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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Ribosomal Peptide Syntheses from Activated Substrates Reveal Rate Limitation by an Unexpected Step at the Peptidyl Site

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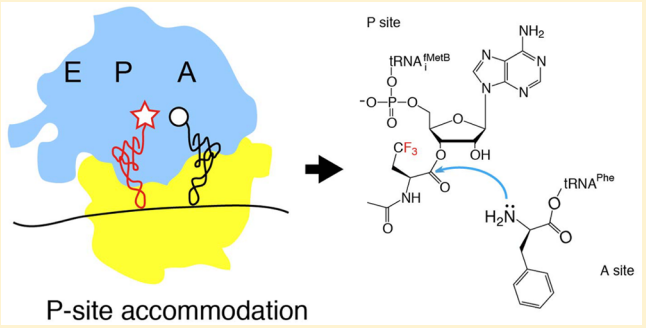
Supporting Information

ABSTRACT: Protein synthesis (translation) is central to cellular function and antibiotic development. Interestingly, the key chemical step of translation, peptide bond formation, is among the slower enzymatic reactions. The reason for this remains controversial because of reliance on studies using highly modified, severely minimized, or unreactive substrate analogues. Here, we investigated this problem by fast kinetics using full-length aminoacyl-tRNA substrates with atomic substitutions that activated the ester electrophile. While trifluoro substitution of hydrogens in nonconserved positions of the peptidyl-site substrate dramatically increased the ester reactivity in solution assays, a large hastening of the combined rates of ribosomal accommodation and peptidyl transfer was observed only with a slowly reacting aminoacyl-site nucleophile, proline. With a fast-reacting A-site nucleophile, phenylalanine, effects did not correlate at all with electrophilicities. As effects were observed using the same, natural, aminoacyl-tRNA at the A site and all rates of accommodation/peptidyl transfer were pH dependent, we concluded that rate limitation was not by A-site accommodation but rather by peptidyl transfer and a hitherto unexpected step at the P site. This new slow step, which we term P-site accommodation, has implications for the activation or inhibition of ribosome function in vitro and in vivo.



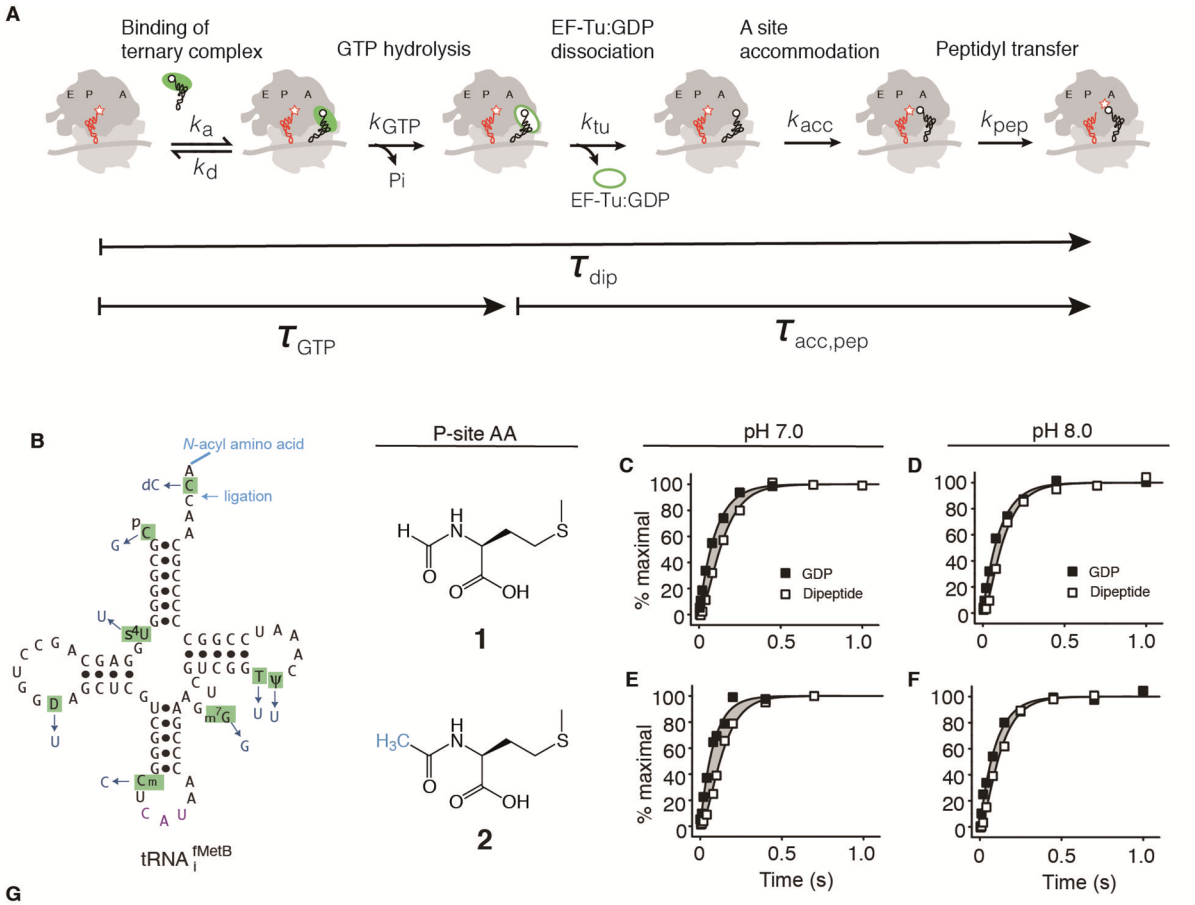
INTRODUCTION

Translation of messenger RNAs (mRNAs) into proteins requires as much as half of the macromolecular mass of a cell and is among the slower enzymatic reactions with an elongation rate of ~22 amino acids (AAs) per second at 37 °C.[1] The speed of translational elongation affects its accuracy,[2] control of gene expression (e.g., by attenuation or frameshifting), protein folding and membrane translocation, codon bias and growth rate,[3] antibiotic susceptibility, and incorporation of unnatural AAs.[4] Rate-limiting steps in elongation are thus bottlenecks for evolutionary optimization, and the rates and mechanisms of the many sequential steps in translation are under intense study.[5−17]

