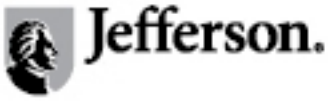

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Amino acid-dependent stability of the acyl linkage in aminoacyl-tRNA.

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Amino acid–dependent stability of the acyl linkage in aminoacyl-tRNA

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ABSTRACT

Aminoacyl-tRNAs are the biologically active substrates for peptide bond formation in protein synthesis. The stability of the acyl linkage in each aminoacyl-tRNA, formed through an ester bond that connects the amino acid carboxyl group with the tRNA terminal 3′-OH group, is thus important. While the ester linkage is the same for all aminoacyl-tRNAs, the stability of each is not well characterized, thus limiting insight into the fundamental process of peptide bond formation. Here, we show, by analysis of the half-lives of 12 of the 22 natural aminoacyl-tRNAs used in peptide bond formation, that the stability of the acyl linkage is effectively determined only by the chemical nature of the amino acid side chain. Even the chirality of the side chain exhibits little influence. Proline confers the lowest stability to the linkage, while isoleucine and valine confer the highest, whereas the nucleotide sequence in the tRNA provides negligible contribution to the stability. We find that, among the variables tested, the protein translation factor EF-Tu is the only one that can protect a weak acyl linkage from hydrolysis. These results suggest that each amino acid plays an active role in determining its own stability in the acyl linkage to tRNA, but that EF-Tu overrides this individuality and protects the acyl linkage stability for protein synthesis on the ribosome.

Keywords: aminoacylation of tRNA; stability of aminoacyl-tRNA; mispaired aminoacyl-tRNA; prolyl-tRNA

INTRODUCTION

The synthesis of peptide bonds is central to cellular life. Up to 22 amino acids are used in peptide bond formation, including the 20 canonical ones, as well as selenocysteine (Bock et al. 1991) and pyrrolysine (Hao et al. 2004). Each of these amino acids by itself is not a substrate for peptide bond formation but instead must be transformed into an aminoacyl-tRNA (aa-tRNA). Although aminoacylation can occur on the 2′- or 3′-OH group of the tRNA terminal ribose, depending on the aminoacyl-tRNA synthetase (aaRS) that catalyzes the reaction (Arnez and Moras 1997), rapid trans-esterification between the two groups leads to aminoacyl esterification to the 3′-OH group (Fig. 1A). The aminoacylation reaction proceeds via the formation of an aminoacyl-adenylate intermediate, using ATP as the energy source. In ribosome-dependent peptide bond formation, the aa-tRNA form provides the basis to physically relate the amino acid to the anticodon of the tRNA and to introduce the amino acid to the ribosome at a codon position matching the tRNA anticodon. In the ribosome-independent peptide bond formation on the N-terminal residue of specific acceptor proteins, catalyzed by aminoacyl-tRNA-protein transferases (e.g., L/F transferase, Arg-tRNA-protein transferase), the aa-tRNA form provides the donor amino acid to mark the conjugated protein products for degradation (Leibowitz and Soffer 1971; Balzi et al. 1990). In the synthesis of cyclodipeptides by cyclodipeptide synthases, two aa-tRNA species are sequentially recognized and their aminoacyl moieties linked and cyclized (Gondry et al. 2009). In other cellular biosynthesis pathways, such as the synthesis of dehydrophos (Bougioukou et al. 2013), the aa-tRNA form is the source of the activated amino acid (Ibba and Soll 2004). However, while the stability of the aa-tRNA form is crucial to biology, the ester linkage is susceptible to spontaneous hydrolysis at the physiological pH, primarily due to its proximity to the 2′-OH of the terminal ribose (Bruice et al. 1962; Hentzen et al. 1972). If hydrolysis

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Abbreviations: aa-tRNA, aminoacyl-tRNA; HEPES, 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid; TLC, thin layer chromatography; $T_{1/2}$, half-life in time; DBE, 3,5-dinitrobenzyl ester; PEP, phosphoenol pyruvate; K_d, equilibrium dissociation constant; k_{on}, on rate of binding; k_{off}, off rate from binding

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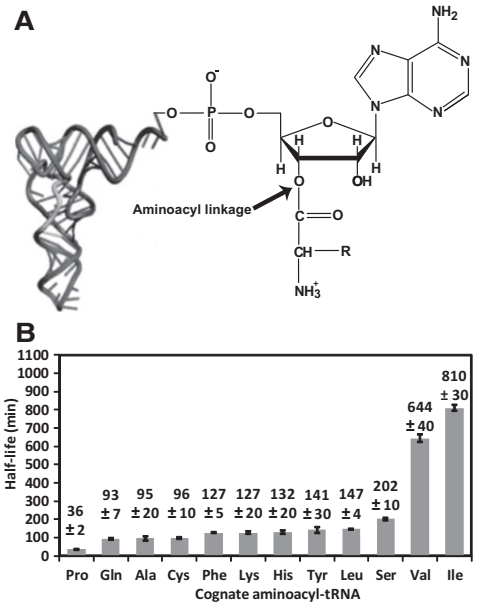


