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Optimization of speed and accuracy of decoding in translation

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The speed and accuracy of protein synthesis are fundamental parameters for understanding the fitness of living cells, the quality control of translation, and the evolution of ribosomes. In this study, we analyse the speed and accuracy of the decoding step under conditions reproducing the high speed of translation *in vivo*. We show that error frequency is close to 10^{-3}, consistent with the values measured *in vivo*. Selectivity is predominantly due to the differences in k_{cat} values for cognate and near-cognate reactions, whereas the intrinsic affinity differences are not used for tRNA discrimination. Thus, the ribosome seems to be optimized towards high speed of translation at the cost of fidelity. Competition with near- and non-cognate ternary complexes reduces the rate of GTP hydrolysis in the cognate ternary complex, but does not appreciably affect the rate-limiting tRNA accommodation step. The GTP hydrolysis step is crucial for the optimization of both the speed and accuracy, which explains the necessity for the trade-off between the two fundamental parameters of translation.

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Introduction

Speed and accuracy of translation determine cell growth and the quality of newly synthesized proteins. The rate of protein elongation in *Escherichia coli* was estimated to be 4–22 s^{-1} per codon at 37°C (Bremer and Dennis, 1987; Sorensen and Pedersen, 1991; Liang *et al*, 2000; Proshkin *et al*, 2010). Estimations of error frequencies range between 10^{-5} and 10^{-3} depending on the type of measurement, concentrations and nature of tRNAs that perform misreading, and the mRNA context (Parker, 1989; Kramer and Farabaugh, 2007; Drummond and Wilke, 2009). Aminoacyl-tRNAs (aa-tRNAs) are delivered to the ribosome in a ternary complex with EF-Tu and GTP. The selection of an aa-tRNA that is cognate to the mRNA codon is accomplished by a kinetic discrimination mechanism (Pape *et al*, 1999; Gromadski and Rodnina, 2004a), which operates in two stages: initial selection and proofreading (Thompson and Stone, 1977; Ruusala *et al*, 1982). Initial selection begins with the codon-independent initial binding of a ternary complex, EF-Tu·GTP·aa-tRNA, to the ribosome (Figure 1A; Rodnina *et al*, 1996; Gromadski and Rodnina, 2004a; Diaconu *et al*, 2005). Initial binding is followed by sampling the A-site codon in the decoding centre by the anticodon of the aa-tRNA (Blanchard *et al*, 2004; Marshall *et al*, 2008). Correct codon–anticodon pairing results in conformational changes of the ribosome, aa-tRNA, and EF-Tu (Rodnina *et al*, 1994; Ogle *et al*, 2001, 2002; Rodnina and Wintermeyer, 2001; Cochella and Green, 2005; Pan *et al*, 2008; Schmeing *et al*, 2009; Schuette *et al*, 2009; Villa *et al*, 2009), which ultimately lead to GTP hydrolysis by EF-Tu (Rodnina *et al*, 1995; Pape *et al*, 1999; Gromadski and Rodnina, 2004a; Lee *et al*, 2007). If the codon–anticodon duplex contains a mismatch, that is, the aa-tRNA is near-cognate to the codon, the concerted rearrangements do not occur, or are different (Ogle *et al*, 2002), and GTPase activation of EF-Tu is slow (Pape *et al*, 1999; Gromadski and Rodnina, 2004a; Gromadski *et al*, 2006; Lee *et al*, 2007). In addition, near-cognate ternary complexes dissociate rapidly from the ribosome, whereas cognate ones are bound very tightly (Thompson and Karim, 1982; Pape *et al*, 1999; Gromadski and Rodnina, 2004a; Cochella and Green, 2005; Daviter *et al*, 2006). Partitioning between GTPase activation and ternary complex dissociation strongly favours acceptance of cognate and rejection of near-cognate ternary complexes. The hydrolysis of GTP irreversibly separates the initial selection stage from the proofreading stage. During the proofreading stage, the acceptor stem of aa-tRNA released from EF-Tu·GDP moves into the ribosome and accommodates at the peptidyl transferase centre. The accommodation of cognate aa-tRNA is rapid and efficient; in contrast, the accommodation of near-cognate tRNA is slow and results in the preferential rejection of near-cognate aa-tRNA (Pape *et al*, 1999). Accommodation is followed by, and in some cases may limit the rate of, irreversible peptide bond formation (Pape *et al*, 1999; Bieling *et al*, 2006).

One major unresolved question concerns the maximum capacity of the ribosome for tRNA discrimination. In the codon-recognition complex, the K_d values for cognate and near-cognate ternary complexes differ by about 1000-fold (Thompson and Karim, 1982; Ogle *et al*, 2002; Gromadski and Rodnina, 2004a; Daviter *et al*, 2006). However, this large inherent $\Delta\Delta G^\circ$ of binding is not fully used. This is because in the cognate complex the following steps of GTPase activation and GTP hydrolysis are rapid, precluding the equilibration of the binding step and increasing the K_M (Gromadski and Rodnina, 2004a). Such design allows for rapid translation; however, the maximum of intrinsically possible discrimination is not achieved, a phenomenon known as trade-off between speed and accuracy (Thompson and Karim, 1982; Lovmar and Ehrenberg, 2006). In fact, despite the large