The rate-limiting step between initial substrate binding to the ribosomal A/T site and peptide bond formation (Figure 1A) is controversial. The time required for GTP hydrolysis on the AA-tRNA carrier, EF-Tu (τ_{GTP}), and for dipeptide synthesis (τ_{dip}) has been measured by fast kinetics in a purified *Escherichia coli* translation system, enabling the combined time for A-site accommodation and peptidyl transfer to be calculated ($\tau_{acc,pep} = \tau_{dip} - \tau_{GTP}$; Figure 1A). However, individual rates of A-site accommodation and peptidyl transfer cannot be measured directly because there is no intermediate chemical reaction and the movement of native substrates on the ribosome cannot be visualized. Kinetics of fluorescence changes using an A-site AA-tRNA labeled chemically with a large fluorophore was interpreted as favoring accommodation as rate-limiting,[16,18,19] although the relative rates of accommodation and peptide bond formation are yet to be resolved. This interpretation was supported by the pH insensitivity of fMet-Phe synthesis at 37 °C[19] because the chemistry of peptidyl transfer (see Figure 2A) is expected to be pH-sensitive due to the required deprotonation of the ammonium group of the A-site AA nucleophile. Peptidyl transfer was presumed to be masked by a slower, rate-limiting accommodation step. Thus, studies on the mechanism of peptidyl transfer were thought to require relatively tiny mimics of the A-site substrate (e.g., puromycin and its analogues) to circumvent accommodation.[8] On the other hand, couplings between full-length substrates revealed a slowing when native Pro-tRNA^{Pro} isoacceptors were at the A site[13] (see Figure 4A), presumably due to the higher steric hindrance and pK_a of proline's imino (alkylated N) nucleophile compared with Phe's amino group.[20] In further support of rate-limiting nucleophilic attack, not accommodation, peptidyl transfer to all six AAs tested (including Pro and Phe) was found to be pH-sensitive at 20 °C in a manner correlating with their pK_a values.[10] The discrepancy between the two pH-sensitivity studies on fMet-Phe synthesis[10,19] is apparently due to the low pK_a of Phe-tRNA^{Phe},[10] making it technically difficult to measure its pH sensitivity at 37 °C.[21]

Received: July 5, 2016
Published: November 10, 2016

DOI: 10.1021/jacs.6b06936
J. Am. Chem. Soc. 2016, 138, 15587−15595



G

No.	P-site AA	pH 7.0			pH 8.0		
		τ_{dip} (ms)	τ_{GTP} (ms)	$\tau_{acc,pep}$ (ms)	τ_{dip} (ms)	τ_{GTP} (ms)	$\tau_{acc,pep}$ (ms)
1	*N*-formyl-Met	163 ± 8	97 ± 5	**66 ± 7**	117 ± 15	83 ± 13	34 ± 6
2	*N*-acetyl-Met	145 ± 14	82 ± 11	**63 ± 9**	134 ± 11	96 ± 10	38 ± 4

Figure 1. Ribosomal dipeptide synthesis from a chemoenzymatically synthesized initiator AA-tRNA and native Phe-tRNA^{Phe}. (A) Scheme for dipeptide synthesis. The average time for GTP hydrolysis on EF-Tu (τ_{GTP}, the time for EF-Tu:GTP-catalyzed delivery of Phe-tRNA^{Phe} to the ribosomal A/T site) and also dipeptide synthesis (τ_{dip}) was measured in the same reaction. This enabled the combined time of release of EF-Tu:GDP, A-site accommodation, and peptidyl transfer ($\tau_{acc,pep}$) to be calculated by subtraction ($\tau_{dip} - \tau_{GTP}$). (B) Secondary structure of tRNA$_i^{fMetB}$ charged chemoenzymatically. Changes from native fMet-tRNA$_i^{fMet}$ are in blue. (C–F) Representative time courses of GTP hydrolyses and dipeptide syntheses from tRNA$_i^{fMet}$ or tRNA$_i^{fMetB}$ charged with AAs **1** or **2**, respectively, and native Phe-tRNA^{Phe}. Gray shading indicates $\tau_{acc,pep}$. (G) Kinetics values from (C–F).

Here, we thus chose 20 °C and fast and slow full-length native substrates at the A site to study the rate-limiting step in dipeptide synthesis, thereby avoiding potential artifacts in accommodation or peptidyl transfer due to highly minimalized[10,22,23] or otherwise altered[18] A-site substrates. The rate-limiting step was probed by chemical activations of the ester electrophile in full-length P-site substrates.

RESULTS

Fast, pH-Dependent Ribosomal Peptidyl Transfer from a Synthetic Initiator tRNA. We first confirmed that the time for peptidyl transfer, $\tau_{acc,pep}$, from native fMet-tRNA$_i^{fMet}$ (Figure 1B, in black and green) to native Phe-tRNA^{Phe} (Figure S1) at 20 °C was both physiologically compatible[24] and pH-dependent (Figure 1C,D,G; compound **1**). This pH sensitivity thus implies that, at least at pH 7.0, the reaction was rate-limited by peptidyl transfer, not accommodation.[10] Similarly, the accommodation time, which is assumed to be independent of pH, should be as short or shorter than the shortest $\tau_{acc,pep}$ that we measured at any pH, i.e., ≤34 ms (Figure 1G). Accommodation times as short as 20 ms at room temperature were measured by FRET in Figure S10 of ref 11. Thus, at pH 7, where $\tau_{acc,pep}$ = 63 ms (~2× the accommodation time or more), we predicted that a much more electrophilic substrate would exhibit a measurably shorter $\tau_{acc,pep}$ time (~2× shorter).

Anticipating synthetic challenges due to faster hydrolysis of activated AA-ribose derivatives,[25] we favored dinucleotide chemical acylation and ligase-catalyzed aminoacyl-tRNA synthesis from an in vitro transcript (Figure 1B, blue)[26,27] over the slower ribozyme-catalyzed acylation methods.[28] However, this choice was subject to validating that native fMet-tRNA$_i^{fMet}$ could be substituted with a synthetic tRNA$_i^{fMet}$-based body[29] (tRNA$_i^{fMetB}$; Figure 1B) without adversely affecting the kinetics

