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A Uniform Response to Mismatches in Codon-Anticodon Complexes Ensures Ribosomal Fidelity

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Summary

Ribosomes take an active part in aminoacyl-tRNA selection by distinguishing correct and incorrect codon-anticodon pairs. Correct codon-anticodon complexes are recognized by a network of ribosome contacts that are specific for each position of the codon-anticodon duplex and involve A-minor RNA interactions. Here, we show by kinetic analysis that single mismatches at any position of the codon-anticodon complex result in slower forward reactions and a uniformly 1000-fold faster dissociation of the tRNA from the ribosome. This suggests that high-fidelity tRNA selection is achieved by a conformational switch of the decoding site between accepting and rejecting modes, regardless of the thermodynamic stability of the respective codon-anticodon complexes or their docking partners at the decoding site. The forward reactions on mismatched codons were particularly sensitive to the disruption of the A-minor interactions with 16S rRNA and determined the variations in the misreading efficiency of near-cognate codons.

Introduction

Aminoacyl-tRNAs (aa-tRNAs) are selected by the ribosome according to the extent of complementarity between the mRNA codon and the tRNA anticodon. Aa-tRNA enters the ribosome in a stable ternary complex with elongation factor Tu (EF-Tu) and GTP. The selection process is kinetically controlled and has two main stages, initial selection and proofreading, that are irreversibly separated by GTP hydrolysis. Discrimination between the correct and incorrect aa-tRNA depends both on the differences in their dissociation rates from the ribosome and on specific acceleration of the forward rate constants by cognate substrate (Rodnina and Wintermeyer, 2001). Crystal structures indicated that correct codon-anticodon complexes are recognized by a network of contacts within the decoding site on the small (30S) ribosomal subunit (Ogle and Ramakrishnan, 2005). The conserved bases A1492, A1493, and G530 of 16S rRNA change their positions to interact with the minor grove of the first two base pairs of the codon-anticodon complex in a fashion that is specific for Watson-Crick geometry, but does not depend on sequence (Ogle et al., 2001). A1493 monitors the base pair between the first codon position and nucleotide 36 in the anticodon of tRNA. G530 and A1492, facilitated by interactions with C518 and the ribosomal protein S12, act in concert to monitor the base pair between the second codon position and nucleotide 35 of tRNA. At the third position, the codon nucleotide interacts with G530 and indirectly interacts, through a Mg^{2+} ion, with C518 and protein S12, whereas C1054 interacts with nucleotide 34 of tRNA (Ogle et al., 2001). The local conformational changes in the decoding site result in a global rearrangement of the head and shoulder of the 30S subunit that close on the correct codon-anticodon duplex (Ogle and Ramakrishnan, 2005). Mismatches distort the correct geometry of the codon-anticodon duplex and impair the interactions with the elements of the decoding site. The closed conformation of the 30S subunit does not form, the shoulder does not move, and the head moves in a different direction than with the correct substrate (Ogle et al., 2002).

The structural insight into the decoding center (Ogle and Ramakrishnan, 2005) provided a number of experimentally testable predictions with respect to the mechanism of tRNA selection. First, formation of the network of contacts to the decoding center should increase the stability of a cognate codon-anticodon duplex on the ribosome compared to that in solution, which is exactly what has been observed in biochemical, rapid kinetics, and single-molecule experiments (Thompson and Dix, 1982; Pape et al., 1999; Blanchard et al., 2004; Gromadski and Rodnina, 2004). Second, near-cognate codon-anticodon complexes should be much less stable than cognate ones both on and off the ribosome, which has been observed for mismatches at the first codon position. However, it is not known whether and how the structural differences in monitoring the first, second, and third position affect the stability of the mRNA-tRNA complex on the ribosome. According to molecular dynamics simulations, the stability of binding should depend on the position and type of mismatch, mediated by the differences in thermodynamic and geometric properties of noncanonical base pairs and distortions of the interaction with the ribosome (Sanbonmatsu and Joseph, 2003). The contacts of the two adenines A1492 and A1493 with the codon-anticodon duplex are A-minor interactions (Nissen et al., 2001). The A-minor motif is a universal mode of RNA helical packing that is found in virtually every known structure of large RNAs (Doherty et al., 2001; Nissen et al., 2001). Thermodynamic and structural analysis of the specificity of A-minor interactions in the *Tetrahymena* group I self-splicing intron showed that the motif gives RNA the ability to recognize Watson-Crick base pairs by shape (Battle and Doudna, 2002). The energetic penalty for a mismatch in the receptor base pair was found to be quite high, 4–10 kcal/mole, and to depend strongly on the nature of the mismatch (Doherty et al., 2001; Battle and Doudna, 2002).

In addition to stabilizing the codon-anticodon complex, the conformational changes in the decoding site that are induced by the formation of the correct codon-anticodon complex correlate with the stimulation of the

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forward reactions, GTPase activation of EF-Tu, and tRNA accommodation on the large ribosomal subunit (the 50S) (Pape et al., 2000; Rodnina and Wintermeyer, 2001; Ogle and Ramakrishnan, 2005). A single C-A mismatch at the first codon position reduced the rate of GTP hydrolysis more than 600-fold, and this effect is crucial for the efficient selection of cognate versus near-cognate tRNA (Gromadski and Rodnina, 2004). It is not known whether mismatches at other positions have a similar effect and to what extent the A-minor interactions at the decoding site are involved in transmitting the conformational signal to the functional sites on the 50S subunit. Furthermore, the irreversible GTPase step is so rapid that it does not allow equilibration at the codon-recognition step. Therefore, the discrimination potential based on stability differences of the codon-anticodon complexes cannot be used in full (Yarus, 1992; Gromadski and Rodnina, 2004). Thus, the actual contribution to tRNA discrimination of specific interactions between ribosomal residues and the codon-anticodon duplex remains unclear.

In the present work, we studied the mechanism by which the interactions of ribosomal residues with the first, second, and third position of the codon-anticodon complex mediate aa-tRNA selection. We disrupted these interactions by introducing mismatches at defined positions and determined the effects on the stability of tRNA binding to the ribosome and the rate constants of GTP hydrolysis and peptide bond formation.

