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Contribution of ribosomal residues to P-site tRNA binding

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ABSTRACT

Structural studies have revealed multiple contacts between the ribosomal P site and tRNA, but how these contacts contribute to P-tRNA binding remains unclear. In this study, the effects of ribosomal mutations on the dissociation rate (k_{off}) of various tRNAs from the P site were measured. Mutation of the 30S P site destabilized tRNAs to various degrees, depending on the mutation and the species of tRNA. These data support the idea that ribosome-tRNA interactions are idiosyncratically tuned to ensure stable binding of all tRNA species. Unlike deacylated elongator tRNAs, *N*-acetyl-aminoacyl-tRNAs and tRNA^{fMet} dissociated from the P site at a similar low rate, even in the presence of various P-site mutations. These data provide evidence for a stability threshold for P-tRNA binding and suggest that ribosome-tRNA^{fMet} interactions are uniquely tuned for tight binding. The effects of 16S rRNA mutation G1338U were suppressed by 50S E-site mutation C2394A, suggesting that G1338 is particularly important for stabilizing tRNA in the P/E site. Finally, mutation C2394A or the presence of an *N*-acetyl-aminoacyl group slowed the association rate (k_{on}) of tRNA dramatically, suggesting that deacylated tRNA binds the P site of the ribosome via the E site.

INTRODUCTION

The ribosomal P site plays a central role in all phases of translation. Initiation involves recognition of the start codon by initiator tRNA in the P site, elongation involves passage of many elongator tRNAs through the P site, termination involves hydrolysis of the mature polypeptide from tRNA in the P site, and ribosome recycling involves release of deacylated tRNA from the P site.

Biochemical experiments have shown that peptidyl-tRNA (or its analog *N*-acetyl-aminoacyl-tRNA; Ac-aa-tRNA) occupies the P site of both subunits (i.e. the P/P site), whereas deacylated tRNA can spontaneously fluctuate between the P/P and P/E sites (1–4). In the P/E configuration, the acceptor end of tRNA occupies the 50S E site, while the anticodon stem loop (ASL) remains in the 30S P site. Based on single-molecule FRET experiments, oscillation of tRNA between the P/P and P/E sites occurs at a rate of 1–5 s^{-1} in solution (3). Movement of tRNA into the P/E site involves an interaction with the L1 stalk and is integral to the mechanism of translocation (1,5–10).

The affinity of tRNA for the P site of the ribosome relies mostly on interactions of the ASL with mRNA and the 30S subunit (11,12). In the presence of mRNA, tRNA can bind either the 70S ribosome or the 30S subunit with a K_d in the nanomolar range (11–15). In contrast, the affinity of tRNA for the P site of the isolated 50S subunit is too low to accurately measure (16). The fact that contacts in the 50S subunit account for much less of the total binding free energy is consistent with a much lower energy barrier for translocation with respect to the 50S subunit (i.e. hybrid-state formation) than for translocation with respect to the 30S subunit (17).

Atomic-resolution crystal structures have revealed multiple ribosomal contacts to tRNA and mRNA in the 30S P site (Figure 1) (18–20). 16S rRNA nucleotides (nt) A790 and m^2G966, and the C-terminal 'tails' of ribosomal proteins S9 and S13 interact with the sugar-phosphate backbone of tRNA. C1400 forms a stacking interaction with the anticodon nt 34 of tRNA, while G926 contacts the mRNA backbone between the P and E codons. G1338 and A1339 of 16S rRNA are positioned to form Type II and Type I minor interactions, respectively, with the anticodon stem of tRNA (18–20). All of these 16S rRNA nucleotides are universally conserved, except for m^2G966, which is conserved in bacteria (21). Mutations at these positions decrease translation *in vivo* to varying degrees, with substitutions at 1339 conferring the largest defects (22,23). Biochemical studies have shown that truncation of S9 and mutations at positions 1338 and 1339 differentially affect binding of specific tRNA species (14,23). Yet, no atomic-resolution structure of ribosomes containing P/E-tRNA is currently available, and it

