{Phe} (A/P state) and deacylated tRNA^{fMet} (P/E state). Complex 2 (bottom row) was prepared similarly, except that deacylated tRNA^{fMet} was used to occupy the P site, leaving Phe-tRNA^{Phe} in the A site. For experimental details, see Methods. ***b***, Dissociation from the A site. The dissociation of f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} (filled circles, empty circles, asterisks; complex 1) or of [^{14}C]Phe-tRNA^{Phe} (filled squares, empty squares; complex 2) from the ribosome was measured in buffer A (7 mM Mg^{2+}; filled circles, filled squares) or in polymix buffer (empty circles, empty squares). For comparison (asterisks), 70S initiation complexes were purified by gel filtration prior to the addition of EF-Tu–GTP–[^{14}C]Phe-tRNA^{Phe}. P site-bound fMetPhe-tRNA^{Phe} in buffer A (filled triangles) or polymix (empty triangles) was measured by puromycin. 100% corresponds to the initial amount of [^{14}C]Phe-tRNA^{Phe} bound (~0.2 μM).

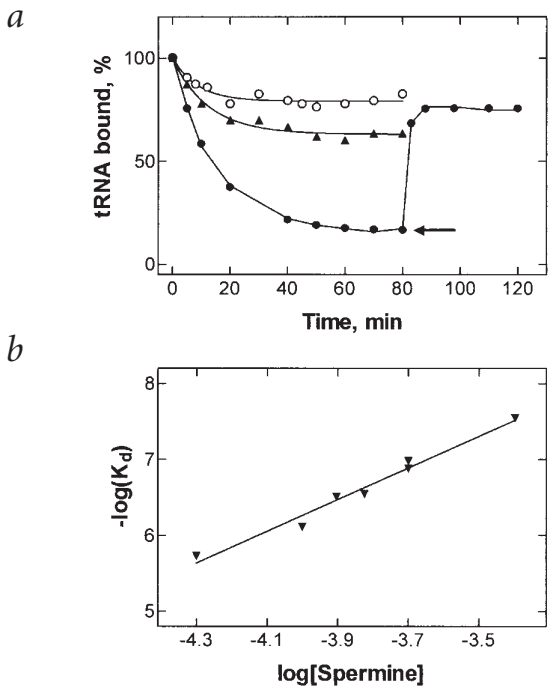

Fig. 2 Effect of spermine on fMetPhe-tRNA^{Phe} binding to the A site. ***a***, Dissociation time courses. Dissociation of f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} in the absence of spermine (filled circles); after 80 min of incubation, 0.4 mM spermine was added (arrow) and rebinding of f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} was measured. Dissociation in the presence of 0.2 mM (filled triangles) and 0.4 mM (empty circles) spermine. 100% corresponds to the initial amount of [^{14}C]Phe-tRNA^{Phe} bound (0.22 μM). ***b***, Spermine dependence of K_d. The slope of the log-log plot indicates that two spermine molecules are directly involved in the interaction.

of the complex. Qualitatively similar results were obtained in a low Mg^{2+} buffer containing polyamines (polymix buffer[5]) (Fig. 1*b*), although the dissociation of fMetPhe-tRNA^{Phe} from the A site was somewhat faster. The stability of Phe-tRNA^{Phe} in the A site was comparably high in the two buffer systems used in the experiments shown in Fig. 1.

Affinity of peptidyl-tRNA in the A site

To determine the affinity of fMetPhe-tRNA^{Phe} for the A site, conditions had to be established where a significant proportion of fMetPhe-tRNA^{Phe} was bound at equilibrium. One way to achieve this was to stabilize the complex by adding spermine (Fig. 2*a*). Equilibrium was reached after ~1 h of incubation, and the equilibrium binding level increased with the concentration of spermine. Furthermore, upon addition of spermine, peptidyl-tRNA that had been released from the ribosome rebound to the A site, demonstrating the reversibility of the equilibrium.

K_d values of A/P complexes were calculated from rate constants obtained by numerical integration of the dissociation time courses (Fig. 2*a*). The same K_d values were also obtained by using the concentrations of the complex and the unbound peptidyl-tRNA at equilibrium. K_d values ranging from 2 μM to 0.03 μM were determined at spermine concentrations from 0.05 mM to 0.4 mM (Fig. 2*b*). Extrapolation yielded a K_d in the absence of spermine of >2 μM.

