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Modulation of the Rate of Peptidyl Transfer on the Ribosome by the Nature of Substrates*

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The ribosome catalyzes peptide bond formation between peptidyl-tRNA in the P site and aminoacyl-tRNA in the A site. Here, we show that the nature of the C-terminal amino acid residue in the P-site peptidyl-tRNA strongly affects the rate of peptidyl transfer. Depending on the C-terminal amino acid of the peptidyl-tRNA, the rate of reaction with the small A-site substrate puromycin varied between 100 and 0.14 s^{-1}, regardless of the tRNA identity. The reactivity decreased in the order Lys = Arg > Ala > Ser > Phe = Val > Asp ≫ Pro, with Pro being by far the slowest. However, when Phe-$tRNA^{Phe}$ was used as A-site substrate, the rate of peptide bond formation with any peptidyl-tRNA was ~7 s^{-1}, which corresponds to the rate of binding of Phe-$tRNA^{Phe}$ to the A site (accommodation). Because accommodation is rate-limiting for peptide bond formation, the reaction rate is uniform for all peptidyl-tRNAs, regardless of the variations of the intrinsic chemical reactivities. On the other hand, the 50-fold increase in the reaction rate for peptidyl-tRNA ending with Pro suggests that full-length aminoacyl-tRNA in the A site greatly accelerates peptide bond formation.

The enzymatic activity of the ribosome is to catalyze peptide bond formation. During the peptidyl transfer reaction, the α-amino group of aminoacyl-tRNA bound to the A site of the ribosome attacks the ester bond of peptidyl-tRNA in the P site, which results in peptidyl-tRNA extended by one amino acid in the A site and deacylated tRNA in the P site. The tRNA substrates are aligned in the active center of the ribosome by interactions of their CCA ends with 23 S rRNA bases (1–3). The ribosome lowers the activation entropy of the reaction (4, 5) by orienting the reacting groups precisely relative to each other (2, 3), providing an electrostatic environment that reduces the free energy of forming the transition state, shielding the reaction against bulk water (6, 7), or a combination of these effects (8).

The peptidyl transfer reaction is modulated by conformational changes at the active site (3, 8–10) as well as by the nature of the substrates. Rapid peptide bond formation requires full-length tRNA in both A and P sites, and the reaction rate is influenced by the length of the tRNA fragments when model substrates are used (8, 10–14). The reaction rate is also influenced by the nature of the amino acid side chain of the A-site substrate (13, 15–17), but is independent of the nucleophilicity of the attacking amino group in model substrates (18). Moreover, the length of the peptidyl chain and the nature of the C-terminal amino acid of the peptidyl-tRNA in the P site seem to have an effect (10, 12, 13, 19). Early studies with 50 S ribosomal subunits indicated that efficient peptidyl transfer was observed with 3′-terminal RNase T1 fragments of *N*-acetyl-Arg-$tRNA^{Arg}$ and fMet-$tRNA^{fMet}$ as model P-site substrates and an analog of aminoacyl-tRNA, puromycin (Pmn[4]; *O*-methyltyrosine linked to N^6-dimethyladenosine via an amide bond), as A-site substrate (20). In contrast, no Pmn reaction was observed with the *N*-acetyl-Asp-$tRNA^{Asp}$ fragment (20). Recently, the importance of the C-terminal amino acid of peptidyl-tRNA was demonstrated for erythromycin-dependent ribosome stalling in synthesis of ErmC protein (21). Peptidyl-tRNA with a C-terminal Pro residue was reported to be exceptionally slow in peptidyl transfer to Pmn on the ribosome (22, 23). Furthermore, the C-terminal Pro is essential for the tryptophan-induced ribosome stalling at the end of the *tnaC* open reading frame (24) or peptide tagging by SsrA (25). However, the effect of different amino acids at the C-terminal position in peptidyl-tRNA on the reaction with aminoacyl-tRNA as A-site substrate has not been studied. Here, we systematically quantified the effect of various C-terminal amino acids in peptidyl-tRNA on the kinetics of peptide bond formation using both Pmn and full-length aminoacyl-tRNA as A-site substrates. Rate constants were measured using the quench-flow technique, and the effects of the amino acid residues and the tRNA identity were distinguished using ribozyme-misacylated tRNAs.

EXPERIMENTAL PROCEDURES

Biochemical Methods—Experiments were carried out in buffer A (20 mM BisTris, 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, and 7 mM $MgCl_2$) at 37 °C. Ribosomes from

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[4] The abbreviations used are: Pmn, puromycin (*O*-methyltyrosine-linked to N^6-dimethyladenosine via an amide bond); BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; EF, elongation factor; CME, cyanomethyl ester; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; HPLC, high pressure liquid chromatography.