Results

Experimental Approach

The specific interactions between bases of 16S rRNA and the codon-anticodon duplex at the decoding site were disrupted by introducing single mismatches at all three positions of the codon-anticodon duplex. We used the ternary complex of EF-Tu·GTP with Phe-tRNA^{Phe} (anticodon, 3′-AAG-5′) and ribosome complexes displaying various cognate or near-cognate codons at the decoding site: UUC (fully matched cognate codon), CUC and GUC (first position C/G-A mismatches), CUU (first position C-A mismatch and U-G wobble pair in the third position), UCC (second position C-A mismatch), and UUA/G (third position A/G-G mismatches). The rate constants of the four key steps that govern aa-tRNA selection are: the dissociation of the ternary complex, k_{-2}, and GTPase activation, k_3, in the initial selection phase and the accommodation of aa-tRNA in the A site, k_5, and the rejection of aa-tRNA, k_7, in the proofreading phase (Figure 1) (Rodnina and Wintermeyer, 2001). The rates of ternary complex binding to the ribosome and of the following rearrangement steps, e.g., codon recognition, were monitored by stopped-flow with a fluorescence reporter group in tRNA^{Phe}, i.e., proflavin attached at positions 16 or 17 of the tRNA, which is sensitive both to the changes in the environment of the dye and to the conformational changes of the tRNA (Rodnina et al., 1994). The rates of the chemistry steps, GTP hydrolysis, and peptide bond formation were measured by quench flow, determining the amounts of the respective products formed with time. The values of the rate constants governing initial selection together with the rate constants of initial binding, k_1 and k_{-1}, and codon

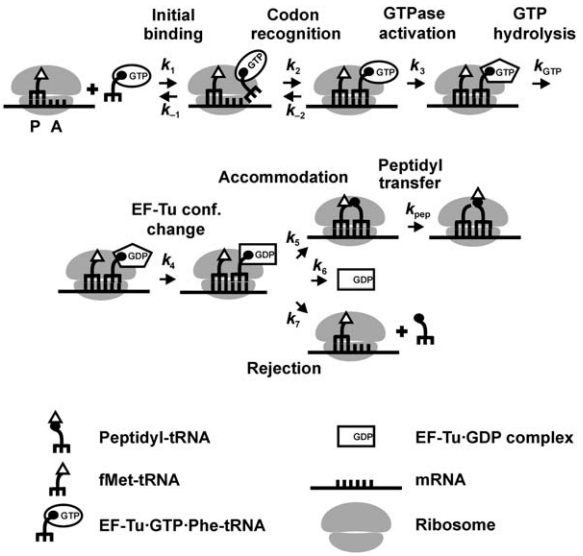

Figure 1. Kinetic Mechanism of EF-Tu-Dependent aa-tRNA Binding to the Ribosomal A Site

Steps are defined as previously described (Rodnina and Wintermeyer, 2001). EF-Tu is depicted in different shapes to indicate different conformations: Ellipse, GTP bound form; pentagon, GTPase-activated state; rectangle, GDP bound form.

recognition, k_2, were measured at high-fidelity (HiFi) conditions (3.5 mM Mg^{2+}, 0.5 mM spermidine, and 8 mM putrescine) (Gromadski and Rodnina, 2004). For all codon-anticodon complexes with mismatches, the rate of GTP hydrolysis was rate-limiting for peptide bond formation, which precluded the determination of k_5 and k_7 at HiFi conditions (Gromadski and Rodnina, 2004; and data not shown). To determine the latter two constants, the complete analysis was repeated at high magnesium concentration (low fidelity [LoFi]; 20 mM Mg^{2+}, no polyamines) at which GTP hydrolysis was not rate limiting and all rate constants could be determined reliably for all codons studied. The rate constants of initial binding, determined as described (Experimental Procedures; Rodnina et al., 1996; Gromadski and Rodnina, 2004), were k_1 = 100–140 $\mu M^{-1}s^{-1}$ and k_{-1} = 80–100 s^{-1} for HiFi (Gromadski and Rodnina, 2004); and k_1 = 60–80 μM^{-1} and k_{-1} = 30–40 s^{-1} for LoFi, regardless of the codon in the A site.

Stability of the Codon-Recognition Complex

To determine the effect of mismatches on the stability of ternary complex binding to the ribosome, we measured the dissociation of the ternary complex EF-Tu·GTP·aa-tRNA from the codon-recognition complex. To prevent the following steps, the mutant EF-Tu(H84A) that is deficient in GTP hydrolysis was used, whereas the rate constants of all preceding steps are not affected (Daviter et al., 2003). The codon-recognition complex was formed by incubating ternary complex containing fluorescent Phe-tRNA^{Phe}(Prf16/17) with the ribosome·mRNA·fMet-tRNA^{fMet} complex. Dissociation rate constants, k_{-2}, were measured by monitoring the fluorescence decrease upon dissociation of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) from the ribosome after mixing with an excess of nonfluorescent ternary complexes in

