# Slow peptide bond formation by proline and other *N*-alkylamino acids in translation

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Proteins are made from 19 aa and, curiously, one *N*-alkylamino acid ("imino acid"), proline (Pro). Pro is thought to be incorporated by the translation apparatus at the same rate as the 19 aa, even though the alkyl group in Pro resides directly on the nitrogen nucleophile involved in peptide bond formation. Here, by combining quench-flow kinetics and charging of tRNAs with cognate and noncognate amino acids, we find that Pro incorporates in translation significantly more slowly than Phe or Ala and that other *N*-alkylamino acids incorporate much more slowly. Our results show that the slowest step in incorporation of *N*-alkylamino acids is accommodation/peptidyl transfer after GTP hydrolysis on EF-Tu. The relative incorporation rates correlate with expectations from organic chemistry, suggesting that amino acid sterics and basicities affect translation rates at the peptidyl transfer step. Cognate isoacceptor tRNAs speed Pro incorporation to rates compatible with in vivo, although still 3–6 times slower than Phe incorporation from Phe-tRNA$^{Phe}$ depending on the Pro codon. Results suggest that Pro is the only *N*-alkylamino acid in the genetic code because it has a privileged cyclic structure that is more reactive than other *N*-alkylamino acids. Our data on the variation of the rate of incorporation of Pro from native Pro-tRNA$^{Pro}$ isoacceptors at 4 different Pro codons help explain codon bias not accounted for by the "tRNA abundance" hypothesis.

non-natural amino acids | ribosomes | tRNA | EF-Tu | GTPase

**N**-alkylation confers special properties on amino acids and polypeptides. The *N*-alkyl group in Pro-tRNA$^{Pro}$ resides directly on the nitrogen nucleophile that forms the peptide bond in translation, yet this is somehow tolerated by the translation apparatus. *N*-alkyl groups in polypeptides and proteins kink 3-dimensional structures, result in slow isomerization between *cis* and *trans* conformations in the case of Pro, and can improve the pharmacological properties of peptides by decreasing their degradation by proteases and increasing their membrane permeability. To investigate these special properties or endow them on translation products, there have been many studies incorporating *N*-alkylamino acids ("imino acids") in translation (1–13). Small linear *N*-alkylamino acids (Fig. 1 *Upper Right*), because of their similarity to Pro, were expected to incorporate well in translation, but the efficiencies were variable (1–13). Incomplete incorporations in purified translation systems (8–13) are particularly puzzling because of the lack of competing reactions, incubation times of tens of minutes, and an average codon translation time comparable with that in *Escherichia coli* (45 ms) (14). Here, we investigate the kinetics of incorporation of Pro and other *N*-alkylamino acids by combining quench-flow translation kinetics and charging of tRNAs with noncognate/unnatural amino acids (Fig. 1).

## Results

A highly flexible method for charging tRNAs with noncognate amino acids and *N*-alkylamino acids is to use T4 RNA ligase to join an unmodified tRNA$^{-CA}$ transcript to a chemically synthesized *N*-NitroVeratrylOxyCarbonyl (NVOC)-aminoacyl-pdCpA; the amino protecting group (NVOC) is then removed by photolysis (15). We first need to ascertain the effect of lack of nucleoside modifications and of a penultimate dC, in those aminoacyl-tRNA constructs (Fig. 1 *Upper*) on translation kinetics. Posttranscriptional modifications have been shown to affect marginally translation kinetics in the case of one tRNA, *E. coli* tRNA$^{PheC3G-G70C}$, at least for *N*-acetyl-Phe-Phe dipeptide synthesis from poly(U) template at 5 °C, which proceeded with a codon translation time of ≈1 s (16). To assess the effects of lack of modifications also at near in vivo conditions (37 °C and codon translation times faster than 45 ms) we employ a version of our purified translation system optimized for speed and accuracy at 37 °C (17, 18). The kinetics of ribosomal synthesis of [$^3$H]fMet-Phe-tRNA$^{PheC3G-G70C}$ from [$^3$H]fMet-tRNA$^{fMet}$ and Phe-tRNA$^{PheC3G-G70C}$, prepared by ligation and photodeprotection (termed Phe-tRNA$^{PheB}$; Fig. 1) are first compared with the corresponding kinetics for the native tRNA$^{Phe}$ charged with Phe by Phe-tRNA synthetase. We measure the average times, $\tau$, for both chemical reactions of codon translation, GTP hydrolysis on EF-Tu ($\tau_{GTP}$; discussed below) and overall dipeptide synthesis ($\tau_{dip}$), in the same reaction mixtures. The similar and short translation times for Phe-tRNA$^{PheB}$ and native Phe-tRNA$^{Phe}$ [Fig. 2 *A* and *B*, Table 1, and supporting information (SI) Table S1] show that the penultimate dC and the absence of all 10 posttranscriptional modifications in tRNA$^{Phe}$ (Fig. 1) have only a minor effect on dipeptide synthesis kinetics, which also validates our combination of methods.