FIGURE 1. The acyl linkage in aa-tRNA. (*A*) The chemical structure of the acyl linkage to the 3′-OH of the terminal ribose of A76 in tRNA, with an arrow indicating the location of the acyl linkage. The size of the tRNA L-shape is not drawn to proportion. (*B*) A summary of $T_{1/2}$ values of cognate pairs of aa-tRNAs determined in this study, showing the average of each by at least three independent measurements. Error bars are standard deviations. The concentration of aa-tRNA in each reaction was as follows: Pro-tRNA^{Pro} (0.18 μM), Gln-tRNA^{Gln} (1.51 μM), Ala-tRNA^{Ala} (0.85 μM), Cys-tRNA^{Cys} (0.42 μM), Phe-tRNA^{Phe} (0.29 μM), Lys-tRNA^{Lys} (1.36 μM), His-tRNA^{His} (0.67 μM), Tyr-tRNA^{Tyr} (0.72 μM), Leu-tRNA^{Leu} (0.27 μM), Ser-tRNA^{Ser} (1.98 μM), Val-tRNA^{Val} (0.90 μM), and Ile-tRNA^{Ile} (0.51 μM).

does occur, the aa-tRNA and the energy of its synthesis would be wasted, requiring the aminoacylation process to be repeated. Thus, the stability of aa-tRNAs toward hydrolysis is an essential factor that determines the speed and efficiency of peptide bond formation.

Surprisingly, while the acyl linkage is locally identical for all aa-tRNAs, earlier work showed that the half-life of the acyl linkage varied from a few minutes to hours for different cognate pairs of amino acid and tRNA (Hentzen et al. 1972; Schuber and Pinck 1974a,b). This finding was unexpected, suggesting that the determinant for the stability of the acyl linkage was not as simple as the chemical nature of the ester bond. However, some earlier work was performed with unusual components (e.g., 0.5 mM $ZnSO_4$) (Matthaei et al. 1966) or at low pH values (e.g., 4.7) (Chousterman et al. 1966). In one of the most thorough early analyses, by Hentzen et al. (1972), measurements of the acyl linkage stability were performed at pH 7.0 but in buffers with nonphysiological salt concentrations (e.g., 1.5 M potassium phosphate). These unusual conditions make it difficult to evaluate the stability of the acyl linkage in a biological context. Most importantly, even with the determination of the relative stability of the acyl linkage of a series of aa-tRNAs, the earlier work gave no insight into what drives the stability of each linkage, thus leaving open the important question of how and why the stability varies.

To address what determines the stability of each acyl linkage, we considered a number of factors, such as the identity of the amino acid, the sequence of the tRNA near the acceptor end, and the modification state of the tRNA. None of these factors had been rigorously evaluated. Importantly, with the recent development of techniques to generate tRNA molecules with mispaired aa-tRNAs (Murakami et al. 2006), with natural and site-specifically mutated tRNA sequences, and with unmodified transcripts (Sampson and Uhlenbeck 1988), we are in a position to clearly define the determinants of the stability of acyl linkage. We show here, by analysis of 12 of the 22 protein amino acids, that the identity of the amino acid makes the single largest contribution to the stability. Specifically, proline confers the least stability, whereas isoleucine and valine confer the highest, while the tRNA nucleotide sequence near the acceptor end makes little contribution to the stability. The finding that the prolyl linkage to tRNA is the least stable relative to others has implications for translation of proline codons on the ribosome.