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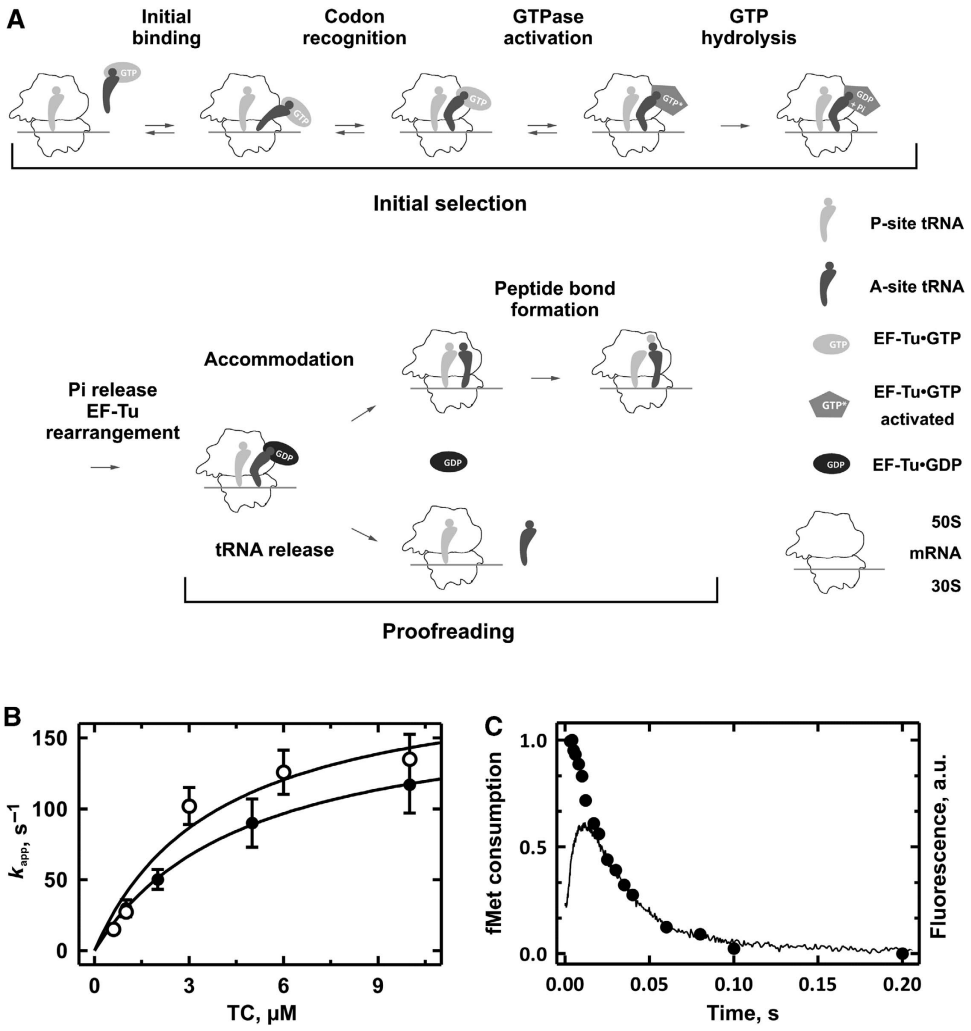

Figure 1 Dipeptide formation on a cognate codon. (**A**) Schematic of the decoding mechanism. Kinetically resolved steps are indicated (Gromadski and Rodnina, 2004a). (**B**) Dipeptide (fMetPhe) formation in HiFi (open circles) or polymix (closed circles) buffer at 37°C. Increasing amounts of ternary complex (TC = EF-Tu · GTP · Phe-tRNA^{Phe}) were added to initiation complex with a UUC codon at the A site. (**C**) Time courses of accommodation and dipeptide formation. Peptide bond formation is shown as consumption of fMet-tRNA^{fMet} substrate (left *y*-axis). Accommodation was monitored by the fluorescence decrease after the binding of EF-Tu · GTP · Phe-tRNA^{Phe}(Prf16/17) to the initiation complex (right *y*-axis); a.u., arbitrary units.

differences in the affinities and in the rates of forward reactions for cognate and near-cognate aa-tRNAs both at initial selection and proofreading stages, the error frequency obtained *in vitro* was close to 10^{-2}–10^{-3} under a variety of conditions (Pape *et al*, 1999; Gromadski and Rodnina, 2004a; Cochella and Green, 2005; Daviter *et al*, 2006; Lee *et al*, 2007). Recently, Ehrenberg and colleagues reported a substantially lower missense error rate of 3×10^{-7}, as calculated from the k_{cat}/K_M values measured for Phe-tRNA on a cognate UUU and a near-cognate CUU codon in 'polymix' buffer at 37°C (Johansson *et al*, 2008). The K_M value for the near-cognate aa-tRNA was quite high at $>20\,\mu M$. These data may have several important implications. First, the high K_M value would suggest that the ribosome is capable of increasing the $\Delta\Delta G^\circ$ of cognate and near-cognate codon–anticodon interactions to an extent that has not been observed before. Second, due to the high K_M value for the near-cognate substrate, there would be practically no competition with the cognate ones, which, as a consequence, would decode their codons at maximum speed. Third, very high accuracy would suggest that there is essentially no trade-off between speed and accuracy of decoding. Finally, the authors argued that, under the conditions used by them, the accommodation step was not rate-limiting for peptide bond formation (Johansson *et al*, 2008). The conceptual importance of these findings for understanding the decoding mechanism has prompted us to re-analyse the kinetics of peptide bond formation under conditions reproducing the high speed of translation *in vivo* and to directly measure the error frequency in polymix buffer at 37°C. For comparison, we used two buffer systems that were reported to mimic conditions of rapid protein synthesis in the cell, the polymix buffer used by Johansson *et al* (2008) and the HiFi buffer used by our group (Gromadski and Rodnina, 2004a). Using rapid kinetics techniques, we compared the rate constants of peptide bond formation for cognate and near-cognate tRNAs, measured error frequencies, and studied the effect of competition on the speed of decoding by the cognate aa-tRNA.

Results

Rate of dipeptide formation with cognate aa-tRNA

Time courses of fMetPhe formation were measured by quench-flow (Materials and methods section), mixing ribosomal initiation complex containing fMet-tRNA^{fMet} at the P site and a UUC codon at the A site with excess of ternary complex, EF-Tu · GTP · Phe-tRNA^{Phe}. In contrast to our routine protocol (Gromadski and Rodnina, 2004a), the ternary complex was formed *in situ* and used without further purification, as described previously (Johansson *et al*, 2008). Apparent rate constants (k_{app}) for peptide bond formation were determined at increasing concentrations of ternary complex. The concentration dependence of k_{app} was hyperbolic and at saturation gave the rate constant of dipeptide formation, k_{dip}, of about 170 s^{-1} in polymix buffer at 37°C (Figure 1B). The titration yielded also $K_M = 4.8$ μM (Figure 1B; Table I), in agreement with the results of Johansson *et al* (2008), who reported values of $k_{dip} = 130\ s^{-1}$ and $K_M = 4.5$ μM. In HiFi buffer at 37°C, the rate of dipeptide formation was higher, 200 s^{-1}, and the K_M value was approximately the same (4 μM). Thus, the rate constant of peptide bond formation at 37°C in either buffer was compatible with the rate of elongation *in vivo*. Analogous experiments in HiFi buffer at 20°C, in which purified and non-purified ternary complexes were compared or an excess of ribosome complex over ternary complex was used, yielded k_{dip} values between 12 (a value close to 7 s^{-1} was obtained previously using purified components and excess of initiation complex over ternary complex (Gromadski and Rodnina, 2004a)) and 22 s^{-1} (excess unpurified ternary complex (Table I)); the latter value reproduced the value reported for polymix buffer at 20°C, 26 s^{-1} (Johansson *et al*, 2008); thus, the two buffer systems yielded comparable rates of peptide bond formation. The K_M values were consistently higher with unpurified ternary complexes, suggesting that the effective concentration of EF-Tu · GTP · Phe-tRNA^{Phe} in the unpurified mixture was overestimated. It should be noted that the rate of peptide bond formation is strongly affected by Mg^{2+} and polyamines; therefore, comparisons with other buffer systems (e.g. without or with different polyamines) have to be carried out with caution.