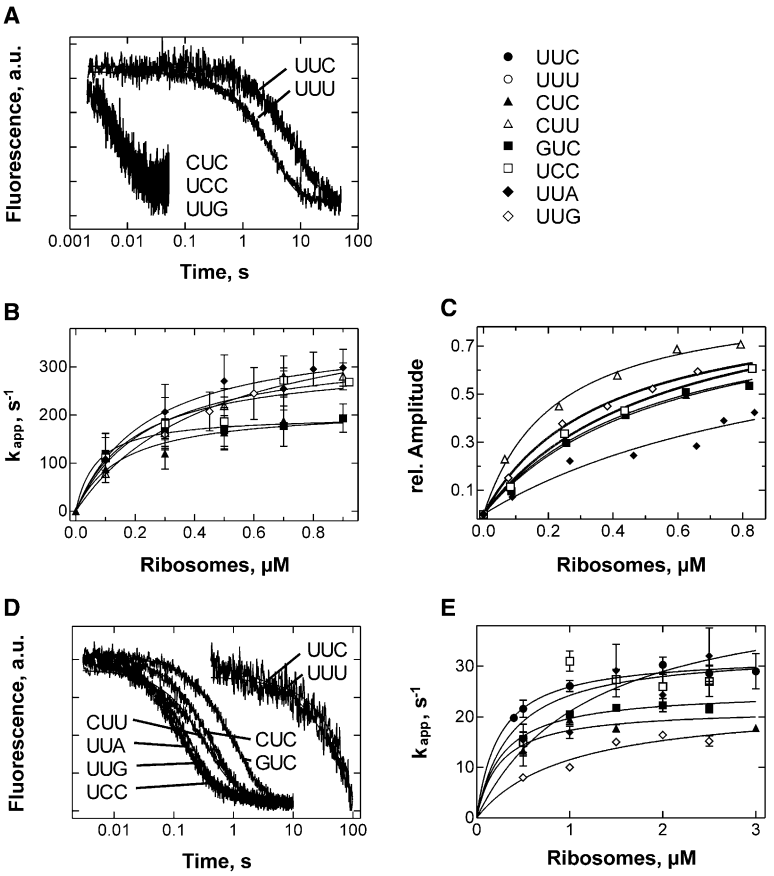

Figure 2. Dissociation of Codon-Recognition Complexes

(A) Selected time courses at HiFi conditions. The dissociation of EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}(Prf16/17) from ribosome complexes with the indicated codons was initiated by mixing with excess nonfluorescent ternary complex.

(B) Concentration dependence of the rate of codon recognition k_{app2} (HiFi). Symbols are explained in the top right panel and are used in all Figures. Error bars represent standard deviation of the measured values.

(C) Concentration dependence of the fluorescence amplitude of codon recognition (HiFi). The data were normalized by setting the saturation of the curves to 1.

(D) Time courses of dissociation at LoFi conditions. Exponential fitting yielded dissociation rate constants, k_{-2}.

(E) Determination of k_{app2} at LoFi conditions.

a stopped-flow device (Figure 2A). The cognate complex dissociated slowly with a rate constant of 0.1–0.2 s^{-1} (HiFi conditions; Table 1). All complexes with mismatches in the codon-anticodon duplex were much less stable than the cognate complex. The dissociation rates of the complexes containing mismatches (100–200 s^{-1}) were close to the value of k_{-1} (85 s^{-1}), which made it impossible to calculate the k_{-2} values with precision, because in this case, k_{-1}, rather than k_{-2}, may be the rate-determining step of dissociation. To overcome this problem, an alternative approach was applied to calculate the k_{-2} values by studying formation of the codon recognition complex in isolation from the following steps. Upon mixing the GTPase-deficient EF-Tu (H84A)·GTP·Phe-tRNA^{Phe}(Prf16/17) with ribosome complexes, only two steps, initial binding and codon recognition, occur, leading to an increase of proflavin fluorescence (Daviter et al., 2003). At a given concentration of ribosomes added in excess over the ternary complexes (pseudo-first-order conditions), an apparent rate constant of codon recognition (k_{app2}) was estimated by exponential fitting of the time courses. The k_{app2} value increased with ribosome concentrations and reached saturation at 200–300 s^{-1} (Figure 2B). For a model with two reversible steps, the value of k_{app2} at saturation is given by k_2+k_{-2} (Fersht, 1998). For cognate codons, $k_{app2} \approx k_2$, because k_{-2} is very small (Table 1). However, for near-cognate codons, k_{-2} is not negligible, and the k_2 and k_{-2} values cannot be determined from k_{app2} alone. Additional information required for calculations is provided by the analysis of the amplitudes of the fluorescence change (Figure 2C). The concentration dependence of the amplitudes was hyperbolic. The concentration of ribosomes at half saturation yields the value $K_{d\ app2}$, which is a direct measure for the thermodynamic stability of the codon recognition complex and was used to calculate ΔΔG° of the interaction (Table 1).

Table 1. Effect of Mismatches in the Codon-Anticodon Complex on the Rate Constants of A Site Binding at HiFi Conditions

Codon	k_2 s^{-1}	k_{-2} s^{-1}	ΔΔG° kcal/mol	k_3 s^{-1}	$\Delta\Delta G_3^{\neq}$ kcal/mol
UUC	180	0.12	–	120	–
UUU	190	0.23	–	260	–
CUC	190	100	4.3	0.6	3.1
CUU	280	100	3.7	0.4	3.4
GUC	100	100	4.3	0.2	3.8
UCC	190	160	4.3	0.06	4.5
UUA	140	240	4.7	0.9	2.9
UUG	190	120	4.0	1.3	2.7

Standard deviation of all measured values was about 15%. ΔΔG° was calculated directly from the $K_{d\ app2}$ values measured with EF-Tu(H84A) according to the equation $\Delta\Delta G° = RT \cdot \ln(K_{d\ near\text{-}cognate}/K_{d\ UUC})$. $\Delta\Delta G_3^{\neq} = -RT\ln(k_{3\ near\text{-}cognate}/k_{3\ UUC})$. Data for the UUU codons are shown for comparison (Gromadski and Rodnina, 2004). The k_3 value measured here on the CUC codon, 0.6 ± 0.1 s^{-1}, is slightly higher than that reported previously, 0.4 ± 0.1 s^{-1} (Gromadski and Rodnina, 2004); however, the deviation is at the border of statistical significance.

Table 2. Effect of Mismatches in the Codon-Anticodon Complex on the Rate Constants of A Site Binding at LoFi Conditions

Codon	k_2 s^{-1}	k_{-2} s^{-1}	$\Delta\Delta G^\circ$ kcal/mol	k_3 s^{-1}	k_5 s^{-1}	$\Delta\Delta G_5^{\neq}$ kcal/mol	k_7, s^{-1}
UUC	32	0.005	–	>500	2	–	<0.1
CUC	20	1.7	3.8	3.5	0.09	1.8	1.1
CUU	18	1.6	3.7	0.8	0.02	2.7	0.8
GUC	25	0.8	3.1	0.6	0.03	2.6	0.7
UCC	33	9	4.4	1.6	0.03	2.6	1.8
UUA	20	5	4.4	21	0.30	1.1	1.7
UUG	24	9	4.6	8	0.27	1.1	2.1

Standard deviation of all measured values was about 15%. $\Delta\Delta G_3^{\neq}$ and $\Delta\Delta G^\circ_7$ were not calculated, because the k_3 and k_7 values for the cognate codon could not be determined with precision.

