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# Inefficient Delivery but Fast Peptide Bond Formation of Unnatural L-Aminoacyl-tRNAs in Translation

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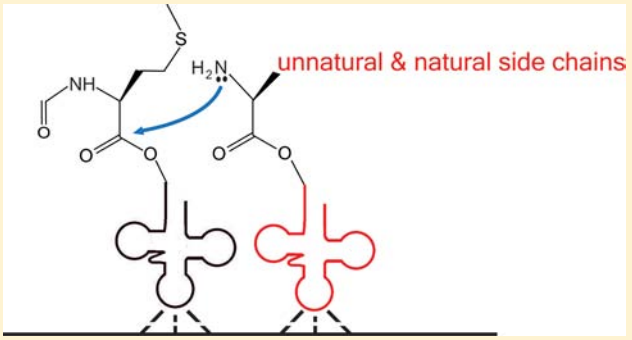
Supporting Information

**ABSTRACT:** Translations with unnatural amino acids (AAs) are generally inefficient, and kinetic studies of their incorporations from transfer ribonucleic acids (tRNAs) are few. Here, the incorporations of small and large, non-*N*-alkylated, unnatural L-AAs into dipeptides were compared with those of natural AAs using quench-flow techniques. Surprisingly, all incorporations occurred in two phases: fast then slow, and the incorporations of unnatural AA-tRNAs proceeded with rates of fast and slow phases similar to those for natural Phe-tRNA$^{Phe}$. The slow phases were much more pronounced with unnatural AA-tRNAs, correlating with their known inefficient incorporations. Importantly, even for unnatural AA-tRNAs the fast phases could be made dominant by using high EF-Tu concentrations and/or lower reaction temperature, which may be generally useful for improving incorporations. Also, our observed effects of EF-Tu concentration on the fraction of the fast phase of incorporation enabled direct assay of the affinities of the AA-tRNAs for EF-Tu during translation. Our unmodified tRNA$^{Phe}$ derivative adaptor charged with a large unnatural AA, biotinyl-lysine, had a very low affinity for EF-Tu:GTP, while the small unnatural AAs on the same tRNA body had essentially the same affinities to EF-Tu:GTP as natural AAs on this tRNA, but still 2-fold less than natural Phe-tRNA$^{Phe}$. We conclude that the inefficiencies of unnatural AA-tRNA incorporations were caused by inefficient delivery to the ribosome by EF-Tu, not slow peptide bond formation on the ribosome.




## INTRODUCTION

Ribosomal incorporation of unnatural amino acids (AAs) into peptides and proteins has found wide utility for probing protein structure and function, studying post-translational modifications, labeling with biophysical probes, and discovering potential therapeutics.[1−4] Product yields from unnatural AA-transfer ribonucleic acid (tRNA) substrates have been improved for some bulky unnatural AAs by mutation of elongation factor Tu (EF-Tu),[5,6] for some smaller unnatural AAs[7] by appropriate matching of AA and tRNA body,[8] or for unnatural AAs in general by using a purified translation system.[9,10] Nevertheless, applications remain severely restricted by low efficiencies of unnatural AA incorporation.[3,11] Two reasonable approaches to investigate and eventually solve the low efficiency problem are determining (i) which features of the unnatural AA-tRNA substrates are detrimental for unnatural AA incorporation, and (ii) which translation steps are affected kinetically by these detrimental features. These approaches may not only lead to improved incorporation efficiencies, but may also elucidate general rules of substrate recognition by the translating ribosome.

Regarding (i) unnatural substrate features, in almost all incorporations of unnatural AAs in translation, unnatural tRNA bodies have been used. The reason is that it is difficult to purify natural tRNA isoacceptors, change their codon specificities and charge them with unnatural AAs. Unnatural AA-tRNAs are generally synthesized from unmodified tRNA transcripts by ligation to pdCpA-AA-NVOC[12−14] or by ribozyme-catalyzed charging with activated AAs.[15] In translation incorporations based on a tRNA$^{Ala}$ adaptor using purified components and an incubation time of 30 min, anticodon swaps or substitution of the penultimate C with dC had little effect on single amino acid incorporations but led to substantially decreased yields for five consecutive incorporations; lack of tRNA modifications or swapping Ala for L-allyl-glycine (aG) or L-methyl-serine (mS; Figure 1a) had little effect on both single and multiple incorporations.[16] However, even single incorporations from several tRNAs of the bulky biotinyl-lysine (Figure 1a) or *N*-alkylated AAs in the same purified translation system were inefficient.[9,10,17]

Regarding (ii), translation kinetics, a pioneering study showed incorporation of a D-AA to be $10^3$ times slower than incorporation of an L-AA.[18] The reasons were lower affinity for EF-Tu and slower accommodation/peptidyl transfer of the D-AA compared to the L-AA, at least at an unphysiological reaction temperature of 0 °C. Substitution of the $\alpha$-$NH_2$ nucleophile of Phe-tRNA$^{Phe}$ by an OH group slowed dipeptide

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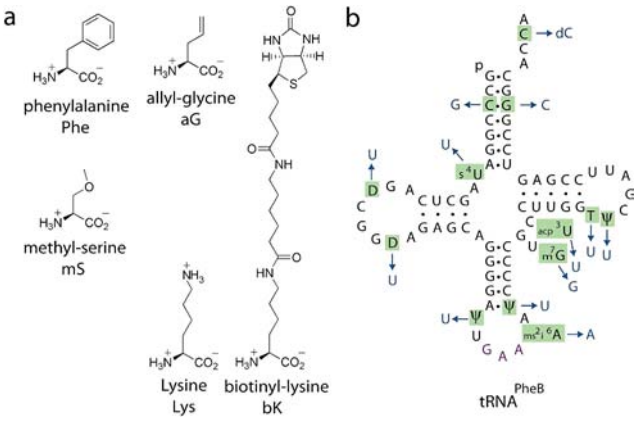


**Figure 1.** A $tRNA^{Phe}$ derivative charged with natural and unnatural AAs. (a) Natural and unnatural L-AAs used in our kinetics studies. (b) Synthetic $tRNA^{PheB}$ which is an unmodified tRNA based on natural *E. coli* $tRNA^{Phe}$ (black with purple anticodon; tRNA modifications are in green) with changes in blue.

