Molecular Cell, Vol. 13, 191–200, January 30, 2004, Copyright ©2004 by Cell Press

Kinetic Determinants of High-Fidelity tRNA Discrimination on the Ribosome

Kirill B. Gromadski and Marina V. Rodnina*
Institute of Physical Biochemistry
University of Witten/Herdecke
D-58448 Witten
Germany

Summary

The ribosome selects aminoacyl-tRNA (aa-tRNA) matching to the mRNA codon from the bulk of nonmatching aa-tRNAs in two consecutive selection steps, initial selection and proofreading. Here we report the kinetic analysis of selection taking place under conditions where the overall selectivity was close to values observed in vivo and initial selection and proofreading contributed about equally. Comparison of the rate constants shows that the 350-fold difference in stabilities of cognate and near-cognate codon-anticodon complexes is not used for tRNA selection due to high rate of GTP hydrolysis in the cognate complex. tRNA selection at the initial selection step is entirely kinetically controlled and is due to much faster (650-fold) GTP hydrolysis of cognate compared to near-cognate substrate.

Introduction

The ribosome discriminates between correct and incorrect aminoacyl-tRNAs (aa-tRNAs) according to the match between anticodon and mRNA codon in the A site. The fidelity of discrimination is very high, with only one incorrect per 1,000–10,000 correct amino acids incorporated into protein. Kinetic and mechanistic studies showed that rejection of incorrect tRNAs occurs in two stages: initial selection of ternary complexes EF-Tu·GTP·aa-tRNA and proofreading of aa-tRNA, separated by irreversible hydrolysis of GTP (Rodnina and Wintermeyer, 2001; Ruusala et al., 1982; Thompson and Stone, 1977). The free energy of base-pairing alone is not sufficient to discriminate between the correct (cognate) and near-cognate tRNAs that may differ by as little as a single mismatch in the codon-anticodon duplex. The ribosome both controls the differences in the stabilities of the codon-anticodon complexes and specifically accelerates the rates of GTPase activation and accommodation of correct substrates, implicating duplex stability and induced fit as sources of selectivity (Pape et al., 1999, 2000; Rodnina and Wintermeyer, 2001).

The relative contributions of initial selection and proofreading to overall selection in vivo are not known. In vitro kinetic and biochemical experiments indicated that during proofreading alone, about 1 amino acid out of 100 near-cognate aa-tRNAs was incorporated into peptide (Dix et al., 1990; Pape et al., 1999; Ruusala et al., 1982; Thompson and Stone, 1977). This suggested that in order to achieve the high overall selectivity, the initial selection step must be about as precise as proofreading, i.e., not more than 1 out of 10–100 near-cognate ternary complexes should pass the initial selection screen. In fact, early estimations suggested that the efficiency of initial selection may indeed be that high (Bilgin and Ehrenberg, 1994; Bilgin et al., 1990), although it appeared to be strongly dependent on the experimental conditions, particularly the concentration of Mg^{2+} (Pape et al., 1999).

Measurements of the stability of codon-recognition complexes, i.e., ternary complex with nonhydrolyzable GTP analog bound to programmed ribosomes, indicated 100- to 1,000-fold different dissociation rate constants of cognate complexes compared to those with a single G-U mismatch at the first codon position (Karim and Thompson, 1986; Pape et al., 1999; Thompson and Dix, 1982). However, to provide the high speed of protein synthesis, all forward reactions leading to incorporation of a correct amino acid into a protein have to be fast. Therefore, the full discrimination potential of intrinsic stability differences cannot be used due to the high rate of the following GTPase step, and thus the accuracy of tRNA selection is reduced (Yarus, 1992). Indeed, there was practically no initial selection at 10 mM Mg^{2+}, despite the 100-fold difference in stability and a 10-fold difference in the rates of GTPase activation of cognate compared to near-cognate ternary complexes (Pape et al., 1999). In principle, high efficiency of initial selection can be achieved in two ways: (1) stability differences can be increased by selective stabilization of cognate, but not near-cognate, codon-anticodon complexes by the ribosome and/or (2) the rates of forward reactions can be modulated depending on the structure of the codon-anticodon complex (Ogle et al., 2002; Pape et al., 1999).

Crystal structures of 30S subunits with anticodon stem-loop (ASL) fragments of tRNA bound to codon triplets in the decoding site show that the codon-anticodon complex forms a network of interactions with 16S rRNA in the decoding site, where bases of 16S rRNA interact with the minor groove of the codon-anticodon complex in a fashion that is specific for Watson-Crick geometry, but sequence independent (Ogle et al., 2001). It seemed likely that these interactions stabilize the complexes of correct geometry more than those with mismatches, thereby enhancing intrinsically small differences in stabilities of codon-anticodon complexes (Ogle et al., 2002). However, kinetic and equilibrium measurements indicated that the ribosome stabilizes near-cognate codon-anticodon complexes with a G-U mismatch at the first codon position to a similar extent as a cognate fully matched one (Rodnina and Wintermeyer, 2001). Stability differences on the ribosome were estimated between 100-fold (Pape et al., 1999), 300-fold (Ogle et al., 2002), and 1000-fold (Thompson and Dix, 1982), which is hardly sufficient to explain the observed discrimination between the two tRNAs. Thus, the importance of preferential stabilization of the cognate codon-anticodon complexes by the ribosome for tRNA selection remained unclear.

*Correspondence: rodnina@uni-wh.de

On the other hand, the crystal structures indicated that the structural rearrangement in the decoding center leads to a global conformational change of the 30S subunit, mainly consisting in a movement of the shoulder and the head domain of the 30S subunit from an open to a closed conformation (Ogle et al., 2002). Those of the 30S rearrangements that take place on the 70S ribosome (Valle et al., 2003; Vila-Sanjurjo et al., 2003) may be related to the regulation of steps of A site binding following codon recognition that take place on the 50S subunit, such as GTPase activation of EF-Tu or proper positioning of aa-tRNA in the 50S A site (accommodation) (Pape et al., 1999). However, the functional relevance of the selective acceleration of GTP hydrolysis for tRNA discrimination also remained obscure, owing to the low efficiency of initial selection at the conditions used in earlier experiments (Pape et al., 1999)

In the present work we determined the respective contribution of the two recognition strategies, i.e., differential stabilization of the cognate substrates and modulation of the forward reactions, to tRNA selection on the ribosome, particularly during the initial selection step. For the present work we choose experimental conditions at which the overall fidelity of selection was high, due to high efficiency of both initial selection and proofreading steps. Elemental rate constants of the steps contributing to initial selection of ternary complex, EF-Tu·GTP·Phe-tRNA^{Phe} (anticodon 3′-AAG-5′), were determined on mRNA-programmed ribosomes with cognate (UUU) or near-cognate (CUC) codons in the A site.

Results

Initial Selection and Proofreading at Conditions of High Fidelity

First, we tested whether high fidelity of translation can be achieved in our experimental system and determined the relative contributions of initial selection and proofreading. Ribosomes were programmed with a 120 nt long mRNA containing a Shine-Dalgarno sequence and an AUG start codon followed by a CUC triplet coding for leucine. To achieve high accuracy of initial selection, a buffer system was chosen with low Mg^{2+} concentration (3.5 mM) and two polyamines, spermidine (0.5 mM) and putrescine (8 mM), mimicking conditions found in bacterial cells (Neidhardt, 1987). The high accuracy of aa-tRNA selection was due to low Mg^{2+}; addition of polyamines had only slight effects, while variations in all other components of the buffer influenced neither accuracy nor speed of translation (not shown). "Polymix" buffer (Ehrenberg et al., 1990), which has the same Mg^{2+} and polyamines, but with phosphate buffer, was not used because of low buffering capacity and solubility problems caused by simultaneous presence of phosphate, NH_4^+, and Mg^{2+} ions.

To measure overall selectivity, two competing ternary complexes, with Leu-tRNA^{Leu} specific for the CUC codon as cognate and Phe-tRNA^{Phe} as near-cognate aa-tRNAs, were added in large excess over initiated ribosomes, and the formation of correct and incorrect dipeptides, fMetLeu and fMetPhe, respectively, was determined. At the present conditions, referred to as high-fidelity conditions in the following, the selectivity against near-cognate Phe incorporation was about 450, i.e., only 1 out of 450 dipeptides formed was incorrect fMetPhe (Figure 1). At low-accuracy conditions (20 mM Mg^{2+}, no polyamines), the efficiency of selection was strongly reduced, resulting in a significant incorporation of incorrect Phe into dipeptides (1 out of 20). In comparison, the selectivity of poly(U)-programmed ribosomes against near-cognate Leu in a buffer containing 10 mM Mg^{2+} and no polyamines was about 70 (Pape et al., 1999).