Furthermore, $K_{d\ app2} = K_{d1} \cdot K_{d2} = k_{-1}/k_1 \cdot k_{-2}/k_2$. Given the known values of k_1 and k_{-1} and the values of k_{app2} and $K_{d\ app2}$ determined from the data of Figures 2B and 2C, respectively, the values of k_2 and k_{-2} were calculated for all codons (Table 1). The rate constants of codon recognition, k_2, were very similar for all codons, cognate and near-cognate. The dissociation of ternary complex from ribosomes with near-cognate codons did not depend systematically on the nature or position of the mismatch and was approximately 1000-fold faster than that from cognate ribosomes.

The experiments carried out at LoFi conditions revealed similar rate differences between cognate and near-cognate substrates, although the absolute values of all constants at HiFi and LoFi conditions were different. The rate of dissociation of the codon recognition complex was 0.005 s^{-1} for cognate and 0.8–9 s^{-1} for near-cognate codons (Figure 2D). The mismatches in the first position destabilized the complex slightly less than those at the second or third position, suggesting that at LoFi, the interactions at the first codon position contribute somewhat less to the stability of the codon-anticodon duplex than those of both second- and third-position base pairs (Table 2). The rate of codon recognition at LoFi was calculated from the concentration dependence of k_{app2} and was k_2 = 20–30 s^{-1} for cognate or near-cognate codons (Figure 2E).

GTPase Activation

The effect of codon-anticodon mismatches on the GTPase activation step (k_3) was determined from the rates of GTP hydrolysis measured with ternary complexes containing wild-type EF-Tu. GTP hydrolysis is inherently very rapid, and its rate is limited by the preceding GTPase activation step (Pape et al., 1999). Therefore, time courses of GTP cleavage yield the rate constants of the GTPase activation step. Time courses of [γ-^{32}P]-GTP cleavage upon ternary complex binding to ribosome complexes were measured by quench flow (Figures 3A and 3B). Values for k_{app3} were estimated from the saturation levels at high concentrations of ribosomes as described in Experimental Procedures (Figures 3C and 3D). Cognate interaction with the UUC codon led to rapid GTP hydrolysis in the ternary complex, k_3 = 120 s^{-1}. Any mismatch in the codon-anticodon complex greatly reduced the rate of GTP hydrolysis with only small variations, depending on the position of the mismatch (Table 1). At HiFi conditions, the second position mismatch appeared to affect GTP hydrolysis particularly strongly. At LoFi, mismatches in both the first or second position strongly reduced the rate constant of GTPase activation, whereas the third-position mismatches had smaller effects (Table 2).

Proofreading

Mismatches at different codon positions were also assessed with respect to their effects on the rate constants governing proofreading, i.e., aa-tRNA accommodation, k_5, and rejection, k_7, at LoFi conditions (Figure 1). Apparent rate constants of dipeptide formation (Figure 4A) and the extent of Phe incorporation (Figure 4B) were measured on various codons, and rate constants of accommodation and rejection were calculated from these data as described in the Experimental Procedures. The effects of mismatches on tRNA accommodation (k_5) follow similar rules as found for GTPase activation (k_3) (Table 2). The rate constant of accommodation, 2 s^{-1} for cognate aa-tRNA, was strongly reduced by the mismatches. The first- and second-position base pairs were more important for accommodation than the third base pair. The stability of aa-tRNA on the ribosome was generally quite low (large k_7) independent of the type and position of the mismatch.

At LoFi conditions, the overall selectivity for correct aa-tRNA in the presence of equal amounts of cognate (Leu) and near-cognate (Phe) ternary complexes in excess over the CUC-programmed ribosome was 20, i.e., one incorrect for 20 correct amino acids was incorporated, contributed by initial selection (selectivity of 2) and proofreading (selectivity of 10) (Gromadski and Rodnina, 2004). At HiFi conditions, the efficiencies of initial selection and proofreading of the same mismatch were 30 and 15, respectively, and only one incorrect in 450 correct amino acids was incorporated into peptide (Gromadski and Rodnina, 2004). The similar efficiency of proofreading at HiFi and LoFi conditions implies that the ratio between the rates of tRNA accommodation and rejection is largely independent of the ionic conditions, in agreement with the results obtained with poly(U)-programmed ribosomes (Pape et al., 1999). This suggests that at any reaction conditions, proofreading is governed by the same rules, based on uniformly high tRNA dissociation rate constants (k_7) versus low and somewhat varying accommodation rate constants (k_5) on near-cognate codons.

Discrimination of Codon-Anticodon Complexes with Single Mismatches

From the rate constants of the kinetic steps prior to GTP hydrolysis (Table 1), the k_{cat}/K_M values for cognate (UUC codon) and all near-cognate codons at HiFi conditions were calculated (Experimental Procedures). Assuming similar binding parameters for all aa-tRNAs to their respective cognate codons (Olejniczak et al., 2005), one can use the k_{cat}/K_M parameters determined above to estimate the efficiency of discrimination against Phe-tRNA^{Phe} on the Leu, Val, and Ser codons used here. Initial selection contributes a factor of 30–800, resulting in error frequencies of $1 \times 10^{-3} - 3 \times 10^{-2}$, depending on the position and type of mismatch, with the error frequency