Escherichia coli MRE600, initiation factors, EF-Tu, EF-G, [^{3}H]fMet-tRNA^{fMet}, and [^{14}C]Phe-tRNA^{Phe} were prepared as described (12, 26, 27). The mRNAs used were 122-nucleotide-long derivatives of m022 mRNA (28) with the coding sequence 5′-AUG-NNN-UUC-3′, where NNN = GCA, CGC, GAU, AAA, UUU, CCG, UCU, or GUG, coding for Ala, Arg, Asp, Lys, Phe, Pro, Ser, or Val, respectively. Initiation complexes were prepared by incubating 70 S ribosomes (1 μM) with a 3-fold excess of mRNA; 1.5 μM initiation factors 1, 2, and 3; 1.5 μM [^{3}H]fMet-tRNA^{fMet}; and 1 mM GTP in buffer A for 30 min at 37 °C. Initiation complexes were purified by centrifugation through 400-μl sucrose cushions (1.1 M sucrose in buffer A) at 260,000 × *g* for 2 h (RC M120 GX ultracentrifuge, Sorvall). Pellets were dissolved in buffer A to a final concentration of 5 μM, shock-frozen in liquid nitrogen, and stored at −80 °C. Total tRNA from *E. coli* was aminoacylated with ^{14}C-labeled amino acids as described (29). To prepare EF-Tu·GTP·aminoacyl-tRNA ternary complexes, EF-Tu (1 μM) was incubated with pyruvate kinase (0.1 mg/ml), phosphoenolpyruvate (3 mM), and GTP (1 mM) in buffer A for 15 min at 37 °C and added to aminoacyl-tRNA in 2-fold excess. Pre-translocation complexes were formed by mixing equal amounts of initiation complexes and ternary complexes. Translocation was initiated by addition of EF-G (0.05 μM) and GTP (0.5 mM) for 20 s at 37 °C. The resulting post-translocation complexes with peptidyl-tRNA in the P site were purified by gel filtration on Sephacryl S-300 (Amersham Biosciences) in buffer A at 4 °C. Fractions containing ribosome complexes were identified by ^{14}C radioactivity and absorbance at 260 nm, frozen in liquid nitrogen, and stored at −80 °C. Ribosome occupancy with peptidyl-tRNA was ~70% as estimated from the ratio of ^{3}H and ^{14}C radioactivity to the ribosome concentration measured by absorbance.

Preparation of Phe-tRNA^{Arg} and Phe-tRNA^{Lys}—*t*-Butoxycarbonyl-Phe-cyanomethyl ester (CME) was synthesized and deprotected as described (30, 31). Purified tRNA^{Arg}, tRNA^{Lys}, and tRNA^{Phe} were aminoacylated by the RNA enzyme Flexizyme (Fx3) using Phe-CME as substrate (32). Fx3 selectively charges the 3′-end of tRNAs; in the absence of Fx3, Phe-CME did not react with tRNA (33). tRNA and Fx3 were dissolved in 50 mM EPPS (pH 7.5) and 12.5 mM KCl, and RNAs were refolded by heating for 3 min at 95 °C followed by cooling to 25 °C within 3 min. After adding $MgCl_2$ (500 mM), tRNA (1 μM) was mixed with Fx3 (2 μM), and the reaction was started by addition of Phe-CME (10 mM) in Me_2SO (final concentration of 20% (v/v)). After incubation for 2 h on ice, the reaction was stopped by ethanol precipitation. After two ethanol precipitations, the resulting aminoacyl-tRNA was used to prepare ternary complexes with EF-Tu·GTP.

Rapid Kinetics—Measurements were performed in buffer A, except for the experiment shown in Fig. 2*C*, which was carried out in polymix buffer (5 mM potassium phosphate (pH 7.5), 0.5 mM $CaCl_2$, 1 mM dithioerythritol, 95 mM KCl, 5 mM magnesium acetate, 5 mM NH_4Cl, 8 mM putrescine, and 1 mM spermidine) (34). Time courses of peptide bond formation were measured at 37 °C in a quench-flow apparatus (KIN-TEK Laboratories, Inc.) upon mixing equal volumes (14 μl) each of post-translocation complex (0.4 μM) and Pmn or EF-Tu·GTP·Phe-tRNA^{Phe}. Reactions were quenched with KOH (0.5 M); peptides were released by alkaline hydrolysis for 45 min at 37 °C, analyzed by reversed-phase HPLC (LiChrospher 100 RP-8, Merck), and quantified by double-label radioactivity counting.