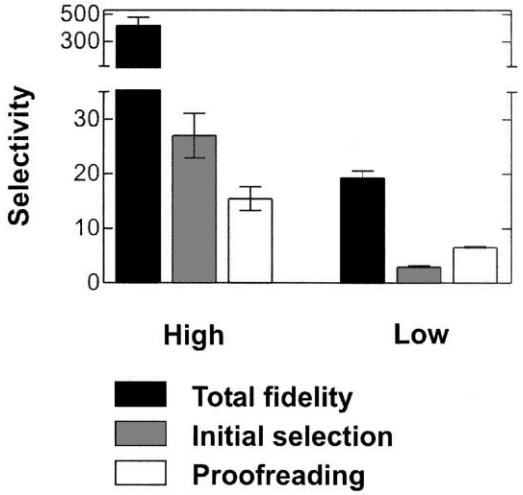

Figure 1. Selectivity at High- and Low-Fidelity Buffer Conditions

Contribution of initial selection and proofreading to the overall selectivity at conditions of high (buffer containing 3.5 mM Mg^{2+}, 0.5 mM spermidine, and 8 mM putrescine) and low (20 mM Mg^{2+}) fidelity.

There is no experimental approach to measure the efficiency of initial selection directly, whereas the efficiency of proofreading alone can be readily determined. The contribution of initial selection can be calculated from the values of overall selectivity and proofreading, because overall selectivity is given by the product of initial selection and proofreading (Fersht, 1998). To measure the efficiency of proofreading, ribosomes programmed with CUC in the A site were mixed with near-cognate ternary complex, EF-Tu·GTP·Phe-tRNA^{Phe}, and the formation of incorrect fMetPhe dipeptide was monitored. In the absence of cognate competitor, all near-cognate ternary complex entered the ribosome and eventually hydrolyzed GTP, thereby passing the initial selection screen, and Phe-tRNA^{Phe} could be rejected in the proofreading phase only. To ensure that only one selection round was measured, the experiments were done in excess of ribosomes over ternary complexes. In parallel, Phe incorporation was measured on the cognate UUU codon. From the incorporation of Phe into dipeptides on near-cognate and cognate codons, the selectivity of proofreading can be calculated (Fersht, 1998). When the efficiency of proofreading was determined by a different method, i.e., from the ratio of GTP hydrolyzed to the amount of dipeptide formed (Ehrenberg et al., 1990), the same results were obtained (not shown).

At high-fidelity conditions, similar efficiencies of initial selection and proofreading were found, contributing selectivities of about 30 and 15, respectively, to the overall

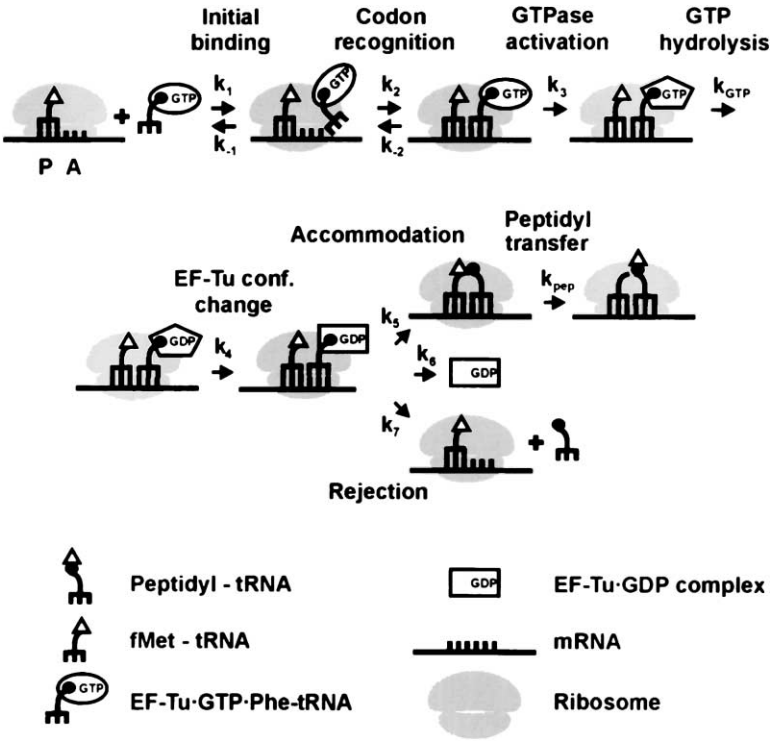

Figure 2. Kinetic Mechanism of EF-Tu-Dependent aa-tRNA Binding to the A Site

Kinetically resolved steps are indicated by rate constants k_1-k_7, k_{-1}, k_{-2}, and the two chemical steps that are rate-limited by the preceding step are designated k_{GTP} and k_{pep}. EF-Tu is depicted in different conformations in the GTP- and GDP-bound form and in the activated GTPase state.

selectivity of approximately 450 (Figure 1). The efficiencies of both selection steps were decreased at low-fidelity conditions, to about 3 (initial selection) and 6.5 (proofreading). In earlier experiments using Leu-tRNA^{Leu} as a near-cognate aa-tRNA on a UUU codon, the respective selectivities were 1.1 and 60 (Pape et al., 1999). The differences between previous and present results obtained at low-fidelity conditions, particularly concerning the efficiency of proofreading, can be largely attributed to different properties of the two tRNAs (unpublished data), as in the present experiments Phe-tRNA^{Phe} was used as near-cognate substrate on CUC, rather than Leu-tRNA^{Leu} on UUU as in the previous experiments.

Experimental Strategy to Measure Elemental Rate Constants

The kinetic analysis was carried out using the kinetic model of A site binding determined previously (Figure 2) (Rodnina and Wintermeyer, 2001). In the first step, the ternary complex forms a labile initial binding complex with the ribosome (rate constants of the forward and backward reaction are defined as k_1 and k_{-1}, respectively). Subsequent codon recognition (k_2, k_{-2}) triggers GTPase activation of EF-Tu (k_3), which is rate-limiting for GTP hydrolysis (k_{GTP}). Release of inorganic phosphate induces the conformational transition of EF-Tu from the GTP to the GDP form (k_4), whereby the factor loses the affinity for aa-tRNA and dissociates from the ribosome (k_6). The aa-tRNA released from EF-Tu is free to accommodate in the 50S A site (k_5) where it takes part in rapid peptide bound formation (k_{pep}). Alternatively, aa-tRNA may be rejected from the ribosome (k_7).

The rate constants of the elemental steps of Figure 2 were determined using ternary complex containing Phe-tRNA^{Phe} and initiated ribosomes with UUU (cognate) or CUC (near-cognate) codons in the A site. A fluorescent tRNA derivative, Phe-tRNA^{Phe} (Prf16/17), was used to monitor the steps of initial binding and codon recognition and determine the rate constants k_1, k_{-1}, and k_2 from the concentration dependencies of the respective apparent rate constants (Pape et al., 1999). Whenever possible, k_2 values were verified by experiments monitoring the fluorescence of wybutine at position 37 of tRNA^{Phe} (Rodnina et al., 1995). To obtain the rate constant of dissociation of the codon-recognition complex, k_{-2}, mutant EF-Tu(H84A) was used, which was virtually inactive in GTP hydrolysis, whereas the rate constants of all steps preceding GTP hydrolysis were not affected (Daviter et al., 2003). The rate constant of GTPase activation, k_3, was measured using a fluorescent GTP derivative, mant-GTP (Rodnina et al., 1995). Fluorescence experiments were carried out by the stopped-flow technique. Rates of GTP hydrolysis and peptide bond formation were measured by quench flow using [γ-^{32}P]GTP or aa-tRNA charged with ^{3}H- or ^{14}C-labeled amino acids. Elemental rate constants of the following steps, k_4-k_7, do not contribute to initial selection. Where possible, values for these rate constants were also calculated (Pape et al., 1999; Experimental Procedures).

