Contribution of the Esterified Amino Acid to the Binding of Aminoacylated tRNAs to the Ribosomal P- and A-Sites†

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ABSTRACT: Crystallographic studies suggest that the esterified amino acid of aminoacyl tRNA make contacts with the ribosomal A-site but not in the P-site. Biochemical evidence indicating a thermodynamic contribution of the esterified amino acid to binding aminoacyl-tRNA to either the ribosomal P- and A-sites has been inconsistent, partly because of the labile nature of the aminoacyl linkage and the long times required to reach equilibrium. Measuring the association and dissociation rates of deacylated and aminoacylated tRNAs to the A-site and P-site of *E. coli* ribosomes afforded an accurate estimate of the contribution of the amino acid. While esterified phenylalanine or methionine has no effect on the affinity of tRNA to the P-site, an esterified pheylalanine stabilizes binding to the A-site by 7 kJ/mol, in agreement with the contacts observed in the X-ray crystal structure. In addition, it was shown that the presence of an esterified amino acid in one ribosomal site does not affect the binding of an aa-tRNA to the other site.

Efficient protein synthesis requires that deacylated tRNAs not compete effectively with aminoacyl-tRNAs for ribosome binding, since binding of deacylated tRNAs to the ribosomal A-site stalls polypeptide elongation (*1*). Discrimination against deacylated tRNAs from binding the ribosomal A-site is primarily achieved by the preferential binding of aa-tRNAs to EF-Tu·GTP (*2*), as the EF-Tu·GTP·aa-tRNA[1] ternary complex binds ribosomes much more rapidly than free deacylated tRNAs (*3, 4*). In addition, under most growth conditions, tRNAs are primarily aminoacylated, resulting in a low concentration of deacylated tRNAs available for ribosomal binding (*5*). However, under growth conditions where amino acids are depleted, the concentration of deacylated tRNAs increases and they can compete for A-site binding, resulting in stringent response (*6*).

It is possible that ribosomes possess an intrinsic ability to discriminate against deacylated tRNAs. This is suggested by the X-ray crystal structure of *H. marismortui* 50S ribosomes with a puromycin derivative in the ribosomal A-site (*7*). Not only does the α-amino group of the esterified tyrosine form a hydrogen bond to either the N3 or the O2′ of A2486 of the rRNA (A2451 in *E. coli*) but the tyrosine side chain may stack on A2486, as well as form a hydrogen bond with G2102 (G2061 in *E. coli*). Biochemical evidence that the esterified amino acid contributes to the binding affinity to the *E. coli* ribosomal A-site is less clear. Early experiments reported that Phe-tRNA^{Phe} bound to the A-site only slightly tighter (1.5−6-fold) than tRNA^{Phe} (*8, 9*). However, a more recent experiment (*10*) suggests that Phe-tRNA^{Phe} binds much tighter than was previously suggested, primarily because the earlier studies had not appreciated the slow rate of factor-independent binding to ribosomes and thus were not at equilibrium. Although deacylated tRNA^{Phe} was not remeasured in the more recent work, the reported K_D of 2 nM for Phe-tRNA^{Phe} was more than 100-fold tighter than previously measured for tRNA^{Phe} (*8, 9*), suggesting a substantial contribution of the esterified phenylalanine group.

Obtaining an accurate thermodynamic assessment of the contribution of the esterified amino acid to either the ribosomal P- or A-sites requires overcoming two experimental challenges. First, spontaneous deacylation of many aa-tRNAs in typical buffer conditions is significant on the time scales required to reach equilibrium. This problem has been addressed in the past by including aminoacyl tRNA synthetases to the ribosome binding reaction to maintain high levels of aa-tRNA (*8, 10*). Second, to assess the binding affinity of an aa-tRNA to the ribosomal A-site, the P-site first needs to be occupied by another tRNA. However, if the P-site tRNA is aminoacylated, peptide bond formation occurs. Normally, this possibility is avoided experimentally by using deacylated tRNA to occupy the P-site. However, this may be inappropriate if the two esterified amino acids bind cooperatively or if the absence of the P-site amino acid leads to incorrect positioning of the A-site amino acid. Here we used several methodological improvements to obtain accurate values for the affinities for aminoacyl- and deacyl-tRNAs binding to both ribosomal sites. This permitted the contribution of the esterified amino acid to the overall affinity to be accurately determined.

MATERIALS AND METHODS:

Materials. Tight coupled 70S ribosomes were isolated from *Escherichia coli* MRE 600 cells grown to mid-log phase

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[1] Abbreviations: aa-tRNA, aminoacyl-tRNA; EF-G, elongation factor G; EF-Tu, elongation factor Tu; Met, methionine; Phe, phenylalanine; TCA, trichloroacetic acid.

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and purified by sucrose density gradient centrifugation (*11*). Final ribosome pellets were suspended in RB buffer (25 mM MES; 25 mM HEPES pH 7.0; 30 mM KCl; 70 mM NH_4Cl; 10 mM $MgCl_2$; 1mM DTT; 0.1 mM EDTA), aliquoted, flash frozen, and stored at −80 °C. Prior to use, a 10 μL aliquot of frozen ribosome stock (5.5 μM 70S) was defrosted, incubated at 42 °C for 10 min, and cooled to 20 °C. The fraction of active ribosomes was determined by titrating mRNA, P-site tRNA, or A-site tRNA. All three assays generally agreed, and typical ribosome preparations were from 55 to 85% active. Reported ribosome concentrations are for active ribosomes.

The mRNA fragments were purchased from Dharmacon (Lafayette, CO) and chemically deprotected and purified by denaturing PAGE.

Purified *E. coli* $tRNA^{fMet}$ and $tRNA^{Phe}$ were purchased from Sigma-Aldrich. The [3′-^{32}P] labeling of tRNA was performed as previously described (*12*) using $(His)_6$-tagged tRNA-terminal nucleotidyl transferase with the following modification. During the last step of labeling in which pyrophosphatase and ATP were added, 2 μM CTP was added and the reaction incubated for 3 min at 37 °C instead of 30 s.

Yeast PheRS was purified as previously described (*13*). P. Schimmel (Scripps Institute) provided purified *E. coli* MetRS. Aminoacylation reactions were performed with 1 μM [3′-^{32}P] tRNA, 4mM ATP, 30 mM KCl, 15 mM $MgCl_2$, 5mM DTT, 30 mM Na-HEPES (pH 7.0), 250 μM methionine or phenyalanine, and 0.2 μM *E. coli* MetRS or yeast PheRS. To improve the yields of the reaction, 0.025 units/μL yeast inorganic pyrophosphase (Sigma) was included (*14*). The extent of aminoacylation of [3′-^{32}P] tRNAs was determined by nuclease P1 digestion and thin-layer chromatography as previously described (*12*). Preparations of Met-$tRNA^{fMet}$ were typically 70−85% aminoacylated, and Phe-$tRNA^{Phe}$ was 50−80% aminoacylated.