The second method used to measure the A site affinity of fMetPhe-tRNA^{Phe} was to monitor the dissociation at Mg^{2+} concentrations between 8 and 17 mM where a measurable amount of complex remained at equilibrium (Fig. 3*a*). From the dissociation time courses, K_d values were obtained that ranged from 1.6 μM to 0.1 μM (Fig. 3*b*). At 7 mM Mg^{2+}, the dissociation of the complex was complete, thus precluding the direct determination of a value for the K_d. Extrapolation to 7 mM Mg^{2+} gave a K_d of 3 μM, corresponding to a free energy of interaction (ΔG°) of -32.8 kJ mol^{-1} (7 mM Mg^{2+}, 37 °C).

Affinity of aa-tRNA in the A site

The high stability of Phe-tRNA^{Phe} binding to the A site was not due to the presence of EF-Tu, as verified by A site binding and dissociation experiments performed in the absence of this factor. Because EF-Tu independent binding of Phe-tRNA^{Phe} to the A site is very slow at 7 mM Mg^{2+}, and is strongly accelerated by increasing Mg^{2+}, a high occupancy of the A site was achieved by binding Phe-tRNA^{Phe} at 20 mM Mg^{2+}. The Mg^{2+} concentration was then lowered to 7 mM and the binding level monitored over time (Fig. 4*a*). Practically no dissociation of Phe-tRNA from the A site was observed within one hour of incubation, indicating that the high stability of the A site complex is independent of EF-Tu.

The reversibility of Phe-tRNA^{Phe} binding to the A site was verified using purified complexes carrying deacylated tRNA^{fMet} in the P site and Phe-tRNA^{Phe} in the A site (see Methods). To avoid the complication by deacylation of Phe-tRNA^{Phe} upon prolonged incubation, recharging conditions were established by adding Phe-tRNA synthetase, ATP and [^{14}C]phenylalanine (see

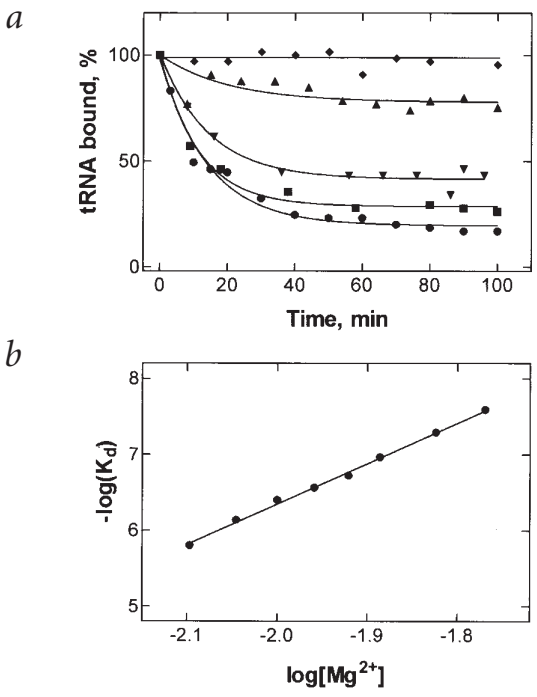

Fig. 3 Effect of Mg^{2+} on fMetPhe-tRNA^{Phe} binding to the A site. ***a***, Dissociation time courses. Dissociation of f[^{3}H]Met[^{14}C]Phe-tRNA^{Phe} in the presence of 7 mM (circles), 8 mM (squares), 10 mM (inverted triangles), 15 mM (upright triangles) and 30 mM (diamonds) Mg^{2+}. 100% corresponds to the initial amount of [^{14}C]Phe-tRNA^{Phe} bound (0.22 μM). ***b***, Mg^{2+} dependence of K_d. The slope of the log-log plot indicates that five Mg^{2+} ions are directly involved in the interaction.