DOI: 10.1021/jacs.6b06936
J. Am. Chem. Soc. 2016, 138, 15587–15595

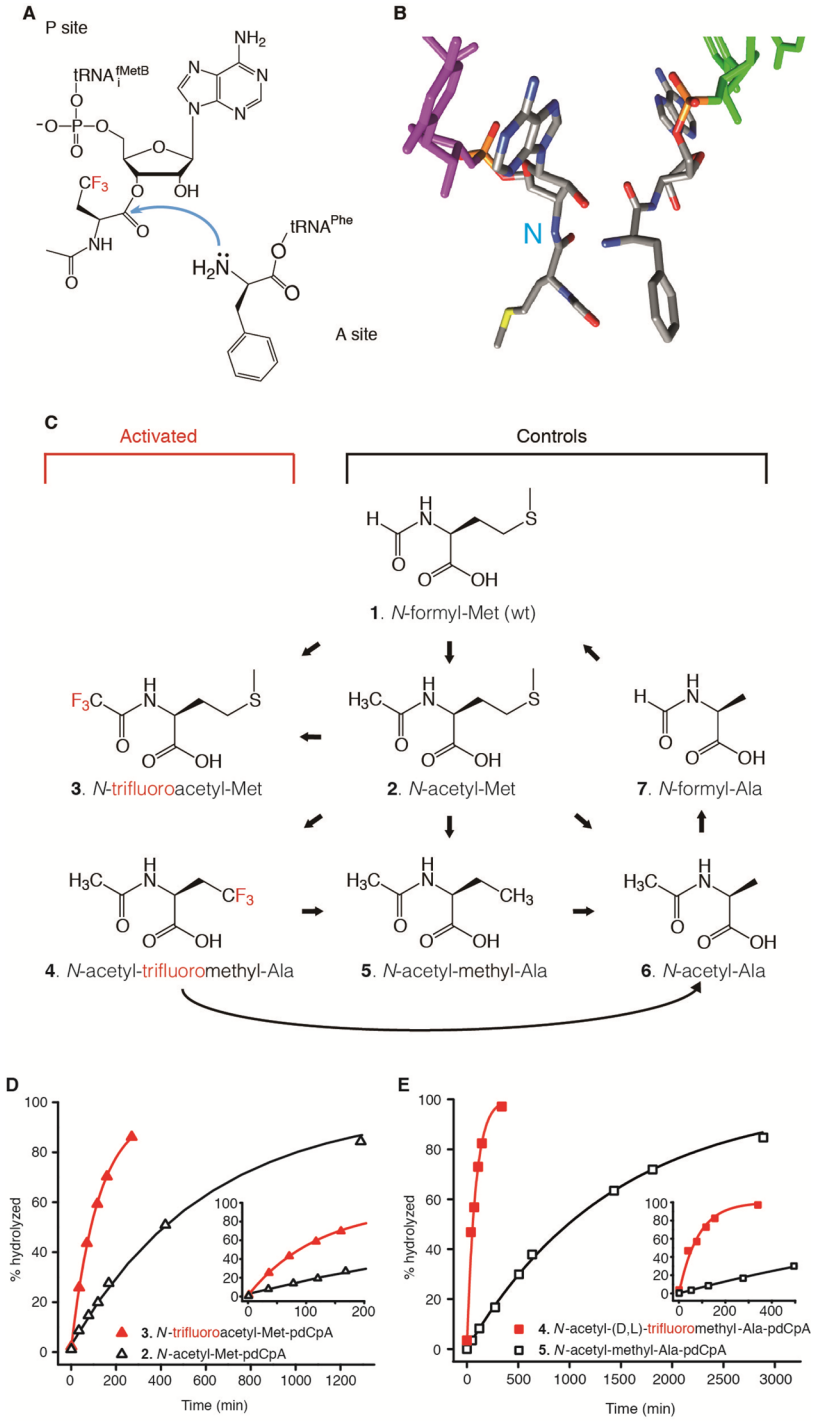


Figure 2. Atomic substitutions in the peptidyl portion of the P-site substrate. (A) Nucleophilic attack at the peptidyl transfer center of the ribosome. The rate of peptide bond formation is pH-sensitive because it is dependent on the fraction of unprotonated amine at the A site, even though ammonium proton exchange is probably much faster[30] than peptide bond formation. The electrophile shown (compound **4**) contains three strongly electron-withdrawing flourine atoms (red). (B) Crystal structure of substrate analogues bound to the 70S ribosome (adapted from PDB 1VY4 from ref 14). N marks the atomic substitution for O in the ester electrophile. (C) N-Acyl amino acids used in this study grouped by similarity (arrows). (D, E) Representative time courses of hydrolysis from the dinucleotide pdCpA of the two activated AAs in (C) compared with their unactivated controls. Insets show short time points.

of dipeptide synthesis. At the same time, we tested the potential effect of N-acetylation of Met (compound **2**), instead of N-formylation (compound **1**), because acetylation is chemically more straightforward and gives more options for activation. The kinetics of dipeptide synthesis from synthetic *N*-acetyl-Met-tRNA$_i^{fMetB}$ was indistinguishable from native *N*-formyl-Met-tRNA$_i^{fMet}$ at both pH values tested, validating our synthetic method (Figure 1C–G and Table S1).

Rationale for Choice of Atomic Substitutions. Given that N-alkylation of the amino nucleophile slows ribosomal dipeptide synthesis,[13] we reasoned conversely that chemical changes activating the ester electrophile[20] at the P site should

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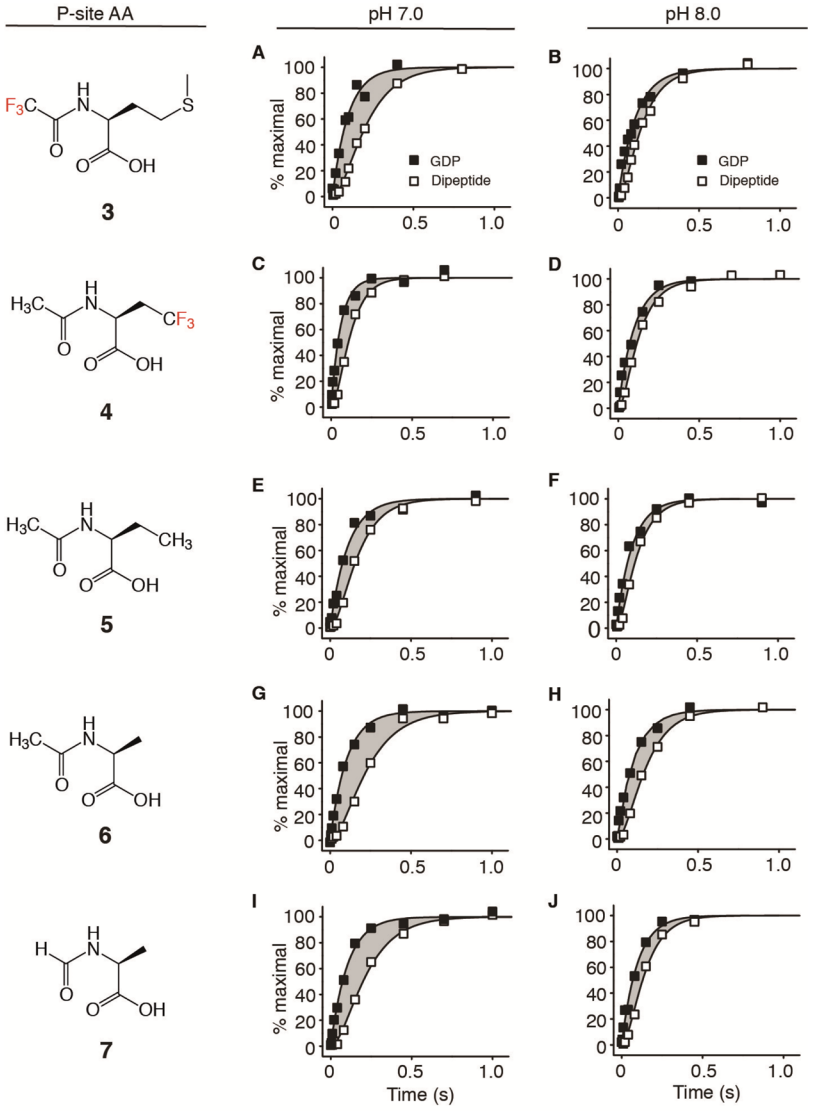
J. Am. Chem. Soc. 2016, 138, 15587–15595