Initial Binding (k_1, k_{-1})

Several steps of A site binding, including initial binding and codon recognition, lead to conformational changes in tRNA, as detected by fluorescence changes of tRNA^{Phe}(Prf16/17) (Pape et al., 1998; Rodnina et al., 1994, 1996). Initial binding of ternary complex to the ribosome was measured by monitoring the fluorescence

change of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) upon mixing with nonprogrammed ribosomes, which is equivalent to initial binding to programmed ribosomes, but technically more reliable (Pape et al., 1998; Rodnina et al., 1996). Values of k_{app} were determined from time courses measured at different ribosome concentrations by exponential fitting and plotted against the ribosome concentration (Figure 3A). From the slope of the linear plot, the bimolecular association rate constant was calculated, $k_1 = (140 \pm 20)\ \mu M^{-1}s^{-1}$. From the intercept with the Y-axis, the dissociation rate constant of the initial binding complex, $k_{-1} = (85 \pm 25)\ s^{-1}$, was determined.

Codon Recognition (k_2)

Upon mixing of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) with UUU-programmed ribosomes, a fluorescence increase was observed, followed by a fluorescence decrease (not shown). Apparent rate constants determined from the fluorescence increase changed with ribosome concentration in a hyperbolic fashion (Figure 3B), consistent with a rearrangement step following a bimolecular association reaction, i.e., the codon-recognition step. The initial binding step had little contribution into the observed fluorescence increase, because codon recognition resulted in a much larger signal change. To confirm the assignment of this step, experiments were performed monitoring the fluorescence of wybutine, a natural fluorophore 3′ to the anticodon of yeast tRNA^{Phe}, which specifically reports the formation of the codon-anticodon complex (Paulsen et al., 1982; Rodnina et al., 1995). This yielded apparent rate constants that were very similar to those measured with proflavin (not shown), indicating that the step in question was in fact codon recognition. From the hyperbolic fit of the concentration dependence of k_{app}, the value of k_2 was estimated using the concept of net rate constants (Cleland, 1975) (see Supplemental Data at http://www.molecule.org/cgi/content/full/13/2/191/DC1). Given that $k_{-2} << k_2$, the saturation level of the hyperbolic fit yielded $k_2 = (190 \pm 20)\ s^{-1}$. The decrease of proflavin fluorescence was shown to be due to a step after GTP hydrolysis, presumably tRNA accommodation in the A site (Pape et al., 1998). At saturation, the apparent rate constant of that step was 25 s^{-1} (not shown).

Ternary complex binding to ribosomes programmed with the near-cognate codon, CUC, resulted in fluorescence changes of tRNA^{Phe}(Prf16/17) that were qualitatively similar to those on the cognate codon, with a fluorescence increase followed by a decrease (not shown). By analogy to the cognate case and based on the previous assignment (Pape et al., 1999), the apparent rate constant of the first step was attributed to codon recognition. From the titration shown in Figure 3B, k_{app} at saturating ribosome concentration was $270 \pm 30\ s^{-1}$. In the near-cognate case, $k_{-2} = 80\ s^{-1}$, which is not negligible, but the rate constant of the following reaction, k_3, is much smaller than both k_2 and k_{-2}. In this case, the saturation of the hyperbolic concentration dependence is given by $k_2 + k_{-2}$ (Fersht, 1998), hence $k_2 = 190 \pm 30\ s^{-1}$. Similar values of k_2 and k_{-2} were obtained in a different experimental setup (described in Supplemental Data). Thus, the presence of a mismatch in the codon-anticodon duplex did not affect the rate constant of codon recognition. The rate constants are summarized in Figure 4. The last step observed upon binding of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) to CUC-programmed ribosomes, the fluorescence decrease was slow, 0.6 s^{-1}, and rate-limited by GTP hydrolysis (see below).

Dissociation of the Codon-Recognition Complex (k_{-2})

The rate constant of ternary complex dissociation from the ribosome prior to GTP hydrolysis (codon-recognition complex), k_{-2}, was measured by chase experiments. The dissociation from the ribosome of ternary complex containing GTPase-deficient EF-Tu(H84A), GTP, and fluorescent Phe-tRNA^{Phe}(Prf16/17) was initiated by adding an excess of nonfluorescent ternary complex, and the fluorescence decrease was monitored with time (Figure 3C). Dissociation rate constants were $0.23 \pm 0.05\ s^{-1}$ (UUU) and $80 \pm 10\ s^{-1}$ (CUC). (The latter value was verified in additional experiments described in Supplemental Data.) Thus, at high-fidelity conditions, the kinetic stabilities of cognate and near-cognate codon-recognition complexes differed by a factor of 350, which is only 4 times larger than that determined at conditions of lower fidelity (Pape et al., 1999). Thus, increased stability differences cannot explain the 30-fold improvement of initial selectivity that resulted from switching from low-fidelity to high-fidelity buffer conditions.

From the rate constants of Figure 4, the affinity differences between cognate and near-cognate ternary complexes, $\Delta\Delta G^\circ$, can be calculated. Because k_1, k_{-1}, and k_2 were the same for cognate and near-cognate ternary complexes (Figure 4), the only rate constant that contributes to the different affinity is k_{-2}; hence, $\Delta\Delta G^\circ = RT\ln(350) = 3.4$ kcal/mol. However, as outlined below, this difference would be effective only if there were no rapid GTP hydrolysis, which is not the case.

GTPase Activation and GTP Hydrolysis (k_3, k_{GTP})

Rates of GTPase activation and GTP hydrolysis were measured by stopped-flow monitoring mant-GTP fluorescence and by quench-flow with [γ-^{32}P]GTP, respectively. Essentially all GTP in the ternary complex was hydrolyzed upon binding to ribosomes, both UUU and CUC-programmed, but the rates were dramatically different (Figure 3D). To obtain rate constants of GTPase activation and GTP hydrolysis, ribosome titrations were carried out. On UUU-programmed ribosomes, k_{app} of GTP hydrolysis increased with ribosome concentration and saturated at $110 \pm 25\ s^{-1}$, whereas on CUC the rate of GTP hydrolysis was $0.4 \pm 0.1\ s^{-1}$ throughout the titration (Figure 3E). Apparent rate constants of GTPase activation measured by mant fluorescence were identical to those of GTP hydrolysis, both on UUU and CUC codons (not shown), indicating that GTPase activation is rate-limiting for the following step, GTP hydrolysis, in agreement with previous results (Pape et al., 1998, 1999). Therefore, k_{app} values measured by GTP hydrolysis represent rate constants of GTPase activation, k_3, provided there is no rate-limiting step preceding GTPase activation. In fact, the rate constant of CUC recognition, 190 s^{-1}, was much higher than the rate of GTP hydrolysis, 0.4 s^{-1}, hence $k_3 = 0.4\ s^{-1}$ on CUC. However, the rate