Next, we examined whether the accommodation step is in fact not limiting the rate of peptide bond formation in polymix at 37°C, as suggested previously (Johansson *et al*, 2008). The time course of the accommodation step was monitored by the fluorescence change in Phe-tRNA^{Phe}(Prf16/17); the label reports conformational changes and movements of the tRNA through the ribosome but is insensitive to peptide bond formation (Pape *et al*, 1998). The resulting time course was biphasic, with the fast step (fluorescence increase) reflecting the binding of the ternary complex to the ribosome and all following steps up to GTP hydrolysis (Figure 1C), whereas the slower phase (fluorescence decrease) reflects the accommodation of the aa-tRNA at the A site (Pape *et al*, 1998; Gromadski and Rodnina, 2004a). The rate of the second step at the present conditions was 40 s^{-1}. The rate of peptide bond formation measured by quench-flow in parallel was the same, 40 s^{-1}, indicating that, at least with Phe-tRNA^{Phe}, accommodation is rate-limiting for peptide bond formation not only in the buffers used previously (Pape *et al*, 1998, 1999), but in polymix buffer at 37°C (pH 7.5) as well. The rate-limiting step of peptidyl transfer to other aa-tRNAs (e.g. prolyl-tRNA for which the reaction seems to be slow (Pavlov *et al*, 2009)) remains to be determined.

Missense error frequency

To estimate the error frequency of decoding, we used two independent methods. First, we used the approach proposed by Johansson *et al* (2008). An excess of ternary complex with Phe-tRNA^{Phe} was added to initiation complex displaying the near-cognate CUC (Leu) codon at the A site; time courses of fMetPhe formation were measured at increasing ternary complex concentrations in polymix buffer at 37°C (Figure 2A). As the reactions were carried out at multiple-turnover conditions, k_{app} values were determined by linear fitting of the initial parts of the time courses. The concentration dependence of k_{app} was hyperbolic with k_{dip} (near-cognate) = 0.26 s^{-1} and $K_M = 1.9$ μM (Figure 2B). From the ratio between the k_{dip} and K_M values for near-cognate and cognate codons, an error frequency of 3×10^{-3} was obtained. This value was four orders of magnitude higher than that reported by Ehrenberg and colleagues (Johansson *et al*, 2008), but in full agreement with our previous data measured at 20°C in HiFi buffer (Gromadski and Rodnina, 2004a; Gromadski *et al*, 2006).

The K_M value of 1.9 μM (Figure 2B), which is quite different from that reported by Ehrenberg and colleagues (near-cognate $K_M > 20$ μM), was further verified by the alternative approach by measuring dipeptide formation under single-turnover conditions with an excess of initiation complex over near-cognate ternary complex (Figure 2C). In the absence of a competing cognate ternary complex, the initial selection step is inefficient and near-cognate substrates are rejected at the proofreading stage. Two parameters can be extracted from the analysis of the time courses: The amplitude of fMetPhe formation (fMetPhe/TC), which gives the error frequency of proofreading alone, and the apparent rate

Table I Rates of cognate peptide bond formation

Buffer	T (°C)	Conditions	k_{dip} (s^{-1})	K_M (μM)	k_{dip}/K_M ($\mu M^{-1}\ s^{-1}$)
Polymix	37	Unpurified, TC > IC	174 ± 4	4.8 ± 0.2	36
Polymix[a]	37	Unpurified, TC > IC	130 ± 10	4.5 ± 0.5	29
HiFi	37	Unpurified, TC > IC	200 ± 40	3.9 ± 1.9	51
Polymix[a]	20	Unpurified, TC > IC	26 ± 1	2.0 ± 0.3	13
HiFi	20	Unpurified, TC > IC	22 ± 4	1.4 ± 0.5	16
HiFi	20	Purified, TC > IC	14 ± 4	0.5 ± 0.4	28
HiFi	20	Purified, IC > TC	12 ± 1	0.3 ± 0.1	40

IC, initiation complex; TC, ternary complex.
[a]From Johansson *et al* (2008).