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Table 1. mRNAs used in this study

mRNA	tRNA species	Sequence[a]
m291	Phe	AA**AGGA**AAUAAAAAUGUUUGUAUACAAAUCU
m292	Val	AA**AGGA**AAUAAAAAUGGUAUACUUUAAAUCU
m293	Tyr	AA**AGGA**AAUAAAAAUGUACUUUGUAAAAUCU
m639	Arg	AA**AGGA**AAUAAAAAUGCGUUACUUUAAAUCU
m640	Glu	AA**AGGA**AAUAAAAAUGGAAUACUUUAAAUCU
m651	fMet/Met	AA**AGGA**AAUAAAAUUCAUGUACUUUAAAUCU

[a]Relevant regions (5′ → 3′) of mRNAs are shown. The Shine–Dalgarno sequence is shown in bold. P-codons are underlined. All mRNAs are derived from the T4 gene *32* mRNA (30).

remains unclear how particular contacts in the 30S P site contribute to the overall binding energy of different tRNA species in either the P/P or P/E site. Here, the effects of several ribosomal mutations on P-tRNA stability are measured. The data obtained shed light on how interactions in the P site are idiosyncratically tuned for stable and uniform binding of an assortment of natural tRNA substrates.

MATERIAL AND METHODS

Strains

The Δ7 prrn strains supported by mutant ribosomes were constructed as described (10,24). An *Escherichia coli* strain engineered to express S7 with truncation of the β-hairpin (S7Δ77–84) has been described (25). Strains expressing S9 and/or S13 with C-terminal truncations (S9Δ113–117, S13Δ128–130 and S9Δ113–117/S13Δ128–130) have been described (14).

Ribosomes, tRNAs and mRNAs

Tight-couple ribosomes and isolated subunits were prepared as described (10,24), dialyzed against polymix buffer (26), and stored at –70°C. 50S subunits harboring G2252U were purified from a mixed population of ribosomes using affinity chromatography as described (27,28). $tRNA^{Phe}$, $tRNA^{Val}$, $tRNA^{Arg}$, $tRNA^{Glu}$, $tRNA^{Met}$ and $tRNA^{fMet}$ were purchased from Chemical Block (Moscow, Russia). $tRNA^{Tyr2}$ was purchased from Sigma. [3′-^{32}P]-tRNA was prepared by re-extending the CCA end of 3′-truncated tRNA in the presence of [α-^{32}P]ATP as described (28). [3′-^{32}P]-tRNAΔA76 was made in an analogous way except that [α-^{32}P]CTP was added in the absence of ATP. The [3′-^{32}P]-tRNA was aminoacylated and acetylated as described (28). Aminoacylation and acetylation were highly efficient (~100%), as assessed by acid gel electrophoresis (29). mRNAs were made by *in vitro* transcription, using T7 polymerase and plasmid templates, and purified as described (30). The initiation regions of the mRNAs employed are shown in Table 1. In each case, the P codon is cognate for the most abundant tRNA isoacceptor (31).

Kinetic experiments and data analysis

The dissociation rate (k_{off}) of tRNA from the P site was measured using a double-membrane filtration method as described (32). Typically, <50 nM [3′-^{32}P]-tRNA (~20 000 cpm/pmol), 0.5 μM mRNA and 0.7 μM ribosomes were incubated in polymix buffer at 37°C for 20 min to bind the P site. At $t = 0$, 2 μl of the reaction was diluted 100-fold in polymix buffer containing 0.2 μM unlabeled tRNA, and 20 μl aliquots were filtered at various time points. Membranes were immediately washed with 200 μl of polymix buffer, dried and exposed to a phosphor screen (Amersham). Data were quantified, corrected for background (based on reactions lacking ribosomes), and normalized to the fraction of radiolabel on the nitrocellulose membrane at $t = 0$. k_{off} was determined by fitting the data to a single exponential function, using KaleidaGraph (Synergy Software). When mutant 50S subunits were used, 30S subunits were first heat-activated at 42°C for 20 min and then incubated in the presence of an equal amount of 50S subunits for 10 min at 37°C to form 70S ribosomes.