RESULTS AND DISCUSSION

Stability of the acyl-linkage in cognate aa-tRNA pairs

While the acyl linkage is the same among cognate pairs of aa-tRNAs, the amino acid side chain differs and the tRNA acceptor end sequence varies. To determine whether the stability of the acyl linkage is the same for all aa-tRNA pairs or varies with the amino acid side chain or with the acceptor end sequence, we prepared 12 pairs of *Escherichia coli* cognate aa-tRNAs in vitro and determined the half-life of each acyl linkage at pH 7.5 in an aqueous solution mimicking the physiological buffer. These 12 pairs included amino acids with aliphatic, aromatic, basic, amide, and sulfur-containing side chains. To minimize the effect of post-transcriptional modifications and to focus on tRNA primary sequences, we produced each tRNA by template-dependent transcription in vitro. To provide more consistent aminoacylation efficiency, we used the ribozyme dFx to catalyze tRNA aminoacylation with chemically synthesized activated aa-DBE (3,5-dinitrobenzyl ester) derivatives (Falorni et al. 2000; Murakami et al. 2006). The dFx ribozyme is a 46-mer catalytic RNA that attaches an activated amino acid to the 3′-OH of the tRNA terminal ribose (Murakami et al. 2006; Xiao et al. 2008). This ribozyme uses its terminal 5′-GGU-3′ sequence to base-pair with the 5′-ACC-3′ sequence in tRNA from positions 73 to 75, which is particularly appropriate for sequences with A73 as the discriminator base. This simple base-pairing requirement allows dFx to aminoacylate virtually all tRNA species. The level of aminoacylation by dFx was generally twofold lower than those reported previously (Supplemental Fig. S1; Murakami et al. 2006).

To provide a sensitive assay for the stability of the acyl linkage, we labeled each tRNA with ^{32}P at the terminal A76 via the exchange reaction of the CCA-adding enzyme (Shitivelband and Hou 2005), converted the labeled tRNA to aa-^{32}P-tRNA by dFx, and monitored the decay of aa-^{32}P-tRNA into ^{32}P-tRNA at 20°C. The hydrolyzed tRNA product was distinguished from the substrate by digestion with S1 nuclease, generating ^{32}P-AMP and aa-^{32}P-AMP, respectively, which were resolved by TLC. The fraction of aa-^{32}P-AMP radioactivity that remained at each time point relative to the total radioactivity [aa-^{32}P-AMP + ^{32}P-AMP] was then calculated to determine the extent of the acylated state vs. the deacylated state. The advantage of the assay was that the fraction was independent of the input radioactivity. In contrast, the earlier assay of Hentzen et al. used ^{14}C-amino acid to generate ^{14}C-aminoacyl-tRNA (Hentzen et al. 1972), which was then quantified as acid precipitable counts on filter pads. Because the fraction of the acylated state must be measured by a scintillation counter and calculated relative to the total radioactivity, the sensitivity of the earlier assay was highly dependent on the input counts. An example of our ^{32}P-based assay is shown for Gln-tRNA^{Gln} (Supplemental Fig. S2), showing the course of deacylation over time. The data were fit to a pseudo-first-order exponential decay equation to determine the half-life $T_{1/2}$.

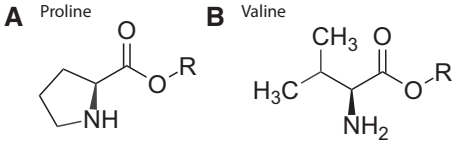
We showed that the $T_{1/2}$ values of the 12 pairs of aa-tRNAs differed significantly over a range of more than 22-fold, from 36 ± 2 min for Pro-tRNA^{Pro} to 640 ± 40 min for Val-tRNA^{Val} and to 810 ± 30 min for Ile-tRNA^{Ile} (Fig. 1B). Interestingly, while our $T_{1/2}$ data differed in absolute values from those reported previously (Hentzen et al. 1972), the relative acyl-linkage stability among these cognate pairs was in good agreement (Supplemental Fig. S3). For example, Hentzen et al. reported that the half-life of Pro-tRNA^{Pro} was relatively short ($T_{1/2}$ = 44 ± 6 min), while that of Val-tRNA^{Val} ($T_{1/2}$ = 690 ± 40 min) and of Ile-tRNA^{Ile} ($T_{1/2}$ = 950 ± 80 min) was long. Also, we showed that the $T_{1/2}$ values of Phe-tRNA^{Phe}, Lys-tRNA^{Lys}, and Tyr-tRNA^{Tyr} were similar (127 ± 5, 127 ± 20, and 141 ± 30 min) (Fig. 1B), consistent with the finding of Hentzen et al. (93 ± 8, 90 ± 6, and 98 ± 8 min, respectively). Thus, despite major differences in buffer compositions and in assay methodologies, the trend of relative stability among aa-tRNA pairs is similar. This result indicates that the buffer composition does not determine the relative stability of aa-tRNAs.

The lack of a buffer effect was further confirmed by analysis of the stability of Tyr-tRNA^{Tyr} in different buffer conditions (Supplemental Fig. S4). Indeed, we showed that the addition of each individual component (150 mM KCl, 3.5 mM $MgCl_2$, or 0.5 mM spermidine) to the basic buffer (100 mM HEPES-KOH, pH 7.5) had no more than a 1.5-fold effect on the $T_{1/2}$ value relative to the buffer that contained all of the components. This result further indicates that the ionic strength of the buffer, which would vary with the addition of each component, did not play a major role in determining the stability of the acyl linkage.