K

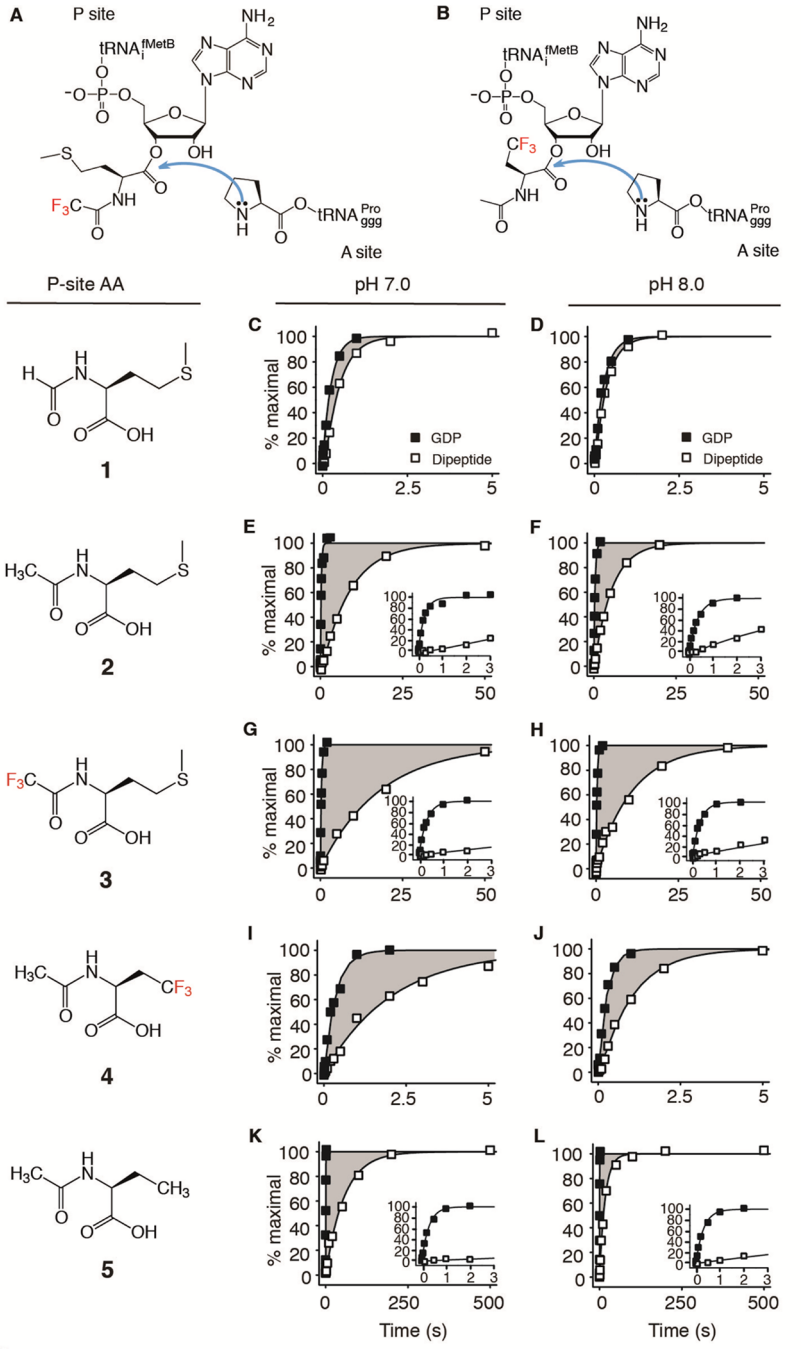
No.	P-site AA	pH 7.0			pH 8.0		
		τ_{dip} (ms)	τ_{GTP} (ms)	$\tau_{acc,pep}$ (ms)	τ_{dip} (ms)	τ_{GTP} (ms)	$\tau_{acc,pep}$ (ms)
1	*N*-formyl-Met	163 ± 8	97 ± 5	**66 ± 7**	117 ± 15	83 ± 13	34 ± 6
2	*N*-acetyl-Met	145 ± 14	82 ± 11	**63 ± 9**	134 ± 11	96 ± 10	38 ± 4
3	*N*-trifluoroacetyl-Met	226 ± 16	98 ± 13	**128 ± 10**	159 ± 10	108 ± 8	51 ± 5
4	*N*-acetyl-trifluoromethyl-Ala	141 ± 6	78 ± 3	**63 ± 5**	142 ± 20	101 ± 19	41 ± 6
5	*N*-acetyl-methyl-Ala	172 ± 15	86 ± 8	**86 ± 12**	141 ± 24	98 ± 24	42 ± 5
6	*N*-acetyl-Ala	253 ± 13	97 ± 5	**156 ± 13**	193 ± 18	108 ± 15	85 ± 9
7	*N*-formyl-Ala	236 ± 12	102 ± 6	**134 ± 10**	156 ± 18	98 ± 15	58 ± 9

Figure 3. Ribosomal dipeptide syntheses from native Phe-tRNA^{Phe} and activated initiator AA-tRNAs compared with their unactivated controls. (A–J) Representative time courses of GTP hydrolyses and dipeptide syntheses. Gray shading indicates $\tau_{acc,pep}$. (K) Kinetics values from (A–J) and Figure 1G.

hasten dipeptide synthesis, provided such changes did not interfere with the ribosomal translation mechanism. Crystal structures of full-length substrate analogues bound to the 70S ribosome suggested that the main conformational state of accommodated native substrates would be alignment poised for peptidyl transfer (Figure 2B). To minimize interference with the translation mechanism, we avoided changing chemical groups common to all P-site substrates, including the terminal ribose 2′-OH, which affects catalysis in some assays.[9,15,17] This left only the AA side chain (of which there are 21 natural proteinogenic possibilities in *E. coli*) and the formyl group of fMet (which can be substituted at the P site by any of the 21 proteinogenic AAs in natural ribosomal polypeptides) for chemical activation. For atomic substitution in these two "nonconserved" positions, substitution of hydrogen by fluorine was deemed ideal because of fluorine's high electronegativity and small size[25] (Figure 2A,C; compounds **3** and **4**).