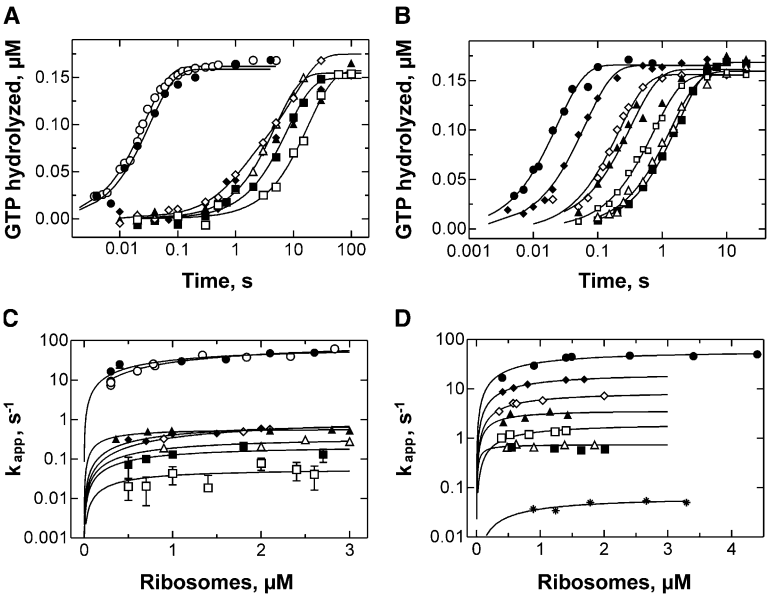

Figure 3. GTPase Activation and GTP Hydrolysis

(A) Time courses of GTP hydrolysis at HiFi conditions (1.5 μM ribosome complexes).

(B) Time courses of GTP hydrolysis at LoFi conditions (1.5 μM ribosome complexes).

(C) Concentration dependence of the rate of GTP hydrolysis (HiFi). Values of k_{app} at saturation were used to calculate the rate constant of GTP hydrolysis, k_3.

(D) Concentration dependence of the rate of GTP hydrolysis (LoFi). Symbols are as in Figure 2. Asterisk indicates GTP hydrolysis on a codon with two mismatches, CUA, shown for comparison.

increasing in the order UCC < CUU, GUC < UUA, UUG, and CUC. A very similar order (UCC, CUU < GUC < CUC < UUG, UUA) was found experimentally for the proofreading step for at LoFi (Figure 4B) and HiFi (not shown) conditions. Proofreading contributed an additional factor of 10–100 to tRNA discrimination. Thus, the overall error frequency of Phe-tRNA^{Phe} misincorporation at Leu, Val, and Ser codons is predicted to be 10^{-5}–10^{-3} for equal concentrations of competing aa-tRNAs.

Discussion

Uniform Effect of Mismatches on the Stability of Codon-Anticodon Complexes

Interactions between the codon-anticodon duplex and bases of 16S rRNA in the decoding center contribute to tRNA selection in two ways: they increase the stability of tRNA binding and enhance the rates of GTPase activation and tRNA accommodation in the A site, i.e., of the rearrangement steps that limit the rate of the irreversible chemical steps of GTP hydrolysis and peptide bond formation. The stability of tRNA binding to the ribosome, which is very high for the cognate tRNA, is dramatically and uniformly reduced by single mismatches, regardless of the position and type, at both the initial selection and the proofreading phases. Generally, the stability of anticodon-anticodon complexes formed between two tRNAs in solution differ significantly, depending on the type and position of a mismatch (Grosjean et al., 1978). The exact thermodynamic penalty due to C-A, G-A, or G-G mismatches is not known, because the respective complexes are so unstable that their lifetimes could not be measured with precision. Mismatches are likely to change the characteristic shape of the Watson-Crick codon-anticodon base pairs (Seeman et al., 1976), cause uncompensated loss of hydrogen bonds formed with elements of the decoding center (Ogle et al., 2002), impair base stacking (Grosjean et al., 1976; Konevega et al., 2004; Murphy et al., 2004), and change the solvation state of the complex. Geometry should be the most effective criterion for discrimination against all but cognate tRNA (Ogle et al., 2002), but the exact contribution of shape discrimination versus loss of hydrogen bonding, stacking, and desolvation is not known. The present data show that all noncanonical codon-anticodon complexes are equally unstable on the ribosome, indicating that potential differences in thermodynamic stabilities between base pairs and details of base pairing interactions with the ribosome are not essential for the stabilization of the tRNA in the A site.

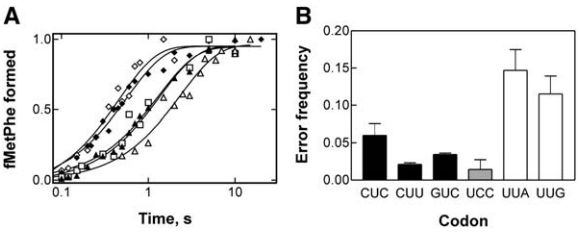

Figure 4. Proofreading

(A) Time courses of dipeptide formation (LoFi, 1.5 μM ribosome complexes). The formation of fMetPhe dipeptide was determined by quench-flow for the indicated codons. Symbols are as in Figure 2. Ordinate values indicate the incorporation of Phe normalized in each case to the respective final level, corresponding to that shown in (B), e.g., about 0.06 fMetPhe per ternary complex added were formed on the CUC codon. Single-exponential fitting of the time courses yielded k_{app} of peptide bond formation.

(B) Error frequency of Phe misincorporation into fMetPhe dipeptides during proofreading; on the cognate UUC codon, the efficiency of Phe incorporation was 1.0 per ternary complex. Error bars represent standard deviation of three independent measurements.

In comparison to discrimination by shape, the contribution to tRNA discrimination of the hydrogen bonds between the codon-anticodon duplex and the 30S subunit seems to be small. A C-A mismatch at the first codon position may abolish the hydrogen bonds between A1493 and the codon nucleotide, but would not be expected to affect the geometry or orientation of the base pairs

at the second or third codon position (Sanbonmatsu and Joseph, 2003). However, the energetic penalty for the C-A mismatch at the first position is found to be as high as for the bulky G-A mismatch at the first position or the C-A mismatch at the second position, or as for purine-purine mismatches at the third position, each of which is expected to disturb the interactions at the neighboring positions and thus cause a stronger destabilization of the codon-anticodon complexes on the ribosome (Sanbonmatsu and Joseph, 2003). Furthermore, substitutions of the ribose 2′OH at positions 34, 35, and 37 in the anticodon of cognate tRNA, i.e., the positions that form hydrogen bonds to the ribosome, reduced the stability of the tRNA·codon·ribosome complex by a factor of 4–13, much less than expected for a loss of an essential hydrogen bond (Phelps et al., 2002). This may be because the removal of a 2′OH group can be accommodated by the ribosome with minimal distortion, which would explain the small measured effects and suggests that shape monitoring plays a dominant role in tRNA recognition by the ribosome. Van-der-Waals contacts are likely to play a major role in recognition; the additional contribution of the solvation effects and base stacking are difficult to assess at present.