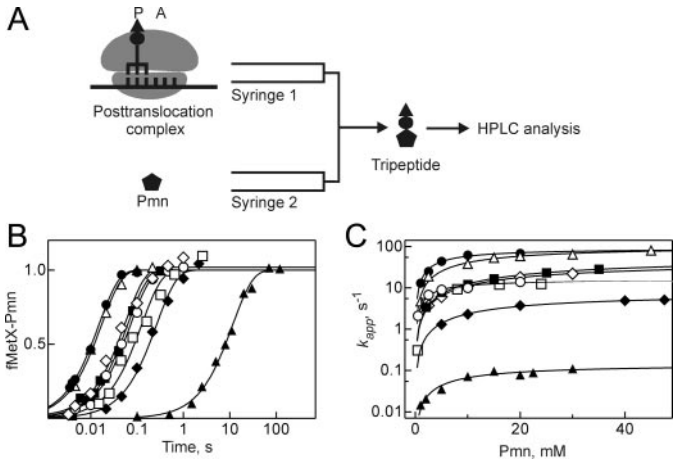

FIGURE 1. **Pmn reaction with different peptidyl-tRNAs.** *A*, experimental approach. Ribosome complexes with dipeptidyl-tRNA (fMet*X*-tRNA^X) in the P site were rapidly mixed with Pmn in the quench-flow apparatus. After the desired incubation time, the reaction was quenched, and the tripeptide fMet*X*-Pmn was analyzed by HPLC. *B*, normalized time courses of the reaction between Pmn (20 mM) and Ala (■), Arg (●), Asp (♦), Lys (△), Phe (○), Pro (▲), Ser (◇), and Val (□) as amino acid *X* in fMet*X*-tRNA^X. Apparent rate constants (k_{app}) were obtained by single-exponential fitting (*continuous lines*). *C*, concentration dependence of k_{app} with different fMet*X*-tRNAs^X. Amino acids *X* are as defined in *B*. Concentration dependences were fitted to a two-step model with a rapid binding step followed by irreversible peptide bond formation, yielding rate constants of peptide bond formation (k_{pep}) and the apparent affinity for Pmn ($K_{1/2}$).

RESULTS

Rate Constants of Pmn Reaction with Different Peptidyl-tRNAs—The reactivity of different peptidyl-tRNAs was first studied with Pmn as A-site substrate. Compared with full-length aminoacyl-tRNA, the reaction with Pmn is slower and more sensitive to pH-dependent changes of the ribosome than that with the native substrate (35). However, at high concentrations, Pmn can be used to monitor the rate of the chemistry step (12). An alternative model substrate that includes the terminal CA of aminoacyl-tRNA (C-Pmn, puromycin attached to a cytidine residue representing the universally conserved C^{75} of aminoacyl-tRNA) has the same (low) affinity for the A site as Pmn (10) and could not be supplied in saturating concentrations required to determine rate constants. At low concentrations, binding of C-Pmn to the A site appeared to be rate-limiting (data not shown), precluding its use at an affordable concentration. The reaction rate with the longer model substrate CC-Pmn (puromycin derivative corresponding to the complete aminoacylated CCA terminus linked to *O*-methyltyrosine) or the full-length substrate aminoacyl-tRNA on the 70 S ribosome is partially or completely limited by accommodation in the A site (10, 35, 36), which makes it difficult to interpret the results for the following peptidyl transfer reaction.

Post-translocation complexes were prepared with P site-bound dipeptidyl-tRNA of the type fMet*X*-tRNA^X, where *X* was Ala, Arg, Asp, Lys, Phe, Pro, Ser, or Val (see "Experimental Procedures") (Fig. 1*A*). The complexes were mixed with excess

TABLE 1
Peptidyl transfer rate constants (k_{pep}) and apparent affinities of Pmn binding ($K_{1/2}$)

P-site substrate	k_{pep}	$K_{1/2}$
	s^{-1}	mM
fMet-Ala-tRNA^{Ala}	57 ± 4	35 ± 4
fMet-Arg-tRNA^{Arg}	90 ± 7	6 ± 1
fMet-Asp-tRNA^{Asp}	8 ± 1	22 ± 2
fMet-Lys-tRNA^{Lys}	100 ± 7	14 ± 3
fMet-Phe-tRNA^{Phe}	16 ± 1	4 ± 1
fMet-Pro-tRNA^{Pro}	0.14 ± 0.02	12 ± 4
fMet-Ser-tRNA^{Ser}	44 ± 2	30 ± 3
fMet-Val-tRNA^{Val}	16 ± 1	6 ± 1

Pmn in a quench-flow apparatus, and the time courses of fMet*X*-Pmn formation were measured at increasing Pmn concentrations (Fig. 1, *B* and *C*). The rate constants of peptide bond formation (k_{pep}) were determined at Pmn saturation (Fig. 1*C*). The rate constants of the Pmn reaction with fMet*X*-tRNA^{X} decreased in the order *X* = (Lys, Arg) > Ala > Ser > (Phe, Val) > Asp ≫ Pro from 100 s^{-1} (Lys) to 0.14 s^{-1} (Pro) (Table 1). The apparent affinities ($K_{1/2}$) of Pmn binding to complexes with different peptidyl-tRNAs varied within the range of 4–35 mM (Table 1).