EF-Tu:EF-Ts complex was isolated by over-expressing $(His)_6$-tagged EF-Ts in *E. coli* and then purifying the EF-Tu:EF-Ts-$(His)_6$ complex as previously described (*15*, *16*). The final complex was dialyzed into RB buffer and 10% glycerol, flash frozen, and stored at −80 °C. To form the EF-Tu·GTP·aa-tRNA ternary complex, EF-Tu was first activated by incubating a sample containing 3 μM EF-Tu:EF-Ts protein complex with 0.5 mM GTP in RB buffer for 15 min at 37 °C. Ternary complex was then formed by incubating 800 nM activated EF-Tu and 400 nM [3′-^{32}P] Phe-$tRNA^{Phe}$ at 4 °C for 20 min in the same buffer.

Filter Binding. A modified (*17*) 96 well dot blot apparatus (Shleicher and Schuell) was used to determine the kinetics of tRNAs binding to ribosomes. An upper nitrocellulose membrane (NitroBind, Osmonics) and a lower nylon membrane (Hybond-N^+, Amersham) were soaked in RB buffer prior to use. The nylon membrane showed greater retention efficiency than DEAE paper previously used (*17*). Reactions containing ribosome complexes were performed in rows of 8 in a 96 well microtiter plate (NUNC conical bottom). For association and dissociation experiments, eight reactions were initiated simultaneously. Subsequent time points were taken using a multichannel pipet and immediately filtered and washed with at least 150 μL of RB buffer. After a series of filtrations were complete, the membranes were removed from the filter apparatus, dried, and exposed to a phosphorimager screen (Molecular Dynamics).

tRNA Binding to the P-Site. Dissociation rates of P-site-bound tRNAs were measured in 15 μL reactions containing 0.3 μM ribosomes, 0.9 μM mRNA, and 10 nM [3′-^{32}P] $tRNA^{fMet}$ (deacylated or aminoacylated) in RB buffer. After incubating for 20 min at 20 °C, 1 μL aliquots of each reaction were transferred to a microtiter well and 250 μL of 100 nM unlabeled $tRNA^{fMet}$ (deacylated or aminoacylated) in RB buffer was added to initiate the reaction. At indicated times, 15 μL aliquots were removed and immediately filtered and washed. Experiments measuring dissociation rates using 2-fold dilutions of the ribosome samples with cold chase (1 μM) gave identical dissociation rates for both P-site and A-site experiments. Large dilutions were generally used to minimize the amount of material required for experiments.

Association rates of tRNA to the P-site of mRNA-programmed ribosomes were investigated by initially preparing ribosome stock solutions containing 3.5 μM 70S tight coupled ribosomes and 10.5 μM mRNA in RB buffer. After serial dilutions of the ribosome:mRNA complex were made to obtain a range of desired concentrations, association reactions were initiated by mixing 15 μL of each ribosome sample with 15 μL of 4.5 nM [3′ ^{32}P]-end-labeled tRNA in RB buffer. At the indicated times, 1.5 μL aliquots were filtered.

tRNA Binding to the A-Site. Dissociation of deacylated *E. coli* $tRNA^{Phe}$ from the ribosomal A-site was measured in 10 μL reactions containing 1.0 μM 70S ribosome, 3.0 μM mRNA, and 5.0 μM $tRNA^{fMet}$ in RB buffer. In some cases, $tRNA^{fMet}$ was replaced with either Met-$tRNA^{fMet}$ or Met-$tRNA_{2A}^{fMet}$. After preincubation for at least 20 min at 20 °C to allow $tRNA^{fMet}$ to fully occupy the ribosomal P-site, [3′-^{32}P] $tRNA^{Phe}$ was added (100 nM final) and further incubated for 30−45 min at 20 °C to allow binding to the A-site. To initiate dissociation, 2 μL aliquots of the above reaction were mixed with 250 μL of chase solution containing 300 nM unlabeled yeast $tRNA^{Phe}$ in RB buffer. Yeast $tRNA^{Phe}$ was used as the chase, as it is readily available and binds *E. coli* ribosomes well. Aliquots of 15 μL were removed at desired times and immediately filtered. Dissociation of Phe-$tRNA^{Phe}$ from the A-site was performed similarly to deacylated tRNAs with the following modifications. Preincubation contained 150 nM ribosomes, 450 nM mRNA, and 600 nM $tRNA^{fMet}$, and 25 nM [3′-^{32}P] Phe-$tRNA^{Phe}$ was added in the second incubation. Dissociation was induced with buffer containing 300 nM Phe-$tRNA^{Phe}$.

Enzymatic loading of Phe-$tRNA^{Phe}$ into the A-site by EF-Tu·GTP was performed in the following way. Ternary complex (6 μL of 400 nM), formed as described above, was combined with 12 μL of 300 nM mRNA-programmed 70S ribosomes containing P-site bound $tRNA^{fMet}$. After incubating for 5 min on ice, ribosome complexes were purified by gel filtration on a MicroSpin S-300 HR column (Amersham) equilibrated in RB buffer. Immediately after elution from the column, the sample was used for dissociation rate measurements from the A-site as described above for Phe-$tRNA^{Phe}$.

Association of deacylated and aminoacylated $tRNA^{Phe}$ to the A-site was performed in a manner similar to P-site association experiments except that the ribosomal stock

sample also contained a 10 μM concentration of either tRNA^{fMet}, Met-tRNA^{fMet}, or Met-tRNA$_{2A}^{fMet}$.

Measuring Deacylation Rates. tRNA deacylation on the ribosome was measured in 150 μL reactions containing 200 nM [^{3}H]-labeled aminoacyl tRNA (specific activity 100 Ci/mmol for Phe, 70 Ci/mmol for Met), 1.0 μM 70S ribosomes, and 3.0 μM mRNA in RB buffer. For A-site experiments, the samples also contained 4 μM tRNA^{fMet}, which was preincubated with the ribosomes prior to the addition of Phe-tRNA^{Phe}. Samples were incubated at 20 °C, and 10 μL aliquots were removed at times ranging from 0 to 200 min and quenched by adding 100 μL of 10% TCA containing 100 ug/mL total yeast tRNA. After the experiment was completed, the quenched samples were filtered on a single-membrane filter (MF-membrane, Millipore), dried, and analyzed by phosphorimagery.