formation $5 \times 10^4$-fold.[19] Dipeptide formation with unnatural *N*-alkylated L-AAs was also inefficient: *N*-butyl-Phe incorporation was undetectable and *N*-methyl-Phe was incorporated $10^4$ times more slowly than Phe.[20,21] The main differences in translation rates between *N*-alkyl AAs and Phe occurred after GTP hydrolysis on EF-Tu, implying that the slow step was not delivery to the ribosome by EF-Tu, but release of the AA-tRNA from EF-Tu:GDP on the ribosome, movement of the AA-tRNA to the ribosomal peptidyl-transferase site (accommodation) and/or the chemistry of peptide bond formation.[21] These and other data[22−25] support a "chemical reactivity hypothesis" of translation,[20] proposing a rate-limiting chemistry of peptide bond formation, which is further slowed down by increased steric bulk on or near the N nucleophile and by increased $pK_a$ of this N nucleophile. However, the steric and $pK_a$ effects for unnatural non-*N*-alkyl L-AAs are expected to be generally smaller than for *N*-alkyl AAs, so we examine here the single incorporation kinetics of non-*N*-alkyl L-AAs, the most commonly used class of unnatural AAs.

## RESULTS AND DISCUSSION

**Measuring Kinetics of Single Incorporations at Different Concentrations of EF-Tu.** In this work, we used our pure physiologically compatible translation system for measuring dipeptide formation from initiator fMet-$tRNA_i^{fMet}$ to various natural and unnatural aminoacylated $tRNA^{Phe}$ derivatives at 37 °C (Figure 1). In preliminary studies, we had previously observed biphasic (double-exponential) kinetics for some unnatural AAs attached to the $tRNA^{PheB}$ body (discussed in the final paragraph of[21]). Here we began by exploring the cause for the slow phase by monitoring over a long time the incorporation kinetics of AA from AA-tRNA:EF-Tu:GTP ternary complex formed in the presence of different EF-Tu concentrations. In these experiments, the ternary complexes were first preformed during a 15 min incubation of EF-Tu:GTP with AA-tRNA at 37 °C and then rapidly mixed with an excess of preinitiated fMet-tRNA:ribosome:mRNA complexes in a quench flow instrument. The reactions were quenched with formic acid at different incubation times.

**Natural Phe-$tRNA^{Phe}$ Exhibits Biphasic Kinetics of Dipeptide Synthesis.** To test whether our standard incorporation at 0.5 $\mu$M EF-Tu of natural Phe-$tRNA^{Phe}$ exhibited biphasic kinetics, we extended the incubation time of the published 0.15 s reaction time course[21] up to 200 times longer (Figure 2a, filled triangles). Though the curve before

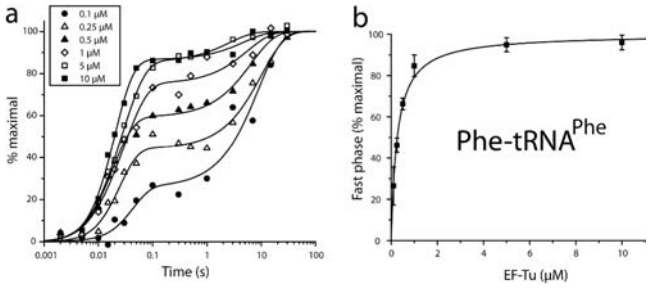


**Figure 2.** Effects of EF-Tu concentration on the kinetics of dipeptide synthesis from fMet-$tRNA_i^{fMet}$ and natural Phe-$tRNA^{Phe}$. (a) Time course for normalized fraction of f[$^3$H]Met-Phe formed at different EF-Tu concentrations. Data were fitted nonlinearly to a double-exponential association model. (b) Curve representing nonlinear fitting of the normalized fast phase fractions estimated from the kinetics of dipeptide synthesis (a) at different EF-Tu concentrations. $K_d$ for the binding of Phe-$tRNA^{Phe}$ to EF-Tu:GTP was estimated to be 0.26 $\mu$M from the fit. Experiments were done in LS3 buffer at 37 °C (see Experimental Section).

0.15 s was very similar to the previously reported monophasic kinetics,[21] prolonged incubation to 30 s clearly reveals a second, slow phase of Phe incorporation. The amplitude of the slow phase was about 40% of the final yield of dipeptide product and its rate about 150 times slower than that of the fast phase with 60% of the amplitude. In considering why such a large proportion of the product from natural Phe-$tRNA^{Phe}$ was formed at a slow rate, such a degree of heterogeneity of AA-tRNA substrate seemed unlikely. Heterogeneity of the purified ribosomes or inefficient delivery by EF-Tu seemed more probable. In order to distinguish between these three possibilities, we measured the kinetics of Phe incorporation from Phe-$tRNA^{Phe}$:EF-Tu:GTP ternary complex formed at a fixed Phe-$tRNA^{Phe}$ concentration and different EF-Tu concentrations.

**Higher Concentrations of EF-Tu Increase the Fast-Phase Fraction for Natural Phe-$tRNA^{Phe}$, Allowing Measurement of Binding Affinity of AA-tRNA to EF-Tu:GTP during Translation.** Measurement of Phe-$tRNA^{Phe}$ incorporation into dipeptide at higher EF-Tu concentrations revealed a more dominant fast phase, contributing almost 90% of total incorporation at the highest EF-Tu concentration (Figure 2a, filled squares). In contrast, at lower EF-Tu concentrations, the kinetics was dominated by the slow phase (Figure 2a, circles and open triangles). These results indicate that the fast phase is due to a burst of preformed ternary complex reacting rapidly with the ribosome, while the slow phase is rate-limited by slow formation of translationally active ternary complex. Consistent with this conclusion, (i) moving the EF-Tu from the ternary complex mixture to the ribosome mixture, thereby abolishing preformed ternary complexes, abolished also the fast phase without changing the rate of the slow phase and the final yield of incorporation (Figure S1); and (ii) the rate of the fast phase, determined by the concentration of 70S ribosomes in excess over ternary complex, remained unaltered at varying EF-Tu concentration (Table 1). But to our surprise, the rate of the slow phase also remained essentially unaltered as the EF-Tu concentration varied (Table 1),