Variance in k_{off} among different tRNA species was calculated from the variance (σ^2) of the natural logarithms of the rate using the following formula:

$$\sigma^2 = \frac{1}{N-1}\sum_{i=1}^{N}(\ln k_i - \overline{\ln k})^2$$

where N is the number of tRNA species, k_i is k_{off} of a certain tRNA species, and $\overline{\ln k}$ is the average of $\ln k_{off}$ for all the tRNAs. This variance is independent of the units in which the k_i are measured. By taking natural logarithms, the data are effectively normalized to allow the variance for each mutant ribosome to be compared. The calculated variance values are proportional to the variance in binding free energies among different tRNA species. In principle, this variance contains two contributions: the true biological variation of the rates k_i and the experimental uncertainty of the measurements of the k_i. Since the uncertainty $\Delta\ln k_i$ of $\ln k_i$ is given by $\Delta\ln k_i = \Delta k_i/k_i$, the contribution of the experimental uncertainty to the variance can be calculated as

$$\sigma^2_{exp} = \frac{1}{N}\sum_{i=1}^{N}\left(\frac{\Delta k_i}{k_i}\right)^2$$

Measured variances that exceed this uncertainty-based variance are statistically significant and thus biologically meaningful.

The association rate (k_{on}) of tRNA with the P site was estimated as described (15). Briefly, 1 μM mRNA and various concentrations of ribosomes were incubated at 37°C for 10 min, mixed with an equal volume of [3′-^{32}P]-tRNA (<2.5 nM final) at $t = 0$, and incubated at 37°C. 2 μl of the reaction was filtered at various time points. Membranes were immediately washed with 20 μl of ice-cold polymix buffer and quantified as described above. Data were corrected for the background based on the fraction of radiolabel on the nitrocellulose membrane without ribosomes ($t = 0$) and fit to a single exponential function. k_{on} was then calculated based on the apparent rates, the ribosome concentrations, and the independently determined k_{off} value.

RESULTS

Mutagenesis of the 30S ribosomal subunit

Mutations in 16S rRNA targeting the A site (position 1054), P site (positions 790, 966, 1338, 1400 and 926), and E site (positions 693 and 795) were introduced into a Δ7 prrn strain of *E. coli* SQZ10 (Figure 1). This strain lacks chromosomal rRNA operons and is supported by a single plasmid-encoded *rrn* operon and hence a homogeneous population of ribosomes. At least two different substitutions at each of these positions were tolerated in Δ7 prrn (Table 2). The effects of these mutations on growth rate were generally modest and only roughly correlated with their effects on *in vivo* translation, as measured in a specialized ribosome system (22). Why a better correlation was not observed remains unclear. However, the fact that the control Δ7 prrn strain contains wild-type ribosomes but grows considerably more slowly than wild-type *E. coli* suggests that its growth is limited by something other than translation *per se*. We attempted to obtain Δ7 prrn strains with mutations C1054G, C1400G, A1339C, A1339G and A1339U, but were unsuccessful. Presumably, the corresponding mutant ribosomes cannot support cell growth. This may be due to a high error rate in the case of C1054G (S. McClory and K.F., unpublished data), and low translation activity (<6%) in the other cases (Table 2) (22). Ribosomes with A790G, m^{2}G966U, C1054A, G1338A, G1338U, C1400A, G926C, G693C or C795G were purified for further analysis. Ribosomes were