Nonhydrolyzable Aminoacyl tRNA^{fMet} Analogue. A non-hydrolyzable analogue of Met-tRNA^{fMet} containing a 2′-amino linkage was prepared using a protocol analogous of that previously described (*18*). A 100 μL reaction containing 1 μM tRNA^{fMet}, 30 μg/mL tRNA-terminal nucleotidyl transferase, 1 mM sodium pyrophosphate in glycine buffer (50 mM glycine-HCl, pH 9.0, 10 mM $MgCl_2$) was incubated for 20 min at 37 °C. The resulting truncated tRNA sample was purified on a 10% denaturing gel, eluted, and precipitated with ethanol. The sample was suspended in 10 μM CTP, 25 μM 2′-amino ATP (IBA), 30 μg/mL tRNA-terminal nucleotidyl transferase, and 10 U/mL pyrophosphatase in glycine buffer. After 20 min at 37 °C, the reaction was extracted twice with acid phenol chloroform (5:1), pH 4.5, and twice with chloroform. The sample was desalted on a Micro Bio-Spin 6 chromatography column and precipitated with ethanol. The resulting modified tRNA^{fMet} possessed a 2′-amino group on the 3′-terminal and was termed (2′-NH_2)tRNA^{fMet}. The (2′-NH_2)tRNA^{fMet} was aminoacylated as described above except that the MetRS concentration was increased to 0.8 μM and subsequently incubated for 30 min at 37 °C. An aliquot of the (2′-NH_2)tRNA^{fMet} was also aminoacylated with [^{3}H]-methionine in order to monitor deacylation kinetics. At pH 8.3, no detectable deacylation was observed after 3 h at 20 °C for the modified [^{3}H]-Met-(2′-NH_2)tRNA^{fMet}, while the similarly labeled unmodified [^{3}H]-Met-tRNA^{fMet} was essentially completely deacylated at this time (data not shown). For association and dissociation rate measurements of Met-(2′-NH_2)tRNA^{fMet} binding to ribosomes, the tRNA was aminoacylated with [S^{35}]-methionine (specific activity > 1000 Ci/mmol).

RESULTS

To evaluate the contribution of the esterified amino acid to the binding of aa-tRNAs to the ribosomal A- and P-sites, tight coupled 70S *E. coli* ribosomes were used with a defined 27 nucleotide mRNA sequence (Figure 1A). A very similar mRNA sequence has been used by others (*19*) and was originally derived from the T4 phage gene 32 mRNA that has been used for X-ray crystallographic studies of mRNA bound to the ribosome (*20*). The placement of an AUG codon in the P-site and an UUC codon in the A-site directs the binding of tRNA^{fMet} and tRNA^{Phe}, respectively, as shown in Figure 1B.

The measurement of tRNA binding to ribosomes may potentially be complicated by the dissociation of the mRNA

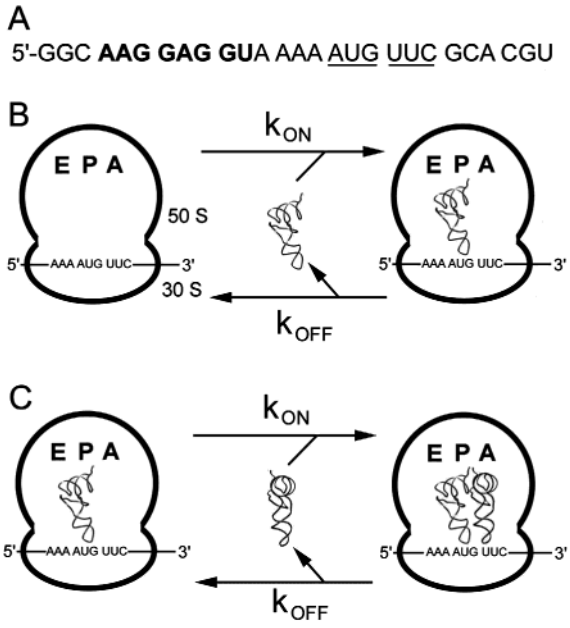

FIGURE 1: (A) The 27 nucleotide mRNA sequence derived from T4 gene 32 mRNA. The Shine−Dalgarno sequence is highlighted in bold, while the P-site Met codon and A-site Phe codon are underlined. (B) P-site association and dissociation by tRNA^{fMet}. (C) A-Site association and dissociation by tRNA^{Phe}.

oligomer under the conditions of the experiment. This was of special concern when measuring the relatively slow dissociation of tRNAs from the ribosome, since it was critical that the observed rate not reflect the simultaneous dissociation of the mRNA. To test this possibility, the mRNA fragment was [5′-^{32}P] labeled and its association and dissociation rate constants to *E. coli* ribosomes measured in the absence of added tRNAs at 20 °C in RB buffer. The association rate was too rapid to measure even at 1.8 nM ribosomes and thus can be estimated to exceed 3×10^8 M^{-1} min^{-1}. The dissociation rate determined using a cold chase protocol was 0.0045 ($\pm$0.0005) min^{-1} (data not shown). This means the K_D of this mRNA for ribosomes is less than 15 pM, indicating that a fragment containing the Shine−Dalgarno mRNA sequence binds much tighter to 70S ribosomes than has been previously determined for a poly U message (*21*). Interestingly, when 0.1 μM deacyl tRNA^{fMet} was added to the reactions, the dissociation rate decreases to 0.00038 ($\pm$0.00012) min^{-1} and the association rate was still too fast to measure. The increased binding affinity (K_D < 1.5 pM) agrees with previous experiments demonstrating cooperative binding of a P-site tRNA and an mRNA (*22*). As both these dissociation rates are slower than the dissociation rates of P-site tRNAs (see below), it appears that mRNA dissociation is not a factor in our experiments.

The equilibrium constants for the binding of tRNAs to ribosomes were calculated from the experimentally determined association and dissociation rate constants. This approach minimizes the hydrolysis of the labile aminoacyl linkage that becomes significant during the long incubation times that are required to reach equilibrium (*10*). The use of [3′-^{32}P]-labeled tRNAs was essential for the execution of these experiments. Not only can the same tRNA preparation be used for deacyl- and aminoacyl-tRNAs, but the extent of the deacylation can be easily monitored using P1 nuclease digestion (*12*). Furthermore, the high specific activity of ^{32}P

Table 1: Deacylation Protection by the Ribosome

		deacylation rate (min^{-1})[a]	fold protection
Met-tRNA^{fMet}	unbound	0.012	
	P-site-bound	<0.001	>12
Phe-tRNA^{Phe}	unbound	0.01	
	A-site-bound	<0.0007	>14

[a] Rates measured in 1 × RB buffer (25 mM MES, 25 mM HEPES, pH 7.0, 70 mM NH_4Cl, 30 mM KCl, 1 mM DTT, and 10 mM $MgCl_2$) at 20 °C.

makes it possible to use low enough ribosome concentrations that the association rate constant can be measured by manual filter binding assays. When lower specific activity [^{3}H]- or [^{14}C]-labeled aminoacyl-tRNAs are used, association rates are too rapid to measure at ribosome concentrations where bound complex can be accurately detected. Finally, the high energy of ^{32}P emission makes it easy to adopt the classic nitrocellulose filter binding assay to the much more accurate dual-membrane format (*17*), where tRNAs that do not bind ribosomes are trapped on a lower nylon membrane. Filtration is performed on a modified 96 well dot blot apparatus, and the two filters are counted separately on the phosphorimager. The fraction of tRNA bound is obtained directly, without needing to know the specific activity of the tRNA with a high degree of precision. Differential quenching of lower energy ^{3}H and ^{14}C emitters on the different types of membranes reduces the utility of double membranes when labeled amino acids are used.