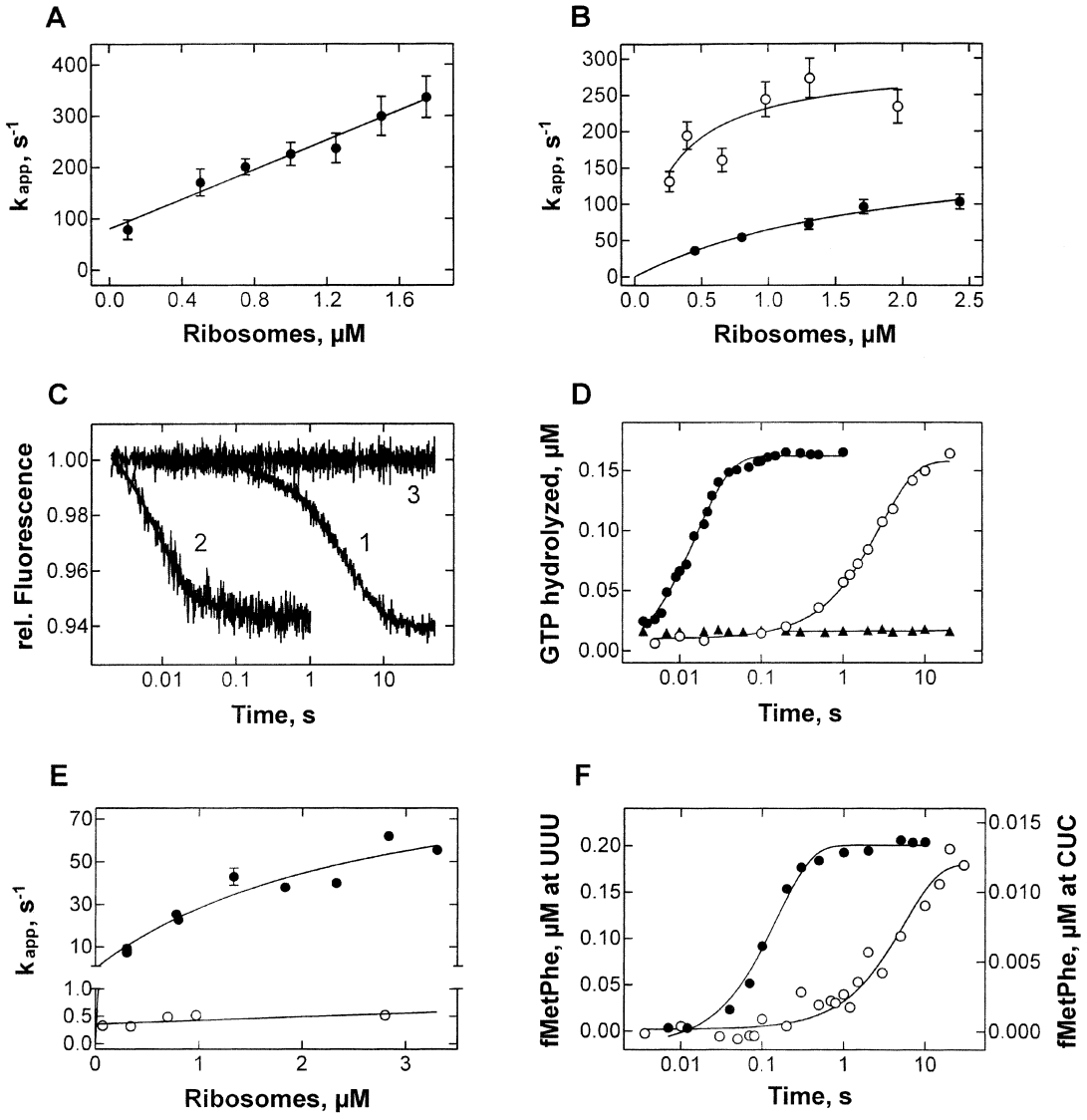

Figure 3. Kinetic Analysis of A Site Binding

(A) Determination of k_1 and k_{-1} of initial binding. k_{app} values were obtained by exponential fitting of time courses of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) binding to nonprogrammed ribosomes present at increasing concentration measured by stopped-flow. The linear concentration dependence of k_{app} gives $k_1 = 140 \pm 20$ $\mu M^{-1}s^{-1}$ (slope) and $k_{-1} = 85 \pm 25$ s^{-1} (ordinate intercept).

(B) Codon recognition (k_2). Concentration dependence of k_{app} of codon recognition measured by fluorescence changes of proflavin on mRNA-programmed ribosomes with fMet-tRNA^{fMet} in the P site and a UUU (closed circles) or CUC (open circles) codon in the A site.

(C) Dissociation of the codon-recognition complex (k_{-2}). Cognate and near-cognate codon-recognition complexes were prepared by mixing EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}(Prf16/17) and mRNA-programmed ribosomes with fMet-tRNA^{fMet} in the P site and a UUU or CUC codon in the A site (0.3 μM each after mixing). Dissociation was initiated by rapidly mixing the codon-recognition complex with a 7-fold excess of nonfluorescent EF-Tu(H84A)·GTP·Phe-tRNA^{Phe} in the stopped-flow apparatus. Exponential fitting yielded $k_{-2} = 0.23 \pm 0.05$ s^{-1} (UUU) (1) and 80 ± 15 s^{-1} (CUC) (2); no dissociation was observed upon addition of buffer (3).

(D) GTP hydrolysis. Time courses of GTP hydrolysis in EF-Tu·[^{32}P]GTP·Phe-tRNA^{Phe} on the ribosomes with a cognate (closed circles) or near-cognate (open circles) codon, or in the absence of ribosomes (triangles). Concentrations were 0.2 μM ternary complex and 2.8 μM initiated ribosomes. Exponential fitting gave $k_{app} = 62 \pm 3$ s^{-1} for the UUU codon and 0.35 ± 0.02 s^{-1} for the CUC codon.

(E) GTPase activation (k_3). Concentration dependence of k_{app} of GTP hydrolysis on the UUU (closed circles) or CUC (open circles) codons.

(F) Peptide bond formation. Time courses of fMetPhe formation were measured on codons UUU (closed circles) or CUC (open circles) with 0.2 μM ternary complex and 1 μM ribosomes. Single exponential fitting yielded $k_{app} = 6.6 \pm 0.4$ s^{-1} (UUU) and $k_{app} = 0.19 \pm 0.04$ s^{-1} (CUC). Note different ordinates for cognate and near-cognate codons.

constant of UUU codon recognition, 190 s^{-1}, was comparable to k_{app} of GTP hydrolysis at ribosome saturation, 110 s^{-1}, suggesting that codon recognition is partially rate-limiting in the cognate case. A value of $k_3 = 260 \pm 60$ was calculated (see Supplemental Data at http://www.molecule.org/cgi/content/full/13/2/191/DC1) from k_2 and k_{app} of GTP hydrolysis using the concept of net rate constants (Cleland, 1975) (Figure 4).

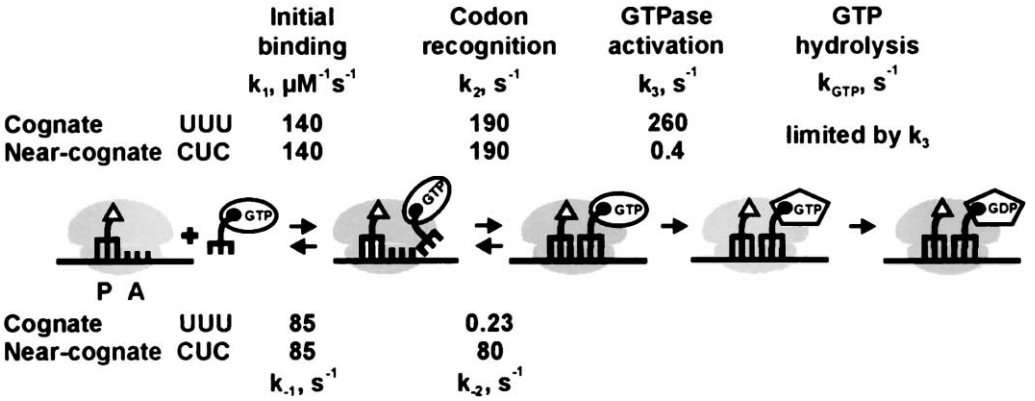

Figure 4. Kinetic Determinants of Initial Selection of aa-tRNA on the Ribosome Measured on Natural mRNA at Conditions of High Fidelity

The rate constants measured on poly(U) at 10 mM Mg^{2+} were the following: $k_1 = 110\ \mu M^{-1}s^{-1}$, $k_{-1} = 25\ s^{-1}$, $k_2 = 100\ s^{-1}$ (same constants for cognate and near-cognate tRNA), $k_{-2} = 0.2\ s^{-1}$ (cognate) or $17\ s^{-1}$ (near-cognate), $k_3 = 500\ s^{-1}$ (cognate) or $50\ s^{-1}$ (near-cognate) (Pape et al., 1999).