Next, we measured the effect on translation of changing the cognate amino acid on tRNA$^{PheB}$, Phe, to a much smaller, noncognate, natural amino acid, Ala. Again, the effect is minor (Fig. 2 *B* and *C* and Table 1).

In marked contrast, changing the cognate Phe to its simplest *N*-alkyl analog, *N*-methyl-Phe (Fig. 1), increases the average dipeptide synthesis time ($\tau_{dip}$) by a factor of 8,000 to 3.5 min (Fig. 2*D* and Table 1). Nevertheless, virtually all *N*-methyl-Phe-tRNA$^{PheB}$ was converted to fMet-*N*-methyl-Phe-tRNA$^{PheB}$ within 30 min (Fig. 2*D*). Such a huge inhibitory effect of a small methyl group on the kinetics of dipeptide formation is surprising, albeit consistent with our previous inability to saturate incorporation at the third position of a tetrapeptide with this substrate (11). Mechanistic insight into the remarkably slow incorporation

**Fig. 1.** Combination of tRNAs charged with noncognate/unnatural amino acids and quench-flow translation kinetics. (*Upper*) The 5 amino- and *N*-alkylamino acids used in this study, one of which is shown charged on tRNA$^{PheB}$. This synthetic, unmodified tRNA is based on natural *E. coli* tRNA$^{Phe}$ (black with purple anticodon; posttranscriptional modifications are in green), with changes in blue (11). (*Lower*) Aminoacyl-tRNA prebound to elongation factor Tu–GTP is mixed with ribosomes preinitiated with fMet-tRNA$_{initiator}$ and then quenched rapidly with formic acid. Reactants are in green; products measured are in red.

of *N*-methyl-Phe comes from measuring $\tau_{GTP}$, the average time for GTP hydrolysis on elongation factor Tu (EF-Tu). GTP hydrolysis is greatly stimulated when the ternary complex of aminoacyl-tRNA–EF-Tu–GTP is bound to a cognate codon at the ribosomal A site. $\tau_{GTP}$ is only 4 times longer for *N*-methyl-Phe-tRNA$^{PheB}$ (Fig. 2*D* and Table 1) than for Phe-tRNA$^{PheB}$. Further, *N*-methyl-Phe incorporation is strictly dependent on the aminoacyl-tRNA carrier, EF-Tu (Fig. S1), excluding the possi-

### Table 1. Average times for dipeptide synthesis from fMet-tRNA$^{fMet}$ and different aminoacyl-tRNAs

| Aminoacyl-tRNA | Codon | $\tau_{dip}$, ms | $\tau_{GTP}$, ms | $\tau_{acc,pep}$, ms |
|---|---|---|---|---|
| Phe-tRNA$^{Phe}$ | UUU | 19.6 ($\pm$2.1) | 7.8 ($\pm$0.6) | 11.9 ($\pm$2.1) |
| Phe-tRNA$^{Phe}$ | UUC | 18.5 ($\pm$1.7) | 7.7 ($\pm$0.6) | 10.8 ($\pm$1.8) |
| Phe-tRNA$^{Bulk}$ | UUC | 20.4 ($\pm$1.7) | 8.9 ($\pm$0.8) | 11.5 ($\pm$1.9) |
| Phe-tRNA$^{PheB}$ | UUC | 24.4 ($\pm$2.3) | 13.0 ($\pm$0.7) | 11.4 ($\pm$2.4) |
| *N*-methyl-Phe-tRNA$^{PheB}$ | UUC | 200,000 ($\pm$12,000) | 52.6 ($\pm$7.3) | 199,900 ($\pm$12,000) |
| *N*-butyl-Phe-tRNA$^{PheB}$ | UUC | Undetectable | 67.7 ($\pm$27) | Undetectable |
| Ala-tRNA$^{PheB}$ | UUC | 37.0 ($\pm$5.1) | 13.2 ($\pm$1.4) | 23.9 ($\pm$7.1) |
| Pro-tRNA$^{PheB}$ | UUC | 571.3 ($\pm$65) | 16.1 ($\pm$3.2) | 555 ($\pm$65) |
| Pro-tRNA$^{Bulk}$ | CCG | 62.5 ($\pm$7.6) | 12.5 ($\pm$1.4) | 50.0 ($\pm$7.7) |
| Pro-tRNA$^{Bulk}$ | CCA | 66.7 ($\pm$8.9) | 18.9 ($\pm$2.8) | 47.8 ($\pm$9.3) |
| Pro-tRNA$^{Bulk}$ | CCU | 71.4 ($\pm$9.7) | 23.8 ($\pm$3.5) | 47.6 ($\pm$10.3) |
| Pro-tRNA$^{Bulk}$ | CCC | 116.3 ($\pm$16.1) | 50.0 ($\pm$5.1) | 66.3 ($\pm$16.9) |