A characteristic feature of the Pmn reaction with fMet-Phe-tRNA^{Phe} is its pH dependence (12), which reveals two ionizing groups, the α-amino group of Pmn and a ribosomal group contributing to the rate observed at a given pH. To test whether the increased k_{pep} observed with the positively charged C-terminal amino acids Lys and Arg may be related to an altered pH/rate profile, we measured the pH dependence of the reaction with fMet-Arg-tRNA^{Arg} (Fig. 2*A*). Measurements in the pH range from 6.5 to 8.5 indicated that fMet-Arg-tRNA^{Arg} and fMet-Phe-tRNA^{Phe} exhibit a similar pH dependence. Assuming a pK_a for Pmn of 6.9 (12), the pK_a for the putative ribosomal ionizing group was ~7.4 for both peptidyl-tRNAs, and the rate difference between the two P-site substrates remained constant over the pH range studied. Thus, the higher reactivity of fMet-Arg-tRNA^{Arg} compared with fMet-Phe-tRNA^{Phe} is not due to an altered pH dependence of the reaction, but rather reflects an intrinsically higher activity of fMet-Arg-tRNA^{Arg} on the ribosome.

The ribosome accelerates the reaction between Pmn and fMet-Phe-tRNA^{Phe} by 10^7-fold compared with the uncatalyzed peptidyl transfer reaction in solution (4, 5). The acceleration is achieved by lowering the activation entropy. The temperature/rate profiles measured with fMet-Arg-tRNA^{Arg} and fMet-Phe-tRNA^{Phe} indicated that the reaction enthalpies were very similar and that the differences in peptidyl transfer rates originated from differences in the activation entropies (Fig. 2*B*). This observation supports the conclusion that peptide bond formation by the ribosome is an entropy-driven process (4). Furthermore, the difference in the rate constants of peptide bond formation for fMet-Arg-tRNA^{Arg} and fMet-Phe-tRNA^{Phe} appears to be almost independent of temperature, as indicated by the parallel lines in the Arrhenius plot, suggesting that the differences are observed at any temperature in the physiological range.

The observed rate of dipeptide formation when initiation complexes and ternary complexes are mixed appears to depend