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**Table 1. Kinetics Values for Dipeptide Synthesis from fMet-tRNA$^{fMet}$ and Different AA-tRNAs**

| AA-tRNA | EF-Tu ($\mu$M) | $k_{fast}$ ($s^{-1}$) | $k_{slow}$ ($s^{-1}$) | $K_d$ ($\mu$M) |
|---|---|---|---|---|
| Phe-tRNA$^{Phe}$ | 0.1 | 23.6 (±18.2) | 0.12 (±0.04) | 0.26 (±0.03) |
| | 0.25 | 39.6 (±7.2) | 0.10 (±0.03) | |
| | 0.5 | 33.3 (±10.4) | 0.43 (±0.28) | |
| | 1 | 31.3 (±4.7) | 0.18 (±0.12) | |
| | 5 | 33.5 (±1.8) | 0.39 (±0.17) | |
| | 10 | 48.1 (±3.2) | 0.21 (±0.13) | |
| unmodified Phe-tRNA$^{Phe}$ | 0.5 | 10.5 (±2.2) | 0.21 (±0.02) | 0.67 (±0.13) |
| | 1 | 32.4 (±6.6) | 0.42 (±0.08) | |
| | 2.5 | 31.5 (±2.1) | 0.24 (±0.04) | |
| | 10 | 31.5 (±3.9) | 0.52 (±0.19) | |
| Phe-tRNA$^{PheB}$ | 0.5 | 43.5 (±16.8) | 0.13 (±0.03) | 0.66 (±0.18) |
| | 1 | 30.3 (±6.4) | 0.34 (±0.11) | |
| | 2.5 | 36.4 (±5.7) | 0.24 (±0.10) | |
| | 10 | 27.7 (±2.9) | 0.19 (±0.07) | |
| aG-tRNA$^{PheB}$ | 0.25 | 52.0 (±56.3) | 0.17 (±0.06) | 0.62 (±0.09) |
| | 0.5 | 45.5 (±23.6) | 0.093 (±0.016) | |
| | 1 | 20.4 (±12.9) | 0.30 (±0.09) | |
| | 2.5 | 46.3 (±8.0) | 0.24 (±0.07) | |
| | 5 | 32.0 (±4.2) | 0.22 (±0.08) | |
| | 10 | 41.9 (±6.9) | 0.49 (±0.20) | |
| mS-tRNA$^{PheB}$ | 0.25 | 68.4 (±77.8) | 0.15 (±0.04) | 0.61 (±0.11) |
| | 0.5 | 48.8 (±14.5) | 0.048 (±0.005) | |
| | 1 | 26.7 (±9.4) | 0.32 (±0.10) | |
| | 2.5 | 34.2 (±2.9) | 0.20 (±0.04) | |
| | 5 | 31.7 (±3.5) | 0.11 (±0.03) | |
| | 10 | 36.5 (±7.8) | 0.74 (±0.41) | |
| bK-tRNA$^{PheB}$ | 0.5 | undetectable | 0.034 (±0.002) | 50[a] |
| | 5 | undetectable | 0.047 (±0.006) | |
| | 10 | 17.1 (±12.0) | 0.24 (±0.04) | |
| Lys-tRNA$^{PheB}$ | 0.25 | 69.0 (±24.9) | 0.14 (±0.02) | 0.57 (±0.13) |
| | 0.5 | 40.0 (±11.8) | 0.12 (±0.02) | |
| | 1 | 35.9 (±7.0) | 0.20 (±0.04) | |
| | 2 | 31.0 (±3.8) | 0.20 (±0.04) | |
| | 5 | 32.9 (±4.8) | 0.22 (±0.06) | |
| | 10 | 24.3 (±2.2) | 0.35 (±0.07) | |
| bK-tRNA$^{PheB}$ at 20 °C | 2 | 8.34 (±2.92) | 0.079 (±0.011) | 3.30 (±0.92) |
| | 3 | 7.76 (±3.57) | 0.18 (±0.07) | |
| | 6 | 8.86 (±1.28) | 0.15 (±0.03) | |
| | 10 | 7.80 (±1.61) | 0.18 (±0.05) | |
| | 15 | 8.97 (±1.13) | 0.36 (±0.08) | |

[a]The $K_d$ value for bK-tRNA$^{PheB}$ was calculated from the fast phase fraction (15%) at 10 $\mu$M EF-Tu assuming that the maximal fast phase fraction was 80% (see Experimental Section).

contrary to the theoretical prediction based on simple one-step formation of active ternary complex from AA-tRNA and EF-Tu:GTP (see Supporting Information). We thus hypothesized that the slow phase was the result of two independent phenomena, (i) incomplete binding of AA-tRNA by EF-Tu:GTP at lower EF-Tu concentrations, and (ii) a slow two-step formation of active ternary complex (even at high EF-Tu concentrations; see Supporting Information). One possible explanation is that the slow phase kinetics reveals a previously unknown slow conformational change in the ternary complex that determines the maximal rate of the slow phase and follows the initial inactive complex formation between AA-tRNA and EF-Tu:GTP. Whatever the explanation for the limited rate of the slow phase, the observed increase in the amplitude of the fast phase with EF-Tu concentration reflects the increase in the concentration of preformed ternary complexes active in dipeptide synthesis, allowing determination of the dissociation (equilibrium) constant $K_d$ for binding of AA-tRNA substrate to EF-Tu:GTP under the exact conditions of the translation reaction at 37 °C (Figure 2b; Table 1; $K_d$ = 0.26 $\mu$M; see


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Supporting Information for comparison with published $K_d$ values, all of which have the potential limitation of not being determined directly during translation). This establishment of kinetic assays for delivery by EF-Tu and dipeptide formation over an unusually long time frame under physiological conditions opened a window for investigating incorporation of unnatural AA-tRNAs, most of which were predicted to incorporate slowly.[26]