Data are expressed as average times for GTP hydrolysis ($\tau_{GTP}$) and dipeptide synthesis ($\tau_{dip}$) measured in the same reaction mixture. The average time for accommodation/peptidyl transfer is calculated as $\tau_{acc,pep} = \tau_{dip} - \tau_{GTP}$. All experiments were conducted at 37 °C in high-accuracy polyamine containing LS3 buffer (see *Materials and Methods*); each experiment was performed at least twice.
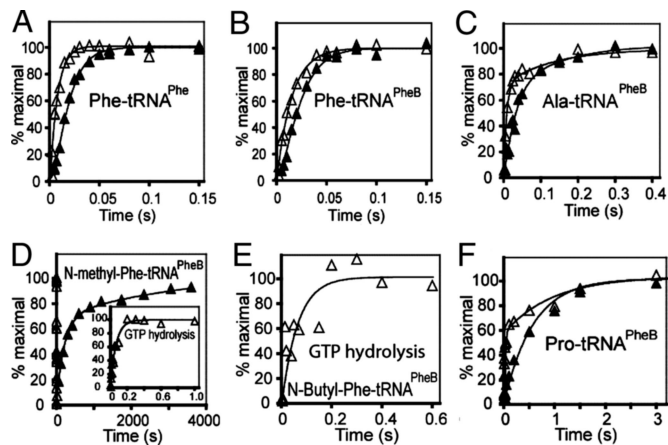
**Fig. 2.** Kinetics of dipeptide synthesis with aminoacyl- and *N*-alkylaminoacyl-tRNAs$_5^{Phe}$. Kinetics of dipeptide synthesis (▲) from fMet-tRNA$_i^{fMet}$ and from Phe-tRNA$^{Phe}$ (*A*), Phe-tRNA$^{PheB}$ (*B*), Ala-tRNA$^{PheB}$ (*C*), *N*-methyl-Phe-tRNA$^{PheB}$ (*D*), *N*-butyl-Phe-tRNA$^{PheB}$ (*E*), and Pro-tRNA$^{PheB}$ (*F*) are shown. Kinetics of the prerequisite, faster GTP hydrolysis (△) by EF-Tu in the same reactions are shown. The ribosome had the Phe codon UUC at the A site. All kinetics were measured at 37 °C in high-accuracy polyamine containing LS3 buffer (see *Materials and Methods*). Representative plots are shown for each assay.

bility of slow, direct binding of *N*-methyl-Phe-tRNA$^{PheB}$ to the A site of the ribosome. The dramatic inhibition of peptide bond formation caused by the *N*-methyl group (Table 1) occurs, therefore, at one or more of the translation steps subsequent to GTP hydrolysis on EF-Tu: release of EF-Tu–GDP from the aminoacyl-tRNA on the ribosome, movement of the aminoacyl-tRNA to the ribosomal peptidyltransferase site (accommodation), and the chemistry of peptide bond formation itself. The average time for these subsequent steps, $\tau_{acc,pep}$ is readily calculated from our data as $\tau_{acc,pep} = \tau_{dip} - \tau_{GTP}$ (Table 1).