The labile aminoacyl linkage could potentially complicate the interpretation of experiments measuring the rates of binding or dissociation of aminoacyl-tRNAs to ribosomes in at least two ways. First, in association rate measurements, the aminoacyl tRNA could deacylate significantly during the time course of the reaction, reducing its concentration and thereby the observed rate. Measuring initial rates over short time periods prevents significant deacylation from occurring. Second, in dissociation rate measurements, aminoacyl-tRNAs could deacylate on the ribosome and subsequently dissociate as deacyl-tRNA. Although the pH of our standard buffer was 7.0 to minimize deacylation, it was critical to measure the deacylation rates of aminoacyl-tRNAs both on and off the ribosome. This was done by preparing *E. coli* [^{3}H]-Met-tRNA^{fMet} and *E. coli* [^{3}H]-Phe-tRNA^{Phe} and determining deacylation rates either free in solution or in the presence of a high concentration of mRNA-programmed ribosomes. Met-tRNA^{fMet} deacylation in the ribosomal P-site was measured with an empty A-site, while Phe-tRNA^{Phe} deacylation in the ribosomal A-site was measured with deacyl tRNA^{fMet} in the P-site. As shown in Table 1, the deacylation rates of both free aminoacyl-tRNAs are on the order of 0.01 min^{-1}, which means that deacylation is negligible for incubations in the time scale of up to 5 min but substantial for incubations of 1 h or more. Deacylations of aa-tRNAs bound to the ribosome were substantially slower with the k_{app} values of <0.001 min^{-1} in the P-site and <0.0007 min^{-1} in the A-site. These data agree with previous experiments showing protection of Met-tRNA^{Met} from deacylation in the P-site (*23*). While these slow deacylation rates are difficult to obtain accurately and may partially reflect deacylation off the ribosome, they are clearly much slower than the corresponding dissociation rates measured for the aminoacyl-tRNAs below. Thus, deacylation on the ribosome does not significantly influence the dissociation rate measurement.

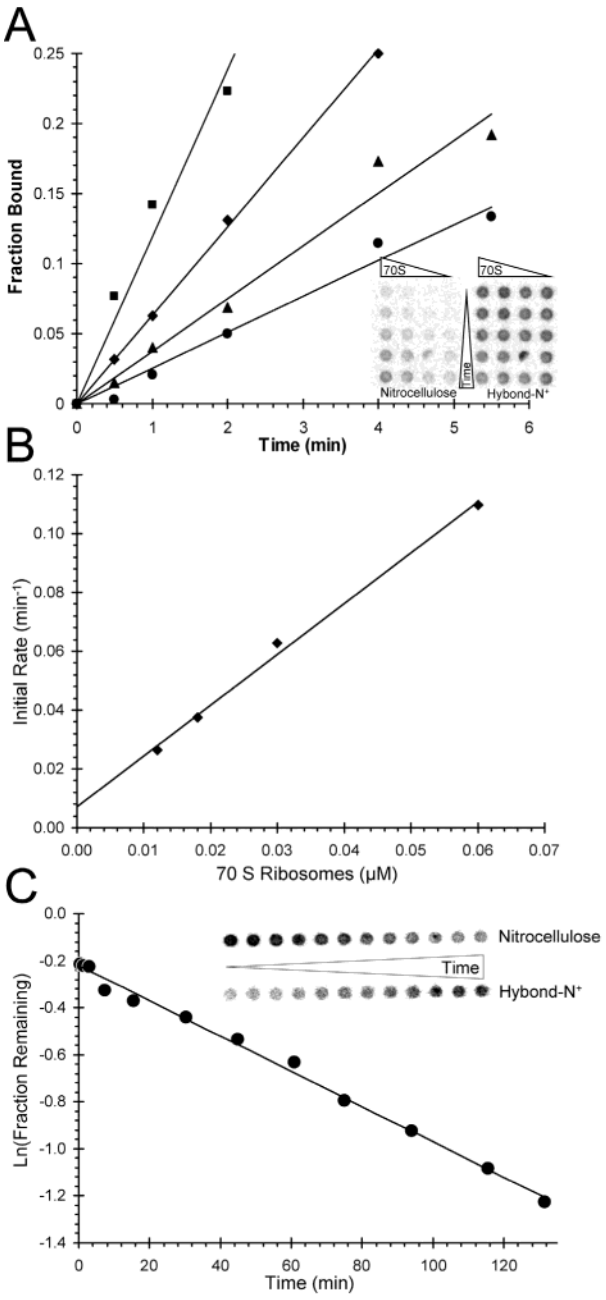

FIGURE 2: Kinetics of association and dissociation of tRNA^{fMet} to the ribosomal P-site at 20 °C in RB buffer. (A) Association of [3′-^{32}P] tRNA^{fMet} to the ribosomal P-site measured at (■) 60, (◆) 30, (▲) 18, and (●) 12 nM 70S ribosomes. Insert reveals raw phosphorimager data of the upper nitrocellulose and lower Hybond-N+ membranes. (B) Second-order plot of the initial rates in A. The line has a slope that corresponds to k_{on} (1.7×10^6 M^{-1} min^{-1}) and an intercept of k_{off} (0.007 min^{-1}). (C) First-order dissociation plot of [3′-^{32}P] tRNA^{fMet} from the ribosomal P-site where the negative of the slope gives a k_{off} of 0.0065 min^{-1}. Insert displays the raw data of the reaction.

tRNA^{fMet} Binding to the P-Site. The association rate constant of [3′-^{32}P] tRNA^{fMet} to the ribosomal P-site was determined by mixing the labeled tRNA with varying concentrations of mRNA-programmed ribosomes and filtering aliquots at various times (Figure 2A). A plot of the initial rates of binding versus ribosome concentration gives an accurate measurement of $k_{on} = 1.7 \times 10^6$ M^{-1} min^{-1} from