Trifluoro Substitutions 4 or 5 Bonds Away from a Carbonyl Group Are Dramatically Activating in Solution. Next, we tested if the above-mentioned standard AA-pdCpA chemoenzymatic synthesis and ligation was compatible with our activated AAs. Activated compounds **3** and **4** (Figure 2C)

DOI: 10.1021/jacs.6b06936
J. Am. Chem. Soc. 2016, 138, 15587–15595



M

No.	P-site AA	pH 7.0			pH 8.0		
		τ_{dip} (s)	τ_{GTP} (s)	$\tau_{acc,pep}$ (s)	τ_{dip} (s)	τ_{GTP} (s)	$\tau_{acc,pep}$ (s)
1	*N*-formyl-Met	0.51 ± 0.02	0.25 ± 0.02	**0.26 ± 0.03**	0.39 ± 0.01	0.28 ± 0.03	0.11 ± 0.03
2	*N*-acetyl-Met	13.02 ± 0.89	0.23 ± 0.01	**12.79 ± 0.89**	5.04 ± 0.13	0.32 ± 0.03	4.76 ± 0.15
3	*N*-trifluoroacetyl-Met	18.26 ± 2.12	0.26 ± 0.02	**18.01 ± 2.12**	11.33 ± 0.96	0.28 ± 0.03	11.06 ± 0.96
4	*N*-acetyl-trifluoromethyl-Ala	2.21 ± 0.10	0.26 ± 0.04	**1.95 ± 0.11**	0.88 ± 0.18	0.25 ± 0.04	0.64 ± 0.19
5	*N*-acetyl-methyl-Ala	53.43 ± 3.91	0.24 ± 0.02	**53.17 ± 3.91**	15.74 ± 0.54	0.23 ± 0.02	15.51 ± 0.54

Figure 4. Ribosomal dipeptide syntheses from native Pro-tRNA$_{ggg}^{Pro}$ and activated initiator AA-tRNAs compared with their unactivated controls. (A, B) Phe was substituted with Pro at the ribosomal A site because it reacts slower. (C–L) Representative time courses of GTP hydrolyses and dipeptide syntheses with Pro-tRNA$_{ggg}^{Pro}$. Gray shading indicates $\tau_{acc,pep}$. Insets show short time points. (M) Kinetics values from (C–L).

were synthesized and then further activated by preparation of their cyanomethyl esters, reacted with pdCpA, purified, ligated to tRNA$_i^{fMetB\ minus\ CA}$, and purified. Hydrolytic losses were able to be minimized by hastening procedures (see Methods).

The relative activations of both aminoacyl-pdCpA esters were quantitated by measuring ribosome-free hydrolysis rates in solution in comparison with control compounds (Figure 2C–E and Table S2). CF_3 substitutions increased the reaction rates 5× for compound **3** and 11× for compound **4**, with the

DOI: 10.1021/jacs.6b06936
J. Am. Chem. Soc. 2016, 138, 15587–15595

substitution closer to the ester having the bigger effect, as might be expected.

Trifluoro Substitutions Do Not Hasten Translation with Phe-tRNA^{Phe}. Armed with the desired experimental system at pH 7 and two highly activated P-site substrates synthesized from compounds **3** and **4**, we then tested if the ribosomal catalytic mechanism could be hastened measurably. To our surprise, it could not (Figure 3A,C,K: compare compounds **1**−**4** τ_{dip} and $\tau_{acc,pep}$ at pH 7). In other words, at a pH where peptidyl transfer, not accommodation, is inferred to be rate-limiting with natural substrates, activated compound **4** yielded the same $\tau_{acc,pep}$ as control compounds **1** and **2**, whereas activated compound **3** yielded a $\tau_{acc,pep}$ that was actually 2× slower. This significant prolongation cannot be attributed to slower accommodation because the A-site native AA-tRNA was the same. Rather, it indicates a slowing of rate-limiting peptidyl transfer.

Additional Control Translations. Controls were performed to further evaluate the impact of our changes in the nonconserved portions of the substrates. Compound **5** was prepared to measure the electronic effect of just the three H to F substitutions in compound **4** independent of the side chain substitution (compared with compound **2**). Activation by compound **4** compared with compound **5** was minimal (Figure 3E,K $\tau_{acc,pep}$ at pH 7), and both of these unnatural side chains had similar rates as those of compounds **1** and **2**, suggesting that the Met side chain was not important. Yet, when compound **6** was prepared to test the effect of swapping two natural side chains, Met and Ala, $\tau_{acc,pep}$ at pH 7 was surprisingly slowed by 2× (Figure 3G,K). The three initiation factors were unimportant in our assay as their omission made no difference to the rates (Table S3). This 2× slowing was not due to potentiation by the unnatural N-acetyl group because compound **7** with a natural N-formyl group gave similar kinetics as that of compound **6** at pH 7 (Figure 3I,K). While this reinforced the above conclusion that N-formyl and N-acetyl groups are interchangeable based on comparison between compounds **1** and **2** on different tRNAs, it made the slowing observed with compound **3** versus compounds **1** and **2** even more puzzling. All translations were thus performed at pH 8.0 to verify that the pH 7.0 translations were rate-limited by the chemistry. Indeed, increased pH approximately halved all $\tau_{acc,pep}$ values (Figures 3B,D,F,H,J,K and S2). The τ_{GTP} values, reflecting A-site delivery rates under the conditions used, were similar in all cases, as expected for delivery of the same Phe-tRNA^{Phe}.