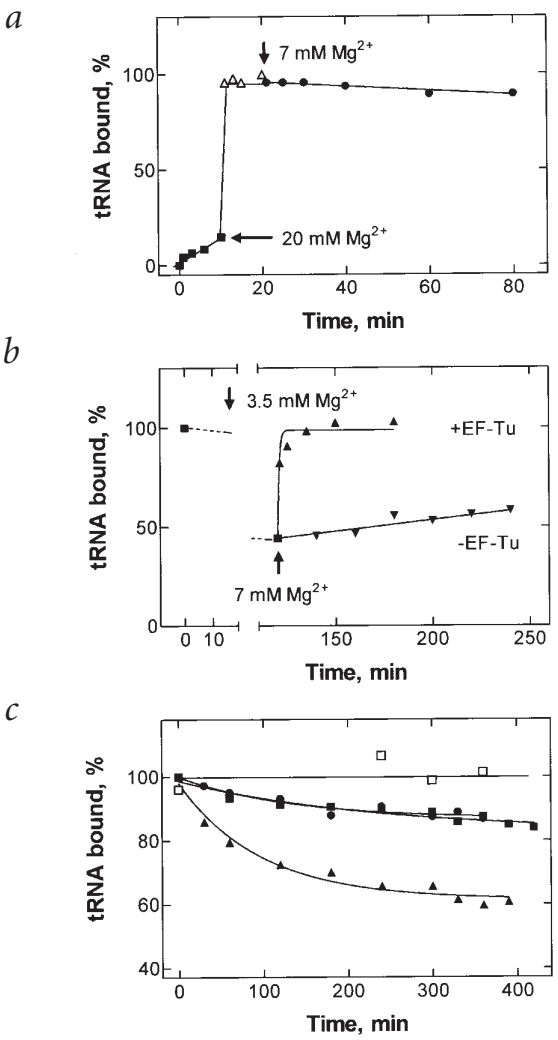

Fig. 4 Stability of Phe-tRNA^{Phe} binding to the A site. ***a***, Nonenzymatic binding and dissociation. mRNA-programmed ribosomes with tRNA^{fMet} in the P site (0.5 μM) were incubated with [^{14}C]Phe-tRNA^{Phe} in the presence of 3 mM ATP, 2 μM [^{14}C]phenylalanine, and a catalytic amount of partially purified Phe-tRNA synthetase at 7 mM Mg^{2+} (0.5 μM ribosomes; squares), after addition of Mg^{2+} to 20 mM (0.43 μM ribosomes; triangles), and after lowering Mg^{2+} to 7 mM Mg^{2+} (0.15 μM ribosomes; circles). ***b***, Reversibility of Phe-tRNA^{Phe} binding. Ribosome complexes containing tRNA^{fMet} in the P site and [^{14}C]Phe-tRNA^{Phe} in the A site at 7 mM Mg^{2+} (squares) were prepared and purified as described in Methods. Dissociation was initiated by dilution to 3.5 mM Mg^{2+} (0.07 μM ribosome complex; 100%) in the presence of additional [^{3}H]tRNA^{fMet} (4 μM), mRNA (2 μM) and the aminoacylation system as above. After 2 h, the Mg^{2+} concentration was increased to 7 mM, and rebinding of [^{14}C]Phe-tRNA^{Phe} measured in the presence of EF-Tu–GTP (0.43 μM) (upright triangles) or in the absence of the factor (inverted triangles). ***c***, Dissociation time courses. Dissociation of Phe-tRNA^{Phe} from the A site at 7 mM (circles, filled squares) or 5 mM (triangles) Mg^{2+}. Binding of [^{14}C]Phe-tRNA to the A site was carried out with EF-Tu·GTP–at 7 mM Mg^{2+} (circles, triangles) or nonenzymatically at 20 mM Mg^{2+} and subsequent dilution to 7 mM Mg^{2+} as in Fig. 4*a* (filled squares). Ribosome complexes were purified by gel filtration. tRNA^{fMet} (2 μM at 7 mM Mg^{2+} or 4 μM at 5 mM Mg^{2+}), mRNA (2 μM), and the aminoacylation system were added; the concentration of the ribosome complexes was 0.075 μM (100%). Empty squares represent the total amount of [^{14}C]Phe-tRNA^{Phe} in the reaction determined by TCA precipitation.

Methods). To induce dissociation of Phe-tRNA^{Phe} from the A site, the Mg^{2+} concentration was lowered to 3.5 mM; ~60% Phe-tRNA^{Phe} dissociated within two hours at 37 °C (Fig. 4*b*). After shifting the Mg^{2+} concentration to 7 mM, rebinding of Phe-tRNA^{Phe} was monitored. In the presence of EF-Tu, Phe-tRNA^{Phe} rapidly rebound at the A site, indicating that the dissociation was fully reversible. Without EF-Tu, Phe-tRNA^{Phe} also rebound to the ribosome, albeit very slowly, in agreement with the results shown in Fig. 4*a*.