The complete, although slow, incorporation of *N*-methyl-Phe into dipeptide prompted us to check the larger *N*-butyl-Phe (Fig. 1), whose incorporation was undetectable in the tetrapeptide system (11). Despite an incubation time of 30 min, *N*-butyl-Phe-tRNA$^{PheB}$ incorporation was undetectable (Fig. S2 and Table 1). However, its GTP hydrolysis time is similar to that of *N*-methyl-Phe-tRNA$^{PheB}$ (Fig. 2*E* and Table 1), again showing that the main inhibition occurs at one or more of the translation steps subsequent to GTP hydrolysis on EF-Tu. This is consistent with measurable codon-specific binding of *N*-butyl-Phe-tRNA$^{PheB}$–EF-Tu–GTP to the ribosomal A site (11).

Given these huge inhibitions by small *N*-alkylations, we wondered whether Pro incorporates significantly more slowly than Phe and Ala. Indeed, incorporation of Pro from Pro-tRNA$^{PheB}$ into dipeptide takes ≈570 ms, 23 times slower than incorporation of Phe from Phe-tRNA$^{PheB}$ and 15 times slower than incorporation of Ala from Ala-tRNA$^{PheB}$ (Fig. 2*F* and Table 1). However, the GTP hydrolysis time is similar for Pro-tRNA$^{PheB}$, Phe-tRNA$^{PheB}$, and Ala-tRNA$^{PheB}$ (Fig. 2 and Table 1), indicating retardation of the accommodation/peptidyl transfer reaction ($\tau_{acc,pep}$), as seen for *N*-methyl-Phe.

The average codon translation time (including translocation) has been estimated for 1 of the 4 Pro codons in vivo as ≈170 ms (rate of 6 s$^{-1}$) at CCG in *E. coli* (19). Although this is substantially longer than the average in vivo codon translation time (45 ms; rate of 22 s$^{-1}$) and our dipeptide synthesis times from Ala-tRNA$^{PheB}$ and Phe-tRNA$^{PheB}$, it is 3 times shorter than our in vitro dipeptide synthesis time from Pro-tRNA$^{PheB}$ at UUC (which does not include the translocation time). We thus hypothesized that the faster rate for Pro in vivo might be caused by its cognate tRNA adaptors and codons (Fig. 3 *A* and *B*). To test

this, we prepared natural aminoacyl-tRNA isoacceptors by charging *E. coli* tRNA$^{Bulk}$ substrate in situ. This substrate was first validated in our system by showing that the natural Phe-tRNA$^{Phe}$ substrates prepared in situ from tRNA$^{Bulk}$ and from pure tRNA$^{Phe}$ incorporate with very similar kinetics (Figs. 2*A* and 3*C* and Table 1). Next, tRNA$^{Bulk}$ was used as the source of the 3 natural Pro-tRNA$^{Pro}$ isoacceptors to measure the kinetics of fMet-Pro synthesis at codon CCG. Interestingly, the dipeptide synthesis time is 62.5 ms (Fig. 3*D* and Table 1), 9 times shorter than for the hybrid substrate Pro-tRNA$^{PheB}$ and reassuringly shorter than the CCG translation time estimated in vivo. Nevertheless, the natural Pro-tRNA$^{Pro}$ isoacceptors at CCG are still 3 times slower than natural Phe-tRNA$^{Phe}$ essentially because of the slower accommodation/peptidyl transfer, not GTPase reaction (Fig. 3*D* and Table 1). Mindful that that each of the 4 Pro codons is translated by a different subset of isoacceptors and that these isoacceptors differ significantly in sequence (73–83% homology; Fig. 3*A*), we also measured the Pro incorporation kinetics at the other 3 Pro codons. The kinetics at CCA and CCU are similar to CCG, but dipeptide synthesis at CCC is twice as slow because of a slower step(s) up to and including GTP hydrolysis (Fig. 3 *E–G* and Table 1). In contrast, translation of the synonymous codons UUC and UUU by the sole *E. coli* Phe-tRNA$^{Phe}$ isoacceptor yields virtually the same kinetics (Figs. 3*H* and 2*A* and Table 1), 6 times faster than translation by Pro-tRNA$_3^{Pro}$ at CCC.