Table 2: P-Site Binding by tRNA[a]

	k_{on} (M^{-1} min^{-1} × 10^{-5})	k_{off} (min^{-1} × 10^3)	K_D (nM) (calcd)
$tRNA^{fMet}$	16 ± 6	5.9 ± 0.9	3.8 ± 1.9
Met-$tRNA^{fMet}$	15 ± 2	5.0 ± 0.9	3.4 ± 1.7
Met-(2′-NH_2)$tRNA_{2A}^{fMet}$	4.4 ± 1.7	3.8 ± 0.6	8.6 ± 4.7
$tRNA^{fMet}$ + Phe-$tRNA^{Phe}$ [b]	nd[c]	3.0 ± 0.6	nd[c]
$tRNA^{Phe}$	29 ± 6	8.5 ± 2.2	3.0 ± 1.4
Phe-$tRNA^{Phe}$	23 ± 0.2	8.4 ± 1.5	3.7 ± 1.0

[a] Rates measured in 1× RB buffer at 20 °C. [b] Measurement of $tRNA^{fMet}$ while A-site is occupied with Phe-$tRNA^{Phe}$. [c] Nd = not determined.

the slope and extrapolates to a k_{off} of 0.007 min^{-1} from the intercept (Figure 2B). A useful feature of the double-filter protocol is that the fraction of the bound tRNA is measured directly from the ratio of counts on the two filters. As a result, the tRNA concentration does not need to be known precisely to calculate the association rate.

A more accurate determination of k_{off} can be obtained by forming a complex with [3′-^{32}P] $tRNA^{fMet}$, adding an excess of unlabeled $tRNA^{fMet}$ and filtering at different times. As shown in Figure 2C, the slow first-order dissociation gives $k_{off} = 0.0065$ min^{-1}, in good agreement with the less accurate value determined above. Table 2 lists average association and dissociation rate constants of deacylated $tRNA^{fMet}$ determined from four replicate experiments.

The absence of an esterified methionine on $tRNA^{fMet}$ raises the possibility that the 3′-terminus of the tRNA does not correctly occupy the P-site on the large subunit. While the crystal structure of deacyl-tRNA bound to the ribosomal P-site (*24*) as well as chemical probing data (*25*) suggest that deacylated $tRNA^{fMet}$ is bound to the P-site, cryo electron microscopy studies have suggested that, in buffers lacking polyamines, the tRNA occupies a hybrid state, with the 3′-terminus in the E-site (*26*). To address this question, the association and dissociation rate constants of Met-$tRNA^{fMet}$ and a nonhydrolyzable methionylated (2′-NH_2)$tRNA^{fMet}$ analogue, where the terminal adenosine residue contains a 2′-amino group, were determined. Since it is known that aa-tRNAs cannot bind the E-site (*8*, *9*, *27*−*29*), these tRNAs are expected to exclusively bind the P-site.

The association and dissociation rate constants determined for Met-$tRNA^{fMet}$ and Met-(2′-NH_2)$tRNA^{fMet}$ are summarized in Table 2. Both the association and dissociation rate constants of Met-$tRNA^{fMet}$ to the ribosomal P-site are essentially identical to the deacylated $tRNA^{fMet}$. The Met-(2′-NH_2)$tRNA^{fMet}$ analogue exhibits modestly reduced association and dissociation rate constants and gives only a slightly weaker K_D. The results suggest that the deacylated and aminoacylated $tRNA^{fMet}$ bind the P-site similarly and that the esterified amino acid does not contribute significantly to the binding affinity.

Dissociation of deacylated $tRNA^{fMet}$ from the ribosomal P-site has also been measured while the A-site is occupied with Phe-$tRNA^{Phe}$. In this case, only a small reduction in the dissociation rate of $tRNA^{fMet}$ is observed (Table 2). This suggests that tRNAs binding to the two sites are independent thermodynamically.

$tRNA^{Phe}$ Binding to the P-Site. Since most previous experiments used $tRNA^{Phe}$ to measure equilibrium tRNA binding to the P-site, it was useful to redetermine these values using the initial rate protocols. This was easy to do because the mRNA fragment has a UUC Phe codon immediately downstream of the AUG codon (Figure 1A), so experiments could be performed with the same mRNA fragment used above. Previous experiments using similar mRNA fragments have demonstrated that a tRNA will bind the P-site despite its codon being much further down stream of the Shine−Dalgarmo sequence (*19*). Association and dissociation experiments for P-site binding by *E. coli* $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ were performed in RB buffer in the same way as those with $tRNA^{fMet}$ and are summarized in Table 2. No significant differences in the binding of $tRNA^{fMet}$ and $tRNA^{Phe}$ to the P-site were observed, and the presence of esterified amino acid did not affect the affinity.

$tRNA^{Phe}$ Binding to the A-Site. Experiments with poly U message indicate that deacylated $tRNA^{Phe}$ will occupy the E-site prior to occupying the A-site (*9*), although this observation is not universal (*8*). Thus, prior to measuring $tRNA^{Phe}$ binding to the ribosomal A site, it was therefore important to test whether it could bind to the E-site when the P-site is occupied with $tRNA^{fMet}$. Since the E-site binds noncognate tRNAs less than cognate tRNAs (*29*), $tRNA^{Phe}$ was not expected to bind well to the noncognate AAA codon upstream of the AUG codon in mRNA. To confirm this experimentally, an alternative mRNA was used where the standard UUC phenylalanine codon was replaced by a GUC codon. Under conditions where $tRNA^{Phe}$ is fully bound with the standard mRNA sequence, no binding to the alternative mRNA was observed (data not shown). Thus, E-site binding of deacyl $tRNA^{Phe}$ to the noncognate AAA codon is negligible under the conditions of the experiments.

To properly evaluate the association and dissociation of *E. coli* $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ to the ribosomal A-site, it is important to consider the identity of the P-site tRNA. To avoid rapid peptide bond formation, the binding of aminoacyl-tRNA to the A-site is traditionally measured with deacyl tRNA in the P-site (*8*−*10*, *28*, *30*). However, since the positions of the esterified amino acids are close in space (*31*), they may influence one another thermodynamically. If this is the case, the absence of an amino acid in the P-site may alter tRNA affinity for the A-site. In addition, it is possible that complexes with a deacyl-tRNA in the P-site may partially or fully occupy a hybrid state where the 3′-ends of the tRNAs have translocated (*26*). To address these issues, the binding of $tRNA^{Phe}$ to the A-site was performed with both deacylated $tRNA^{fMet}$ and the nonhydrolyzable analogue of $tRNA^{fMet}$, which is incapable of acting as a donor in peptide bond formation (*32*).