Table 2. Effects of 16S rRNA mutations on cell growth in Δ7 prrn

Site[a]	Mutation[b]		Growth rate (doublings/h)[c]	Translation activity (% of control)[d]
	Control		1.7 ± 0.05	100 ± 5
A	C1054	A	1.4 ± 0.1	52 ± 4
		G	NO	27 ± 2
		U	1.6 ± 0.1	94 ± 7
P	A790	C	1.6 ± 0.1	81 ± 9
		G	1.4 ± 0.04	38 ± 5
		U	1.6 ± 0.1	20 ± 3
	m^{2}G966	A	1.7 ± 0.04	32 ± 2
		C	1.5 ± 0.02	10 ± 1
		U	1.6 ± 0.01	14 ± 3
	G1338	A	1.6 ± 0.04	82 ± 6
		C	NA	9 ± 1
		U	1.2 ± 0.1	22 ± 5
	C1400	A	1.6 ± 0.1	9 ± 2
		G	NO	6 ± 0.4
		U	1.4 ± 0.1	53 ± 9
P, E	G926	A	1.6 ± 0.04	12 ± 0.3
		C	1.3 ± 0.1	12 ± 2
		U	1.6 ± 0.1	12 ± 1
E	G693	A	1.7 ± 0.03	ND
		C	1.7 ± 0.02	ND
		U	1.7 ± 0.03	ND
	C795	A	1.6 ± 0.004	ND
		G	1.5 ± 0.04	ND
		U	1.7 ± 0.02	ND

NO, strain not obtained; NA, strain construction not attempted; ND, not determined.
[a]The ribosomal site(s) in the closest proximity to mutations.
[b]The original residue, residue number, and introduced residue are indicated.
[c]Growth rates in LB medium at 37°C are reported as the mean ± SEM from at least three independent experiments.
[d]From ref. 22.

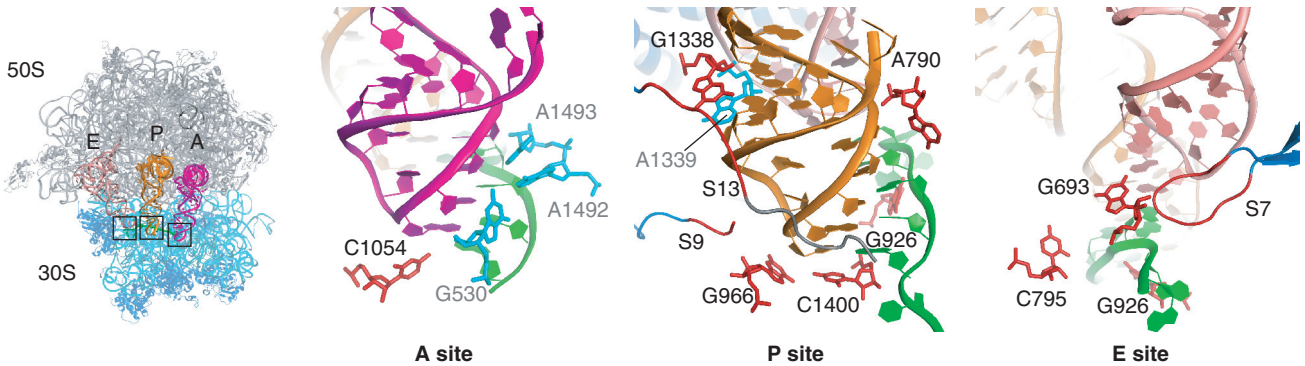

Figure 1. Ribosomal contacts to tRNA and mRNA in the A, P and E sites. In the overview (left panel), 16S rRNA is colored cyan; small-subunit ribosomal proteins, marine blue; 23S rRNA and large-subunit ribosomal proteins, gray. In the close-up (right three panels), residues of the 30S subunit targeted in this study are shown in red. Essential 16S rRNA nucleotides are shown in cyan. A C-terminal extension present in S13 of *Thermus thermophilus* but not in *E. coli* is labeled in gray. A-site tRNA is shown in magenta; P-site tRNA, orange; E-site tRNA, salmon; mRNA, green. This figure was created from the X-ray structure of the *T. thermophilus* ribosomal complex (PDB ID code 2J00 and 2J01) (18).