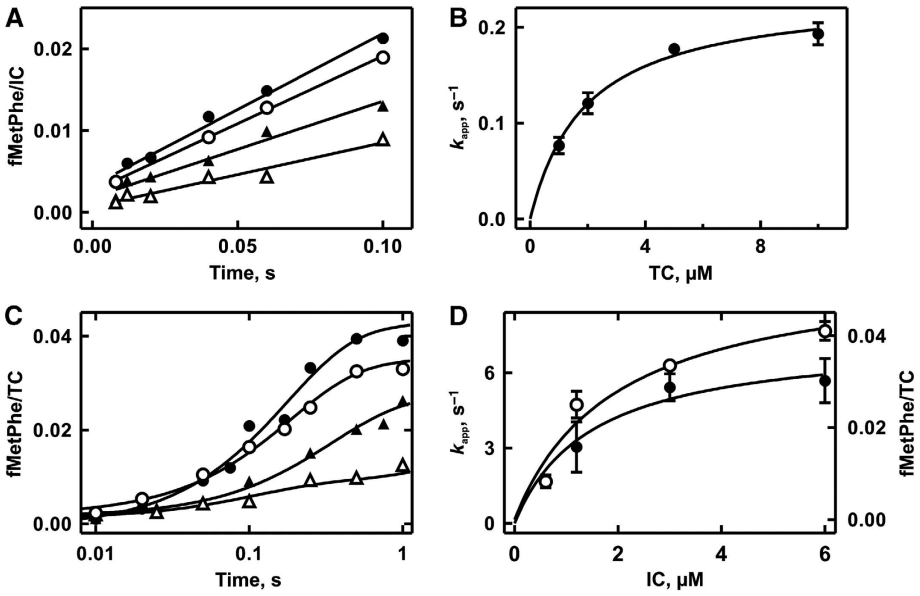

Figure 2 Dipeptide formation on a near-cognate codon. (**A**) Time courses of fMetPhe formation under multiple-turnover conditions in polymix buffer. A fixed amount of initiation complex (CUC codon at the A site) was mixed with varying concentrations of EF-Tu · GTP · Phe-tRNA^{Phe}: 10 (closed circles), 5 (open circles), 2 (closed triangles), and 1 μM (open triangles). (**B**) Concentration dependence of dipeptide formation under multiple-turnover conditions. (**C**) Time courses of dipeptide formation under single-turnover conditions measured with limiting concentration of purified ternary complex and varying concentrations of initiation complex: 6 (closed circles), 3 (open circles), 1.2 (closed triangles), and 0.6 μM (open triangles). (**D**) Rate (closed circles, left *y*-axis) and amplitude (open circles, right *y*-axis) dependence of dipeptide formation under single-turnover conditions.

constant (k_{app}), which at saturation is the sum of the $k_{dip} + k_{rejection}$; the concentration dependence of either k_{app} or of the amplitude yields an independent estimate of the K_M value for near-cognate binding. The time courses of dipeptide formation were single-exponential, and the concentration dependencies of both amplitude and k_{app} were hyperbolic, yielding K_M values of 1.5–2.0 μM (Figure 2D), consistent with the K_M value determined above under conditions of multiple turnover. The yield of dipeptide at saturation, that is, the error frequency of proofreading, was 0.055, in agreement with our previous value, 0.06 (Gromadski and Rodnina, 2004a). An alternative calculation using the equation $k_{app} = k_{dip} + k_{rejection}$, combined with the value k_{dip}(near-cognate) $= 0.26\,s^{-1}$, gives $k_{rejection} = 7.1\,s^{-1}$ and an error frequency of proofreading of 0.035; in agreement, within experimental error, with the value measured directly (also see below).

As a second independent approach to determine the error frequency, we directly measured the incorporation of Phe and Leu on a CUC codon in a competition assay in which the respective ternary complexes with [^{3}H]Phe-tRNA^{Phe} and [^{14}C]Leu-tRNA^{Leu}(CUC) were present in excess over ribosomes, both in polymix and HiFi buffer at 37°C. For this experiment, the two ternary complexes were purified by gel filtration. Misincorporation was quantified by determining the amounts of fMetLeu (cognate) and fMetPhe (near-cognate) formed. The overall error frequency normalized for equal concentrations for Phe-tRNA^{Phe} and Leu-tRNA^{Leu} was 5.6×10^{-3} for polymix (Figure 3), in agreement with the value of 3×10^{-3} determined above from the ratio of k_{dip}/K_M values. The misincorporation error frequency in HiFi buffer at 37°C was slightly lower (4×10^{-3}) and similar to that measured in HiFi buffer at 20°C (2×10^{-3}; (Gromadski and Rodnina, 2004a)). The relative contributions of initial selection (I) and proofreading (P) stages were quite similar in HiFi and polymix buffers ($I = 0.12$ and 0.22 in HiFi and polymix, respectively; P is about 0.03 in both buffers). Thus, the results obtained by the two different approaches yield error frequencies in the range of $3–6 \times 10^{-3}$.

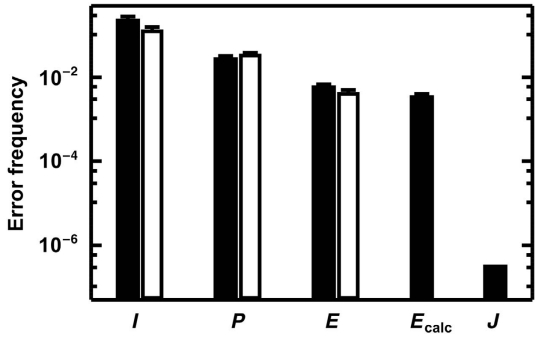

Figure 3 Error frequencies in HiFi and polymix buffers. Error frequencies were determined for polymix (black bars) and HiFi (white bars) buffers. The contribution of initial selection (*I*) was calculated from the overall error frequency (*E*) and the measured error frequency of proofreading (*P*). The calculated error frequency (E_{calc}) was determined from the ratio of k_{cat}/K_M values determined for fMetPhe dipeptide formation on near-cognate and cognate codons. The overall error frequency reported by Johansson *et al* (2008) is shown for comparison (*J*).

Potential sources of apparent very high fidelity

The fact that very similar error frequencies were observed in polymix and HiFi buffers excludes buffer composition as a source of the marked fidelity differences observed in our experiments and in those of Ehrenberg and colleagues. Furthermore, rate constants of cognate dipeptide formation reported by the two groups are very similar, arguing against

significant differences in the activities of ribosomes, EF-Tu, or tRNAs. Differences due to the mRNA constructs were examined and excluded previously (Johansson *et al*, 2008). Furthermore, the experimental setup (excess of initiation complexes over ternary complexes or *vice versa*) does not seem to have a strong effect. However, we note that Johansson *et al* (2008) applied different experimental protocols for the cognate and near-cognate decoding. In the cognate case, ternary complex and initiation complex mixtures were prepared under the same conditions, that is, in the presence of 1 mM ATP, 1 mM GTP, and 10 mM phosphoenol pyruvate (PEP). However, when working with the near-cognate complexes, Johansson *et al* (2008) used twice the concentrations of ATP, GTP, and PEP (2 mM, 2 mM, and 20 mM, respectively) in the ternary complex mixture. When we measured the concentration dependence of the initial velocity of dipeptide formation on a near-cognate codon using ATP, GTP, and PEP at these high concentrations, we also observed very low k_{app} values that did not saturate even at 15 μM ternary complex (Figure 4A, inset), indicating a very high K_M value similar to the data obtained by Ehrenberg and colleagues. From the ratio of near-cognate and cognate k_{cat} and K_M values obtained from these data, we could reproduce the apparent very low error frequency of CUU decoding reported by Johansson *et al* (2008) (Figure 4A).