Analysis of mispaired aa-tRNA stabilities

To understand the observed differences among the half-lives of the cognate aa-tRNAs, we considered the chemical structure of the amino acid side chains. In particular, the large differences in the hydrolytic stability between Pro-tRNA^{Pro} (least stable) and Val-tRNA^{Val} and Ile-tRNA^{Ile} (most stable) may be due to inductive and/or steric effects. Specifically, the proline side chain possesses a cyclic secondary amine moiety (Fig. 2A), which has an appreciably higher pK_a than primary congeners (α amine pK_a for Pro, Val, Ile = 10.60, 9.72, 9.76, respectively) (Jenks and Regenstein 1976). Lower contribution of the ionized ammonium form under the experimental pH would explain the significantly higher rate of hydrolysis for the proline ester (Wolfenden 1963; Vig et al. 2003). Additionally, both the valine and isoleucine side chains possess secondary substitution at the β carbon (Fig. 2B) and are, therefore, more sterically inhibited toward hydrolytic attack of the ester carbonyl relative to other amino acid side chains. Indeed, among the three aliphatic side chains (Leu, Val, and Ile), while leucine differs from valine by having a γ carbon side-chain, isoleucine differs from valine by having a bulkier branched β carbon side-chain. We showed that the stability of the acyl linkage is Leu-tRNA^{Leu} < Val-tRNA^{Val} < Ile-tRNA^{Ile}, in an order consistent with the steric effect of the amino acid side chain at the β carbon position. This relative stability was also observed previously (Hentzen et al. 1972), showing $T_{1/2}$ = 73 ± 7 min for Leu-tRNA^{Leu}, 690 ± 40 min for Val-tRNA^{Val}, and 950 ± 80 min for Ile-tRNA^{Ile}. Thus, further computational studies of the steric effect at the β carbon position for natural and unnatural amino acids should be beneficial to establish a framework that can be used to predict and test the acyl linkage stability of a wide variety of aa-tRNAs.

As an example to test the correlation of the β-side chain steric effect with the stability of the acyl linkage, we focused on proline and valine and used dFx to introduce proline to tRNA^{Val} and valine to tRNA^{Pro} (Fig. 2C), such that the



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aa-tRNA	Mean $T_{1/2}$ ± SD (min)	N
Pro-tRNA^{Pro}	36 ± 3	3
Pro-tRNA^{Val}	39 ± 3	3
Val-tRNA^{Val}	644 ± 40	4
Val-tRNA^{Pro}	637 ± 30	2

FIGURE 2. Determinants of acyl stability. (*A*) Structure of a proline side chain with the cyclic secondary amine structure. (*B*) Structure of a valine side chain with a branched β carbon group. (*C*) $T_{1/2}$ values of noncognate pairs of aa-tRNAs compared to the cognate pairs. Error bars are standard deviations. (N) Number of independent measurements. The concentration of aa-tRNA in each reaction was as follows: Val-tRNA^{Val} (0.90 μM), Pro-tRNA^{Pro} (0.18 μM), Pro-tRNA^{Val} (0.75 μM), and Val-tRNA^{Pro} (0.45 μM).

most and the least stable linkage was examined in the context of noncognate aa-tRNAs. The results showed that the half-life of Pro-tRNA^{Val} was identical to that of Pro-tRNA^{Pro} and that the half-life of Val-tRNA^{Pro} was identical to that of Val-tRNA^{Val}. Thus, the attachment of proline to tRNA^{Val} and the attachment of valine to tRNA^{Pro} each drove the acyl stability in a different direction. The virtually complete recapitulation of half-lives according to the nature of the amino acid emphasizes that the side chain is the single most important determinant of acyl stability and that the sequence of tRNA plays no major role.