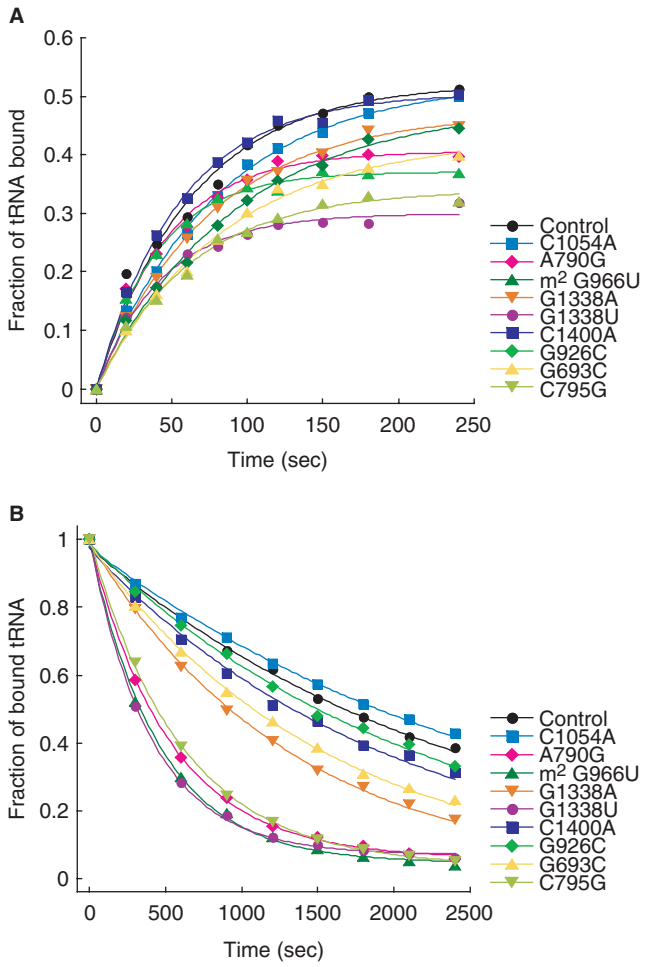

Figure 2. Effects of 16S rRNA mutations on the binding of $tRNA^{Val}$. (**A**) Examples of experiments measuring association of $tRNA^{Val}$ with the P site of control and mutant ribosomes at 7 nM. (**B**) Examples of experiments measuring dissociation of $tRNA^{Val}$ from the P site of control and mutant ribosomes. Data were normalized at $t = 0$. For values of the dissociation and association rate constants, see Table 3.

also purified from strains that express S9 and S13 with C-terminal truncations (14) and S7 with a partial deletion of the β-hairpin element (25).

Effects of 16S rRNA mutations on $tRNA^{Val}$ binding

Uhlenbeck and colleagues measured the dissociation rate (k_{off}) and association rate (k_{on}) of a number of different tRNA species in the ribosomal P site, using a double-membrane filter binding method (12,32,33). In those studies, it was found that k_{on} was nearly identical among nine natural tRNAs. Using the same method, we found that k_{on} of $tRNA^{Val}$ in polymix buffer was virtually unchanged by the 30S mutations (<2-fold), whereas a number of these mutations increased k_{off} substantially (Figure 2; Table 3). Thus, k_{off} values correlated well with calculated K_d values (Table 3).