## Discussion

We have measured the kinetics of dipeptide formation between fMet-tRNA$_i^{fMet}$ in the P site and several different natural and unnatural aminoacyl-tRNAs under near-physiological conditions (Table 1). The rate of amino acid incorporation may vary with the identity of peptidyl-tRNA in the P site and with the length of the nascent polypeptide. However, the kinetics we observe here for native aminoacyl-tRNAs are fully compatible with the rate of bulk protein elongation in vivo, suggesting that our results are biologically relevant. Moreover, the measured times of accommodation/peptide bond formation for our 5 different amino/*N*-alkylamino acids (Table 1), irrespective of tRNA adaptor or codon, fit well with organic chemistry theory and experiment for nonenzymatic peptide synthesis. In solid-phase peptide synthesis, amino acids and Pro are more reactive than *N*-methylamino acids, which are more reactive than *N*-butylamino acids (20). This demonstrates, at least in organic solvent, that the increasing nucleophilicity of the respective nitrogen nucleophiles caused by the electron-releasing inductive effects is outweighed by the decreasing nucleophilicity caused by the steric effects: the N nucleophiles in Ala and Phe are less hindered than in Pro, which is less hindered than the freely rotating N in *N*-methyl-Phe, which is less hindered than the N in *N*-butyl-Phe (Fig. 1). Furthermore, amino groups are generally less basic (have lower pK$_a$'s values) in amino acids than in *N*-alkylamino acids, so amino acids may be less protonated and hence more nucleophilic also in aminoacyl-tRNAs on the ribosome. These correlations suggest that amino acid sterics and basicities may play a major role in governing translation incorporation rates of natural and unnatural amino acids and that the major inhibition caused by *N*-alkylation is at the stage of peptide bond formation, not accommodation.

Translations with *N*-alkylamino acids, and perhaps many other unnatural amino acids, may give low yields because the time for peptide bond formation increases to become longer than the dropoff time of ≈1 min for P site-bound, short peptidyl-tRNAs (21, 22), thereby allowing dropoff from the ribosome to occur before coupling. Decreasing dropoff experimentally may thus increase yields with unnatural amino acids (23). This could facilitate ribosomal synthesis and directed evolution of protease-

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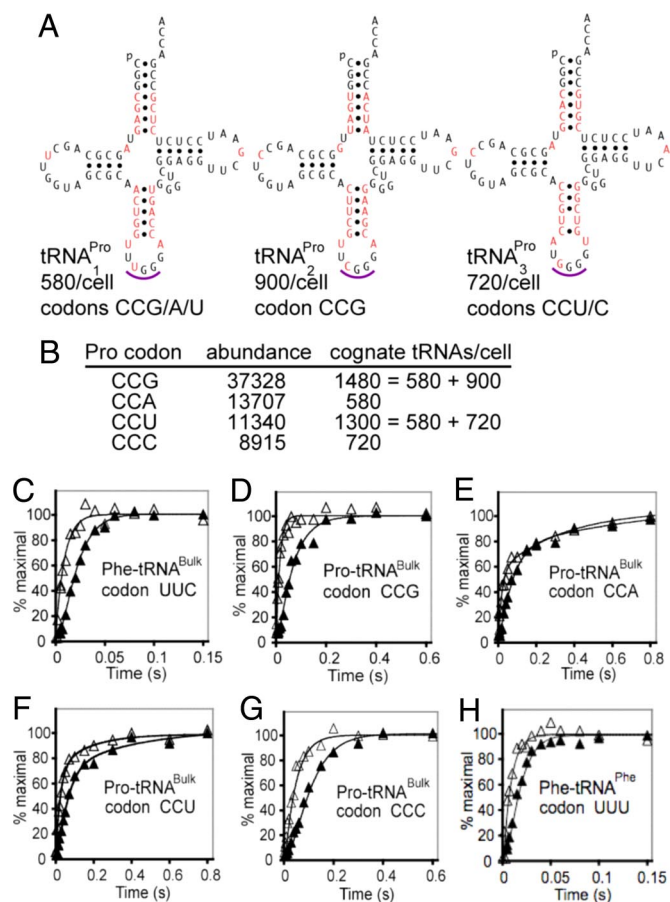
**Fig. 3.** Kinetics of dipeptide synthesis with the native Pro-tRNA^Pro isoacceptors. (*A*) Sequences of the 3 *E. coli* tRNA^Pro isoacceptors. Nonconserved sequences are in red, and anticodons are underlined. The measured number of molecules per *E. coli* cell (28) and accepted codon specificities are shown (the posttranscriptional modifications have not been reported, but the Pro-1 anticodon is accepted to be modified at U34 to cmo^5U) (31). (*B*) Lack of correlation between abundance of Pro codons in *E. coli* genes and measured number of cognate isoacceptor tRNAs. (*C–H*) Kinetics of dipeptide synthesis (▲) and accompanying GTP hydrolysis (△) from native isoacceptors at all 6 codons for Phe and Pro. All kinetics were measured at 37 °C in high-accuracy polyamine containing LS3 buffer (see *Materials and Methods*). Representative plots are shown for each assay.

resistant, membrane-permeable, peptide ligands containing multiple *N*-methylamino acids (23).