**Lack of tRNA Modifications Lowers the Binding Affinity of AA-tRNA to EF-Tu:GTP.** Adaptor tRNAs for unnatural AAs are typically unmodified transcripts, so next we ascertained the effect of the lack of tRNA modifications by preparing unmodified $tRNA^{Phe}$ with the wild type sequence by *in vitro* transcription and charging it with Phe by PheRS. As in the case of natural Phe-$tRNA^{Phe}$, the incorporation of Phe from unmodified Phe-$tRNA^{Phe}$ measured at different EF-Tu concentrations exhibited biphasic kinetics with unaltered rates of the fast phases (Figure 3a, Table 1). Moreover, the rate of

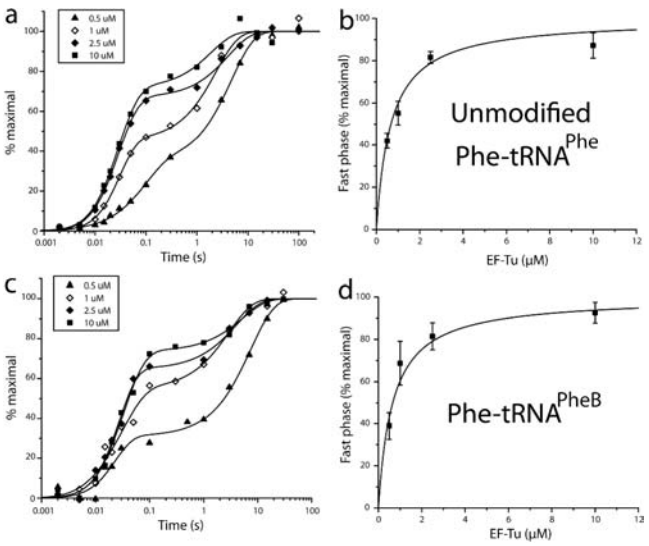


**Figure 3.** Effects of EF-Tu concentration on the kinetics of dipeptide synthesis from fMet-$tRNA_i^{fMet}$ and unmodified Phe-$tRNA^{Phe}$ (a,b) or Phe-$tRNA^{PheB}$ (c,d).

the fast phases for unmodified Phe-$tRNA^{Phe}$ was similar to that of natural Phe-$tRNA^{Phe}$, confirming the published result that the lack of modifications had no significant effect on peptide bond formation on the ribosome.[21] However, the fraction of the fast phase of unmodified Phe-$tRNA^{Phe}$ was significantly smaller than that of natural Phe-$tRNA^{Phe}$ at the same EF-Tu concentrations (compare Figures 2a and 3a). The $K_d$ for binding of unmodified Phe-$tRNA^{Phe}$ to EF-Tu:GTP was estimated as 0.67 $\mu$M (Figure 3b; Table 1), which is about 2.5 fold higher than for natural Phe-$tRNA^{Phe}$. We conclude that the lack of modifications measurably reduced the binding affinity of AA-tRNA to *E. coli* EF-Tu:GTP, comparable with the published difference.[27]

In order to compare the kinetics of incorporation of unnatural AAs with that of the related natural Phe-$tRNA^{Phe}$, we ligated AA-dinucleotides onto $tRNA^{PheC3G\text{-}G70C\ minus\ CA}$ ($tRNA^{PheB}$): this mutation of the GC base pair increases the efficiency of transcription by T7 RNA polymerase without affecting translation incorporation kinetics.[27] *N*-Nitroveratryloxycarbonyl (NVOC)-aminoacyl-pdCpA derivatives (Figure 1a) were chemically synthesized and ligated onto the $tRNA^{minusCA}$ *in vitro* transcript using T4 RNA ligase, followed by removal of the NVOC amino protecting group by photolysis.[28] The ligated $tRNA^{PheB}$ body thus has three types of changes compared to that of natural $tRNA^{Phe}$ (Figure 1b): substitutions at positions 3 and 70, lack of tRNA modifications, and a penultimate dC to enable charging with unnatural AAs. We previously showed that these changes have minimal effects on dipeptide synthesis kinetics on the ribosome in our standard 0.15 s time interval;[21] here we used much longer incubation times and also varied EF-Tu concentration to check for putative effects of these combined changes on AA-tRNA binding to EF-Tu. We found the kinetics to be indistinguishable from that of unmodified Phe-$tRNA^{Phe}$ (Figure 3c,d, Table 1).

**Small Unnatural L-AAs Form Dipeptides Fast on the Ribosome.** aG-$tRNA^{PheB}$ and mS-$tRNA^{PheB}$ also exhibited biphasic incorporation kinetics at 0.5 $\mu$M EF-Tu (Figure 4a,c,

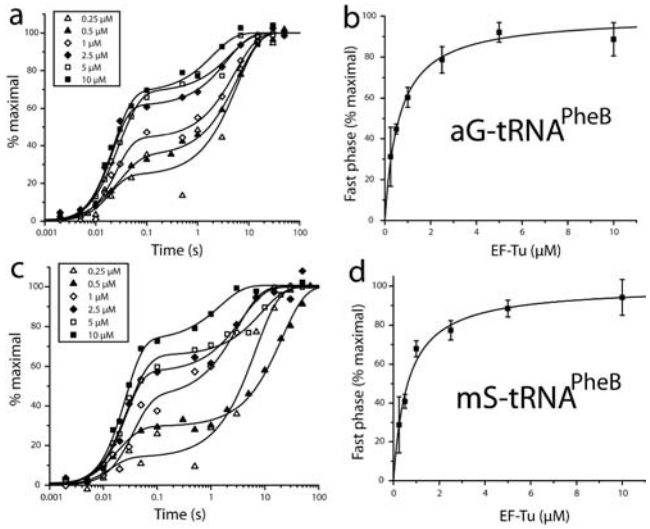


**Figure 4.** Effects of EF-Tu concentration on the kinetics of dipeptide synthesis from fMet-$tRNA_i^{fMet}$ and aG-$tRNA^{PheB}$ (a,b) or mS-$tRNA^{PheB}$ (c,d).

filled triangles). Unexpectedly, the rates of their fast and slow phases were very similar to those of the Phe-$tRNA^{PheB}$ control (Table 1), so we conclude that unnatural AAs can be incorporated at similar rates as natural AAs. Because these unnatural AA incorporations exhibited biphasic kinetics similar to that of Phe, we again measured the kinetics of incorporation at different EF-Tu concentrations to test if this would similarly increase the fast phase fractions with unnatural AAs.