Second-order plots for the association of $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ to ribosomes containing either $tRNA^{fMet}$ or Met-(2′-NH_2)$tRNA^{fMet}$ in the P-site are shown in Figure 4A. The values of $k_{on} = 1.3 \times 10^5$ are about 10-fold slower than the k_{on} values for tRNAs binding to the P-site. Since the rates extrapolate close to the origin at low ribosome concentrations, estimates of k_{off} cannot be determined from these data

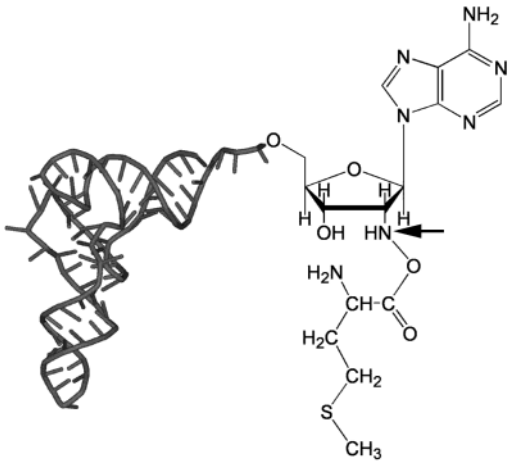

FIGURE 3: Aminoacylated 2′-amino-modified tRNA^{fMet} Met-(2′-NH_2)tRNA^{fMet}). The arrow indicates the nitrogen atom linking the amino acid to the 3′-terminal adenosine of the tRNA.

but must be quite slow. The presence of the esterified phenylalanine shows a modest 2-fold increase in k_{on}. Association rates are identical whether the P-site is occupied with deacylated tRNA^{fMet} or the Met-(2′-NH_2)tRNA^{fMet} analogue.

Aminoacylation makes a more significant impact on A-site dissociation. The dissociation rate of deacylated [3′-^{32}P]-tRNA^{Phe} from the A-site when either deacylated tRNA^{fMet}, Met-tRNA^{fMet}, or Met-(2′-NH_2)tRNA^{fMet} was present in the P-site was determined by adding an excess of nonradioactive tRNA^{Phe} and filtering at different times (Figure 4B). Approximately the same k_{off} of 0.055 min^{-1} is obtained in all three cases. Similar experiments using Phe-[3′-^{32}P]-tRNA^{Phe} (Figure 4C) and either deacylated tRNA^{fMet} or Met-(2′-NH_2)-tRNA^{fMet} in the P-site gave about 9-fold slower k_{off} values (0.006 min^{-1}). This indicates that, unlike in the P-site, the esterified amino acid contributes to the dissociation rate of tRNA^{Phe} from the A-site. In addition, this experiment indicates that the presence of an amino acid in the P-site has no effect on the binding of aminoacyl tRNA in the A site. This agrees with the reciprocal experiment that measured binding of tRNAs in the P-site and shows that the esterified amino acids do not interact thermodynamically.

In the dissociation experiments, the Phe-tRNA^{Phe} was initially bound to the A-site by simply incubating for 30 min at 4 °C. Although this incubation did not lead to substantial deacylation, it was of interest to test whether the dissociation rate was any different when the Phe-tRNA^{Phe} was loaded into the A-site as an EF-Tu·GTP·Phe-tRNA^{Phe} ternary complex. As expected, loading of the Phe-tRNA^{Phe} into the A site was very rapid by this enzymatic route. The dissociation rate constant of the resulting Phe-tRNA^{Phe} from the A-site was 0.004 ± 0.0011 min^{-1} (data not shown) and is essentially the same as with the tRNA loaded nonenzymatically. This confirms that the enzymatic and nonenzymatic A-site complexes are thermodynamically identical, as others have shown (*10*).

The more rapid dissociation of Phe-tRNA^{Phe} observed when Met-tRNA^{fMet} occupies the P-site, as in Figure 4C, is the result of peptide bond formation and subsequent dissociation of the dipeptidyl-tRNA, Met-Phe-tRNA^{Phe}. Relatively rapid dipeptidyl-tRNA dissociation from the A-site has been previously reported for conditions in which translocation does not occur due to the absence of EF-G (*10*, *33*). It has also been shown that after peptide bond formation, the dipeptidyl-tRNA is bound differently due to the appearance of puromycin reactivity, suggesting that it is in a hybrid state (*34*). Our observed rate constant of dissociation of 0.026 ± 0.005 min^{-1} was slower than previously reported rates, but this probably reflects the fact that Met-tRNA^{fMet} was not loaded into the P-site with initiation factors. Factor-independent loading will not discriminate between aminoacylated and deacylated tRNA^{fMet}, which exhibit similar binding affinities (Table 2). As a result, the observed dissociation rate is likely to be a combination of the fast dissociation of Met-Phe-tRNA^{Phe} and slower dissociation of Phe-tRNA^{Phe} when deacylated tRNA^{fMet} contaminates the P-site.