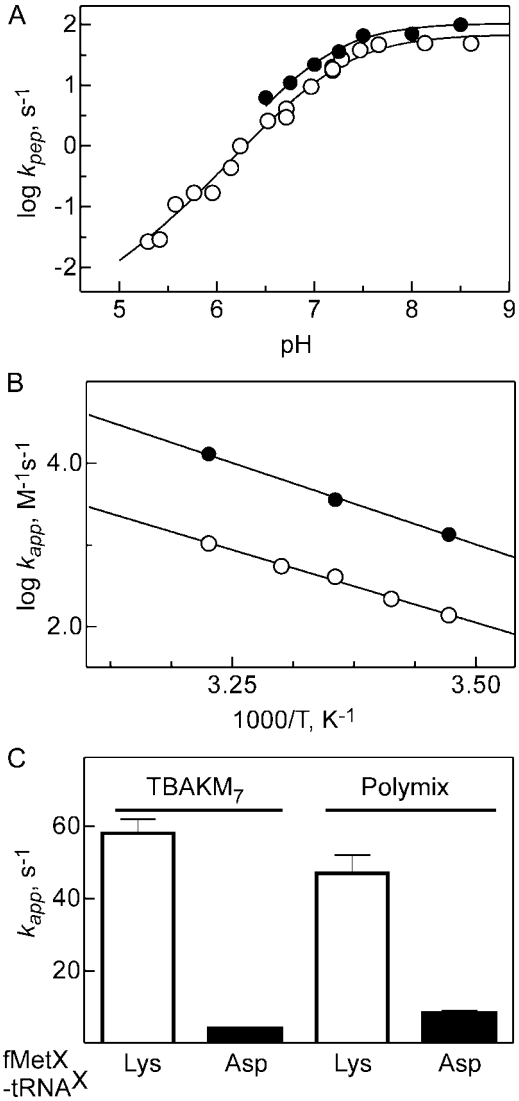

FIGURE 2. **Characteristics of the Pmn reaction.** *A*, comparison of the pH dependences of the Pmn reaction with fMet-Arg-tRNA^{Arg} (●) and fMet-Phe-tRNA^{Phe} (○) (12). *Continuous lines* represent fits assuming two ionizing groups. *B*, Arrhenius plot of the second-order rates of fMet-Arg-Pmn (●) and fMet-Phe-Pmn (○) formation. Activation parameters (25 °C, in kcal/mol) of the Pmn reaction were $\Delta G^{\ddagger}$ = 12.6 ± 1.8, $\Delta H^{\ddagger}$ = 17.7 ± 1.4, and $T\Delta S^{\ddagger}$ = 5.1 ± 0.8 with fMet-Arg-tRNA^{Arg} and $\Delta G^{\ddagger}$ = 13.9 ± 1.4, $\Delta H^{\ddagger}$ = 16.0 ± 0.4, and $T\Delta S^{\ddagger}$ = 2 ± 0.2 with fMet-Phe-tRNA^{Phe} (4). *C*, rates of Pmn (20 mM) reaction in buffer A (*TBAKM*$_7$) and polymix buffer.

on the buffer conditions, as an ~10-fold higher rate has been reported for polymix buffer (37) compared with buffer A used in this work. However, the rate of the Pmn reaction was comparable in polymix buffer and buffer A, and the large difference between the Pmn reaction rates with fMet-Lys-tRNA^{Lys} and fMet-Asp-tRNA^{Asp} was observed in both buffers (Fig. 2*C*), suggesting that the rate differences observed for various peptidyl-tRNAs are robust and are not limited to particular buffer conditions.

Amino Acid Versus tRNA Identity—In addition to the amino acid, the tRNA molecule plays an important role during various steps of elongation, *e.g.* in determining the rate of decoding (38, 39). In the above experiments, peptidyl-tRNAs differed not only in the C-terminal amino acid, but in the nature of the tRNA as well. Therefore, it was important to test whether the

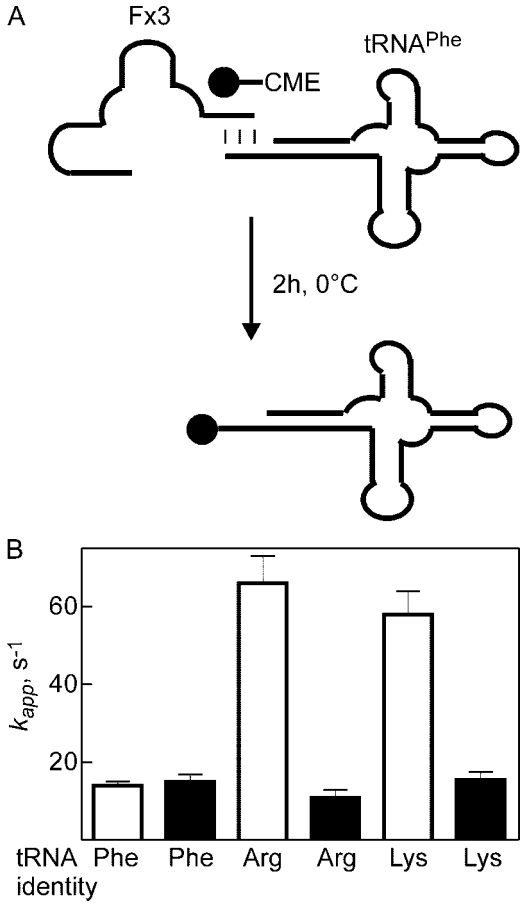

FIGURE 3. **Pmn reaction with misaminoacylated tRNA.** *A*, schematic of the misaminoacylation procedure utilizing ribozyme Fx3 (32) and the activated aminoacyl ester Phe-CME. *B*, rates of Pmn (20 mM) reaction with fMet-Phe-tRNA^X, where X = Phe, Lys, or Arg. *White bars* show the rates for aminoacyl-tRNAs that were aminoacylated by aminoacyl-tRNA synthetases. *Black bars* show the rates for aminoacyl-tRNAs that were charged by Fx3.