To confirm that the tRNA sequence has no major role, we used Tyr-tRNA^{Tyr} as a reference and placed Tyr onto eight tRNA species with different sequences near the acceptor end (tRNA^{Leu}, tRNA^{Tyr}, tRNA^{Ser}, tRNA^{His}, tRNA^{Gln}, tRNA^{Lys}, tRNA^{Ala}, and tRNA^{Cys}) (Fig. 3A). These tRNA species differ at position 73 and at the first base pair 1-72, both of which have the ability to influence the flexibility of the CCA end (Lee et al. 1993; Hou et al. 1998) to which amino acid is attached. For example, tRNA^{Tyr}, tRNA^{Lys}, tRNA^{Leu}, and tRNA^{Ala} share in common A73 and a G1-C72 pair, whereas tRNA^{Ser} and tRNA^{Gln} share G73 but contain G1-C72 and U1-A72, respectively. The U73 nucleotide, with the ability to confer flexibility to the CCA end, is used by tRNA^{Cys}, whereas an extra G^{-1} base is present in tRNA^{His} to form a base pair with C73. Despite these variations, we showed that the half-lives of different pairs of Tyr-tRNA^{xx} were closely similar within 1.6-fold of each other (Fig. 3B), supporting the notion that the tRNA sequence near the acceptor end plays little role in determining the acyl stability.

To further examine the role of tRNA sequences, we analyzed the effect of point substitutions in tRNA^{Pro}. Using the GGG isoacceptor of *E. coli* tRNA^{Pro} as an example (Fig. 3C), we showed that the tRNA in the native state isolated from cells and in the transcript state prepared in vitro exhibited no major difference in the acyl stability (Fig. 3D), indicating that the natural base and backbone modifications present in the native state had no effect. In the transcript state, we performed a more global analysis to include substitutions both at the acceptor end and at the distal anticodon end, including the A73U substitution at the discriminator position, the exchange of the first base pair from C1-G72 to G1-C72, and the replacement of A32-U38 in the anticodon loop with U32-A38. None of these sequence replacements had a major effect on the acyl stability (Fig. 3D).

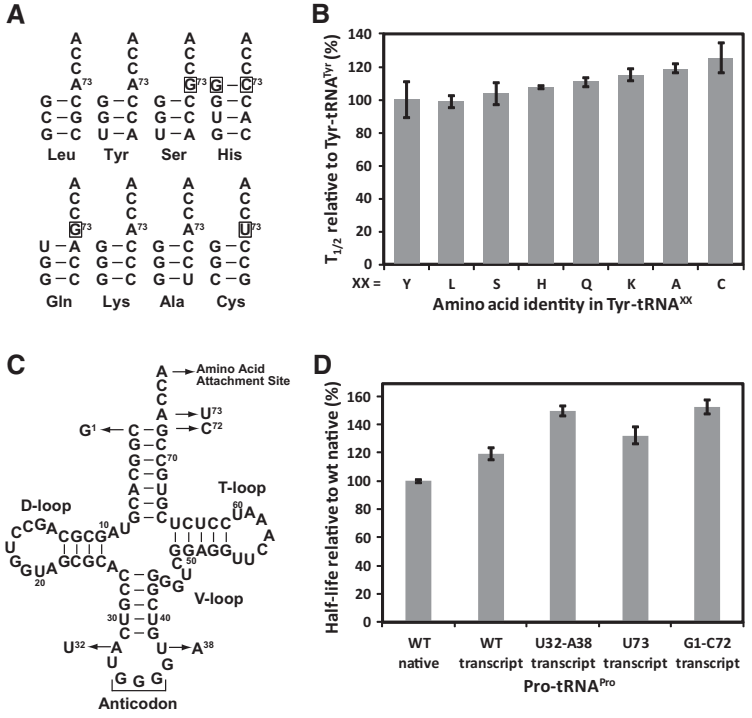

FIGURE 3. No major contribution of tRNA to acyl stability. (*A*) Analysis of $T_{1/2}$ in Tyr-tRNA^{XX}, where the amino acid Tyr is charged to tRNA^{XX} by dFx. Each tRNA^{XX} is shown only for the first four base pairs of the acceptor stem. (*B*) Values of $T_{1/2}$ of Tyr-tRNA^{XX}. Each tRNA was tested as a transcript. The concentration of aa-tRNA^{XX} in each reaction was as follows: Tyr-tRNA^{Leu} (0.20 μM), Tyr-tRNA^{Ser} (0.70 μM), Tyr-tRNA^{His} (0.45 μM), Tyr-tRNA^{Gln} (0.60 μM), Tyr-tRNA^{Lys} (0.67 μM), Tyr-tRNA^{Ala} (0.60 μM), and Tyr-tRNA^{Cys} (0.58 μM). (*C*) The location and nature of the mutations introduced to the sequence of *E. coli* tRNA$^{Pro/GGG}$ as shown by arrows. (*D*) Values of $T_{1/2}$ of Pro-tRNA$^{Pro/GGG}$ for WT and variants of the tRNA. (WT) Wild-type sequence of the tRNA, (t) transcript of the tRNA, without modifications, (n) native form of the tRNA purified from *E. coli*, with all natural modifications. Values are the average of at least two independent measurements. Error bars are standard deviations. The concentration of Pro-tRNA^{Pro} in each reaction was as follows: WT transcript (0.18 μM), WT native (1.48 μM), U32-A38 transcript (0.37 μM), U73 transcript (0.99 μM), and G1-C72 transcript (0.28 μM).