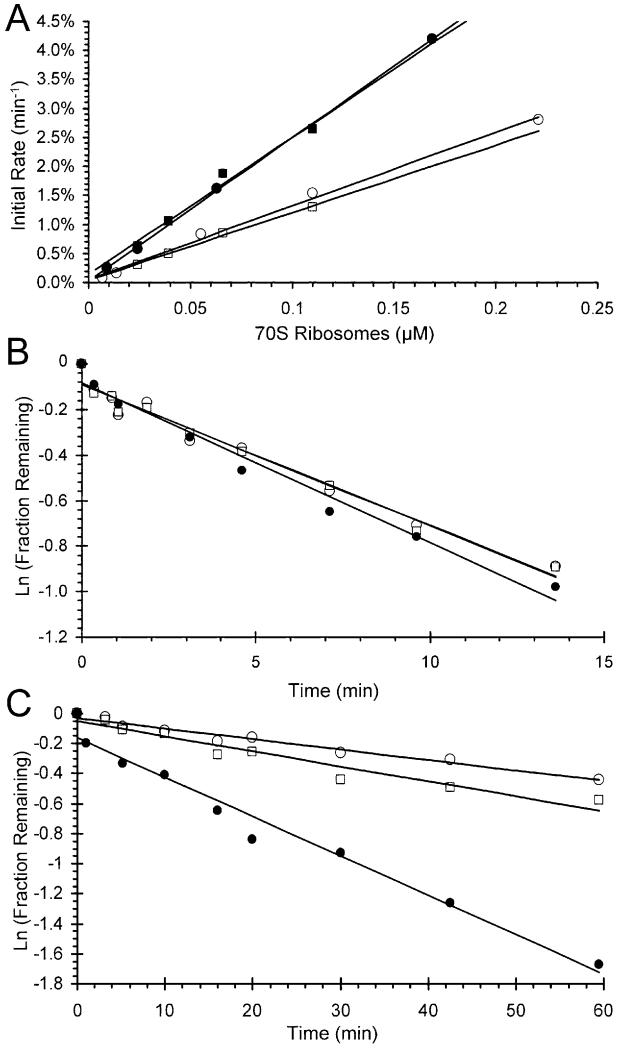

FIGURE 4: Association and dissociation of tRNA^{Phe} from the ribosomal A-site at 20 °C in RB buffer. (A) Association of aminoacylated (●,■) and deacylated (○,□) [3′-^{32}P] tRNA^{Phe} with either deacylated (●,○) or Met-(2′-NH_2)tRNA^{fMet} (■,□) in the P-site. (B) First-order dissociation plot of deacylated [3′-^{32}P] tRNA^{Phe} from the ribosomal A-site. Measurements made when the P-site is occupied with; deacylated tRNA^{fMet} (○), Met-tRNA^{fMet} (●), or the Met-(2′-NH_2)tRNA^{fMet} analogue (□). (C) First-order dissociation plot of aminoacylated [3′-^{32}P] tRNA^{Phe} from the ribosomal A-site. Measurements made when the P-site is occupied with deacylated tRNA^{fMet} (○), Met-tRNA^{fMet} (●), or the Met-(2′-NH_2)tRNA^{fMet} analogue (□).

A summary of the association and dissociation rate constants to the ribosomal A-site are shown in Table 3. The

Table 3: Comparison of Phe-tRNA^{Phe} and Deacyl tRNA^{Phe} Binding to the A-Site[a]

P-site	A-site	k_{on} (M^{-1} min^{-1} × 10^{-5})	k_{off} (min^{-1} × 10^{3})	K_D (nM) (calcd)
tRNA^{fMet}	tRNA^{Phe}	1.3 ± 0.2	50 ± 12	380 ± 150
Met-tRNA^{fMet}	tRNA^{Phe}	1.4 ± 0.3	55 ± 11	390 ± 160
Met-(2′NH_2)tRNA^{fMet}	tRNA^{Phe}	1.2 ± 0.3	63 ± 18	525 ± 280
tRNA^{fMet}	Phe-tRNA^{Phe}	2.6 ± 0.6	5.8 ± 1.2	22 ± 10
Met-tRNA^{fMet}	Phe-tRNA^{Phe}	nd[b]	26 ± 5	nd[b]
Met-(2′NH_2)tRNA^{fMet}	Phe-tRNA^{Phe}	2.7 ± 0.8	8.2 ± 1.4	30 ± 14

[a] Rates measured in 1× RB buffer at 20 °C. [b] Nd = not determined; peptide bond formation occurs.

resulting calculated equilibrium constants show that unlike the P-site, the esterified amino acid causes about 17-fold tighter binding in the A-site. This corresponds to an increase in free energy of binding from −36 to −43 kJ/mol.

DISCUSSION

The kinetics and thermodynamics of binding of tRNAs to *E. coli* ribosomal A- and P-sites were reinvestigated with the goal of evaluating the contribution of esterified amino acids to the overall binding affinity. Several methodological improvements were employed to improve the accuracy of the measurements and to evaluate whether the binding of esterified amino acids are thermodynamically linked. The use of high specific activity [3′-^{32}P]-labeled tRNAs and a dual-membrane filtration system permits accurate association and dissociation rate constants to be obtained in shortened time scales that the deacylation of aa-tRNAs were negligible. The preparation of the 2′-NH_2 analogue of Met-tRNA^{fMet} permits aa-tRNA to be bound to both the A- and P-sites without peptide bond formation.

The association and dissociation of tRNAs directly to the ribosomal A- and P-sites are not equilibria that are part of the protein synthesis mechanism. Instead of direct association, aa-tRNAs normally enter the A-site after GTP hydrolysis of ribosomally bound ternary complex. Instead of direct dissociation, tRNAs leave the A-site for the P-site by translocation catalyzed by EF-G. Similarly, tRNAs enter the P-site either with the aid of initiation factors or by translocation from the A-site and leave the P-site for the E-site during translocation. Thus, the major motivation for studying these nonnatural equilibria is that they can be used to understand the basis of the interaction between tRNAs bound to these ribosomal sites. This is especially valuable because the available X-ray crystal structure permits structure−function analysis where changes in the free energy of binding due to defined alterations of a tRNA molecule can be compared with the structure. In this paper, the focus is on the esterified amino acid.

tRNAs associate to the ribosomal P- and A-sites much slower than the expected rate for a diffusion-controlled reaction, suggesting that structural rearrangements are required after initial association. The measured association rate constants of aa-tRNAs binding to the ribosomal P-site are 1.5 × 10^6 and 2.3 × 10^6 M^{-1} min^{-1} for Met-tRNA^{fMet} and Phe-tRNA^{Phe}, respectively, while association of Phe-tRNA^{Phe} to the A-site is 2.6 × 10^5 M^{-1} min^{-1}. When compared to their aminoacylated counterparts, deacylated tRNAs exhibit identical association rates for the P-site and only about 2-fold slower for the A-site. Because of the high specific activities of radioactive aa-tRNAs required to obtain accurate second-order association rates at a series of ribosome concentrations, relatively few values for k_{on} have been reported using a filter binding assay. One report of k_{on} = 2 × 10^5 M^{-1} min^{-1} (*35*) for Phe-tRNA^{Phe} to the ribosomal A-site agrees well with the data in Table 3. However, stopped-flow fluorescent experiments with proflavin-labeled tRNA^{Phe} binding to the P-site have revealed a very fast second-order association rate followed by a slower rate of rearrangement (*4*) that are both more rapid than the second-order association rate measured by filter binding. These experiments suggest that one or more short-lived intermediates are formed before the tRNA is bound to ribosomes in a complex that is stable to nitrocellulose filtration. These intermediates presumably would reflect the conformational changes required by the ribosome in order to envelop the tRNAs as seen in the crystal structures (*20*).