From the measured stabilities of codon-anticodon complexes, the thermodynamic costs of mismatches can be calculated. At the initial selection step, the energetic penalty for mismatches in the first and second position, which both lead to a loss of an A-minor interaction, is about 4 kcal/mol (Table 1). This value can be compared to the effects of mismatches on the stability of type I A-minor interactions in the *Tetrahymena* group I self-splicing intron. In that system, thermodynamic destabilization greatly depends on the type of mismatch, ranging from $\Delta\Delta G^{\circ}$ = 8.3 kcal/mol for a C-A mismatch to $\Delta\Delta G^{\circ}$ = 2.8 kcal/mol for a G-A mismatch (Battle and Doudna, 2002). Thus, the two experimental systems seem to yield quantitatively different results, which probably have to be attributed to differences in conformational coupling between A-minor recognition and further rearrangements in the intron and the ribosome. On the ribosome, additional contacts as well as conformational rearrangements in the decoding center may compensate for deficiencies in A-minor recognition and energetic differences between base pairs. Furthermore, the free energy gained from interactions with the first and second base pair may not be fully available for stabilizing the complex, because part of it is used for the conformational work required to expose adenines 1492 and 1493 from helix 44 of 16S rRNA and to change the conformation of the 30S subunit from open to closed (Ogle et al., 2001, 2002, 2003). Although the decoding site is not rigid and accommodates a number of unconventional geometries, such as wide purine-purine base pairs in the wobble position (Murphy and Ramakrishnan, 2004) or—stabilized by paromomycin—an unconventional G-U base pair at the second position, mismatches may impair rearrangements that lead to stabilization of the closed conformation of the 30S subunit. Thus, the observed uniform effect of mismatches on the stability of tRNA binding may be explained by the failure to restrict the conformational mobility of the 30S subunit and, consequently, the failure to stabilize the form that is active in binding and catalysis.

Acceleration of Forward Reactions

The present data show that acceleration of GTPase activation requires Watson-Crick base pairs at the first and second codon-anticodon positions and a regular base pair (Watson-Crick or wobble) at the third position. A single mismatch at any position reduced the rate of GTPase activation significantly, corresponding to an increase in the activation energy barrier of 2.7–4.5 kcal/mol. Double or triple mismatches, which characterize the noncognate situation, increased the barrier even more dramatically, i.e., by 6 to 7 kcal/mol (Figure 3D and [Rodnina et al., 1996; Pape et al., 2000]). Similar effects were found for tRNA accommodation, where single mismatches increased the activation energy by 1.1–2.7 kcal/mol. Although any mismatch strongly decreased the rate of the two forward reactions, mismatches at the first and second codon position had somewhat larger effects than at the third position, particularly at LoFi conditions. The acceleration of forward steps correlates with the formation of A-minor interactions of A1492 and A1493 with these base pairs (Ogle et al., 2001), indicating that these interactions have a major role in the induction of the conformational signal that is communicated from the decoding site to the functional sites on the 50S subunit to accelerate GTPase activation and tRNA accommodation.

Given the concentrations of ribosomes (10 μM) and ternary complexes (100 μM) in vivo, the present set of rate constants can be used to predict the speed and efficiency of incorporation of cognate, near-cognate, and noncognate amino acids into proteins. Assuming about 2% of cognate Phe-tRNA^{Phe} in the total tRNA pool, 10%–20% near-cognate tRNAs (single mismatches between codon and anticodon), and the remaining portion of noncognate tRNAs, competition between the respective ternary complexes is expected to decrease the actual rate of GTP hydrolysis for the cognate ternary complex from 120–260 s^{-1} to 3–4 s^{-1} (for the calculations, see Experimental Procedures). This rate of initial selection turns out to be rate limiting for amino acid incorporation into peptide and is in good agreement with the estimated rates of protein synthesis in vivo. In comparison, the incorporation of a near-cognate aa-tRNA is low due to efficient tRNA rejection and is very slow due to retarded GTP hydrolysis by EF-Tu.

The small differences between the rates of forward reactions on various near-cognate codons may play a significant role in modulating the misreading frequency of codons differing by a position or type of a mismatch. As all rate constants, except for k_3 and k_5, are practically identical for codons with single mismatches, variations in error frequencies on near-cognate codons that depend on the position and type of the mismatch must result from the differences in the rates of forward reactions rather than from different stabilities of near-cognate aa-tRNA binding. For all codons, the stringency of initial selection is similar to that of proofreading, allowing for the incorporation of only one incorrect amino acid in 10–100 correct ones in each selection step. Consequently, the total error frequency of Phe misincorporation on various near-cognate codons in the presence of an equal amount of the respective cognate aa-tRNA was estimated to be 10^{-5}–10^{-3} (see Results), close to values measured in vivo (for references, see Rodnina and Wintermeyer, 2001). The observed error frequency

will be further modulated by the actual concentrations of aa-tRNAs in the cell that influence the competition between cognate, near-cognate, and noncognate ternary complexes.

The efficiency of misincorporation during proofreading was 1 in 10–15 incorrect aa-tRNA at both HiFi and LoFi conditions. A higher efficiency of proofreading, about 1 in 100, was found in earlier work with poly(U)-programmed ribosomes (Thompson and Dix, 1982; Pape et al., 1999). One potential explanation for this difference is that previously, a different tRNA, AcPhe-tRNA^{Phe}, was present in the P site rather than fMet-tRNA^{fMet} employed in the present work. This suggests the interesting possibility that the accommodation of tRNA in the A site is affected by the tRNA bound to the P site, which would explain a number of in vivo observations on the effects of neighboring tRNAs on the efficiency of tRNA selection.