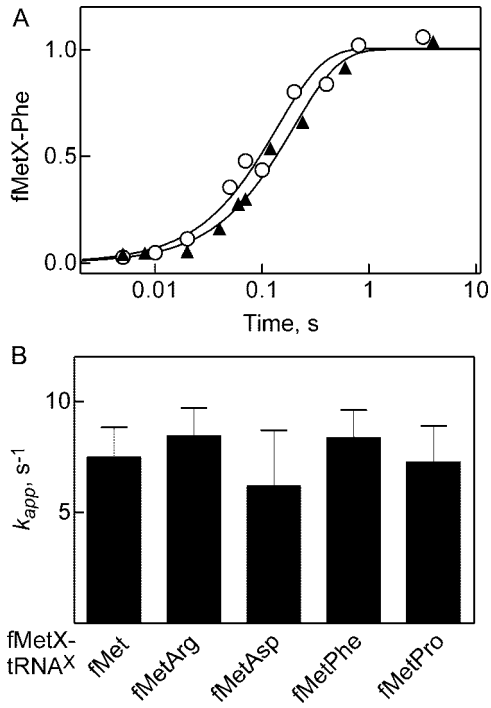

FIGURE 4. **Reaction between fMet*X*-tRNA^X and Phe-tRNA^{Phe}.** *A*, formation of fMet-Phe-Phe (○) and fMet-Pro-Phe (▲). Ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} (1.2 μM) was added in 3-fold excess over the post-translocation complex. *B*, comparison of k_{app} values for different P-site substrates.

effects on the rate of Pmn reaction described above were due to the C-terminal amino acid or the tRNA identity. We addressed this question by studying the reaction with different tRNAs (tRNA^{Phe}, tRNA^{Lys}, and tRNA^{Arg}) that were charged with the same amino acid, Phe (see "Experimental Procedures") (Fig. 3*A*). The resulting Phe-tRNAs were used to prepare post-translocation ribosomes with fMet-Phe-tRNA^{Phe}, fMet-Phe-tRNA^{Arg}, or fMet-Phe-tRNA^{Lys} in the P site. Aminoacylation by the ribozyme produced functionally unaltered aminoacyl-tRNA because the rate of Pmn reaction was identical on ribosomes with peptidyl-tRNA containing Phe-tRNA^{Phe} aminoacylated by Phe-tRNA synthetase or by the ribozyme (Fig. 3*B*). Replacing the C-terminal amino acid in fMet-Arg-tRNA^{Arg} or fMet-Lys-tRNA^{Lys} by Phe resulted in a decrease in the Pmn reaction rate to the value measured for fMet-Phe-tRNA^{Phe} (Fig. 3*B*). These results indicate that the differences in the rates of the Pmn reaction with various peptidyl-tRNAs are due to the nature of the C-terminal amino acid rather than the tRNA identity.

Reaction between Native Substrates—The rates of the peptidyl transfer reaction were also measured between P site-bound fMet*X*-tRNA^X and Phe-tRNA^{Phe} as A-site substrate. Strikingly, very similar time courses (k_{pep} = 7 s^{-1}) were obtained with peptidyl-tRNAs containing X = Phe or Pro (Fig. 4*A*), for which the rates of the Pmn reaction differed by >100-fold, suggesting a large acceleration of the peptidyl transfer reaction from fMet-Pro-tRNA to the natural A-site substrate. Based on the comparison of the rates of the reaction of fMet-Pro-tRNA^{Pro} with Pmn and Phe-tRNA^{Phe}, the rate enhancement brought about by the full-length tRNA substrate in the A site must be at least 50-fold. Furthermore, practically the same reaction rates were obtained for X = Arg, Asp, Phe, and Pro or with fMet-tRNA^{fMet} in the P site (Fig. 4*B*). The observed rates reflect the accommodation of Phe-tRNA in the A site, which is rate-limiting for the peptidyl transfer reaction (35, 40) and ensures uniform velocity of peptide synthesis for all peptidyl-tRNAs regardless of the C-terminal amino acid of the peptidyl-tRNA.

DISCUSSION

Modulation of Reaction Rates by the Nature of the P-site Substrate—The reactivity of peptidyl-tRNA in the peptidyl transfer reaction is modulated by the length of the peptide and the nature of its C-terminal amino acid. Peptidyl-tRNAs with longer peptide chains reacted with Pmn more rapidly than those with shorter ones (12, 19), which may result from reduced mobility of the longer peptide chains and thus better positioning of the reactive groups in the catalytic center of the ribosome. The length of the peptide had no effect on the rate of peptidyl-tRNA hydrolysis in solution (41), suggesting that the effect on the ribosome is due to the environment of either the peptidyltransferase center or the peptide exit tunnel. In fact, certain nascent peptides were shown to affect the activity of the peptidyltransferase center, indicating specific interactions between the nascent peptide and residues of the

ribosome tunnel that are signaled to the catalytic center (reviewed in Ref. 42).