**Higher Concentrations of EF-Tu Increase the Fast Phase Fraction for Small Unnatural AAs.** As for Phe-$tRNA^{PheB}$ and natural Phe-$tRNA^{Phe}$, incorporation of aG-$tRNA^{PheB}$ and mS-$tRNA^{PheB}$ into dipeptide at higher EF-Tu concentrations increased the fast phase fractions (Figure 4a,c, squares and filled diamonds). Again, the slow phases dominated at low EF-Tu concentrations (Figure 4a,c, triangles) and the rates of the fast and slow phases were unaffected by the EF-Tu concentration within experimental error (Table 1). $K_d$ values for binding of aG-$tRNA^{PheB}$ and mS-$tRNA^{PheB}$ to EF-Tu:GTP were estimated as 0.62 and 0.61 $\mu$M, respectively (Figure 4b,d; Table 1), coincidentally similar to that measured for Phe-$tRNA^{PheB}$ (0.66 $\mu$M in Table 1; Figure 3d). These results suggest that the binding affinities to EF-Tu:GTP of the aG and mS portions of the AA-tRNAs were very similar to that of


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natural Phe. According to the "thermodynamic compensation" hypothesis,[29] optimal AA-tRNA design for translation[8] requires similar affinities to EF-Tu of all AA-tRNAs. Thus, when a particular tRNA body binds tightly to EF-Tu, its AA should bind weakly, and vice versa. Phe is neither a tight nor a weak AA, having intermediate affinity to EF-Tu.[30,31] Likewise, the body of $tRNA^{Phe}$ is neither tight nor weak, also having intermediate affinity to EF-Tu.[32] This indicates that the affinity-matching of AA and tRNA body that occurs for all natural elongator AA-tRNAs in *E. coli* within a 14-fold range[33] also occurs fortuitously for aG and mS on $tRNA^{PheB}$, with both unnatural AAs having intermediate affinities for EF-Tu, similar to that of Phe (although all three AA-tRNAs have about a 2-fold higher $K_d$ for EF-Tu than natural Phe-$tRNA^{Phe}$).

We conclude from the similar rates of dipeptide formation for Phe and small unnatural AAs that small unnatural AAs may react just as efficiently at the peptidyl transferase center of the ribosome and by the same mechanism as natural AAs. However, the unmodified tRNA used for charging with unnatural AAs had a lower affinity to EF-Tu:GTP that resulted in a larger fraction of slow phase kinetics. This may help explain why small unnatural AAs are sometimes incorporated less efficiently than AAs from natural AA-tRNAs, and the lower efficiency is likely exacerbated when the AA has a low affinity for EF-Tu. Although even the slow phase is fast compared with typical translation incubation times, it may be too slow to prevent competing reactions during elongation, such as peptidyl-tRNA drop-off.[26] Our results suggest that incorporation of some small unnatural AAs might be improved by using a high concentration of EF-Tu and preincubation of the ternary complex mixture before translation. Improvement of unnatural AA incorporation efficiencies with a high concentration of EF-Tu has so far only been well-documented for large unnatural AAs.[5]

**A Large Unnatural L-AA Exhibits Only Slow Incorporation Kinetics up to 5 $\mu$M EF-Tu.** Bulky unnatural L-AAs such as biotinyl-lysine (bK, Figure 1a) are known to be incorporated generally less efficiently than small unnatural L-AAs.[10,34] In particular, bK had a substantially lower incorporation efficiency than aG and mS in prior studies in a purified translation system.[9,10] In line with this, the present kinetics of dipeptide synthesis from bK-$tRNA^{PheB}$ exhibited only a slow phase at both 0.5 and 5 $\mu$M EF-Tu (Figure 5, triangles and open squares). Furthermore, the slow phase rate was about 10 times smaller than the slow phase rates of the other substrates (Table 1). Nevertheless, in contrast to prior work, quantitative incorporation of this large AA was achieved in our optimized translation system based on measurements of the charged fraction of input substrate.

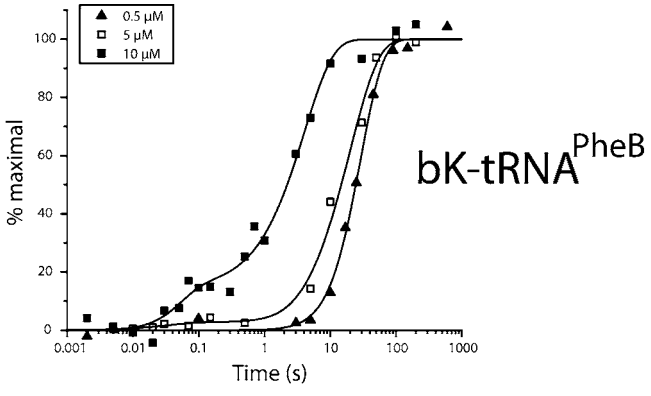


**Figure 5.** Effect of EF-Tu concentration on the kinetics of dipeptide synthesis from fMet-$tRNA_i^{fMet}$ and bK-$tRNA^{PheB}$.