Analysis of amino acid enantiomerism and EF-Tu effects on stabilities

Given that the chemical identity of the amino acid side chain plays the dominant role in the stability of the acyl linkage, we then determined whether the chirality of the side chain has a role. Both D- and L-forms of amino acids can be charged onto the cognate tRNA by natural aaRS enzymes, although only the L-enantiomer is used for peptide bond formation. High concentrations of D-Tyr cause cellular toxicity, in part due to accumulation of D-Tyr-tRNA^{Tyr}, which limits the pool of tRNA^{Tyr} available for synthesis of L-Tyr-tRNA^{Tyr} for the ribosome. This toxicity is ameliorated if cells maintain an active deacylase to remove D-Tyr-tRNA^{Tyr}, or express tRNA^{Tyr} to high levels to increase the supply of the L-enantiomer (Soutourina et al. 2004). These observations implied that the D-form of Tyr-tRNA^{Tyr} is stable enough to exhaust

the available pool of tRNA^{Tyr} and to challenge the synthesis of the L-form. We tested this hypothesis, using the D- and L-forms of Phe-tRNA^{Phe} as an example. Indeed, the two forms showed parallel decay over time with a similar $T_{1/2}$ value (130 ± 40 min and 125 ± 3 min, respectively) (Fig. 4A), in agreement with an earlier analysis of Tyr-tRNA^{Tyr} (Calendar and Berg 1967). Even in the study of N-acetyl-Phe on AMP as a model for the 3′-terminal adenosine of tRNA, the D- and L-forms differed in stability by only a small effect (Wickramasinghe and Lacey 1993).

For Pro-tRNA^{Pro}, we determined if EF-Tu stabilizes the acyl linkage. EF-Tu is a GTP-dependent bacterial elongation factor (with a homolog EF-1α in eukaryotes), which recognizes all canonical aa-tRNAs and escorts each to the matching codon position on the ribosome and, upon GTP hydrolysis, provides the aa-tRNA as the substrate for protein synthesis. The equivalent protein factor for selenocysteine (Sec)-tRNA^{Sec} in bacteria is SelB, which possesses similar properties as EF-Tu (Paleskava et al. 2010). In its crystal structures in complex with a cognate aa-tRNA (Nissen et al. 1995, 1999), EF-Tu recognizes the acyl linkage using a conserved β-barrel motif that has the ability to adapt to variations in the amino acid side chain. This recognition mechanism suggests the possibility to protect the acyl linkage of all aa-tRNAs from hydrolysis. Indeed, we showed that the presence of a 30-fold molar excess of EF-Tu-GTP stabilized Pro-tRNA^{Pro} up to 800 min (Fig. 4B), indicating protection of the acyl linkage ($T_{1/2}$ = 36 ± 2 min) by more than 20-fold, to a level similar to that of Ile-tRNA^{Ile} (Fig. 1B). Based on high cellular concentrations of EF-Tu (100–200 μM) (Burnett et al. 2013) and the entire population of aa-tRNAs (50–200 μM) (Dong et al. 1996), the protection of EF-Tu-GTP at a high molar ratio to a single Pro-tRNA^{Pro} species is physiologically relevant. This protection is consistent with an earlier observation of EF-Tu-GTP protecting the acyl stability of Phe-tRNA^{Phe} by more than 10-fold (from $T_{1/2}$ of 56 to 800 min) (Beres and Lucas-Lenard 1973).

To determine the significance of the EF-Tu protection of prolyl linkage, we analyzed the stability of the ternary complex EF-Tu-GTP-Pro-tRNA^{Pro}. Using an RNase A assay (LaRiviere et al. 2001), in which Pro-tRNA^{Pro} released from the complex would be cleaved by the nuclease, we determined the k_{off} of the release as $(1.57 \pm 0.01) \times 10^{-2}$ s^{-1} (Supplemental Fig. S5), ~10-fold faster compared to the k_{off} of yeast Phe-tRNA^{Phe} [$(1.0 \pm 0.1) \times 10^{-3}$ s^{-1}] measured by the same method (data not shown), the latter of which was in agreement with data of others (Schrader et al. 2009). Because the on-rate of Pro-tRNA^{Pro} and Phe-tRNA^{Phe} to EF-Tu-GTP is similar between the two (Louie and Jurnak 1985), the off-rate drives the difference in affinity in the ternary complex. Thus, a 10-fold weaker affinity of Pro-tRNA^{Pro} in the ternary complex relative to Phe-tRNA^{Phe} would sensitize the former to rapid hydrolysis.