Parameters such as pH, ionic strength, or free Mg^{2+} concentration can be altered when reaction components are added to the polymix buffer. In particular, the free Mg^{2+} concentration decreases compared with the initial concentration (5 mM in polymix buffer) on the addition of 1 mM ATP, 1 mM GTP, and 10 mM PEP due to the chelation of Mg^{2+}. As ATP and GTP each bind Mg^{2+} at a 1:1 ratio, Ehrenberg and colleagues pointed out that the free Mg^{2+} concentration decreased correspondingly on addition of NTPs (Pavlov *et al*, 2009). However, also PEP binds Mg^{2+} ions with millimolar affinity, with a K_d value of about 10 mM at 100 mM monovalent salt (Manchester, 1980). In fact, it was shown that PEP shifts the Mg^{2+} optimum of poly(Phe) synthesis by 0.5 mM Mg^{2+} for each additional 1 mM PEP, suggesting a binding ratio of approximately 0.5 Mg^{2+}/PEP at 5–10 mM PEP (Manchester and Alford, 1979). To test the effect of chelating Mg^{2+} ions, we mixed purified initiation complex with a CUU codon at the A site with an excess of near-cognate EF-Tu·GTP·Phe-tRNA^{Phe} complex and measured time courses of product formation in polymix buffer (Figure 4B, note the logarithmic *y*-axis). Under conditions of multiple turnover, that is, excess of near-cognate ternary complexes over ribosomes, all ribosomes should eventually form a dipeptide with the near-cognate aa-tRNA, reaching the end level close to one fMetPhe/IC. The addition of 1 mM ATP and 1 mM GTP not compensated by additional Mg^{2+} results in a much lower extent of near-cognate dipeptide formation; the addition of 10 mM PEP had a comparable effect. The effect of adding GTP, ATP, and PEP without compensating Mg^{2+} was cumulative and virtually abolished near-cognate dipeptide formation. Notably, from the 5 mM Mg^{2+} added, 2 mM

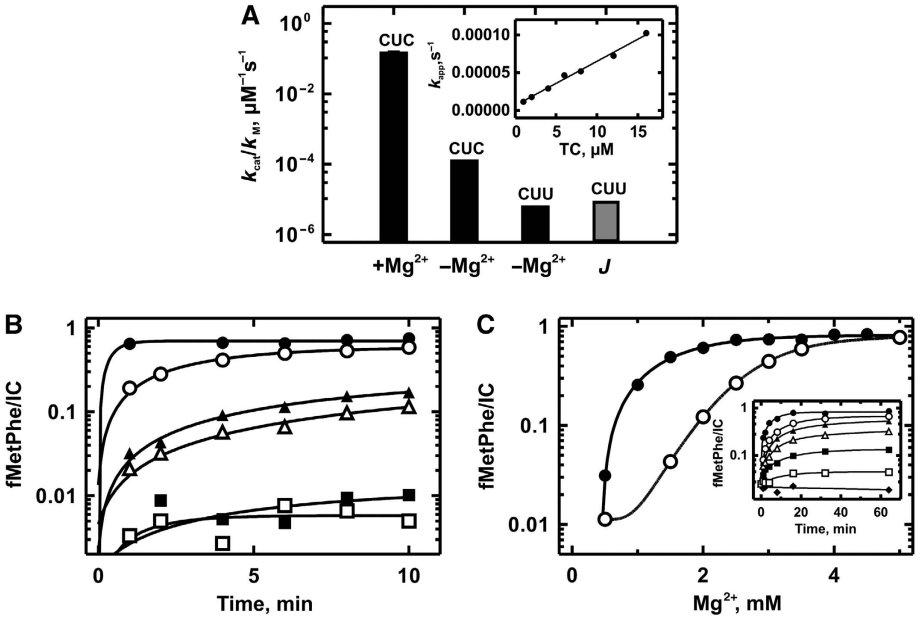

Figure 4 Effect of the Mg^{2+} concentration on dipeptide formation on near-cognate codons. (**A**) Catalytic efficiency (k_{cat}/K_M) of dipeptide formation on CUC or CUU codons with (+Mg^{2+}) or without (−Mg^{2+}) addition of Mg^{2+} to compensate for Mg^{2+}-chelating compounds (ATP, 1 mM; GTP, 1 mM; PEP, 10 mM; black bars); the published value (Johansson *et al*, 2008) obtained under the latter conditions is also indicated (*J*, grey bar). Inset: Dipeptide formation (k_{app}) in the presence of uncompensated NTPs and PEP (CUU codon). (**B**) Effect of Mg^{2+}-binding compounds on dipeptide formation. Ternary complex with Phe-tRNA^{Phe} was added to initiation complex (CUU at the A site) in polymix buffer without further additions (open circles) or after addition of 1 mM ATP/GTP each (closed triangles); 10 mM PEP (open triangles,); or 1 mM ATP/GTP each and 10 mM PEP (open squares). ATP, GTP, and PEP were added with the ternary complex without compensating by Mg^{2+} as described previously (Johansson *et al*, 2008). As a control, the same amounts of non-compensated ATP, GTP, and PEP were added to the cognate reaction (UUC codon) either with the ternary complex (as in Johansson *et al.*, 2008; closed circles) or with the initiation complex (closed squares). (**C**) Mg^{2+} dependence of fMetPhe dipeptide formation on cognate (UUC, closed circles) and near-cognate (CUU, open circles) codons. Amplitudes are plotted against the concentration of free Mg^{2+}. Inset: time courses of dipeptide formation on the near-cognate CUU codon at increasing concentration of Mg^{2+} (mM): 0.5 (closed diamonds), 1.5 (open squares), 2 (closed squares), 2.5 (open triangles), 3 (closed triangles), 3.5 (open circles) and 5 (closed circles).

Mg^{2+} were chelated by ATP and GTP, and addition of 10 mM PEP can be expected to further reduce free Mg^{2+} concentration to close to 1.5 mM assuming a K_d value of 10 mM (Manchester, 1980) or even lower assuming the binding ratio 0.5 Mg^{2+}/PEP (Manchester and Alford, 1979). The cognate reaction (UUC codon at the A site) was not affected appreciably when the NTPs and PEP were added with the ternary complex, but was strongly impaired by pre-incubation with the initiation complex, suggesting that initiation complexes are particularly sensitive to very low concentrations of Mg^{2+}. The effect can be reversed by compensating NTPs and PEP with Mg^{2+} (Figure 4B, open circles and data not shown), suggesting that the low efficiency of dipeptide formation observed with near-cognate aa-tRNA can be attributed to the Mg^{2+}-chelating properties of NTPs and PEP.