Peptide Bond Formation and Proofreading

The kinetic analysis was completed by measuring rates of peptide bond formation on ribosomes programmed with UUU or CUC (Figure 3F). On the cognate UUU codon, Phe was incorporated quantitatively at a rate of $7 \pm 1\ s^{-1}$. As there was no measurable rejection of cognate aa-tRNA, k_7 was below the detection limit, $<0.3\ s^{-1}$ (estimated as in Pape et al. [1999]), and the observed rate of peptide bond formation represented the rate constant of accommodation, $k_5 = 7\ s^{-1}$. In contrast, on the near-cognate CUC codon, only about 6% Phe from initially bound ternary complex was found in dipeptide, indicating a selectivity of proofreading of about 15; k_{app} of dipeptide formation was $0.3\ s^{-1}$ (Figure 3F). Previously, rate constants of tRNA accommodation and rejection, k_5 and k_7, could be calculated from the extent of amino acid incorporation into peptide, given by $k_5/(k_5+k_7)$, and the apparent rate constant of peptide bond formation, which is equal to $k_5 + k_7$. However, in the present near-cognate case, the rate of peptide bond formation, $0.3\ s^{-1}$, was not significantly different from that of GTP hydrolysis, $0.4\ s^{-1}$, indicating that GTP hydrolysis was rate-limiting for all following steps. Therefore, the true value for $k_5 + k_7$, and, with that, individual values of k_5 and k_7, could not be determined for the near-cognate case at the present high-fidelity conditions.

Kinetic Determinants of aa-tRNA Discrimination

On the basis of the rate constants of Figure 4, the efficiency of discrimination can be calculated for conditions at which rapid GTPase activation and GTP hydrolysis do not allow for the equilibration of the codon recognition step. In this case, the efficiency of initial selection is given by the ratio of k_{cat}/K_M values of the GTPase reaction for cognate and near-cognate substrates. For the cognate ternary complex, codon recognition, k_2, is rate-limiting for all subsequent forward steps, hence $k_{cat} = k_2 = 190\ s^{-1}$. The K_M value reflects the distribution of the substrate (ternary complex) between all populated states of the ribosome prior to GTP hydrolysis, i.e., initial binding complex, codon recognition complex, and GTPase-activated state. As both k_3 and k_{GTP} are much larger than k_2, $K_M = (k_2 + k_{-1})/k_1 = 2.0\ \mu M$ (Figure 4; Supplemental Data), consistent with the K_M value for GTP hydrolysis obtained experimentally (Figure 3E). From these relationships and the values of the rate constants (Figure 4), k_{cat}/K_M for the cognate ternary complex is calculated to $100\ \mu M^{-1}s^{-1}$, close to the value determined previously for cognate ternary complex at conditions of low accuracy, $88\ \mu M^{-1}s^{-1}$ (Pape et al., 1999). For the near-cognate ternary complex, the calculation is different, because now the rate-limiting step is GTPase activation, k_3, hence $k_{cat} = k_3 = 0.4\ s^{-1}$ (Table 1). The expression for K_M includes the rate constants of all steps preceding GTPase activation; however, because k_3 is small, K_M can be estimated as $k_{-1}/k_1 \cdot k_{-2}/k_2 = 0.25\ \mu M$, leading to $k_{cat}/K_M = 1.6\ \mu M^{-1}s^{-1}$ for the near-cognate ternary complex (Supplemental Data; Pape et al., 1999). This low value of k_{cat}/K_M is to be compared to the value of $80\ \mu M^{-1}s^{-1}$ determined previously for a near-cognate ternary complex (Leu on UUU codon) under low-fidelity conditions (Pape et al., 1999).

Since K_M for the near-cognate ternary complex is lower than that for the cognate one, i.e., the apparent affinity is higher for the near-cognate substrate, the ribosome cannot discriminate ternary complexes on the basis of apparent affinities. However, from the ratio of k_{cat}/K_M values for cognate and near-cognate ternary complexes, the efficiency of initial selection was 60, which is not far from the measured value of 30 (Figure 1). This indicates that the selectivity due to different stabilities is ineffective because of the high rate of forward reactions, and that the major source of selectivity is the difference in the rates of GTP hydrolysis in the cognate and near-cognate substrates.

The 2-fold difference between calculated (60) and measured (30) initial selection efficiencies is attributed to slightly different kinetic properties of Phe-$tRNA^{Phe}$, for which the kinetic mechanism was solved, and Leu-$tRNA^{Leu}$ used as cognate substrate in the experiments of Figure 1 (data not shown). In addition to initial selection, 94% of near-cognate aa-tRNAs are rejected at the proofreading stage (Table 1), yielding efficient overall discrimination against near-cognate aa-tRNA, while cognate aa-tRNA is accommodated rapidly.

Table 1. Parameters of aa-tRNA Discrimination

	Initial Selection			Proofreading
Codon	k_{cat}	K_M	k_{cat}/K_M	$k_5/(k_5+k_7)$
UUU	190 ± 20	2.0 ± 0.6	100 ± 20	1.0 ± 0.1
CUC	0.4 ± 0.1	0.25 ± 0.1	1.6 ± 0.5	0.06 ± 0.02
			$F_{initial\ selection}$ = 60 ± 20	$F_{proofreading}$ = 15 ± 5

Fidelity of initial selection, $F_{initial\ selection}$, was calculated from $(k_{cat}/K_M)_{cognate}/(k_{cat}/K_M)_{near\text{-}cognate}$ for GTP hydrolysis assuming equal concentrations of cognate and near-cognate substrates. Fidelity of proofreading, $F_{proofreading}$, was calculated from the ratio of product formation with cognate versus near-cognate substrate as described (Fersht, 1998).

Discussion

Initial Selection and Proofreading under High-Fidelity Conditions

The conditions used in the present paper allow for high-fidelity discrimination against codon-anticodon complexes with a single A-C mismatch in the first codon position. The measured incorporation of 1 incorrect per 450 correct amino acids, which corresponds to an error frequency of $2.2{\cdot}10^{-3}$, is well within the range of error frequencies measured in vivo, from $6{\cdot}10^{-4}$ (Edelmann and Gallant, 1977) to $5{\cdot}10^{-3}$ on internal mRNA codons (Precup et al., 1989), the average value being around $3{\cdot}10^{-3}$ (Bouadloun et al., 1983; Khazaie et al., 1984; Parker and Holtz, 1984; Precup et al., 1989; Rice et al., 1984). At high-fidelity conditions, the respective contributions of initial selection and proofreading to discrimination were comparable, 30- and 15-fold. Similar conclusions, i.e., that efficient tRNA discrimination requires two selection steps contributing to a similar extent, were reached previously on the basis of results obtained in a poly(U) translation system and poly(U)-dependent A site binding assays, both at high- and low- fidelity conditions (Dix et al., 1990; Kurland et al., 1990). Thus, two steps of tRNA selection, initial selection prior to and proofreading after GTP hydrolysis, are required for efficient tRNA discrimination under any experimental conditions, and it is therefore very likely that the two steps operate also in vivo.

Kinetic versus Thermodynamic Discrimination

The important question is why the differences in the thermodynamic stabilities between the cognate and near-cognate aa-tRNA should not be sufficient for high-fidelity selection. In each selection step, initial selection and proofreading, not more than 1 incorrect in 10–100 correct amino acids is allowed, in order to achieve the total selectivity of 1,000–10,000. At equilibrium, this would require a ΔG° difference of about 1.3–2.7 kcal/mol in the thermodynamic stabilities of cognate and near-cognate aa-tRNAs. The present data show that the energetic penalty for a first-position C-A mismatch, $\Delta\Delta G^\circ$ = 3.4 kcal/mol, would be sufficient to discriminate between the correct and incorrect substrate, provided the binding step reached equilibrium. However, A site binding is a nonequilibrium process that is driven by the rapid, irreversible forward reactions of GTP hydrolysis and peptide bond formation. Therefore, the stability differences cannot be used for discrimination, and K_M values, rather than K_d, of cognate and near-cognate substrates have to be compared (Fersht, 1998). Due to the large differences in GTPase rates, K_M values for cognate substrates are even larger than for near-cognate, hence there is no discrimination on the basis of K_M. Rather, discrimination is based exclusively on the large differences in forward reaction rates, which are high with correct and low with incorrect substrates, resulting in differences in k_{cat}/K_M values between cognate and near-cognate species that are sufficient to explain the observed levels of discrimination.