Effects of 30S subunit mutations on tRNA dissociation

Next, we measured the effect of each 30S mutation on k_{off} of $tRNA^{Phe}$, $tRNA^{Val}$ and $tRNA^{Tyr}$ from the P site of the 70S ribosome. Mutations A790G, m^2G966U, G1338U, C795G and S9Δ113–117 increased k_{off} of all three tRNA species in the P site (Figure 3A; Supplementary Table 1). With the exception of C795G, these mutations directly target the 30S P site. Mutations G693C, G926C and G1338A had smaller effects on k_{off}. G1338A modestly decreased k_{off} of $tRNA^{Phe}$, as might be predicted from earlier observations (22,24), and modestly increased k_{off} of the other tRNAs. Several mutations (C1054A, C1400A, S13Δ128–130 and S7Δ77–84) had no appreciable effect on k_{off} of these tRNAs in the P site. Dissociation of Ac-aa-tRNAs was virtually unaffected by any of the 30S mutations, with slight increases in k_{off} of AcTyr-$tRNA^{Tyr}$ conferred by some of the mutations that also destabilized deacylated tRNAs (Figure 3B; Supplementary Table 1).

Table 3. Effects of mutations in the 30S subunit on the binding of $tRNA^{Val}$ to the P site

Component[a]	Mutation	Site[b]	k_{off} ($sec^{-1} \times 10^4$)[c]	k_{on} ($\mu M^{-1} sec^{-1}$)[c]	K_d (nM) (calculated)
16S rRNA	Control	–	4.4 ± 0.4	2.2 ± 0.2	0.20
	C1054A	A	3.9 ± 0.3	1.9 ± 0.1	0.21
	A790G	P	20 ± 2	3.2 ± 0.2	0.63
	m^2G966U	P	24 ± 2	3.1 ± 0.1	0.78
	G1338A	P	8.1 ± 0.4	1.9 ± 0.1	0.42
	G1338U	P	27 ± 1	3.2 ± 0.2	0.85
	C1400A	P	5.2 ± 0.1	2.6 ± 0.02	0.20
	G926C	P, E	5.1 ± 0.5	1.8 ± 0.2	0.29
	G693C	E	6.9 ± 0.3	1.7 ± 0.1	0.41
	C795G	E	19 ± 1	2.2 ± 0.1	0.88
r Proteins	Control	–	4.4 ± 0.3	2.6 ± 0.3	0.17
	S9Δ127–129	P	33 ± 3	4.5 ± 0.5	0.73
	S13Δ113–117	P	4.7 ± 0.4	2.3 ± 0.4	0.20
	S9Δ127–129/S13Δ113–117	P	30 ± 3	4.2 ± 0.4	0.71
	S7Δ77–84	E	4.8 ± 0.2	2.7 ± 0.1	0.17

[a]Components of the 30S subunit that bear mutations.
[b]Ribosomal sites in the closest proximity to mutations.
[c]k_{off} and k_{on} values are reported as the mean ± SEM from at least three independent experiments.