**A Very High Concentration of EF-Tu Reveals Fast Peptide Bond Formation for bK-$tRNA^{PheB}$.** To investigate if the slow kinetics for bK-$tRNA^{PheB}$ incorporation also reflects inefficient binding to EF-Tu:GTP, we measured the incorporation kinetics at even higher EF-Tu concentration of 10 $\mu$M. Interestingly, this led to a small but detectable fast-phase fraction of 15%, confirming this hypothesis (Figure 5, filled squares). Also, in contrast to other AA-tRNAs, the rate of the slow phase increased significantly with the concentration of EF-Tu. Thus, the rates of the fast and slow phases for bK-$tRNA^{PheB}$ at 10 $\mu$M EF-Tu were comparable to those for natural Phe-$tRNA^{Phe}$ (Table 1), meaning that even a bulky unnatural AA can be incorporated at a rate similar to that of natural AAs. The presence of biphasic kinetics at 10 $\mu$M EF-Tu enabled calculation of the $K_d$ for binding of bK-$tRNA^{PheB}$ to EF-Tu:GTP: the $K_d$ was 50 $\mu$M (Table 1), 200 times higher than that of natural Phe-$tRNA^{Phe}$. These results show that a bulky unnatural AA is delivered to the ribosome much less efficiently than small unnatural AAs. This deficiency could only be partially compensated by a high concentration of EF-Tu, which is consistent with the reported improvement of bulky unnatural AA incorporation using high concentrations of EF-Tu.[5]

**The Bulky Side Chain of bK-$tRNA^{PheB}$ Is Responsible for the Substrate's Very Low Affinity for EF-Tu:GTP.** In order to ascertain if the very high $K_d$ of bK-$tRNA^{PheB}$ was due to the bulky side chain (biotin and linker group), we measured the kinetics of dipeptide synthesis from Lys-$tRNA^{PheB}$. The incorporation of Lys-$tRNA^{PheB}$ at different EF-Tu concentrations showed similar biphasic kinetics to Phe-$tRNA^{PheB}$ with similar fast and slow rates (Figure 6a; Table 1). The $K_d$ for

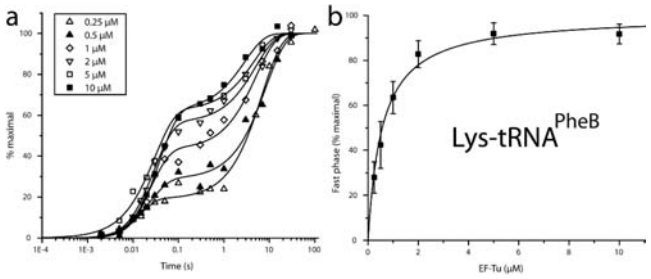


**Figure 6.** Effect of EF-Tu concentration on the kinetics of dipeptide synthesis from fMet-$tRNA_i^{fMet}$ and Lys-$tRNA^{PheB}$.

binding of Lys-$tRNA^{PheB}$ to EF-Tu:GTP was estimated to be 0.57 $\mu$M (Figure 6b; Table 1), similar to Phe-$tRNA^{PheB}$ and consistent with Lys and Phe having comparable affinities for EF-Tu:GTP.[30] We conclude that the bulky side chain of the unnatural AA severely weakened the binding affinity to EF-Tu:GTP.

**Decreasing Temperature Increases the Fast Phase Fraction for bK-$tRNA^{PheB}$.** Due to the very low affinity of binding of bK-$tRNA^{PheB}$ to EF-Tu:GTP, the incorporation of bK was dominated by slow ternary complex formation even at high EF-Tu concentration, making kinetic studies of the fast phase difficult. Given that binding affinity normally increases with decreasing temperature,[29,35,36] we measured incorporation of bK from bK-$tRNA^{PheB}$ at different EF-Tu concentrations also at 20 °C. This showed a dramatic increase in the fast phase


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fraction compared to 37 °C: the kinetics was dominated by the fast phase at 6 $\mu$M and higher concentrations of EF-Tu (Figure 7a, circles, squares and pentagons). The $K_d$ of binding of bK-tRNA$^{PheB}$ to EF-Tu:GTP at 20 °C was measured as 3.3 $\mu$M (Figure 7b; Table 1), which is 15 times lower than the 37 °C estimate. The rate of the fast phase (about 8 $s^{-1}$; Table 1), statistically unaffected by variation of the EF-Tu concentration, was comparable to that for incorporations of natural Phe from Phe-tRNA$^{Phe}$,[22] and other natural AAs from their cognate AA-tRNAs[25] into dipeptides at 20 °C. These results demonstrate that when an unnatural AA is incorporated with very slow kinetics due to a very high $K_d$ value for EF-Tu:GTP, the % incorporation at short times can be greatly improved by reduction of the reaction temperature (e.g., compare incorporations at 5 s using 5−6 $\mu$M EF-Tu in Figures 7a versus 5a). Also, the incorporation of bulky unnatural AAs can be as fast as natural AAs at low temperature, contrary to the prediction of the "chemical reactivity hypothesis" of adverse affects of sterics on the rate of peptide bond formation.[20]

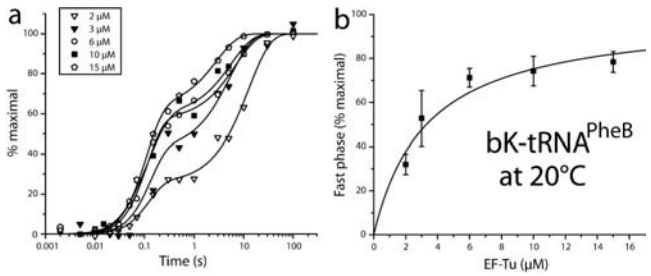


**Figure 7.** Effect of EF-Tu concentration on the kinetics of dipeptide synthesis from fMet-tRNA$_i^{fMet}$ and bK-tRNA$^{PheB}$ at 20 °C.

## CONCLUSION

We have demonstrated that unnatural AAs can react as fast as natural AAs in dipeptide formation, which was unexpected. We also developed an assay for measuring the affinity of EF-Tu for AA-tRNA directly during translation. Though aG and mS have the same affinities for EF-Tu as natural Phe, unmodified tRNA$^{PheB}$, which is required for charging with unnatural AAs, has a lower affinity for EF-Tu than natural tRNA$^{Phe}$. This may help explain the lower efficiencies of incorporation of some small unnatural AAs in translation, particularly where the AA has a low affinity for EF-Tu, and incorporation efficiencies may be improved by increasing the concentration of EF-Tu. In addition, bK has a very low affinity for EF-Tu, which can be significantly improved by lowering the reaction temperature; this provides another method to improve the incorporation of unnatural AAs. Our results opened a kinetic window to understand the rate-limiting steps in protein synthesis with unnatural AAs and to improve their incorporation.