Trifluoro Substitution Can Dramatically Hasten a Slow Translation with Pro-tRNA$_{ggg}^{Pro}$. Given our results above, we wondered if our fluorine substitutions were somehow incapable of hastening ribosomal peptidyl transfer despite the large increases in hydrolytic rates measured in solution for the parent compounds (Figure 2D,E). For example, the fluorines might not orient the substrate at the P site as well as the hydrogens, or ester electrophilicity may never be rate-limiting in ribosomal dipeptide syntheses. In order to rule out these possibilities, we returned to a natural peptide synthesis reaction found to be substantially slower than fMet-Phe synthesis: dipeptide synthesis from P-site native fMet-tRNA$_i^{fMet}$ and A-site native Pro-tRNA^{Pro}.[13]

We first confirmed under our conditions that Pro-tRNA$_{ggg}^{Pro}$ (Figure S1) coupled slower than native Phe-tRNA^{Phe} (3×: compare P-site compound **1** in Figures 1G and 4C,M) in a pH-sensitive manner (Figure 4D,M and S2). Then, as above, we substituted native fMet-tRNA$_i^{fMet}$ with synthetic tRNA$_i^{fMetB}$ followed by N-acetylation. Surprisingly, in stark contrast to Figure 1G, this double swap had a huge effect (26× slower: compare control compounds **1** and **2** in Figure 4C,E,M). As this slowing was exclusively in $\tau_{acc,pep}$, not τ_{GTP}, was pH-sensitive (Figure 4F,M), and occurred without changing the A-site substrate, we concluded that the inhibited step was peptidyl transfer to Pro. As the electronic changes to the P-site electrophile were minimal, we suggest that peptidyl transfer to Pro was much more sensitive to minor positional changes in the P-site substrate than was transfer to Phe because Pro is a poorer nucleophile. Coupling to control compound **5** was even slower (Figure 4K,L,M). Nevertheless, this gave us the desired large kinetic window for potential hastening with more electrophilic substrates (the accommodation time should be ≤0.11 s of the much larger $\tau_{acc,pep}$ values: Figure 4M).

Notably, a comparison between compounds **4** (Figure 4B,I,J,M) and **5** with Pro showed that trifluorination shortened $\tau_{acc,pep}$ by an astonishing amount (∼27×) at both pH values. This hastening was of the same order of magnitude, although even larger than, the 11× activation to hydrolysis measured in solution (Figure 2E and Table S2). Thus, a trifluoro substitution was capable of dramatically hastening a translation reaction. Unexpectedly, our less-activated type of trifluorination did not hasten the translation with Pro: A comparison between compounds **3** (Figure 4A,G,H,M) and **2** instead revealed a slight slowing, reminiscent of the results with Phe above.

■ DISCUSSION

Our measurement of combined accommodation and peptidyl transfer times ($\tau_{acc,pep}$) that were influenced by atomic substitutions in the P-site AA in both fast and slow ribosomal dipeptide syntheses has opened a new window into rate-limiting steps in translation. The pH sensitivity of $\tau_{acc,pep}$ indicated rate-limiting peptidyl transfer, not accommodation, at pH 7 for all couplings. Furthermore, the changes in $\tau_{acc,pep}$ found for a particular A-site substrate at any pH could not be attributed to effects on accommodation or A-site artifacts because the A-site substrate was completely natural and held constant. Nevertheless, it should be noted that our data do not rule out rate-limiting accommodation at pH 8 in some of the fastest reactions.

The results, many of which were unexpected, would seem to defy a simple explanation such as our "chemical reactivity hypothesis"[31] that accounted for incorporation rates with N-alkyl AAs. Nevertheless, this hypothesis apparently still explains some results with slow couplings, including some from a recent study detailed in the next section.

Effects of Activations on Slow Translations. There is one publication pertinent to chemically hastening a slow translation.[6] That study measured the kinetic effects of 12 Pro analogues on the exceptionally slow coupling between P-site fMet-Pro-tRNA^{Pro} and A-site Gly-tRNA^{Gly}. Interestingly, like our analogues, their analogues also exhibited hastening and slowing effects that did not correlate well with their relative ester electrophilicities. The authors summarized appropriately that the steric, not electronic, properties of Pro make it an exceptionally poor substrate at the P site, calling the major determinant "positioning". Mindful that interpretation is complicated by additional effects of their analogues on Pro ring pucker and fMet-Pro cis−trans isomerization, it should be noted that their two analogues that coupled fastest on the ribosome were the most activated chemically (also by

DOI: 10.1021/jacs.6b06936
J. Am. Chem. Soc. 2016, 138, 15587−15595

fluorination). Thus, considering both their and our results with slow translations, apparently some analogues obeyed our chemical reactivity hypothesis, whereas others obeyed their P-site positioning hypothesis.

Effects of Activations on Fast Translations. As we are unaware of another attempt to chemically hasten a fast translation, interpretation of results with the fast reacting A-site Phe-tRNA^{Phe} is more speculative. On the basis of the results with intrinsically slow translations discussed in the previous paragraph together with control results in Figure 1, A-site Phe-tRNA^{Phe} coupling should have been hastened by our most-activated trifluorination compound **4** and slowed by activated trifluorination compound **3** and control compound **5**. Of these three rationalizations, only the one with compound **3** was realized (Figure 2). Furthermore, compounds **6** and **7** were unpredictably slow (Figure 2). Thus, our subtle changes to the sterics of the P-site acetyl group or the side chain clearly outweighed effects due to increased electrophilicity of the electrophile on peptidyl transfer times.

How then can our surprising nonhastening of $\tau_{acc,pep}$ in any of the fast translations with A-site Phe be explained? We hypothesize that we have detected a hitherto unexpected rate-limiting step at the ribosomal P site that is slower than A-site accommodation and comparable with the fastest peptidyl transfer rates. It is unexpected because ribosomal crystal structures with bound, full-length substrate analogues show "a fully accommodated A-site nucleophile poised to react with the P-site substrate"[14] (Figure 2B). However, our hypothesis is not incompatible with this structural study because the authors warned that the catalytically active state may differ from their "thermodynamically equilibrated sample containing unreactive substrate analogs".

It is, of course, impossible to prove that peptidyl transfer will never be hastened in the future by some other specially activated P-site substrate. Nevertheless, it is now appropriate to discuss what our translational "speed barrier" at the P site might be. It was neither overcome by prebinding of the P-site substrate nor influenced by initiation factors IF1−3 (Table S3). One possibility is that the P-site ester is predominantly in the unproductive (ribose 2′-O) isomer and that transacylation to the productive (3′-O) isomer is slow on the ribosome. Indeed, spontaneous transacylation of peptidyl-tRNA in solution can be comparible with translation rates,[32] and crystal structures suggest that the ribosome may stabilize one isomer.[12,14,33] However, the isomer stabilized in the crystals is the productive (3′-O) one, and teleologically, the ribosome should have evolved to stabilize it. Also, P-site 2′ deoxy analogues can inhibit, not activate, translation.[9,15,17] Consistent with this, another possibility is rate-limiting deprotonation of the 2′-OH,[34] an unfavorable reaction due to its high pK_a. Given the positional effects of P-site Pro analogues on slow rates,[6] now generalized in our study to positional effects of non-N-alkyl (normal) AA side chain analogues on slow and fast rates, we propose that the rate-limiting step in dipeptide synthesis is a transient conformational change of the 3′-isomer substrate stably bound at the P site and/or a conformational change of the P-site portion of the 23S rRNA, which we term P-site accommodation.