The large stability (k_{-2}) differences between cognate and near-cognate ternary complexes do not enter discrimination, due to very rapid GTP hydrolysis in the cognate ternary complex, which is much faster (k_3 = 260 s^{-1}) than one might expect to be necessary to sustain an amino acid incorporation rate of about 10 s^{-1}. This raises the question why the rate constant of GTP hydrolysis is not tuned down in order to allow for partial use of k_{-2} differences, thereby increasing selectivity. The likely reason is that in the cell one particular cognate ternary complex competes with a large excess of near-cognate and noncognate ternary complexes for A site binding, which results in a significant decrease of the actual rate of GTP hydrolysis in the cognate ternary complex. Thus, in order to maintain an actual rate of GTP hydrolysis of >10 s^{-1}, the intrinsic rate constant of GTP hydrolysis must be much higher than that in order to compensate for the roughly 20-fold excess of competing noncognate ternary complexes. Thus, the attainable fidelity of aa-tRNA selection is compromised in order to achieve rapid translation.

Differences in aa-tRNA stabilities in the proofreading phase cannot be determined under high-fidelity conditions, because GTP hydrolysis is rate-limiting under these conditions and values of k_5 and k_7 cannot be determined separately. However, because the efficiency of proofreading varies only within a factor of three at quite different ionic conditions (Figure 1; (Pape et al., 1999; Thompson et al., 1981)), $\Delta\Delta G^\circ$ in proofreading can be estimated using previous data obtained at low-fidelity conditions (Pape et al., 1999). During proofreading, cognate aa-tRNA was bound to the A site with at least 20 times higher stability than near-cognate, hence $\Delta\Delta G^\circ$>1.8 kcal/mol. Interestingly, ternary complex dissociation from the A site during initial selection is much faster than that of aa-tRNA during proofreading where aa-tRNA is no longer bound to EF-Tu. It is possible that the difference in dissociation rates results from the different orientation of tRNA on the ribosome in the complex with EF-Tu (Stark et al., 2002; Valle et al., 2002) and during accommodation (Valle et al., 2003; Yusupov et al., 2001).

The comparison of $\Delta\Delta G^{\circ}$ of ternary complexes during initial selection obtained previously at 10 mM Mg^{2+} (Pape et al., 1999; Thompson and Dix, 1982) suggested an energetic penalty of 2.7–4.0 kcal/mol for a G-U mismatch at the first codon position. A similar value, 2.7 kcal/mol, was obtained for tRNA-tRNA duplexes with the same mismatch in the anticodon-anticodon complex (Grosjean et al., 1978), assuming similar association rate constants (Stage-Zimmermann and Uhlenbeck, 1998). Again, if A site binding were an equilibrium process, this difference would be sufficient for efficient tRNA discrimination, provided there are two stages of selection separated by GTP hydrolysis. However, rapid GTP hydrolysis does not allow for equilibration of the codon recognition step.

Our earlier results obtained on poly(U) and at 10 mM Mg^{2+} (Pape et al., 1999) indicated that, despite 100-fold faster dissociation from the ribosome and 10-fold slower GTP hydrolysis in the near-cognate compared to cognate codon-recognition complex, there was virtually no initial selection between Leu- and Phe-tRNA. The stability differences, $\Delta\Delta G^{\circ}$, were similar at high- and low-fidelity conditions, 3.4 and 2.7 kcal/mol, respectively. The main difference comes from GTP hydrolysis in the near-cognate complex, which was much slower at high-fidelity conditions, compared to low-fidelity conditions, or compared to the cognate complex, where GTP hydrolysis was rapid at all conditions.

Energy of Interactions at the Decoding Center

The crystal structure of the 30S subunit in the complex with a codon and a cognate ASL bound to the A site revealed extensive interactions between the codon-anticodon duplex and several residues of 16S rRNA (Ogle et al., 2001). Two adenines in helix 44 of 16S rRNA, A1493 and 1492, form A-minor interactions with the first and second positions, respectively, of the codon-anticodon complex. The second position is also contacted by G530 from helix 18 of 16S rRNA, which is positioned by binding to A1493. The third position is surrounded by several groups, including G1054 from helix 34, G518 from helix 18, and part of ribosomal protein S12. Most of these interactions are sensitive to the correct geometry of the codon-anticodon complex and cannot form on mismatched base pairs, particularly in the first and second codon positions.

A-minor interactions are a common motif in RNA structure. Thermodynamic measurements using the *Tetrahymena* group I self-splicing intron showed that substituting the G-C receptor base pair, which is monitored by A-minor type I interactions, with U-G, C-A, or G-A mismatches led to large energetic penalties, 10.2, 8.3, and 2.8 kcal/mol, respectively, whereas replacements of the G-C pair with other Watson-Crick base pairs had no effect (Battle and Doudna, 2002). These interactions should be comparable to those made by A1493 of 16S rRNA with the first position of the codon-anticodon complex. However, the energetic penalties due to first-position mismatches on the ribosome were found to be much smaller, 2.7 kcal/mol for U-G (Pape et al., 1999) and 3.4 kcal/mol for C-A (this paper); in one case the penalty was comparable, 3.0 kcal/mol for G-A (T. Daviter and M.V.R., unpublished data) to the results obtained with *Tetrahymena* intron. This suggests that on the ribosome the full selection potential of the A-minor interactions is not used, probably because of efficient binding at neighboring positions or energetic losses due to induced conformational changes. On the other hand, the rates of forward reactions, particularly that of GTPase activation, are strongly impaired by those mismatches (Pape et al., 1999; this paper; T. Daviter and M.V.R., unpublished data). Thus, the energy of the interactions at the decoding center of ribosomal residues with the codon-anticodon duplex is used mainly for modulating the rates of forward reactions by induced conformational changes, and less for the preferential stabilization of the correct codon-anticodon complexes, thereby contributing to selectivity for the cognate aa-tRNA.

Induced Fit and Substrate Discrimination on the Ribosome

The theory of induced fit postulates that "(a) a precise orientation of catalytic groups is required for enzyme action; (b) the substrate may cause an appreciable change in the three-dimensional structure of the active site; and (c) the changes in protein structure caused by a substrate will bring the catalytic groups into the proper orientation for reaction, whereas a nonsubstrate will not" (Koshland, 1958). Evidence for substrate-induced conformational changes was found for many enzymes, and the ribosome is one more example of an enzyme using induced fit. In fact, any ribosome action, including GTPase activation of EF-Tu or peptide bond synthesis requires the precise alignment of the catalytic groups in the respective active sites. The ribosome activates GTP hydrolysis in EF-Tu, probably by inducing a conformational change in the factor resulting in the precise orientation of the catalytic groups in EF-Tu (Daviter et al., 2003; Rodnina et al., 2000). Likewise, the precise alignment of the P and A site tRNAs in the peptidyl transferase center accounts for the major part of the acceleration of peptide bond formation on the ribosome (Beringer et al., 2003; Katunin et al., 2002).

Also the second postulate of the induced-fit theory holds for the ribosome. As suggested by the 30S·ASL crystal structures, binding of the substrate, tRNA, induces changes, both locally within the decoding center and globally by switching the overall conformation of the 30S subunit (Ogle et al., 2001, 2002). Furthermore, when ternary complex binds to the ribosome, the positions of several intersubunit bridges change, compared to ribosomes with empty A site or with tRNA accommodated in the A site (Stark et al., 2002; Valle et al., 2002; Yusupov et al., 2001). Thus, it is possible that the changes brought about by tRNA and mRNA binding within the 30S decoding site are translated into global structural changes that extend into the 50S subunit and affect catalytic centers there.

Changes of ribosome structure that are caused by the correct substrate do not take place, or are structurally different, with an incorrect substrate (Ogle et al., 2001, 2002). These differences probably reflect the observation from kinetics that rate constants of GTPase activation and tRNA accommodation in the A site are much faster for the correct than for the incorrect substrates (Pape et al., 1999; Rodnina and Wintermeyer, 2001; this

paper). This indicates that binding of the correct substrate brings the catalytic groups into the proper orientation for reaction, whereas an incorrect substrate does not. This also implies that the ribosome is capable of preferential stabilizing the complexes with the correct substrate in both ground state and transition state, whereas incorrect substrates are poorly or not at all stabilized. In this respect the ribosome, like other induced-fit enzymes and unlike simple rigid Michaelis-Menten enzymes, increases the selection potential by checking the correct structure of intermediates formed on the reaction pathway by an induced fit mechanism.