## EXPERIMENTAL SECTION

Large-scale chemical synthesis of pdCpA was performed in our laboratory according to existing procedures[28] with the following minor modification. The deprotected material was purified by preparative anion exchange chromatography, desalted and passed through a cation exchange column loaded with tetrabutylammonium (TBA) ions in order to convert this dimer into a TBA form, which is more soluble in organic, water-free media. Contrary to literature procedures, no extra tetrabutylammonium hydroxide was added to this material. The isolated material was evaporated; the residue was dissolved in DMF and dried by sequential co-evaporation with dry acetonitrile and stored as a stock solution in DMF.

N-Nitroveratrylooxycarbonyl (N-NVOC) protected cyanomethyl esters of AAs were prepared according to standard methodology.[28] Derivatives of L-phenylalanine (Phe),[17] L-allylglycine (aG)[17] and biotinyl-lysine (bK)[37] were prepared as described.

***N*-NVOC-L-Me-serine Cyanomethyl Ester.**[38] Light yellow solid was synthesized starting from L-Me-serine (FChemicals Limited, Shanghai, China) in 72% overall yield: $^1$H NMR (500 MHz, $CDCl_3$) d 7.72(s, 1H), 7.04(s, 1H), 5.78(d, 1H), 5.57(dd, 2H), 4.80(dd, 2H), 4.59(m, 1H), 4.01 (s, 3H), 3.96(s, 3H), 3.79(dd, 2H), 3.39(s, 3H).

**Bis-*α*,*ε*-*N*-NVOC-L-lysine Cyanomethyl Ester.** Light yellow solid was obtained by acylation of L-lysine using a double amount of NVOC-Cl and the base in 64% overall yield: $^1$H NMR (500 MHz, $CDCl_3$) d 7.69(ss, 2H), 6.98(ss, 2H), 5.64(d, 1H), 5.51(dd, 4H), 4.99(m, 1H), 4.79(dd, 2H), 4.41(m, 1H), 3.98 (m, 12H), 3.22(m, 2H), 1.92−1.46(m, 6H).

The acylation of the pdCpA was done at 10 micromol scale in the presence of 5 mol equiv of the activated AA. Both components were dissolved in a 2 mL Soersted tube in 0.2 mL of DMF followed by addition of dry acetonitrile (0.5 mL). The partially precipitated reaction mixture was dried by azeotropic co-evaporation of water using a vacuum evaporator (SpeedVac), dissolved in dry DMF (0.2 mL) and acylation was started by addition of dry triethylamine (25 mL). The tube was incubated at 50 °C for 16 h, and all volatile matter was evaporated. The residue was dissolved in 0.5 M ammonium acetate (pH 4.5) and acetonitrile 1:1, analyzed on reversed-phase HPLC column, and purified preparatively.[28]

***N*-NVOC-L-Me-Ser-pdCpA.** Light yellow solid: MALDI-TOF $m/z$ calculated from $C_{33}H_{42}N_{10}O_{21}P_2$ 976.20, found $(M+H)^+$ 977.175, $(M+Na)^+$ 999.167.

**Bis-*α*,*ε*-*N*-NVOC-L-lysine-pdCpA.** Yellow solid: MALDI-TOF $m/z$ calculated from $C_{45}H_{56}N_{12}O_{26}P_2$ 1242.29, found $(M+H)^+$ 1243.315, $(M+Na)^+$ 1265.303.

***N*-NVOC-L-allyl-Gly-pdCpA.** Light yellow solid: MALDI-TOF $m/z$ calculated from $C_{34}H_{42}N_{10}O_{20}P_2$ 972.21, found $(M+H)^+$ 973.226, $(M+Na)^+$ 995.224.

***N*-NVOC-L-Phe-pdCpA.** Light yellow solid: MALDI-TOF $m/z$ calculated from $C_{38}H_{44}N_{10}O_{20}P_2$ 1022.22, found $(M+H)^+$ 1023.189, $(M+Na)^+$ 1045.203.

***α*-*N*-NVOC-*ε*-*N*-Bio-Hex-L-lysine-pdCpA.** Light yellow solid: MALDI-TOF $m/z$ calculated from $C_{51}H_{72}N_{14}O_{23}P_2S$ 1342.41, found $(M+H)^+$ 1343.449, $(M+Na)^+$ 1365.441.

**Synthesis of Unnatural AA-tRNAs.** tRNA$^{Phe}$ transcripts and 3′CA-truncated tRNA$^{PheB}$ transcripts were prepared as previously described.[20] *N*-NVOC-AA-pdCpAs derivatives of Phe, Lys, aG, mS, and bK (Figure 1b) were ligated to unmodified 3′CA-truncated tRNA$^{PheB}$ transcripts using T4 RNA ligase. The products were purified on a Q-column as previously described.[21] The amino protecting group, NVOC, was removed by photolysis prior to the translation experiments. The ligation yields were estimated as >50% of the input tRNA$^{PheB}$ based on urea polyacrylamide gel electrophoresis at pH 5.

**Components of the *in Vitro* Translation System.** The purified components for the *in vitro* translation system including 70S ribosomes (purified from *E. coli* strain MRE 600), synthetic mRNAs, initiation factors, elongation factors, f[$^3$H]Met-tRNA$_i^{fMet}$, tRNA$^{Phe}$, and PheRS were prepared as previously described.[39] EF-Tu and EF-Ts were highly active based on native PAGE of ternary complexes and exchange of EF-Tu-bound GDP, respectively. The slow phase was unaffected by omission of EF-Ts (results not shown).

**Preparation of mRNA.** mRNA encoding fMet-Phe-Ile-Ser-Stop was prepared by *in vitro* transcription with T7 RNA polymerase, purified by an oligo(dT) column, and has a strong Shine-Dalgarno sequence (uaaggaggu) in the upstream sequence. The mRNA sequence is shown with sense codons in capitals:

gggaauucgggcccuuguuaacaauuaaggagguauauc AUG UUC AUU

UCG uaauugcagaaaaa aaaaaaaa


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**Buffer Conditions.** All experiments were conducted in polymix-like buffer, LS3, containing 95 mM KCl, 5 mM $NH_4Cl$, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, 30 mM HEPES (pH 7.5), 1 mM dithioerythritol, 2 mM phosphoenolpyruvate (PEP), 5 mM Mg-$(OAc)_2$, 1 mM ATP, and 1 mM GTP,[40] supplemented with 1 $\mu$g/mL pyruvate kinase and 0.1 $\mu$g/mL myokinase for energy regeneration. As PEP chelates $Mg^{2+}$ with a $K_d$ value of 10 mM[41] and assuming that one ATP or GTP molecule chelates one $Mg^{2+}$, the free $Mg^{2+}$ concentration in buffer LS3 was estimated as 2.4 mM.