That Pro is the sole *N*-alkylamino acid in the genetic code may seem mysterious because several other *N*-alkylamino acids are major products of synthetic prebiotic experiments and meteorite analyses (24). However, it follows from Table 1 and our discussion above that Pro may have been selected because it is a privileged cyclic structure that forms peptide bonds much faster than linear *N*-alkylamino acids.

A lower reactivity of Pro compared with amino acids in peptide bond formation could explain why we find the natural tRNA^Pro isoacceptors to be superior adaptors for Pro (Table 1): the very special properties of Pro may require a very special design of the tRNA^Pro isoacceptors. The optimal tRNA design for the 19 aa may be less idiosyncratic than for Pro, so that in vivo-like incorporation rates do not always require that the amino acids are paired with their cognate tRNAs, as exemplified by the Ala incorporation from the hybrid Ala-tRNA^PheB with only a 2-fold smaller rate than the Phe from the cognate Phe-tRNA^PheB (Table 1). An extension of the "thermodynamic compensation" hypothesis (25–27), that evolution has optimized

the pairing of each amino acid with its tRNA body for ribosomal binding and performance in translation, is therefore supported by our data in Table 1, especially for Pro.

Another hypothesis, the "uniform decoding rate" hypothesis, differs from the thermodynamic compensation hypothesis by proposing that all natural aminoacyl-tRNAs have uniform rates of incorporation in translation (26, 27). The uniform decoding rate hypothesis is supported by recent kinetic data with 10 of the 45 aminoacyl-tRNAs of *E. coli* at 10 codons (27), albeit at rates much smaller than those in vivo. However, the 3-fold difference in translation rate estimated for the same tRNA^Glu isoacceptor at its 2 different codons in vivo (19) is an apparent exception to this hypothesis. Furthermore, our kinetic data, showing a 3- to 6-fold faster incorporation of Phe from the natural Phe-tRNA^Phe isoacceptor than of Pro from the natural Pro-tRNA^Pro isoacceptors (Table 1), demonstrate additional exceptions to the uniform decoding rate hypothesis.

Interestingly, kinetic differences between different tRNA isoacceptors cognate to the same amino acid, here exemplified by the Pro-tRNA^Pro isoacceptor case (Table 1), may be a hitherto missing determinant of codon bias. The "tRNA abundance" theory of codon bias (28), which correlates tRNA isoacceptor concentrations with codon usage, breaks down for Pro codons in *E. coli* (Fig. 3*B*). Instead, codon usage for Pro correlates much better when also considering our measured incorporation times (bottom 4 $\tau_{dip}$ values in Table 1). In particular, CCC may be the rarest of the 4 Pro codons because it interacts least efficiently with a Pro-tRNA–EF-Tu–GTP ternary complex (shown by the long $\tau_{GTP}$ in Table 1). The relatively slow rate of incorporation of Pro at all 4 codons may also help explain why Pro has the second highest rate of loss of the 20 aa in evolution (29).

In conclusion, our data reveal kinetic differences of Pro and Phe incorporation from their native aminoacyl-tRNAs not expected from the uniform decoding rate hypothesis (27). In addition, our results may help explain codon bias not accounted for by the tRNA abundance hypothesis (28). The surprisingly slow accommodation/peptidyl transfer with Pro and other *N*-alkylamino acids fits remarkably well with organic chemistry theory. This provides a framework for understanding the rate-limiting steps in protein synthesis and improving the incorporation of unnatural amino acids.