Aminoacyl tRNAs dissociate very slowly from the ribosomal P- and A-sites, also supporting the idea that the ribosome must rearrange for the tRNA to dissociate. Dissociation of aa-tRNAs from the P- and A-sites are similar with rate constants ranging from 0.005 to 0.0084 min^{-1}. The presence of an esterified amino acid has no effect on P-site dissociation for either tRNA^{fMet} or tRNA^{Phe}. However, the presence of an esterified phenylalanine slows A-site dissociation by about 9-fold. There have been no previous reports that directly compare the dissociation rates of aminoacylated and deacylated tRNAs from the P- and A-sites. One study measuring the dissociation of deacylated tRNA^{Phe} from poly U-programmed ribosomes under similar conditions (*8*) agrees that deacylated tRNAs dissociate faster from the A-site than the P-site but report rates 5−10-fold slower than observed here. The reasons for the differences are unclear but may reflect differences in experimental procedures. The dissociation rate of Phe-tRNA^{Phe} from the ribosomal A-site has been reported to be 0.0012 min^{-1} at 10 mM $MgCl_2$ and 0 °C (*35*), in reasonable agreement with the value of 0.0058 min^{-1} measured here at the higher temperature of 20 °C.

Measurements of association and dissociation rate constants permit the calculation of equilibrium binding constants for tRNAs binding the ribosomal P- and A-sites. Aminoacylated and deacylated tRNAs bind to the ribosomal P-site with similar K_Ds of 3−3.7 nM. Aminoacylated tRNA^{Phe} binds the A-site with a K_D of 22 nM, which is ∼17-fold tighter than deacylated tRNA (K_D = 380 nM). Comparing P- and A-site affinities, as well as their kinetics of association, clearly demonstrates why tRNAs preferentially bind the P-site of mRNA-programmed ribosomes. P-site binding is tighter and faster, which agrees with early observations (*4*, *28*, *36*). The calculated values for deacylated tRNA^{Phe} affinities to the ribosomal sites agree well with previously reported values of 7−16 nM for P-site affinity and 200−

590 nM for A-site affinity in buffers containing 10 mM $MgCl_2$ (*8*, *9*). When comparing aminoacylated tRNAs, much more significant divergence in binding affinities are observed where either no differences (*9*), a small increase (*8*), or a large increase (*10*) in binding affinity was observed in A-site binding when $tRNA^{Phe}$ was esterified with phenylalanine. Variations in binding affinities are likely due to the previously discussed difficulties when using aminoacylated tRNAs.

Aminoacylation of a P-site-bound tRNA does not affect binding, nor does it affect binding of an additional tRNA to the ribosomal A-site. Identical kinetics of association and dissociation of aminoacylated and deacylated tRNAs suggest that the tRNAs are similarly bound while the esterified amino acid is not involved in P-site binding. Alternately, the deacylated tRNA may bind in an altered state (i.e., hybrid state) but exhibits identical binding kinetics to aminoacyl-tRNA binding the P-site. Binding kinetics of the ribosomal A-site by $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ are unaffected when either deacyl-$tRNA^{fMet}$ or the nonhydrolyzable Met-(2′-NH_2)-$tRNA^{fMet}$ are used to block the P-site. Two main points can be made from these A-site experiments. First, it is unlikely that $tRNA^{fMet}$ is binding the P-site in a hybrid state under our conditions, as subsequent differences in A-site binding would be predicted. Second, there are no cooperative interactions between the esterified amino acids of the P- and A-site-bound tRNAs. If the amino acids were interacting, binding by Phe-$tRNA^{Phe}$ to the A-site would vary depending on the tRNA used to block the P-site. The lack of cooperative interactions between the esterified amino acids of the P- and A-site-bound tRNAs does not preclude the possibility that the tRNAs themselves may be binding cooperatively.

X-ray crystal structures of ribosomally bound aminoacyl-tRNA analogues support the observations of the esterified amino acid only contributing to A-site binding. The crystal structure of CCA-Phe−caproic acid−biotin bound to the P-site of 50 S ribosomes does not reveal potential contacts to the esterified amino acid (*31*). Furthermore, the lack of a P-site amino acid effect agrees well with aminoacylated tRNAs supporting neither translocation to the extent of peptidyl-tRNAs (*37*) nor efficient binding of various translational factors (*38*). In contrast to the P-site, the crystal structure of 50 S ribosomes bound to a puromycin−minihelix conjugate in the A-site reveals a potential contact between the α-amino group and the O2′ or N3 of A2486 (*7*) as shown in Figure 5. When the X-ray structures of ribosomes with P- and A-site-bound aminoacyl-tRNA analogues are superimposed (*31*), the α-amino group of the esterified amino acid can also potentially hydrogen bond with the 2′-OH group of adenosine 76 (A76) of the P-site-bound tRNA. This contact to the P-site-bound tRNA agrees with the requirement of a 2′-OH on residue A76 of the P-site-bound tRNA for peptidyl transfer (*39*). These contacts by the α-amino group to either A2486 or the P-site-bound tRNA of the ribosomal RNA could explain the 7 kJ/mol increase in binding affinity to the ribosomal A-site observed when comparing $tRNA^{Phe}$ versus Phe-$tRNA^{Phe}$. The environment of the side chain of the esterified amino acid in the ribosomal A-site (Figure 5) reveals that the tyrosine side chain may hydrogen bond with G2102 and stack on A2486. The C-2 amino group of G2102 is less than 2.5 Å from the hydroxyl group of the tyrosine side chain. These contacts would likely increase the stability of a tRNA aminoacylated with tyrosine, but obviously phenylalanine is unable to make the hydrogen bond with G2102 but can still stack on A2486. Differences in how esterified amino acids can interact at the A-site raise the possibility of different esterified amino acids influencing binding of their tRNAs to different extents.

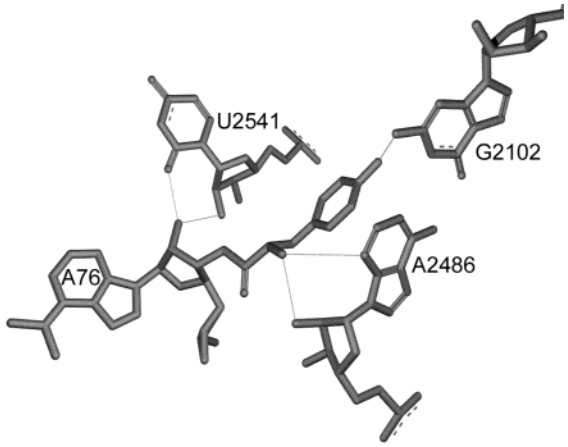

FIGURE 5: Interactions of an esterified amino acid of an aminoacyl-tRNA analogue (*N*-aminoacyl minihelix) to the ribosomal A-site. A76 refers to the terminal 3′-adenosine of an aminoacyl-tRNA. Adenosine 2486 potentially hydrogen bonds with the α-amino group of the esterified amino acid of the A-site-bound tRNA. The structure reveals potential side chain specific contacts between tyrosine and G2102, and the side chain may also stack on A2486. Structure is from the X-ray crystal structure reported by Nissen et al. 2000 (*7*).

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