Experimental Procedures

Buffer and Reagents

All experiments were performed in buffer A (50 mM Tris-HCl [pH 7.5], 70 mM NH_4Cl, 30 mM KCl, 3.5 mM $MgCl_2$, 0.5 mM spermidine, 8 mM putrescine, and 2 mM DTT) at 20°C. Chemicals were from Roche Molecular Biochemicals or Merck. Mant-GTP was purchased from JenaBioScience. Radioactive compounds were from ICN.

Ribosomes, EF-Tu, and tRNAs

Ribosomes from *E. coli* MRE600, wild-type EF-Tu, and tRNAs were prepared as described (Pape et al., 1998; Rodnina et al., 1994). EF-Tu(H84A) (Daviter et al., 2003) was expressed and purified using an established protocol (Gromadski et al., 2002). For the kinetic experiments, ternary complex, EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe}(Prf 16/17), was prepared by incubating EF-Tu (wt or H84A) (30 μM), EF-Ts (0.02 μM), GTP (1 mM), phosphoenol pyruvate (3 mM), pyruvate kinase (0.1 mg/ml), tRNA^{Phe}(Prf16/17) (10 μM), ATP (3 mM), ^{14}C-phenylalanine (30 μM), and yeast S100 fraction as a source of Phe-tRNA synthetase (2% v/v) and purified by gel filtration as described (Pape et al., 1998; Rodnina et al., 1994). Ternary complexes containing mant-GTP or [γ-^{32}P]GTP were prepared in the same way, except that purified [^{14}C]Phe-tRNA^{Phe} (10 μM) was used and the concentration of nucleotides was 300 μM or 30 μM of mant-GTP or [γ-^{32}P]GTP, respectively.

70S initiation complex was prepared in buffer B (50 mM Tris-HCl [pH 7.5], 70 mM NH_4Cl, 30 mM KCl, and 7 mM $MgCl_2$) by incubating ribosomes (1 μM) with f[^{3}H]Met-tRNA^{fMet} (1.5 μM) and mRNA (4 μM) in the presence of initiation factors IF1, IF2, and IF3 (1.5 μM each) for 70 min at 37°C. mRNAs were derivatives of the 122 nt mRNA m022 (Calogero et al., 1988; Rodnina and Wintermeyer, 1995) that included a Shine-Dalgarno sequence and an AUG start codon, and UUU or CUC as the second codon. Ribosome complexes were purified and concentrated by centrifugation through 1.1 M sucrose cushions in buffer A at 259,000 × g for 2 hr on a Sorvall M120GX ultracentrifuge. Binding of tRNAs to the A and P sites was quantified by nitrocellulose filtration.

Selectivity Measurements

70S initiation complexes were prepared as above, except that non-radioactive fMet-tRNA^{fMet} was used. [^{3}H]Phe-tRNA^{Phe} or [^{14}C]Leu-tRNA^{Leu} were prepared using partially purified Phe- or Leu-tRNA synthetases from *E. coli* and purified by HPLC on a LiChrospher WP300 RP-18 column (Merck). Ternary complexes were prepared by incubating 20 μM EF-Tu, 1 mM GTP, 3 mM phosphoenol pyruvate, 0.1 mg/ml pyruvate kinase, and 10 μM aa-tRNA. To measure the overall selectivity, 70S initiation complexes with the CUC codon in the A site (0.5 μM, 50 pmol per assay) were mixed with unpurified ternary complexes containing Leu-tRNA specific for the CUC codon (1.5 μM) and Phe-tRNA as the near-cognate aa-tRNA (1.5, 2.5, or 5 μM). After 1 min incubation at 20°C, the reactions were stopped by addition of 0.5 M KOH, incubated 15 min at 37°C, neutralized with acetic acid, and dipeptides were analyzed by HLPC on a LiChrospher 100 RP-8 column (Merck). A gradient of 0%–65% acetonitril in 0.1% TFA was adapted in such a way to separate the peaks of ^{3}H-Phe, ^{14}C-Leu, fMet[^{3}H]Phe and fMet[^{14}C]Leu. Fractions from HPLC were collected and the radioactivity was counted after addition of Lumasafe Plus scintillation cocktail (Packard). Proofreading was measured with the same materials, except that only one type of ternary complex, either cognate or near-cognate, was added (0.25 μM), and ribosomes (0.5 μM) were present in excess.

Kinetic Experiments

Fluorescence stopped-flow experiments were performed in a SX-18MV spectrometer (Applied Photophysics) as described (Knudsen et al., 2001; Pape et al., 2000). Fluorescence of proflavin was excited at 470 nm, of wybutine at 325 nm, and of mant-GTP at 362 nm, and measured after passing a KV500 filter (Schott) for proflavin or KV408 for wybutine and mant-GTP emission. Experiments were performed in buffer A at 20°C by mixing equal volumes of reactants (60 μl each) and monitoring the time courses of fluorescence changes. Time courses depicted in the figures were obtained by averaging 3–7 individual transients.

Quench-flow assays were performed in a KinTek apparatus. Equal volumes (14 μl) of ribosome complex and ternary complex were rapidly mixed at 20°C. To determine the rates of peptide bond formation, reactions were quenched with KOH (0.8 M) and f[^{3}H]Met[^{14}C]Phe dipeptides were analyzed as described (Katunin et al., 2002). To measure GTP hydrolysis, reactions were quenched with 40% formic acid and the fraction of [γ-^{32}P]GTP hydrolyzed was analyzed by thin layer chromatography using Polygram CEL300 TLC plates.

Determination of Rate Constants

Apparent rate constants were determined by exponential fitting, using up to three exponential terms (characterized by variable time constant, k_{app} and respective amplitudes) and a variable for the final signal. Calculations were performed and standard deviations for apparent constants k_{app} were calculated using TableCurve software (Jandel Scientific). The rate constants of the elemental steps of A site binding were determined as described in the text and Supplemental Data.

Acknowledgments

We thank Wolfgang Wintermeyer, Venki Ramakrishnan, Eric Westhof, and Scott Strobel for critical reading and valuable comments on the manuscript, Vladimir Katunin and Yuri Semenkov for tRNA preparations, Ute Kothe for the preparation of EF-Tu(H84A), and Petra Striebeck, Astrid Böhm, Carmen Schillings, and Simone Möbitz for expert technical assistance. The work was supported by the Deutsche Forschungsgemeinschaft, the Alfried Krupp von Bohlen und Halbach-Stiftung, and the Fonds der Chemischen Industrie.

Received: July 29, 2003
Revised: October 28, 2003
Accepted: November 17, 2003
Published: January 29, 2004

References

Battle, D.J., and Doudna, J.A. (2002). Specificity of RNA-RNA helix recognition. Proc. Natl. Acad. Sci. USA *99*, 11676–11681.

Beringer, M., Adio, S., Wintermeyer, W., and Rodnina, M. (2003). The G2447A mutation does not affect ionization of a ribosomal group taking part in peptide bond formation. RNA *9*, 919–922.

Bilgin, N., and Ehrenberg, M. (1994). Mutations in 23 S ribosomal RNA perturb transfer RNA selection and can lead to streptomycin dependence. J. Mol. Biol. *235*, 813–824.

Bilgin, N., Richter, A.A., Ehrenberg, M., Dahlberg, A.E., and Kurland, C.G. (1990). Ribosomal RNA and protein mutants resistant to spectinomycin. EMBO J. *9*, 735–739.

Bouadloun, F., Donner, D., and Kurland, C.G. (1983). Codon-specific missence errors in vivo. EMBO J. *2*, 1351–1356.

Calogero, R.A., Pon, C.L., Canonaco, M.A., and Gualerzi, C.O. (1988). Selection of the mRNA translation initiation region by *Escherichia coli* ribosomes. Proc. Natl. Acad. Sci. USA *85*, 6427–6431.

Cleland, W.W. (1975). Partition analysis and the concept of net rate constants as tools in enzyme kinetics. Biochemistry *14*, 3220–3224.

Daviter, T., Wieden, H.-J., and Rodnina, M.V. (2003). Essential role

of histidine 84 in elongation factor Tu for the chemical step of GTP hydrolysis on the ribosome. J. Mol. Biol. *332*, 689–699.

Dix, D.B., Thomas, L.K., and Thompson, R.C. (1990). Codon choice and gene expression:synonymous codons differ in translational efficiency and translational accuracy. In The Ribosome. Structure, Function and Evolution, W.E. Hill et al., eds. (Washington, DC: American Society for Microbiology), pp. 527–533.