These results have implications for ongoing attempts to understand the mechanism of translation. An extra limiting step at the P site may be a target for antibiotic action, and it may be functional, e.g., by protecting the peptidyl-tRNA from hydrolysis[33] *post* A-site accommodation or by providing extra time for proofreading[2] because A-site accommodation is reversible.[21] Alternatively, as RNA-catalyzed reactions are inherently slow,[35] it may have been too difficult chemically to evolve a faster peptidyl transfer rate. This might explain why biology stuck with aminoacylated substrates that are not so highly activated compared with other substrates in enzymology. Furthermore, initiation and translocation can also be rate-limiting.[5] Indeed, ribosome mutants that translate faster have never been created in a test tube. Nevertheless, the ability to dramatically hasten slow AA couplings on the ribosome may be applied for further mechanistic studies and for improving ribosomal incorporation of unnatural AAs.

MATERIALS AND METHODS

Materials. Oligonucleotides were ordered from Integrated DNA Technology. Tritium-labeled GTP, Phe, and Pro were purchased from PerkinElmer. D,L-Trifluoromethylalanine was from Manchester Organics Ltd. Other chemicals and reagents were from Sigma-Aldrich and Merck. A physiologically compatible purified *E. coli* translation system[36] was used for kinetic studies. All translation factors, overexpressed fMet-tRNA$_i^{fMet}$, and *E. coli* MRE600 70S ribosomes were prepared as described.[24] [^{3}H]Phe-tRNA^{Phe} was prepared by charging *E. coli* overexpressed tRNA^{Phe} with excess [^{3}H]Phe by 0.6 μM PheRS in a charging buffer containing 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 40 mM NaCl, and 1 mM DTE supplemented with 1 mM ATP, 10 mM PEP, 0.006 U/mL inorganic pyrophosphatase, 0.02 μg/mL pyruvate kinase, and 0.002 μg/mL myokinase. [^{3}H]Pro-tRNA^{Pro} was prepared by charging *E. coli* total tRNA (Roche) with excess [^{3}H]Pro by 0.6 μM ProRS in the same charging buffer. The charged tRNA^{Phe} or tRNA^{Pro} was purified on an anion exchange QFF column as described.[13] Synthetic mRNA mMFA and mMPF were prepared as described and had the following sequence: 5′-gggaauucgggcccuu-guuaacaauuaaggaggguauauc**xxxxxxxxx**uaauugcagaaaaaaaaaaaa-3′, where the coding regions (in bold) were **auguuugca** for mMFA and **augcccuuc** for mMPF.

Syntheses of Various AA-pdCpA Analogues. *N*-Formyl-L-alanine was obtained by acylation of alanine with in situ generated acetic-formic anhydride, following the low-temperature methodology.[37] Acetylations of all L-amino acids and (D,L)-2-amino-4,4,4-trifluorobutyric acid ((D,L)-trifluoromethylalanine) were performed according to a general description.[38] *N*-Trifluoroacetyl L-methionine was prepared following previous methodology.[39] All acylated amino acids were converted to corresponding cyanomethyl esters by reaction with chloroacetonitrile as previously described.[26] Conjugates of pdCpA and activated amino acids were obtained according to a previously described method[40] but with slight modification to minimize the contact time of our highly reactive AA-pdCpA derivatives with water: The original two-step purification procedure of preparative HPLC separation in ammonium acetate buffer followed by desalting HPLC in acidified acetonitrile[40] was shortened to a single HPLC separation in a gradient of water/acetonitrile containing a minimal amount of acetic acid (without ammonium acetate).

*N-Acetyl-L-methionine-pdCpA (Containing Compound **2**).* White solid; TOF MS ES$^+$ m/z calculated from $C_{26}H_{37}N_9O_{15}P_2S$, 809.16; found $(M + H)^+$, 809.98.

*N-Trifluoroacetyl-L-methionine-pdCpA (Containing Compound **3**).* White solid; TOF MS ES$^+$ m/z calculated from $C_{26}H_{34}F_3N_9O_{15}P_2S$, 863.13; found $(M + H)^+$, 863.95.

*N-Acetyl-(D,L)-trifluoromethylalanine-pdCpA (Containing Compound **4**).* White solid; TOF MS ES$^+$ m/z calculated from $C_{25}H_{32}F_3N_9O_{15}P_2$, 817.14; found $(M + H)^+$, 817.98.

*N-Acetyl-L-methylalanine-pdCpA (Containing Compound **5**).* White solid; TOF MS ES$^+$ m/z calculated from $C_{25}H_{35}N_9O_{15}P_2$, 763.17; found $(M + H)^+$, 764.01.

*N-Acetyl-L-alanine-pdCpA (Containing Compound **6**).* White solid; TOF MS ES$^+$ m/z calculated from $C_{24}H_{33}N_9O_{15}P_2$, 749.16; found $(M + H)^+$, 750.01.

DOI: 10.1021/jacs.6b06936
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N-Formyl-L-alanine-pdCpA (Containing Compound 7). White solid; TOF MS ES$^+$ m/z calculated from $C_{23}H_{31}N_9O_{15}P_2$, 735.14; found $(M + H)^+$, 736.03.

Hydrolysis Rates of AA-pdCpA Compounds. Obtaining aminolysis rates in aqueous solution proved to be difficult due to much faster hydrolysis. Therefore, we determined hydrolysis rates instead because these have proved to be excellent surrogates for aminolysis rates.[6] Hydrolysis rates of *N*-trifluoroacetyl-Met-pdCpA, *N*-acetyl-Met-pdCpA, *N*-acetyl-(D,L)-trifluoromethyl-Ala-pdCpA, and *N*-acetyl-methyl-Ala-pdCpA were measured at 0.4 mM in 0.2 M phosphate buffer, pH 7.5, at 20 °C. Aliquots of reaction mixtures were taken at various time points, and the formation of pdCpA was followed by reverse-phase HPLC. The time evolution of the fraction of hydrolyzed AA-pdCpA was fitted to a single-exponential equation to estimate the mean time of hydrolysis (Table S2).