Although the length of nascent peptide affects peptide bond formation only in the ribosomal environment, the nature of the C-terminal amino acid of peptidyl-tRNA affects both ribosome-catalyzed and uncatalyzed reactions (this work and Refs. 13, 22, and 43). Experiments with $tRNA^{Pro}$ misaminoacylated with different Pro derivatives suggested that the C-terminal amino acid has an influence on the reaction with Pmn (22). In this work, we have quantified these effects and showed that the rate of peptide bond formation is determined solely by the C-terminal amino acid and is independent of the tRNA identity (Fig. 3*B*). Similarly, the hydrolysis rate of aminoacyl-tRNAs in solution is dominated by the amino acid and not influenced by the nature of the tRNA (44, 45). In addition to its effect on the reaction rate, the amino acid at the C terminus of the nascent peptide affected the apparent affinity for Pmn; the origin of the latter effect is unclear. In this context, the role of the tRNA is to properly position the peptidyl residue at the peptidyltransferase center (46–49) and, possibly, to induce a reactive conformation of the catalytic center (3, 14, 35, 49).

How the Amino Acid Side Chain May Influence the Rate of Peptide Bond Formation—The effects of the amino acids at the end of the peptidyl-tRNA must originate from the characteristics of their side chains, which can be considered as substitutions at the aminoacyl ester. The side chain may directly affect the reactivity of the peptidyl-tRNA ester by modulating the electrophilicity of the carbonyl carbon, or it may influence the catalytic center of the ribosome, *e.g.* by inducing conformational changes or altering the hydrogen bond network-stabilizing intermediates.

Steric properties of acyl substituents are known to modulate the reactivity of esters (50, 51). The side chains of Ala, Phe, Val, and Ser are uncharged, but sterically different (52–54). Depending on the C-terminal amino acid, the hydrolysis rate decreases in the order Ala > (Ser, Phe) > Val on the ribosome and in solution (43), which correlates with the bulkiness of the side chain (52). Similar effects were observed with other model aminoacyl esters (55–57). Steric factors affect the rates of catalyzed (Table 1) and uncatalyzed (43) reactions to a similar extent, with a rate difference of ~3.5-fold between Ala and Val. This suggests that the sensitivity to steric effects is likely to be intrinsic to aminolysis and not altered by the ribosome to any appreciable extent. One potential reason for steric effects of amino acids may be the distortion by bulky side chains of the tRNA A^{76} ribose sugar pucker, thus affecting the positioning of the catalytically important 2′-OH of A^{76} on the ribosome (58). Furthermore, bulky groups may disturb the optimum trajectory for nucleophilic attack (59), which would decrease the number of successful attacks on the electrophile, or cause steric repulsion or strain upon formation of the tetrahedral intermediate.

In addition to steric contributions, charge effects modulate the rate of peptide bond formation. Although Asp, Arg, and Lys have steric side chain parameters similar to Phe (52, 53), they carry a charge at physiological pH. The rate of peptide bond formation was faster ($k_{pep} \sim 100\ s^{-1}$) with the positively charged Arg and Lys and somewhat slower with the negatively charged Asp ($k_{pep} = 8\ s^{-1}$) than with Phe ($k_{pep} = 16\ s^{-1}$) as C-terminal amino acid. In principle, positively charged side chains could enhance the electrophilicity of the carbonyl carbon. Electronegative acyl substituents were shown to increase the rate of aminolysis of model esters in solution by enhancing the electrophilicity (60, 61), although it is not clear whether the effect would also pertain to the aminolysis of peptidyl-tRNA, for which no data are available. Quantum mechanical calculations suggested an electron-withdrawing effect by the side chains of Arg and Lys, whereas Asp was predicted to be electron-donating (62), consistent with the effects observed with model esters in solution (60). Furthermore, charged amino acid side chains may take part in electrostatic interactions that may change the electrostatic network in the peptidyltransferase center (6). The rate effect of charged amino acid side chains reported here is larger than that reported for the hydrolysis of aminoacyl-tRNA in solution (43), possibly because electrostatic effects may be enhanced and act over a longer range in the environment of the peptidyltransferase center than in aqueous solution.

Unusual Effect of Pro—Peptidyl-tRNA with the C-terminal Pro reacted with Pmn particularly slowly, ~700-fold slower compared with Arg or Lys. These data are consistent with the recent finding of the inefficient Pmn reaction of OmpA-(1–153)-Pro-$tRNA^{Pro}$ (22) and of TnaC-Pro-$tRNA^{Pro}$ (23). Because a longer peptide chain may interact with the ribosome peptide exit tunnel (22, 23), the relative contributions of the nascent chain *versus* the C-terminal amino acid could not be estimated in those experiments. Our data suggest that the Pro side chain alone, without a larger regulating nascent chain reaching into the exit tunnel, greatly reduces the Pmn reactivity of peptidyl-tRNA on the ribosome. In contrast, in solution, Pro-$tRNA^{Pro}$ is no less reactive than other aminoacyl-tRNAs and is hydrolyzed rapidly (43). This implies that the strong inhibitory effect of Pro is facilitated by the environment of the peptidyltransferase center. Interestingly, structurally altered Pro derivatives, *e.g.* the *cis*-Pro analog thiaproline (63), accelerate the Pmn reaction of OmpA-(1–153)-Pro-$tRNA^{Pro}$ (22), suggesting that the unusually low reactivity of Pro may be due to its restricted conformational flexibility.