CONCLUSIONS

We show here that, under physiological buffer conditions, the stability of the acyl linkage of cognate pairs of aa-tRNAs differs markedly, with Pro-tRNA^{Pro} being most sensitive to hydrolysis and Val-tRNA^{Val} and Ile-tRNA^{Ile} being most resistant. While a similar trend was observed in nonphysiological buffer conditions, our study is significant, revealing that it is the amino acid itself that determines the stability of each aa-tRNA. Neither the sequence nor the post-transcriptional modification state of the tRNA contributes to the stability. The finding that only the chemical structure, not even the chirality, of an amino acid is the major determinant of stability emphasizes an active role of the structure in controlling the half-life of the amino acid on the tRNA. Thus, in the aa-tRNA form, a division of labor is evident between the two moieties of the molecule: While the tRNA moiety determines where and when the amino acid is used for protein synthesis on the ribosome, the amino acid moiety, in turn, determines the stability of the tRNA to perform this function. In broader perspectives, this finding provides fundamental insight into the evolution of amino acids and the development of aa-tRNAs for cellular activities. While the great majority of aa-tRNAs are utilized for protein synthesis on the ribosome, a fraction (e.g., Leu-tRNA^{Leu}, Arg-tRNA^{Arg}, Tyr-tRNA^{Tyr}, and Phe-tRNA^{Phe}) is also diverted to biosynthesis of metabolites (Ibba and Soll 2004). However, Pro-tRNA^{Pro} is so far known only for utilization on the ribosome, perhaps due to the need for protection of its relatively unstable acyl linkage by the

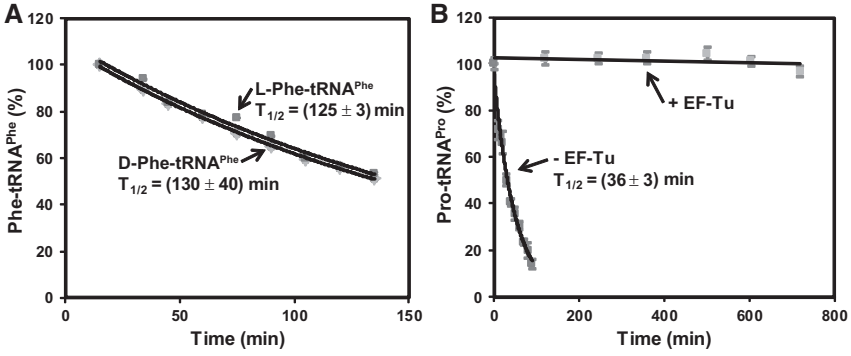

FIGURE 4. Effects of enantiomerism and EF-Tu on acyl linkage stability. (*A*) Analysis of the hydrolytic decay of the D- and L-form of Phe-tRNA^{Phe} over time. The calculated $T_{1/2}$ for each is indicated. The concentration of Phe-tRNA^{Phe} was as follows: L-Phe-tRNA^{Phe} (0.29 μM), D-Phe-tRNA^{Phe} (1.5 μM). (*B*) Analysis of the hydrolytic decay of Pro-tRNA^{Pro} (0.2 μM) over time in the presence and absence of EF-Tu (9 μM). The calculated $T_{1/2}$ in the absence of EF-Tu is shown. The concentration of Pro-tRNA^{Pro} in each reaction was as follows: with EF-Tu (0.3 μM) and without EF-Tu (0.18 μM).

large and elaborate protein synthesis machinery. Indeed, Pro-tRNA^{Pro} is less favorable relative to other aa-tRNAs in many aspects of peptide bond formation: Upon synthesis it is released from the charging enzyme prolyl-tRNA synthetase directly into solution rather than being channeled to EF-Tu-GTP (Zhang et al. 2006); in solution it has a weaker affinity to bind to EF-Tu-GTP (Supplemental Fig. S5); and on the ribosome it performs peptidyl transfer at a rate slower by five- to 10-fold than others (Pavlov et al. 2009). Thus, the finding that the prolyl linkage, once bound to EF-Tu-GTP, is protected from hydrolysis is significant, indicating that the linkage is stabilized and is enabled to participate in peptide bond formation on ribosome-mRNA complexes. Because EF-Tu is a dedicated factor to the ribosome machinery, this provides an explanation for why Pro-tRNA^{Pro} is solely used by the ribosome. It is, therefore, reasonable to expect that, once protein synthesis is initiated on an mRNA sequence, the overall rate of synthesis will depend on the translation of proline codons due to the less reactive nature of Pro-tRNA^{Pro}, which, in turn, depends on the stability of the prolyl linkage.