Effect of Ionic Conditions on Decoding

The rate and fidelity of decoding is long known to depend on the Mg^{2+} concentration and the presence of polyamines. The present data give direct insight into the underlying mechanism. The rate constants of ternary complex binding and dissociation, k_1, k_{-1}, k_2, and k_{-2}, decrease with increasing Mg^{2+} concentration, whereas the rate of GTPase activation, k_3, increases, both on cognate and near-cognate codons. In structural terms, the observed Mg^{2+} effects may have a number of reasons. Mg^{2+} binding can alter the conformation of RNA and stabilize its tertiary structure, thereby affecting the propensity for undergoing conformational changes. Some, but not all, Mg^{2+} binding sites may also be occupied by polyamines. As they may have a different effect on structure stabilization, their competition for binding sites may be a means to fine-tune recognition events. Crystal structures identified a number of Mg^{2+} ions bound to the tRNA^{Phe} molecule (Jovine et al., 2000; Shi and Moore, 2000). There are also three Mg^{2+} ions that mediate contacts between the codon-anticodon complex and 16S rRNA: two are bound within helix 44 of 16S rRNA in the decoding center, but only in the flipped-out conformation of A1492 and A1493, and one takes part in binding interactions with the third base pair of the codon-anticodon complex (Carter et al., 2000; Ogle et al., 2001). Binding of the ternary complex to the A site entails a number of rearrangements, e.g., a transient interaction of the ternary complex with the ribosomal protein L7/12 (Kothe et al., 2004; Diaconu et al., 2005), movement of the tRNA anticodon into the decoding site on the 30S subunit and codon recognition (Blanchard et al., 2004), local conformational changes of the decoding site involving A1492 and A1493, and a global closing of the 30S subunit (Ogle et al., 2001, 2002). It is likely that by stabilizing a particular conformation of rRNA and tRNA, Mg^{2+} ions interfere with the movements of ribosomal substructures that are necessary for adjustment of the anticodon in the active site, which would explain the lower rates for codon recognition at LoFi conditions. However, once formed, the contacts between the ribosome and the codon-anticodon duplex are stabilized by Mg^{2+}, either directly by taking part in the contact or indirectly by stabilizing a binding conformation of the tRNA and rRNA.

The rate of GTPase activation increases with Mg^{2+} and polyamines. As Mg^{2+} ions do not seem to be essential for the intrinsic or the kirromycin-stimulated GTPase activity of EF-Tu (Ivell et al., 1981), the Mg^{2+} dependence of the GTPase activation step is likely to reflect the requirement for divalent ions in either communicating the signal to the GTP binding pocket of EF-Tu or in the interaction between EF-Tu with GTPase-activating elements of the ribosome, e.g., protein L7/12, the sarcin-ricin loop, and possibly protein L11. Alternatively, the effect of Mg^{2+} and polyamines on the GTPase activation may be explained by a stabilization of the tertiary structure of aa-tRNA, consistent with the notion that the tRNA molecule plays an active role in communicating signals from the decoding site on the 30S subunit to the functional sites on the 50S subunit (Piepenburg et al., 2000; Cochella and Green, 2005) and with the important role of tRNA conformation in regulating the speed and the fidelity of decoding (Yarus and Smith, 1995).

Experimental Procedures

Buffers and Reagents

HiFi buffer is comprised as follows: 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. LoFi buffer is as follows: 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, and 20 mM $MgCl_2$. Buffer A contains 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, and 7 mM $MgCl_2$. Buffer B contains 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, and 3.5 mM $MgCl_2$. Chemicals were from Roche, Merck, or Sigma, and radioactive amino acids and [γ-^{32}P]GTP were from ICN. All experiments were carried out at 20ºC if not stated otherwise.

Components and Preparation of Ternary and Initiation Complexes

Ribosomes, EF-Tu, and fMet-tRNA^{fMet} from *E. coli* were prepared as described (Rodnina et al., 1994; Rodnina and Wintermeyer, 1995; Gromadski and Rodnina, 2004); and tRNA^{Phe}(Prf16/17) was prepared as in (Rodnina et al., 1994). mRNAs (122 nt long) with various codons after the AUG start codon were derived from MFF-mRNA (La Teana et al., 1993; Rodnina and Wintermeyer, 1995). mRNAs were prepared by T7 RNA-polymerase transcription and purified by ion-exchange chromatography on MonoQ (Pharmacia). Initiation complexes were formed in buffer A by incubating ribosomes (1 μM), mRNA (3 μM), f[^{3}H]Met-tRNA^{fMet} (1.5 μM), IF1, IF2, IF3 (1.5 μM each), and GTP (1 mM) for 1 hr at 37ºC and purified by centrifugation through 400 μl 1.1 M sucrose cushions in buffer A or LoFi. After centrifugation at 55,000 rpm for 2 hr in a Sorvall M120GX ultracentrifuge, pellets were resuspended in buffer A or LoFi, shock-frozen in liquid nitrogen, and stored at −80ºC. Immediately before the experiments, complexes that were stored in buffer A were diluted with buffer A without Mg^{2+} and polyamines were added to achieve the final concentrations of the HiFi buffer.

For kinetic experiments, ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} was prepared by incubating EF-Tu (wild-type or H84A) (20–50 μM), GTP (1 mM), phosphoenol pyruvate (3 mM), pyruvate kinase (0.05 mg/ml), tRNA^{Phe}(Prf16/17) (5–15 μM), ATP (3 mM), [^{14}C]-phenylalanine (30 μM), and partially purified phenylalanyl-tRNA synthetase (3% v/v) in buffer B or LoFi and purified by gel filtration on a tandem Superdex 75 HR column (Pharmacia) in the same buffer. Polyamines were added to the concentrations contained in HiFi buffer (see above) immediately before use in stopped-flow or quench-flow experiments. Ternary complexes containing [γ-^{32}P]GTP were prepared in the same way except that the concentration of [γ-^{32}P]GTP was 30 μM.