The affinity of Phe-tRNA^{Phe} for the A site was measured by dissociation (Fig. 4*c*). By adding the recharging system, the amount of Phe-tRNA^{Phe} could be kept constant during the prolonged incubations required for the dissociation. Phe-tRNA^{Phe} was released from the A site very slowly, regardless of whether it was bound to the A site with EF-Tu or nonenzymatically. A K_d value of 2.0 ± 0.5 nM was estimated using the equilibrium concentrations of ribosome-bound and unbound Phe-tRNA^{Phe}, corresponding to a ΔG° of -51.7 kJ mol^{-1} (7 mM Mg^{2+}, 37 °C). The affinity strongly decreased with decreasing Mg^{2+} concentration, to 20 nM at 5 mM Mg^{2+}. Previous K_d values for Phe-tRNA^{Phe} dissociation from the A site were generally up to 100 times higher (ref. 6, and refs therein). According to the present results, this is probably due to the fact that incubation times, although long (for instance, 90 min, 10 mM Mg^{2+}, 20 °C; ref. 6), were too short to reach the binding equilibrium.

The affinity constants for A site binding by fMetPhe-tRNA^{Phe} and Phe-tRNA^{Phe} differ by a factor of 1,500 (7 mM Mg^{2+}). The corresponding difference in the free energies of binding (ΔΔG° = 18.9 kJ mol^{-1}) represents the fraction of the free energy of transpeptidylation on the ribosome that is stored in the complex.

Kinetics of translocation of peptidyl- and aa-tRNA

Time courses of translocation were measured by stopped-flow fluorescence[7], monitoring the fluorescence of proflavin attached to the D loop of tRNA^{Phe} (ref. 8). Pretranslocation complexes were prepared as above (Fig. 1*a*; Methods) and contained deacylated tRNA^{fMet} in the P site, and either fMetPhe-tRNA^{Phe}(Prf16/17) (complex 1) or Phe-tRNA^{Phe}(Prf16/17) (complex 2) in the A site. Translocation was initiated by rapidly mixing the respective pretranslocation complex with EF-G and GTP. The rate of translocation of fMetPhe-tRNA^{Phe} was 18 s^{-1} (Fig. 5*a*), while practically no translocation of Phe-tRNA^{Phe} was observed in the short time window. However, upon longer incubation, all Phe-tRNA^{Phe} was translocated (Fig. 5*b*), although at a much lower rate of 0.14 s^{-1} — that is, 130 times more slowly than fMetPhe-tRNA^{Phe}. The same rate resulted from monitoring PhePhe-tRNA formation following rapid (<1 s) binding of the ternary complex to the A site vacated by translocation (Fig. 5*b*).

In the kinetic experiments, translocation was measured at EF-G concentrations (1 μM) close to saturation; therefore, the measured rates closely approximate the rate constants of (irreversible) translocation. This allows the calculation of the free energies of activation of translocation according to the equation[9]:

$$\Delta G^{\neq} = -RT \cdot \ln (k \cdot h / k_B / T)$$

where k is the rate constant of the reaction, T the temperature (K), k_B Boltzmann's constant, and h Planck's constant. $\Delta G^{\neq}$ values of 68.6 and 81.1 kJ mol^{-1} were calculated from the translocation rate constants of fMetPhe-tRNA^{Phe} and Phe-tRNA^{Phe}, respectively.