The reduced end points of near-cognate reactions at the artificially low concentration of free Mg^{2+} caused by adding NTPs and PEP suggest inactivation of the reaction components. To determine the lowest free Mg^{2+} concentrations that can be tolerated without significant inactivation, reaction end points were measured at decreasing Mg^{2+} concentrations at low PEP concentrations (0.5 mM that is sufficient for the conversion to GTP of the submicromolar amounts of GDP formed in the reaction) and GTP (0.5 mM, compensated by Mg^{2+}). At Mg^{2+} concentrations above 3 mM, the end point of dipeptide formation was similarly high for cognate and near-cognate reactions (Figure 4C), as expected for a well-behaved system. At lower Mg^{2+} concentrations, the end points of the near-cognate reaction were decreased more than those of the cognate one, and both reactions were abolished at 1 mM free Mg^{2+}. These data demonstrate that in the multiple-turnover assay substrates are partially inactivated at free Mg^{2+} concentrations below 3 mM. Thus, the apparent low error frequency reported by Johansson *et al* (2008) at free Mg^{2+} concentration close to 1.5 mM seems to be due to the preferential impairment of the reaction with near-cognate substrates due to the chelation of Mg^{2+} in the presence of a large excess of NTPs and PEP.

Effect of ternary complex competition on GTP hydrolysis and peptide bond formation

Our present data confirmed that the K_M values for near-cognate ternary complexes can be comparable to those for the cognate complexes (Gromadski and Rodnina, 2004a). This raises the question of how the ribosome deals with the competition between cognate and excess near- and non-cognate ternary complexes prevailing *in vivo*. To address this question, we measured the rates of GTP hydrolysis and peptide bond formation for mixtures of cognate, near-cognate, and non-cognate ternary complexes on ribosomes containing f[^{3}H]Met-tRNA^{fMet} at the P site and a UUC (Phe) codon at the A site. Separate ternary complexes were prepared and purified by gel filtration: the cognate ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} and the mixture of near- and non-cognate ternary complexes prepared from total tRNA by aminoacylation with a mixture of 19 non-radioactive amino acids excluding phenylalanine (Materials and methods section). The ternary complex mixture was prepared in such a way that the concentration of cognate Phe-tRNA^{Phe} ternary complex was fixed and the concentrations of near-cognate and cognate complexes were varied. The ternary complex mixture was added to the initiation complex, and time courses of GTP hydrolysis and peptide bond formation were measured by quench-flow. The observed rate reflects GTP hydrolysis in the cognate ternary complex, because GTP hydrolysis in the near- and non-cognate ternary complexes is much slower (>100-fold), even in the absence of the cognate competitor (Gromadski and Rodnina, 2004a; Gromadski *et al*, 2006), and is not significant in the time window used in this study. The rate of GTP hydrolysis in the cognate complex decreased more than 10-fold on addition of a 30-fold excess of competing ternary complexes (Figure 5). However, the rate of peptide bond formation with cognate aa-tRNA was not affected by the addition of near- and non-cognate competitors (Figure 5). Thus, the competition between cognate and near- or non-cognate ternary complexes reduced the rate of GTP hydrolysis, but not that of subsequent peptide bond formation.

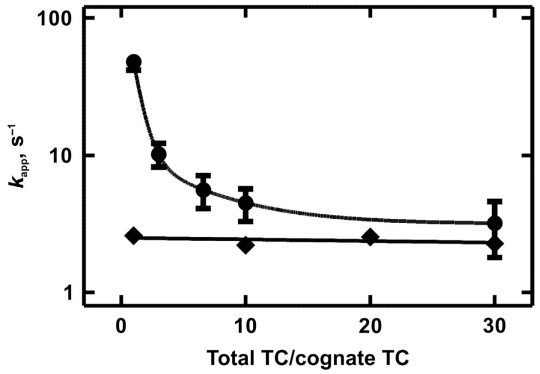

Figure 5 Effect of competition on cognate reaction. Rates (k_{app}) of GTP hydrolysis (closed circles) and dipeptide formation (closed diamonds) were measured on addition of increasing concentrations of competing near- and non-cognate ternary complexes. *x*-axis, molar excess of near- and non-cognate over cognate ternary complexes.

Discussion

Accuracy of translation

The overall accuracy of protein synthesis on the ribosome is determined by three basic selection stages: the selection of ternary complexes before GTP hydrolysis at the initial selection stage; preferential rejection of near-cognate aminoacyl-tRNAs at the proofreading stage after GTP hydrolysis (Thompson and Stone, 1977; Ruusala *et al*, 1982; Rodnina and Wintermeyer, 2001) and preferential release of near-cognate peptidyl-tRNAs by the termination factors (Zaher and Green, 2009). The missense error frequency of translation in the cell depends on the combined efficacy of these three stages and on the abundance of the aa-tRNA cognate to the given codon relative to the near-cognate competitors. The combined error frequency of initial selection and proofreading *in vitro* was observed to be close to 10^{-3} at different temperatures in both polymix and HiFi buffers (this study and Gromadski and Rodnina, 2004a, 2004b; Gromadski *et al*, 2006). Consistently, fidelity measurements conducted in the full range of published buffer systems with tRNA mixtures on heteropolymeric mRNA suggested that *in vitro* protein synthesis proceeds with an error rate of 2×10^{-3}–1×10^{-2} (Zaher and Green, 2009). A single initial miscoding event is then amplified by preferential incorporation of a wrong amino acid

at the following codon, followed by premature chain termination, thereby reducing the observed error frequency of synthesis of a full-length protein approximately 10-fold (Zaher and Green, 2009). Thus, the overall error frequency is expected to be around 10^{-4}, which may be further modulated by the tRNA concentrations in the cell. Assuming an average protein length in *E. coli* of about 300 amino acids (Netzer and Hartl, 1997), this means that most of the proteins in the cell are synthesized correctly.

According to the present results, both rate and fidelity of decoding measured *in vitro* are fully compatible with the values observed *in vivo* and provide new insight into the mechanism of aa-tRNA selection at the proofreading stage. In polymix buffer at 37°C, the rate of accommodation is 650-fold higher with cognate ($170\,s^{-1}$) than with near-cognate ($0.26\,s^{-1}$) aa-tRNA. Conversely, rejection of near-cognate aa-tRNA during accommodation is rapid ($7.1\,s^{-1}$), resulting in efficient proofreading, whereas cognate aa-tRNA does not dissociate to any significant extent during accommodation.