Edelmann, P., and Gallant, J. (1977). Mistranslation in *E. coli*. Cell *10*, 131–137.

Ehrenberg, M., Bilgin, N., and Kurland, C.G. (1990). Design and use of a fast and accurate *in vitro* translation system. In Ribosomes and Protein Biosynthesis. The Practical Approach Series, G. Spedding, ed. (Oxford: IRL Press), pp. 101–129.

Fersht, A. (1998). Structure and Mechanism in Protein Science (New York: W.H. Freeman and Company).

Gromadski, K.B., Wieden, H.J., and Rodnina, M.V. (2002). Kinetic mechanism of elongation factor Ts-catalyzed nucleotide exchange in elongation factor Tu. Biochemistry *41*, 162–169.

Grosjean, H.J., de Henau, S., and Crothers, D.M. (1978). On the physical basis for ambiguity in genetic coding interactions. Proc. Natl. Acad. Sci. USA *75*, 610–614.

Karim, A.M., and Thompson, R.C. (1986). Guanosine 5′-O-(3-thiotriphosphate) as an analog of GTP in protein biosynthesis. J. Biol. Chem. *261*, 3238–3243.

Katunin, V.I., Muth, G.W., Strobel, S.A., Wintermeyer, W., and Rodnina, M.V. (2002). Important contribution to catalysis of peptide bond formation by a single ionizing group within the ribosome. Mol. Cell *10*, 339–346.

Khazaie, K., Buchanan, J.H., and Rosenberger, R.F. (1984). The accuracy of Q beta RNA translation. 1. Errors during the synthesis of Q beta proteins by intact *Escherichia coli* cells. Eur. J. Biochem. *144*, 485–489.

Knudsen, C., Wieden, H.J., and Rodnina, M.V. (2001). The importance of structural transitions of the switch II region for the functions of elongation factor Tu on the ribosome. J. Biol. Chem. *276*, 22183–22190.

Koshland, D.E. (1958). Application of a theory of enzyme specificity to protein synthesis. Proc. Natl. Acad. Sci. USA *44*, 98–104.

Kurland, C.G., Jörgensen, F., Richter, A., Ehrenberg, M., Bilgin, N., and Rojas, A.-M. (1990). Through the accuracy window. In The Ribosome. Structure, Function, and Evolution, W.E. Hill et al., eds. (Washington, D.C: American Society for Microbiology), pp. 513–526.

Neidhardt, F.C. (1987). Chemical composition of *Escherichia coli*. In *Escherischia coli* and *Salmonella typhimurium*. Cellular and Molecular Biology, F.C. Neidhardt et al., eds. (Washington, D.C.: American Society for Microbiology), pp. 3–6.

Ogle, J.M., Brodersen, D.E., Clemons, W.M., Jr., Tarry, M.J., Carter, A.P., and Ramakrishnan, V. (2001). Recognition of cognate transfer RNA by the 30S ribosomal subunit. Science *292*, 897–902.

Ogle, J.M., Murphy, F.V., Tarry, M.J., and Ramakrishnan, V. (2002). Selection of tRNA by the ribosome requires a transition from an open to a closed form. Cell *111*, 721–732.

Pape, T., Wintermeyer, W., and Rodnina, M.V. (1998). Complete kinetic mechanism of elongation factor Tu-dependent binding of aminoacyl-tRNA to the A site of the *E. coli* ribosome. EMBO J. *17*, 7490–7497.

Pape, T., Wintermeyer, W., and Rodnina, M.V. (1999). Induced fit in initial selection and proofreading of aminoacyl-tRNA on the ribosome. EMBO J. *18*, 3800–3807.

Pape, T., Wintermeyer, W., and Rodnina, M.V. (2000). Conformational switch in the decoding region of 16S rRNA during aminoacyl-tRNA selection on the ribosome. Nat. Struct. Biol. *7*, 104–107.

Parker, J., and Holtz, G. (1984). Control of basal-level codon misreading in *Escherichia coli*. Biochem. Biophys. Res. Commun. *121*, 487–492.

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

Precup, J., Ulrich, A.K., Roopnarine, O., and Parker, J. (1989). Context specific misreading of phenylalanine codons. Mol. Gen. Genet. *218*, 397–401.

Rice, J.B., Libby, R.T., and Reeve, J.N. (1984). Mistranslation of the mRNA encoding bacteriophage T7-0.3 protein. J. Biol. Chem. *259*, 6505–6510.

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

Rodnina, M.V., and Wintermeyer, W. (2001). Fidelity of aminoacyl-tRNA selection on the ribosome: kinetic and structural mechanisms. Annu. Rev. Biochem. *70*, 415–435.

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

Rodnina, M.V., Fricke, R., Kuhn, L., and Wintermeyer, W. (1995). Codon-dependent conformational change of elongation factor Tu preceding GTP hydrolysis on the ribosome. EMBO J. *14*, 2613–2619.

Rodnina, M.V., Pape, T., Fricke, R., Kuhn, L., and Wintermeyer, W. (1996). Initial binding of the elongation factor Tu·GTP·aminoacyl-tRNA complex preceding codon recognition on the ribosome. J. Biol. Chem. *271*, 646–652.

Rodnina, M.V., Stark, H., Savelsbergh, A., Wieden, H.J., Mohr, D., Matassova, N.B., Peske, F., Daviter, T., Gualerzi, C.O., and Wintermeyer, W. (2000). GTPases mechanisms and functions of translation factors on the ribosome. Biol. Chem. *381*, 377–387.

Ruusala, T., Ehrenberg, M., and Kurland, C.G. (1982). Is there proofreading during polypeptide synthesis? EMBO J. *1*, 741–745.

Stage-Zimmermann, T.K., and Uhlenbeck, O.C. (1998). Hammerhead ribozyme kinetics. RNA *4*, 875–889.

Stark, H., Rodnina, M.V., Wieden, H.-J., Zemlin, F., Wintermeyer, W., and van Heel, M. (2002). Ribosome interactions of aminoacyl-tRNA and elongation factor Tu in the codon recognition complex. Nat. Struct. Biol. *9*, 849–854.

Thompson, R.C., and Stone, P.J. (1977). Proofreading of the codon-anticodon interaction on ribosomes. Proc. Natl. Acad. Sci. USA *74*, 198–202.

Thompson, R.C., and Dix, D.B. (1982). Accuracy of protein biosynthesis. A kinetic study of the reaction of poly(U)-programmed ribosomes with a leucyl-$tRNA_2$-elongation factor Tu-GTP complex. J. Biol. Chem. *257*, 6677–6682.

Thompson, R.C., Dix, D.B., Gerson, R.B., and Karim, A.M. (1981). Effect of Mg^{2+} concentration, polyamines, streptomycin, and mutations in ribosomal proteins on the accuracy of the two-step selection of aminoacyl-tRNAs in protein biosynthesis. J. Biol. Chem. *256*, 6676–6681.

Valle, M., Sengupta, J., Swami, N.K., Grassucci, R.A., Burkhardt, N., Nierhaus, K.H., Agrawal, R.K., and Frank, J. (2002). Cryo-EM reveals an active role for aminoacyl-tRNA in the accommodation process. EMBO J. *21*, 3557–3567.

Valle, M., Zavialov, A., Li, W., Stagg, S.M., Sengupta, J., Nielsen, R.C., Nissen, P., Harvey, S.C., Ehrenberg, M., and Frank, J. (2003). Incorporation of aminoacyl-tRNA into the ribosome as seen by cryo-electron microscopy. Nat. Struct. Biol., on-line.

Vila-Sanjurjo, A., Ridgeway, W.K., Seymaner, V., Zhang, W., Santoso, S., Yu, K., and Cate, J.H. (2003). X-ray crystal structures of the WT and a hyper-accurate ribosome from *Escherichia coli*. Proc. Natl. Acad. Sci. USA *100*, 8682–8687.

Yarus, M. (1992). Proofreading, NTPases and translation: constraints on accurate biochemistry. Trends Biochem. Sci. *17*, 130–133.

Yusupov, M.M., Yusupova, G.Z., Baucom, A., Lieberman, K., Earnest, T.N., Cate, J.H., and Noller, H.F. (2001). Crystal structure of the ribosome at 5.5 Å resolution. Science *292*, 883–896.