Syntheses of Various Unnatural AA-tRNA$_i^{fMetB}$ Substrates. We used the pdCpA chemoenzymatic ligation method to prepare various unnatural AA-tRNA$_i^{fMetB}$ substrates.[26] The DNA template for in vitro transcription of 3′-CA-truncated tRNA$_i^{fMetB}$ was prepared by primer extension after hybridizing the following two oligonucleotides: 5′-GGTACCGAAATTAATACGACTCACTATAGGCGGGGT-GGAGCAGCCTGGTAGCTCGTCG-3′ and 5′-GTTGCGGGG-GCCGGATTTGAACCGACGATCTTCGGGTTATGAGCCCGA-CGAGCTACCAGGCTGCTCCACCC-3′. After transcription with T7 RNA polymerase in the presence of 20 mM GMP, the synthetic RNA was purified on a QFF column. Unnatural aminoacylation of tRNA$_i^{fMetB}$ was done by ligating the chemically synthesized unnatural AA-pdCpA to the 3′-CA-truncated tRNA$_i^{fMetB}$ with T4 RNA ligase at 37 °C, pH 7.5, for only 5 min to limit hydrolysis. After ligation, the product was purified on a QFF column.

Simultaneous Measurement of Rates of GTP Hydrolysis and Dipeptide Formation in the Same Reactions. Kinetics measurements were performed at 20 °C instead of 37 °C for better precision[10] and for stability of P-site substrates, which were preincubated with initiation factors and ribosomes. Ternary complex was not saturating, so the component in excess out of the ternary complex or ribosomes limited the rate. The fast ribosomal syntheses of dipeptides from tRNAs charged with *N*-acetyl-(D,L)-trifluoromethyl-Ala and L-Phe or L-Pro both produced single HPLC dipeptide product peaks that were assumed to be derived from the L-AA isomers because D isomers react very slowly at the P site.[7]

The kinetics was done at pH 7.0 or 8.0 in standard 1× polymix buffer[41] containing 95 mM KCl, 5 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate, and 1 mM DTE. Two mixtures were prepared for the reactions. A ribosomal mix was prepared in 1× polymix buffer with 0.8 μM ribosomes, 0.8 μM IF1, 1.6 μM IF2, 0.8 μM IF3, 2 μM mMFA, 1 mM ATP, 1 mM GTP, 10 mM PEP, 1 μg/mL pyruvate kinase, and 0.1 μg/mL myokinase with 1 μM of the wild type fMet-tRNA$_i^{fMet}$ or unnatural AA-tRNA^{fMetB}. A ternary complex mix was prepared in 1× polymix buffer with 2 μM EF-Tu, 2.4 μM [^{3}H]Phe-tRNA^{Phe}, 2 μM [^{3}H]GTP, 2 mM ATP, 10 mM PEP, 1 μg/mL pyruvate kinase, and 0.1 μg/mL myokinase. For reactions done with [^{3}H]Pro-tRNA^{Pro} as the A-site substrate, the ribosomal mix contained 2.4 μM ribosomes, 2.4 μM IF1, 4.8 μM IF2, 2.4 μM IF3, 4 μM mMPF, 1 mM ATP, 1 mM GTP, 10 mM PEP, 1 μg/mL pyruvate kinase, and 0.1 μg/mL myokinase with 2 μM of the fMet-tRNAifMet or unnatural AA-tRNA$_i^{fMetB}$. The ternary complex mix was prepared in 1× polymix buffer with 1 μM EF-Tu, 1 μM [^{3}H]Pro-tRNA^{Pro}, 1 μM [^{3}H]GTP, 2 mM ATP, 10 mM PEP, 1 μg/mL pyruvate kinase, and 0.1 μg/mL myokinase. The pH of the mixes was adjusted with a pH meter at room temperature (MiniTrode, Hamilton). After 5 min preincubation of the ribosomal mix and 30 min preincubation of the ternary complex mix at 37 °C, the two mixtures were loaded on a temperature-controlled quench-flow apparatus (RQF-3, KinTeck Corp.). Equal volumes of the two mixtures were rapidly mixed in the reaction loop, and the reactions were quenched with final 17% formic acid at varying time points.

Reaction products were centrifuged at 20 000*g* at 4 °C for 15 min. The supernatant containing the [^{3}H]GDP product and unhydrolyzed [^{3}H]GTP was analyzed on a Mono Q column in an HPLC coupled with a β-RAM model 3 radioactivity detector (IN/US Systems) as described.[13] To the pellet was added 0.5 M KOH for a 10 min incubation at room temperature. (This hydrolyzes all peptides and unreacted AAs from the tRNAs and likely also "deprotects" the *N*-trifluoroacetyl-Met amino group.) Then, formic acid was added to 17% to precipitate the deacylated tRNAs. After centrifugation at 20 000*g* at 4 °C for 15 min, the supernatant containing the unnatural AA-[^{3}H]Phe (or Pro) dipeptide and the unreacted [^{3}H]Phe (or Pro) was analyzed on C18 RP-HPLC coupled with the radioactivity detector. The yield of either the [^{3}H]GDP or unnatural AA-[^{3}H]Phe (or Pro) dipeptide product at each time point was quantified as the fraction of the product signal to the total [^{3}H] signal. By fitting the time evolution of [^{3}H]GDP to a single-step kinetic model and that of unnatural AA-[^{3}H]Phe (or Pro) dipeptide to a two-step kinetic model, the mean time required for GTP hydrolysis on EF-Tu and dipeptide formation could be estimated.[10,21] The overall mean time for release of the CCA 3′ end of the A-site AA-tRNA from EF-Tu:GDP, the accommodation of the A-site AA-tRNA, and the peptidyl transfer reaction could be calculated by subtracting the mean time of GTP hydrolysis from that of dipeptide formation. The inverse of the overall mean time is defined as the k_{pep} value.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06936.

Rates of ribosomal dipeptide synthesis in the presence of initiation factors; ribosome-free solution hydrolysis rates of aminoacyl-pdCpA esters; effect of omission of the three initiation factors on the rates of ribosomal dipeptide synthesis in pre-initiated assays; native *E. coli* tRNAs used at the A-site in this study; and pH sensitivities of dipeptide syntheses with P-site compounds **1**−**7** and A-site Phe-tRNA^{Phe} or Pro-tRNA^{Pro} (PDF)

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Notes

The authors declare the following competing financial interest(s): A.C.F. owns patent US6977150 licensed to Ra Pharmaceuticals, Inc., owns shares in the company, and is a member of its scientific advisory board.

■ ACKNOWLEDGMENTS

We thank M. Ehrenberg for discussions and comments on the manuscript, J. Chattopadhyaya, M. Johansson, and J. Åqvist for discussions, and R. Fowler for technical assistance. This work was supported by grants from the Swedish Research Council (NT project grants 2011-5787 and 2016-1 to A.C.F. and Linnaeus Uppsala RNA Research Centre 349-2006-267).

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