Optimization of the speed of protein synthesis

A significant trade-off between speed and accuracy is a consequence of the necessity for rapid protein synthesis in the cell. If ribosomes functioned in the way proposed by Johansson *et al* (2008), the trade-off between the speed and accuracy would be negligible, because the K_M value for the near-cognate ternary complex would be much higher than that for the cognate one, and thus the discrimination between aa-tRNAs would use the (huge) $\Delta\Delta G^\circ$ of binding, in addition to potential differences in k_{cat} values. Our present results exclude this scenario and further support the kinetic discrimination mechanism derived from kinetic measurements performed under a variety of conditions (Pape *et al*, 1999; Gromadski and Rodnina, 2004a; Gromadski *et al*, 2006; Kothe and Rodnina, 2007). Although k_{cat} values for the peptidyl transfer reaction are grossly different between cognate and near-cognate aa-tRNAs, K_M values are very similar, indicating that the inherent affinity differences between cognate and near-cognate complexes are not used for selection (see below and Gromadski and Rodnina, 2004a). Thus, the potential for very accurate substrate selection in translation is sacrificed in favour of speed.

It has been argued that if the K_M values for cognate aa-tRNA were comparable to those for near-cognate and non-cognate substrates, this would lead to slow cognate amino-acid incorporation due to competition by the large excess of incorrect ternary complexes (Bilgin *et al*, 1988; Lovmar and Ehrenberg, 2006). The present data suggest why this does not occur. Although excess near- and non-cognate ternary complexes reduce the rate of GTP hydrolysis in the cognate ternary complex, the overall faster rates of GTP hydrolysis relative to peptide bond formation buffer the inhibitory effect such that a 10-fold decrease in the rate of GTP hydrolysis, due to a high excess of competing ternary complexes, is not reflected in a decrease of the rate of cognate peptide bond formation (Figure 5). This suggests that the predicted seven-fold decrease in the rate of dipeptide formation due to competition (Lovmar and Ehrenberg, 2006) will not be observed. Indeed, a more realistic model in which there is competition between ternary complexes at the codon recognition step, but not after GTP hydrolysis, gives results that are consistent with the experimental data obtained at 20°C. Predictions for 37°C are more challenging, because many parameters, in particular the K_M values for the GTPase reaction, cannot be determined experimentally due to the very high reaction rate for the cognate substrate. Nevertheless, estimates can be made based on the K_M values for the peptidyl transfer reaction and the rate constants of GTP hydrolysis and peptide bond formation reported in this study and in the study by Johansson *et al* (2008). Assuming conservatively that (i) the K_M values for the GTPase reaction are similar to or higher than that for peptide bond formation and (ii) the K_M values for cognate (2% of total aa-tRNA) are roughly the same as for the mixture of near-cognate (about 10% of total aa-tRNA) and non-cognate ternary complexes, the rate of peptide bond formation on a cognate codon in the presence of a 30-fold excess of competing near- and non-cognate ternary complexes is estimated to be close to $20\,s^{-1}$. This value is fully consistent with the *in vivo* rate of protein synthesis. Thus, the *in vitro* data that lead to the kinetic discrimination model of decoding are fully compatible with the *in vivo* measurements of the rate and accuracy of protein synthesis, and thus are likely to hold for the conditions *in vivo* as well. The high speed of GTPase activation and GTP hydrolysis in the cognate ternary complex causes a loss in the fidelity of selection, but at the same time precludes that the rate of cognate peptide bond formation is decreased by competition with bulk ternary complexes. This suggests that it is the rate of GTP hydrolysis, and not that of peptide bond formation, which governs the evolution and the optimization of speed and accuracy of translation, and explains why the maximum accuracy intrinsic in the system is not achieved.

Materials and methods

Buffers and reagents

HiFi buffer: 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. Polymix buffer: 5 mM potassium phosphate (pH 7.5), 95 mM KCl, 5 mM NH_4Cl, 5 mM magnesium acetate, 0.5 mM $CaCl_2$, 1 mM spermidine, 8 mM putrescine, and 1 mM DTE. Buffer A: 50 mM Tris–HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, and 7 mM $MgCl_2$. Buffer B: 20 mM HEPES–HCl (pH 7.5), 95 mM KCl, 5 mM NH_4Cl, 5 mM magnesium acetate, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, and 1 mM DTE. Chemicals were from Roche Molecular Biochemicals, Sigma Aldrich, or Merck. Radioactive compounds were from Hartmann Analytic. MNV mRNA (5′-GGCAAGGAGG UAAAUA*AUGNNNACGAUU*-3′, in which the coding sequence is in italics and the A-site codon (underlined) is NNN = UUC, CUC, or CUU, coding for Phe or Leu, was purchased from Microsynth.

Ribosomes from *E. coli* MRE600, initiation factors, EF-Tu, and tRNAs were prepared as described previously (Gromadski and Rodnina, 2004a). Initiation complexes were prepared by incubating ribosomes (1 μM) with a three-fold molar excess of mRNA, initiation factors 1, 2, and 3 (1.5 μM each), f[^{3}H]Met-tRNA^{fMet} (1.5 μM), and GTP (1 mM) in buffer A (for experiments in HiFi buffer) or B (for experiments in polymix buffer) for 45 min at 37°C. The complexes were purified by centrifugation through 400 μl sucrose cushions (1.1 M sucrose in buffer A or B, respectively) at 260 000 *g* for 2 h. Pellets were dissolved in HiFi or polymix buffer to a final concentration of 12 μM, shock-frozen in liquid nitrogen, and stored at −80°C. Ternary complexes, EF-Tu·GTP·Phe-tRNA^{Phe} or EF-Tu·GTP·Leu-tRNA^{Leu} (specific for CUC codon) were prepared by incubating EF-Tu (30 μM), GTP (1 mM), PEP (3 mM), pyruvate kinase (0.05 mg ml^{-1}), purified Phe-tRNA^{Phe} (^{14}C- or ^{3}H-labelled) or [^{14}C]Leu-tRNA^{Leu} (10–20 μM) and EF-Ts (0.02 μM) in polymix or HiFi buffer. Where indicated, ternary complexes were purified by gel filtration on two tandem Superdex 75 columns (Rodnina *et al*, 1995) in polymix or HiFi buffer. Ternary complexes were diluted to the final concentration immediately before the experiment. Where

indicated (e.g. in multiple-turnover experiments), additional Mg^{2+} was added to compensate for Mg^{2+} binding to GTP (1:1 Mg^{2+}/GTP), ATP (1:1 Mg^{2+}/ATP), and PEP (0.3 Mg^{2+}/PEP).