## Materials and Methods

**Reagents.** We have published the preparation and characterization of the 5 *N*-NVOC-aminoacyl-pdCpAs (9, 11) and the purified *E. coli* translation system (17). Ligated tRNAs were purified by ion exchange chromatography instead of ethanol precipitation because the latter decreased maximal incorporation by severalfold (16). Buffer LS3 contains 95 mM KCl, 5 mM NH$_4$Cl, 0.5 mM CaCl$_2$, 8 mM putrescine, 1 mM spermidine, 30 mM Hepes (pH 7.5), 1 mM dithioerythritol, 2 mM phosphoenolpyruvate, 5 mM Mg(OAc)$_2$, 1 mM GTP, and 1 mM ATP (30). Buffer LS3A is identical except it lacks GTP and contains 2 mM ATP, 1 μg/mL pyruvate kinase, and 0.1 μg/mL myokinase. Assuming that ATP and GTP each chelate 1 Mg$^{2+}$, the free Mg$^{2+}$ concentration in both buffers is 3 mM.

**Kinetic Assay for Simultaneous Measurement of GTPase Activity and Dipeptide Synthesis.** Final concentrations after combining equal volumes of the 2 mixes are given. Ribosomal mix, containing 2 μM 70S ribosomes, 3 μM mRNA (sequences in Table S2), and 2 μM [³H]fMet-tRNA^fMet in buffer LS3, was preincubated at 37 °C for 30 min. For precharged tRNAs, factor mix, containing 0.5 μM EF-Tu and 0.5 μM [³H]GDP in LS3A buffer, was preincubated at 37 °C for 40 min (to ensure complete conversion of GDP to GTP in solution and on EF-Tu), then 0.6 μM chemoenzymatically synthesized, photodeprotected aminoacyl-tRNA was added, and the incubation continued for 5 min. For uncharged tRNAs, factor mix, containing 0.5 μM EF-Tu, 0.5 μM [³H]GDP, and 0.1 mM Phe or Pro in LS3A buffer, was preincubated for 30 min, and then 0.1 unit/μL purified PheRS or ProRS and 0.6 μM purified tRNA^Phe or tRNA^Phe or tRNA^Pro isoacceptors within tRNA^Bulk (20 μM and 16 μM tRNA^Bulk respectively) were added, and the incubation continued for 10 min. Ribosomal and factor mixes were mixed rapidly at 37 °C in a quench-flow apparatus (RQF-3; KinTek Corp.), and the reaction was quenched rapidly with 50% formic acid at various

times (see Fig. 1). Measured rates were independent of the exact tRNA concentration in the factor mix because the ribosomes were present in excess.

**Analyses of Kinetic Assays.** Translation reactions quenched with 50% formic acid were centrifuged at 20,000 $g$ for 15 min, and the [$^3$H]GDP and [$^3$H]GTP in the supernatants were quantified by Mono Q HPLC using a 0–1 M NaCl multistep gradient in 20 mM Tris (pH 7.5) and a $\beta$-RAM model 3 radio HPLC detector (IN/US Systems). The pellets containing [$^3$H]dipeptidyl-tRNAs and unreacted [$^3$H]fMet-tRNA were washed once with 20% formic acid to remove residual [$^3$H]GDP/[$^3$H]GTP and treated with KOH (to 0.5 M) at room temperature for 10 min to release the dipeptide from tRNA, and then formic acid was added (to 17%). The acid-precipitated deacylated tRNAs were then pelleted by centrifugation at 20,000 $g$ for 15 min, and the [$^3$H]dipeptide in the supernatant was quantified by C18 reversed-phase HPLC. Elution was as described in Fig. S2, except fMet-Ala was eluted with 0.1% trifluoroacetic acid in water, and fMet-Pro with 20% methanol/0.1% trifluoroacetic acid. All 4 dipeptides had different elution times, and these times were unaffected by the source of the elongator amino acid (free amino acid vs. *N*-NVOC-aminoacyl-pdCpA).

$\tau_{GTP}$ and its standard deviation were estimated with the nonlinear regression program Origin 7 (OriginLab Corp.). $\tau_{dip}$ and its standard deviation were estimated by nonlinear regression fitting to a 2-step kinetic model (18). In some cases, measured kinetics displayed double-exponential behavior, with the amplitude of the fast phase generally exceeding 70% of the total amplitude (Figs. 2 and 3). In those cases, the average times reported in Table 1 were estimated for the dominant, fast phase, representing rapid, direct binding of the aminoacyl-tRNA–EF-Tu–GTP ternary complex to the A site of the ribosome followed by GTP hydrolysis and accommodation/peptidyl transfer. The slow exponential phase represents in each case a minor, alternative pathway of dipeptide formation far too slow to be significant for the rapid protein elongation in the living cell.

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