Kinetic Experiments

Fluorescence stopped-flow experiments were performed by using an Applied Photophysics stopped-flow apparatus and monitoring the proflavin fluorescence of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17); excitation was at 470 nm and fluorescence was measured after

passing a 500 nm cutoff filter. Initial binding was monitored by an increase of proflavin fluorescence after mixing constant amounts of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) (0.2 μM) with increasing concentrations of nonprogramed or incorrectly programed ribosomes (Rodnina et al., 1996). Alternatively, the information about the initial binding step was extracted from the first exponential phase of the signal change observed with cognate or near-cognate codons; the two approaches yielded identical values of k_{app1}. The rate constants of initial binding, k_1 and k_{-1}, were calculated from the slope and y axis intercept, respectively, of the linear plot of k_{app1} versus ribosome concentration.

Codon recognition was monitored by an increase of proflavin fluorescence upon addition of EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}(Prf16/17) (0.2 μM) to increasing amounts of ribosome complexes with fMet-tRNA in the P site and different codons exposed in the A site. The k_{app2} was calculated by hyperbolic fitting from saturation of the titration curve obtained at increasing ribosome concentration. The apparent affinity of the ternary complex to the ribosomes, $K_{d\ app2}$, was determined at half saturation of the fluorescence amplitude increase (see Results) by hyperbolic fitting. Rate constants of codon recognition, k_2 and k_{-2}, were estimated from k_{app2} and $K_{d\ app2}$ by using the equations $k_{app2} = k_2+k_{-2}$ and $K_{d\ app2} = k_{-1}/k_1 \cdot k_{-2}/k_2$ (Fersht, 1998). Chase experiments were carried out by mixing EF-Tu(H84A)·GTP·Phe-tRNA^{Phe}(Prf16/17) with an equimolar amount of initiation complex and rapidly mixing the resulting A site complex with ternary complex containing unlabeled Phe-tRNA^{Phe} to reach final concentrations of 0.3 μM and 2.1 μM, respectively. Dissociation of labeled ternary complex from the ribosome was monitored by a decrease of proflavin fluorescence.

Quench-flow experiments were performed with a KinTek apparatus. GTP hydrolysis was measured with 0.2 μM EF-Tu·γ[^{32}P]GTP·Phe-tRNA^{Phe} ternary complex and varying concentrations of initiation complex. Samples were quenched by the addition of formic acid (25%) and analyzed by thin-layer chromatography on polyethyleneimine-cellulose plates in 0.5 M potassium phosphate (pH 3.5) and quantified with a Bio-Rad phosphorimager. Apparent rate constants, k_{app3}, were estimated from the saturation of the hyperbolic fit of the concentration dependence. Rate constants of GTPase activation were calculated from the difference in time required for GTP hydrolysis and codon recognition, $k_3 = 1/(1/k_{app3}-1/k_2)$ (Cleland, 1975).

Dipeptide formation was measured with 0.5 μM ternary complex and saturating concentrations of initiation complex (1.5 μM). Samples were quenched with 0.5 M KOH, hydrolyzed for 30 min at 37ºC, and neutralized with acetic acid and amounts of f[^{3}H]Met-[^{14}C]Phe dipeptides were determined by HPLC on a LiChrospher 100 RP-8 column (Merck) (Pape et al., 1999). The apparent rate constant of dipeptide formation, $k_{app\ dip}$, was obtained by fitting time courses of peptide bond formation with a single exponential function. The rate constants of peptide bond formation were calculated from the difference in time required for peptide synthesis and GTP hydrolysis ($k_{dip} = 1/[1/k_{app\ dip} - 1/k_3]$). At saturation, k_{dip} is given by the sum of the accommodation and rejection rates (Fersht, 1998), $k_{dip} = k_5+k_7$. The error frequency of dipeptide formation, F_e, measured as the efficiency of Phe misincorporation on a particular codon, can be expressed by using the accommodation and rejection rate constants k_5 and k_7: $F_e = k_5/(k_5+k_7)$. By using these equations, elemental rate constants for accommodation and rejection, k_5 and k_7, can be calculated from experimental values of error frequencies and apparent rate constants of peptide bond formation can be determined at saturating ribosome concentration.

Determination of Error Frequencies

Selectivity measurements were performed as described (Gromadski and Rodnina, 2004). To measure the overall selectivity, initiation complexes with the CUC codon in the A site (0.5 μM, 50 pmol per assay) were mixed with an excess of 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 incubation for 1 min at 20ºC, the reactions were stopped by the addition of 0.5 M KOH, incubated 15 min at 37ºC, and neutralized with acetic acid, and dipeptides were analyzed by HLPC as described above. A gradient of 0%–65% acetonitril in 0.1% TFA was adapted in such a way as to separate [^{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.

The efficiency of initial selection was calculated from the values of rate constants k_1, k_{-1}, k_2, k_{-2}, and k_3 (Table 1) in the following way. The k_{cat}/K_M parameters for the GTPase reaction were calculated according to the equation $k_{cat}/K_M = k_1 \cdot k_2 \cdot k_3/((k_{-1}+k_2) \cdot (k_{-2}+k_3) - k_2 \cdot k_{-2})$ (Pape et al., 1999) for the cognate codon (UUC) and each of the near-cognate codons. The selectivity at the initial selection step, I, was estimated as $I = (k_{cat}/K_M)_{cognate}/(k_{cat}/K_M)_{near\text{-}cognate}$. Overall selectivity, S, is the product of I (calculated) and the efficiency of proofreading, P (measured for all codons at both HiFi and LoFi). Error frequency at equal concentrations of cognate and near-cognate ternary complexes is E = 1/(1+A). The observed error frequency depends on the tRNA concentrations as well, $E_{obs} = E \cdot [near\text{-}cognate]/[cognate]$.

The rates of GTP hydrolysis in cognate ternary complex in the presence of excess of nearcognate and noncognate ternary complexes were calculated according to the following equation: $V_{cognate} = (k_{cat}/K_M)_{cognate} \cdot [cognate]/(1+[cognate]/(K_M)_{cognate} + [near\text{-}cognate]/(K_M)_{near\text{-}cognate} + [noncognate]/(K_M)_{noncognate})$. The k_{cat} and K_M values were estimated from the rate constants of Table 1 as described (Gromadski and Rodnina, 2004). The K_M for noncognate ternary complex was 2 μM (to be published elsewhere).

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