Kinetic experiments

All experiments were carried out at 37°C, if not stated otherwise. The formation of cognate dipeptides was measured on rapid mixing of equal volumes (14 μl) of initiation complexes (with UUC as second codon; 0.2 μM) with excess ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} (1–10 μM) in a quench-flow apparatus (RQF-3, KinTek Corporation; Figure 1B). Samples were quenched with 0.5 M KOH, hydrolyzed for 30 min at 37°C, and neutralized with acetic acid. Amino acids and dipeptides were separated by HPLC on a Chromolith 100 RP 8 column (Merck) using a gradient of 0–65% acetonitrile in 0.1% TFA. Radioactivity in the eluate was counted after addition of Lumasafe Plus scintillation cocktail (Perkin Elmer). The time courses showed a delay of product formation followed by a single exponential phase and were fitted by numerical integration (Scientist, MicroMath Scientific Software), using a model with two consecutive irreversible steps, in which the second step reflects dipeptide formation (k_{app}), and the delay comprises all preceding reactions. The rate of dipeptide formation was plotted versus ternary complex concentration and fitted to a hyperbolic function.

Product formation on a near-cognate codon in excess of ternary complex over ribosomes, that is, at multiple turnover conditions, was monitored by quench flow as above, using initiation complexes (CUC at the A site; 0.14 μM) and excess ternary complex (1–10 μM) containing [^{14}C]Phe-tRNA^{Phe}. The k_{app} values were determined from the initial part of the time courses (0.004–0.1 s) as described previously (Johansson *et al*, 2008) and the concentration dependence of k_{app} values was evaluated by hyperbolic fitting (Figure 2A and B). Time courses obtained at low Mg^{2+} concentration were fitted to straight lines and their slopes were plotted versus ternary complex concentration, yielding k_{cat}/K_M values (Figure 4A). To monitor the formation of near-cognate dipeptides by quench-flow at single-round conditions (Figure 2C and D), initiation complexes (0.6–6 μM, CUC as second codon) were rapidly mixed with purified ternary complexes (0.2 μM) containing [^{3}H]Phe-tRNA^{Phe}. Time courses were analysed by single exponential fitting. Rates and end points of dipeptide formation were plotted versus ternary complex concentration and fitted to a hyperbolic function.

To examine the Mg^{2+} dependence of peptide bond formation (Figure 4B), initiation complexes (0.14 μM, UUC, CUU, or CUC as second codon) were mixed with EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} (1 μM) in polymix buffer at 37°C. Where indicated, Mg^{2+} was added to compensate for added nucleoside triphosphates or PEP. The Mg^{2+} dependence of the amplitudes (Figure 4C) was examined at the same conditions in HiFi buffer without ATP and in the presence of GTP (0.5 mM compensated by 0.5 mM Mg^{2+}) and PEP (0.5 mM) and pyruvate kinase (0.05 mg ml^{-1}). Ternary complexes were formed in HiFi buffer (3.5 mM Mg^{2+}, as described above) and diluted to the Mg^{2+} concentration of the particular experiments immediately before the start of the reaction, taking into account the Mg^{2+} added with ternary complex, initiation complex, and EF-Ts (0.02 μM). The amplitude determined at 64 min incubation was taken to represent the final level of product formation.

To test the effect of excess of near- and non- cognate ternary complexes on the rate of GTP hydrolysis (Figure 5), two ternary complexes were prepared. The cognate ternary complex was prepared by mixing EF-Tu (50 μM), EF-Ts (0.02 μM), [γ-^{32}P]GTP (3 μl of undiluted commercial stock solution), [^{14}C]Phe-tRNA^{Phe} (30 μM), PEP (3 mM), and pyruvate kinase (0.05 mg ml^{-1}) in HiFi buffer at 37°C. The mixture of near- and non-cognate ternary complexes was prepared from EF-Tu (180 μM), EF-Ts (0.1 μM), total aa-tRNA (120 μM) aminoacylated with a mixture of 19 amino acids except Phe, GTP (1 mM), PEP (3 mM), and pyruvate kinase (0.05 mg ml^{-1}). The two complexes were purified by gel filtration; mixed at indicated ratios and added to initiation complex (0.14 μM). Reactions were quenched with 40% formic acid, dried, and dissolved in 20 μl H_2O from which 4 μl were used for TLC analysis (Polygram CEL300). To measure rates of peptide bond formation, ternary complexes were prepared in the same manner, except that unlabelled GTP (1 mM) was used.

The overall error frequency was monitored by a competition assay (Gromadski and Rodnina, 2004b) using purified ternary complexes (Figure 3). Initiation complexes (0.5 μM; CUC codon at the A site) were incubated with a mixture of cognate Leu (constant concentration of 1 μM) and near-cognate Phe (1, 1.5, or 3 μM) ternary complexes for 10, 20, and 30 s. The reaction was quenched and the reaction products were analysed as described above. The ratio of dipeptides fMetPhe/(fMetLeu + fMetPhe), normalized to equal concentrations of ternary complexes, represents the observed error frequency (E). To measure proofreading (P), initiation complex (0.5 μM, CUC codon at the A site) was incubated with near-cognate ternary complex (Phe, 0.2 μM) for 10, 20, and 30 s. The reaction was stopped and products were analysed as described above. The amount of Phe in dipeptides represents the error frequency of proofreading (P). From the overall error frequency (E) and the error frequency of proofreading (P), the efficiency of initial selection (I) was calculated: $(I) = (E)/(P)$.

Fluorescence stopped-flow experiments were performed on an Applied Photophysics stopped-flow apparatus, monitoring the proflavin fluorescence of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) (Pape *et al*, 1998), using an excitation wavelength of 463 nm. The experiments were performed by rapidly mixing equal volumes (60 μl each) of ternary complex (0.2 μM final concentration) and initiation complex (2 μM final concentration, UUC codon). The time courses were evaluated by two-exponential fitting. The time course of dipeptide formation, measured in parallel, was normalized by adjusting the start point to 1 and the end point to 0 and fitted by numerical integration.

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Conflict of interest

The authors declare that they have no conflict of interest.

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