MATERIALS AND METHODS

Substrate tRNAs

Most of the tRNA substrates were prepared by in vitro transcription, using T7 RNA polymerase to transcribe synthetic DNA templates (Hou et al. 1993). In some cases, native tRNA species were prepared by isolation from *E. coli* cells that overexpressed the tRNA species (Liu et al. 2011).

Preparation of aa-tRNA

Aminoacylation of tRNA was performed using the ribozyme dFx with most aminoacyl-DBE species or using the ribozyme eFx for Phe-DBE (Murakami et al. 2006). The ribozyme charging reaction was performed with radiolabeled tRNA (nominally 0.5 μM), 5 mM aa-DBE, and 18.75 μM ribozyme in 90 mM HEPES-KOH, pH 7.5, 90 mM KCl, and 0.6 M $MgCl_2$ on ice and incubated from 30 min to 12 h, depending on the aa-DBE. The yield of aminoacylation of tRNA was generally ~10% (Supplemental Fig. S1). The synthesis of the aa-DBEs is described in detail in Supplemental Material. For Pro-tRNA^{Pro}, the synthesis of aa-tRNA was also prepared by using the cognate ProRS with similar yields.

Assay for the stability of the acyl linkage

The tRNA substrate in an aa-tRNA was labeled with α-^{32}P-ATP at the A76 nucleotide by the CCA-adding enzyme of *Bacillus stearothermophilus* (Shitivelband and Hou 2005). The labeled tRNA (0.2–1.5 μM) was then acylated by dFx or eFx to generate the aa-tRNA, which was ethanol-precipitated and used directly for the stability analysis. Analysis of the stability of the acyl linkage was performed at 20°C in a physiological buffer of 100 mM HEPES-KOH, pH 7.5, 150 mM KCl, 3.5 mM $MgCl_2$, 1.0 mM DTT, and 0.5 mM spermidine. Aliquots of 1 μL were taken over time for 1–2 half-lives, quenched in 3 μL of 50 mM NaCl and 200 mM NaOAc, pH 5.0, and stored at −20°C until final processing. The tRNA was digested to mononucleotides by incubation with 4 units of S1 nuclease in the presence of 0.2 mM $ZnSO_4$ for ~20 min at 37°C. The digested samples were run on 10-cm PEI cellulose plastic TLC sheets with 0.1 M NH_4Cl and 5% HOAc buffer until the solvent front reached the end of the sheet. After drying, the TLC sheets were phosphorimaged, and bands of aa-^{32}P-AMP and ^{32}P-AMP were quantified using Image Quant. The ratio of aa-^{32}P-AMP signal to the total signal of [aa-^{32}P-AMP + ^{32}P-AMP] was used to determine the fraction that had been deacylated. The data were fit to the pseudo-first-order exponential decay by least-squares regression to determine the $T_{1/2}$ from the decay rate constant k.

$$[\text{aa-tRNA}] = [\text{aa-tRNA}]_0 e^{-kt} \quad T_{1/2} = \ln(2)/k.$$

EF-Tu binding analysis

Stability of the EF-Tu-GTP-Pro-tRNA^{Pro} ternary complex was measured with Pro-tRNA^{Pro} (0.3 μM) and *Thermus thermophilus* EF-Tu (9 μM). The protein factor was activated in a buffer (50 mM HEPES-KOH, pH 7.0, 20 mM $MgCl_2$, 5 mM DTT, 0.5 M NH_4Cl) containing 20 μM GTP, 1.2 mM PEP (phosphoenolpyruvate), and 50 μg/mL pyruvate kinase for 3 h at 4°C and then incubated with ^{32}P-labeled Pro-tRNA^{Pro} (0.3 μM) in an ice bath. A titration of EF-Tu showed that concentrations higher than 5 μM did not further improve the ability of the factor to protect the prolyl linkage, whereas concentrations in the range of 0.5 to 5 μM showed concentration-dependent protection effect, and concentrations at 0.2 μM or below showed limited effect. In the off-rate k_{off} analysis, aliquots were quenched with RNase A (10 μL of 1 mg/mL) over time, and the tRNA was precipitated by acid. Counts that remained acid-precipitable were measured and plotted as a function of time to determine the k_{off} (Schrader et al. 2009). Assays for stability of the acyl linkage and for the off-rate analysis were performed at 20°C and 4°C, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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