**Kinetic Assays for Measurement of Dipeptide Synthesis.** Below, concentrations are given as final values, after mixing equal volumes of ribosome and ternary complex mixtures. To avoid dilution in the mixing step, EF-Tu was present in each mixture at equal concentrations as indicated for each experiment. The ribosome mixture was prepared by incubating 1 $\mu$M 70S ribosomes, 1.5 $\mu$M IF1, 0.5 $\mu$M IF2, 1.5 $\mu$M IF3, 2 $\mu$M mRNA, 1.2 $\mu$M f[$^3$H]Met-$tRNA_i^{fMet}$, 0.5 $\mu$M EF-Ts, and EF-Tu (variable concentrations) in buffer LS3 at 37 °C for 15 min. The ternary complex mixture was prepared in two alternative ways. For natural $tRNA^{Phe}$ or unmodified $tRNA^{Phe}$ transcript the ternary complex mixture was prepared by incubating EF-Tu (variable concentrations), $tRNA^{Phe}$ or unmodified $tRNA^{Phe}$ (in concentrations between 0.04 and 0.2 $\mu$M), 0.5 $\mu$M EF-Ts, 0.2 mM phenylalanine, and 0.1 unit/$\mu$L PheRS in buffer LS3 for 15 min at 37 °C. For Phe, Lys, and unnatural AAs ligated to $tRNA^{PheB}$, the ternary complex mixture was prepared by incubating EF-Tu (variable concentrations), 0.5 $\mu$M EF-Ts, and chemoenzymatically synthesized and photodeprotected AA-$tRNA^{PheB}$ (at concentrations between 0.04 and 0.2 $\mu$M) in buffer LS3 for 15 min at 37 °C. Equal volumes (20 $\mu$L) of the ternary complex and ribosome mixtures were rapidly mixed in a temperature controlled quench-flow apparatus (RQF-3; KinTeck Corp.). The reaction was stopped at different incubation times by rapidly quenching with 50% formic acid (17% final concentration). Unless specified otherwise, all kinetics measurements were at 37 °C. EF-Tu was present in excess over AA-tRNAs in ternary complex mixture, so the amount of preformed ternary complexes was limited by the amount of AA-tRNAs. Since ribosomes were in excess over ternary complexes, the rates of dipeptide synthesis were limited by the ribosome concentration.

**Analyses of Kinetics Assays.** After quenching, the reaction samples were centrifuged at 20000*g* for 15 min at 4 °C. The pellets containing [$^3$H]dipeptidyl-tRNA and unreacted f[$^3$H]Met-$tRNA_i^{fMet}$ were first dissolved in 120 $\mu$L of 0.5 M KOH for 5 min at 37 °C to release [$^3$H]dipeptide and f[$^3$H]Met from tRNA. Formic acid was then added to 17% to precipitate the deacylated tRNAs, and the samples were centrifuged at 20000*g* for 15 min at 4 °C. The [$^3$H]dipeptide and f[$^3$H]Met in the supernatants were quantified by C18 reversed-phase HPLC equipped with a $\beta$-RAM model 3 radioactivity detector (IN/US Systems).[21] Figure S2 (Supporting Information) shows HPLC conditions and profiles for separation of f[$^3$H]Met and [$^3$H]dipeptide for natural and unnatural substrates.

The data were analyzed by the nonlinear regression program Origin 7.5 (OriginLab Corp.). The rates and the fractions of the fast and slow phases and their standard deviations were estimated by nonlinear regression fitting to a two-phase exponential association model: the fast phase, reflecting dipeptide formation from preformed active ternary complexes, was fitted to a two-step kinetic model,[22] while the slow phase, reflecting active ternary complex formation, was fitted to a single-step exponential model. Except for the reaction of bK-$tRNA^{PheB}$ at 37 °C, the dissociation constants ($K_d$) for the binding of AA-tRNA to EF-Tu:GTP were estimated by fitting the data of the fast phase fraction ($P_{fast}$) versus EF-Tu concentration ([EF-Tu]) to the hyperbolic function, $P_{fast} = P_{fast,\,max}[\text{EF-Tu}]/(K_d + [\text{EF-Tu}])$, where $P_{fast,\,max}$ was the maximal $P_{fast}$ value from the fit ($P_{fast,\,max}$ is the asymptote of $P_{fast}$ as the EF-Tu concentration increases indefinitely), based on (1) our experimental condition that [EF-Tu] ≫ [AA-tRNA] (at least 5-fold excess) and (2) the assumption that $P_{fast}$ corresponded to the fraction of active ternary complex that could be formed under those conditions. The $P_{fast}/P_{fast,max}$ values versus EF-Tu concentration are plotted in Figures 2b, 3b,d, 4b,d, 6b, and 7b. $K_d$ values were estimated from the above hyperbolic function. For the reaction of bK-$tRNA^{PheB}$ at 37 °C, $K_d$ was estimated from the $P_{fast}$ value (15%) at 10 $\mu$M EF-Tu, assuming that $P_{fast,\,max}$ was 80%, due to the undetectable fast phase at lower EF-Tu concentrations.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Supplementary text for comparison of published $K_d$ values of Phe-$tRNA^{Phe}$ for EF-Tu, Figure S1 (slow kinetics of ternary complex formation), Figure S2 (HPLC profiles for separation of fMet and fMet-AA from natural and unnatural AAs), and theoretical analysis of the experimental data. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare the following competing financial interest(s): A.C.F. owns U.S. patent 6977150 licensed to Ra Pharmaceuticals, Inc., owns shares in the company, and is a member of its scientific advisory board.

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