A site tRNA destabilization and translocation

The structural arrangement of the 3′ end of A site bound

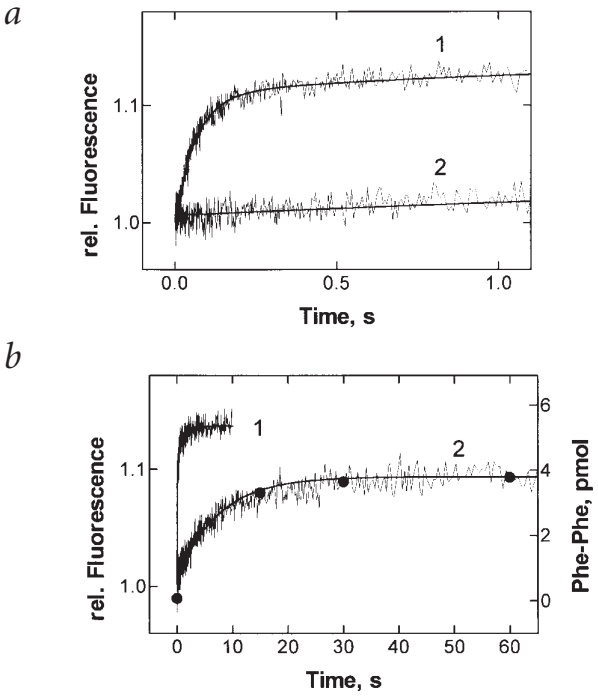

Fig. 5 Rapid kinetics of translocation. Translocation time courses of proflavin labeled fMetPhe-tRNA^{Phe} (1) or Phe-tRNA^{Phe} (2) were measured by stopped flow fluorescence. ***a***, Short time window. ***b***, Long time window. Filled circles, translocation of Phe-tRNA^{Phe} measured by Phe-Phe formation. Translocation rates were 18 ± 1 s^{-1} and 0.14 ± 0.01 s^{-1} for fMetPhe-tRNA^{Phe} and Phe-tRNA^{Phe}, respectively.

aa-tRNA on the 50S ribosomal subunit changes upon transpeptidylation[2,10]. Prior to peptide bond formation, the 3′ end of aa-tRNA interacts with 23S rRNA[11,12], and this contact is important for peptidyl transfer[13,14]. During or after peptide bond formation, the 3′ end moves towards the P site, so that the peptidyl-tRNA assumes the A/P hybrid position[2]. The formation of the A/P hybrid state of peptidyl-tRNA as well as of the P/E hybrid state of deacylated tRNA in the P site were shown to be important for efficient translocation[2,4,15].

Aminoacyl-tRNAs (and peptidyl-tRNAs) are high energy compounds. For the overall equilibrium constant of the amino acylation reaction:

$$E + \text{amino acid} + ATP + tRNA \rightleftharpoons E + aa\text{–}tRNA + AMP + PP_i$$

values in the range of 0.25–0.37 were reported[16–18]. The corresponding standard free energy change, ~3 kJ mol^{-1}, is to be compared with -32.2 kJ mol^{-1} measured for the free energy released on hydrolysis of ATP to AMP and PP_i (ref. 19), indicating that the group transfer potential of the aminoacyl ester bond of aa-tRNA amounts to approximately -29 kJ mol^{-1} (25 °C). The free energy contained in the ester bond of fMet-tRNA^{fMet} (and of peptidyl-tRNAs) is probably comparable. Upon peptide bond formation, the A site tRNA is destabilized by 18.9 kJ mol^{-1} (37 °C). This suggests that approximately two-thirds of the free energy of the ester bond in peptidyl-tRNA, which is cleaved during transpeptidylation, is used for conformational work that results in the destabilization of tRNA-ribosome interactions. It is likely that the formation of the hybrid A/P state, and possibly also the P/E state, constitutes a major part of the conformational work.

Structurally, the difference in the affinity constants must be due to changes in the contacts between the ribosome and the tRNA. The association of the codon–anticodon complex with the 30S A site seems to be similar in the two states, as suggested both by chemical footprinting and cryoelectron microscopy[2,20–22]. Thus, the observed affinity difference probably results from differences in the interaction with the 50S subunit of the 3′ end of aa-tRNA *versus* peptidyl-tRNA. The loss of two or three hydrogen bonds would be sufficient to explain the observed ΔΔG° of 18.9 kJ mol^{-1}. The differences may arise from the loss of specific binding interactions of the free α-amino group of aa-tRNA, which becomes blocked upon transpeptidylation. This would be consistent with the fact that the affinity of N-acetylated Phe-tRNA^{Phe} for the A site, measured under different but roughly comparable conditions, is in the μM range[6,23,24], similar to that of fMetPhe-tRNA^{Phe}. Additionally, hydrogen bonding between C75 of A site bound aa-tRNA and G2553 of 23S rRNA[12] may be disfavored or disrupted upon transpeptidylation.

The acceleration of EF-G dependent translocation that is correlated with the destabilization of tRNA binding in the A site due to transpeptidylation may be attributed to 'ground state destabilization', that is, an increase in the energy level of the pre-translocation state that decreases the free energy of activation, $\Delta G^{\neq}$. As peptidyl-tRNA was translocated ~130 times faster than aa-tRNA, the free energies of activation of translocation differed by 12.5 kJ mol^{-1}. The fact that $\Delta\Delta G^{\neq}$ amounts to only about two-thirds of the free energy difference (ΔΔG°) may indicate that the transition states of translocation are different for aa-tRNA and peptidyl-tRNA.