The C-terminal Pro residue in peptidyl-tRNA appears to have a role in modulating protein synthesis in the cell. A Pro codon preceding a termination codon enhanced the read-through efficiency, presumably by impairing termination (64). Pro is essential for programmed ribosome stalling, such as observed upon TnaC synthesis (65). A conformational change of the ribosome induced by the binding of tryptophan inhibits both TnaC-Pro-$tRNA^{Pro}$ hydrolysis and TnaC-Pro peptidyl transfer. The translating ribosome therefore remains attached to the leader transcript, where it blocks Rho factor binding and subsequent transcription termination (65). Furthermore, the presence of the C-terminal Pro in peptides sometimes leads to "full-length tagging" by the SsrA tagging system (25). These effects can be attributed to slow peptide release during termination of protein synthesis, when peptidyl-tRNA in the P site is hydrolyzed in the peptidyltransferase center with the help of termination factors. Another example where Pro-$tRNA^{Pro}$ plays a crucial role is the inhibition of translation elongation of the SecM protein. In this case, the critical Pro^{166} residue is not incorporated into the nascent chain, although Pro-$tRNA^{Pro}$

appears to bind to the A site of the ribosome (66, 67). The presence of Pro-tRNA^{Pro} in the peptidyltransferase center may predispose the ribosome to stall upon synthesis of SecM (68). Such a model would imply that a slow reaction involving Pro-tRNA^{Pro} gives the signal coming from the peptide tunnel sufficient time to trigger further conformational changes in the active site that lead to complete inhibition of the reaction (42).

Accelerating Effect of Aminoacyl-tRNA in the A site—The reaction with Pmn has been extensively used to study the peptidyl transfer reaction. It yields reaction products of peptidyl-tRNA aminolysis similar to those obtained in the reaction with native substrates and is sensitive to the same inhibitors (69, 70). Thus, the basic features of the mechanism are probably similar for Pmn and aminoacyl-tRNA as A-site substrates. Nevertheless, the presence of full-length tRNA in the A site strongly accelerates peptide bond formation (this work and Ref. 35). The rate of peptidyl transfer from fMet-Pro-tRNA^{Pro} was increased 50-fold, from 0.14 to 7 s^{-1}, when Pmn was replaced with Phe-tRNA^{Phe}. Similarly, fMet-tRNA^{fMet} reacted with Pmn much more slowly (0.8 s^{-1}) (12) than with Phe-tRNA^{Phe} (8 s^{-1}) (this work and Ref. 35). The acceleration may be attributed to more robust substrate positioning (10), induced fit in the active site (3), or effects of tRNA-ribosome interactions outside the peptidyltransferase center on the precise positioning of the substrates (49) or because the reactivity of Pmn is altered by coupling the amino acid and the nucleotide through an amide bond instead of an ester bond. On the other hand, about the same rate of peptide bond formation (5–10 s^{-1}) was observed with all peptidyl-tRNAs tested (Fig. 4*B*), including those ending with Arg, Lys, and Ala, which reacted with Pmn at rates of 50–100 s^{-1}. The rate of 5–10 s^{-1} corresponds to the accommodation of Phe-tRNA^{Phe} in the A site of the ribosome, which precedes and limits the rate of peptide bond formation (35), thus masking any differences in reactivity of P site-bound peptidyl-tRNA. Therefore, the 50-fold increase in the rate of peptide bond formation between fMet-Pro-tRNA and Phe-tRNA compared with Pmn is likely to represent the lower limit of the reaction acceleration by the aminoacyl-tRNA. The intrinsic rate of peptide bond formation is probably much higher and was estimated to be >300 s^{-1} for fMet-Phe formation (35). Given the similar accommodation rates of different aminoacyl-tRNAs (71–73), peptide bond formation with different peptidyl- and aminoacyl-tRNAs takes place at similar rates, regardless of the variations in their chemical reactivities. Thus, the variations in translation rates of particular codons and translational pausing (reviewed in Ref. 74) are more likely to be due to different concentrations of the respective aminoacyl-tRNA or to specific signals in the mRNA and nascent peptide, such as observed upon translation of SecM, recoding of the UGA termination codon by the selenocysteine incorporation machinery, or during frameshifting and bypassing events.

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