The present results were obtained with fMetPhe-tRNA^{Phe}, the shortest peptidyl-tRNA. It is possible that the affinity of peptidyl-tRNA for the A site is influenced by the length of the peptidyl residue. However, because the nascent peptide has to be mobile in order to allow uninhibited movement through the exit channel of the ribosome, it seems unlikely that the interaction of the nascent peptide with the ribosome contributes appreciably to the stabilization of peptidyl-tRNA in the A site. One may expect, therefore, that the results obtained by comparing aa-tRNA with dipeptidyl-tRNA also apply to longer chain peptidyl-tRNAs.

Methods

Sample preparation. Ribosomes from *E. coli* MRE 600, initiation and elongation factors, mRNAs, and tRNAs were prepared as described[25]. Experiments were performed in buffer A (50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM Mg^{2+}, 1 mM DTT) at 37 °C, if not stated otherwise. To prepare 70S initiation complexes, ribosomes (0.5 μM) were programmed with a four-fold excess of MFT-mRNA (coding sequence Met-Phe-Thr) in the presence of a 1.5-fold excess of initiation factors IF1, IF2, IF3, and f[^{3}H]Met-tRNA^{fMet}, and 1 mM GTP for 30 min at 37 °C. The ternary complex EF-Tu–GTP–[^{14}C]Phe-tRNA^{Phe} was prepared by incubating 1.4 μM EF-Tu with 1 mM GTP, 3 mM phosphoenol pyruvate, 0.5 mg l^{-1} pyruvate kinase and 0.7 μM [^{14}C]Phe-tRNA^{Phe} for 15 min at 37 °C. The ternary complex (final concentration 0.23 μM) was added to the initiation complex (final concentration 0.33 μM). The amount of ribosome bound fMet-tRNA^{fMet} (> 95%) was determined by nitrocellulose filtration[25], and all bound Phe-tRNA^{Phe} entered peptide bond formation, as verified by HPLC analysis[1]. To prepare complexes with [^{14}C]Phe-tRNA^{Phe} in the A site, initiation complexes were prepared as above, except that [^{3}H]tRNA^{fMet} was used (four-fold excess over ribosomes). After adding initiation complex (final concentration 0.8 μM) to EF-Tu–GTP–[^{14}C]Phe-tRNA^{Phe} (1.2 μM), binding was allowed for

2 min at 37 °C. The ribosome complex was purified by gel filtration on Sephacryl S300. After purification, the occupancy of the P site with [^{3}H]tRNA^{fMet} and of the A site with [^{14}C]Phe-tRNA^{Phe} was close to 100%. To prevent dissociation of the complexes upon dilution to low Mg^{2+} concentrations, additional [^{3}H]tRNA^{fMet} and mRNA were added (see Figure legends). To maintain a constant aminoacylation level, 3 mM ATP, 2 μM [^{14}C]phenylalanine and a catalytic amount of partially purified phenylalanyl-tRNA synthetase, which was free of elongation factors, were added.

Determination of binding affinity. K_d values were determined from the concentrations of bound and free ribosomes and tRNA at equilibrium. Alternatively, K_d values were calculated from the rate constants, k_{-1} and k_1, as determined by numerical integration of dissociation time courses expressed in concentrations (Scientist for Windows, Micromath).

Translocation experiments. Translocation was induced by adding EF-G (1 μM) and GTP (1 mM) to pretranslocation complexes. The extent of translocation was measured by the formation of f[^{3}H]Met[^{14}C]Phe-puromycin (1 mM puromycin, 10 s at 37 °C; ref. 7). In experiments where translocation was monitored by subsequent formation of PhePhe-tRNA^{Phe}, an initiation complex programmed with MFF-mRNA (coding sequence Met-Phe-Phe) and containing tRNA^{fMet} in the P site (0.25 μM) was mixed with EF-Tu–GTP–[^{14}C]Phe-tRNA^{Phe} (0.63 μM) and EF-G (1 μM) with GTP (1 mM). Dipeptide formation was analyzed by HPLC[1].

Stopped-flow experiments. Rapid kinetics of translocation were measured by stopped-flow fluorecence (SX-18MV, Applied Photophysics), monitoring the fluorescence of proflavin attached to positions 16/17 of fMetPhe-tRNA^{Phe} or Phe-tRNA^{Phe} following rapid mixing of the ribosome complexes (0.1 μM after mixing) with EF-G–GTP (1.0 μM)[7]. Time courses were evaluated by single-exponential fitting.

Acknowledgments

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