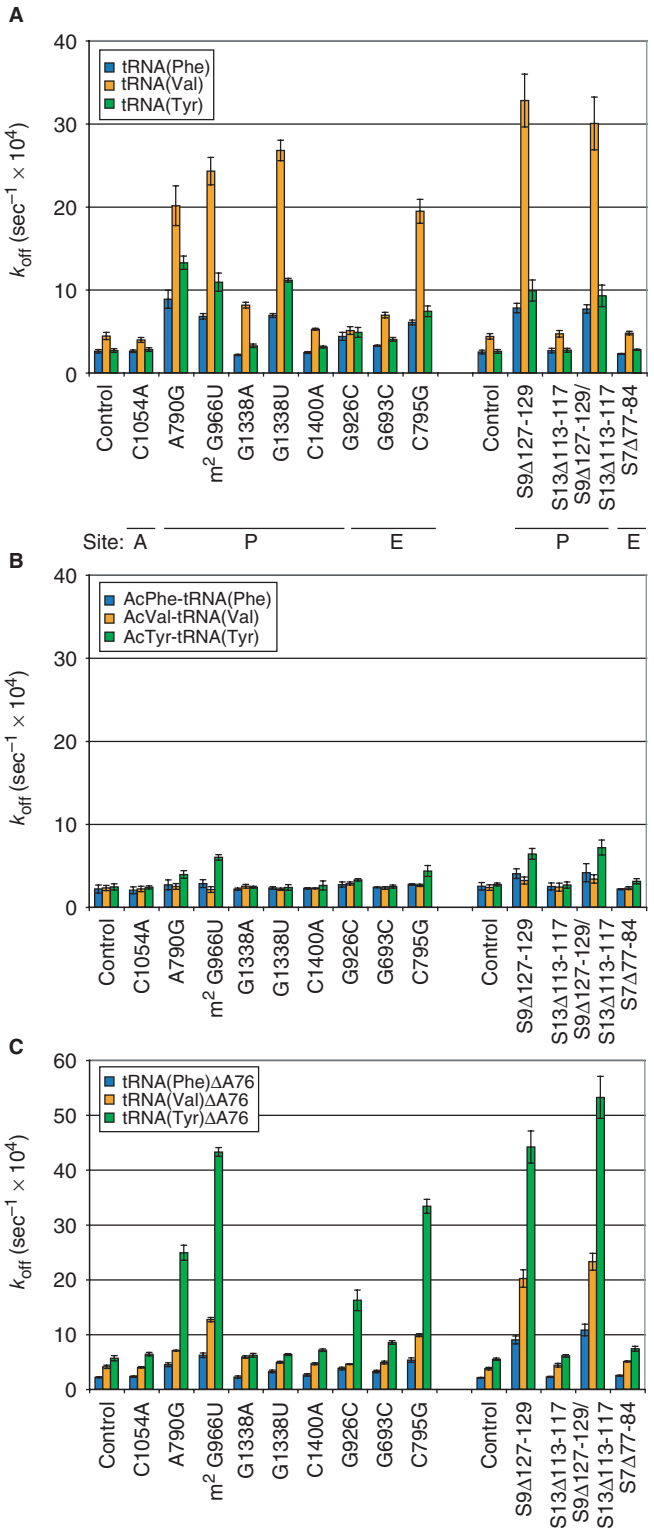

Figure 3. Effects of 30S mutations on the dissociation rate (k_{off}) of tRNA from the P site. k_{off} of deacylated (**A**), *N*-acetyl-aminoacylated (**B**) or A76-truncated (**C**) tRNA^{Phe} (blue), tRNA^{Val} (orange) and tRNA^{Tyr} (green) from the P site in various mutant ribosomes. Data represent mean ± SEM from at least three independent experiments.

Removal of A76 from tRNA stabilizes its binding in the presence of G1338U

Deacylated tRNA is known to spontaneously oscillate between the P/P and P/E sites, while acylated tRNA is restricted in the P/P site (2,3,34,35). The fact that many of the 30S mutations failed to increase k_{off} of Ac-aa-tRNA raised the possibility that their effects on deacylated tRNA involved P/E-site binding. To investigate this possibility, we used tRNAs with truncation of nt A76. A76 is the universally conserved 3' terminal nt of tRNA. In the 50S P site, A76 interacts with A2450 and A2451 of 23S rRNA. In the 50S E site, A76 intercalates between G2421 and A2122 and interacts with C2394 of 23S rRNA (18,19,36). Removal of A76 (ΔA76) from tRNA^{Phe} reduces its affinity for the E site by >100-fold but does not affect its affinity for the P site (37). Based on these observations, ΔA76 should shift the P/P ⇆ P/E equilibrium to the left (5,6,28). With this in mind, we measured dissociation rates of tRNA^{Val}ΔA76, tRNA^{Phe}ΔA76 and tRNA^{Tyr}ΔA76 from the P site of ribosomes harboring the 30S mutations (Figure 3C). We found that ΔA76 generally decreased k_{off} of tRNA^{Val} from the P site of mutant ribosomes and had a similar, albeit lesser, effect on tRNA^{Phe}. In contrast, ΔA76 increased k_{off} of tRNA^{Tyr} in all cases except G1338U. Notably, k_{off} of all 3′-truncated tRNAs in the presence of G1338U was virtually the same as the control (Figure 3C), even though this mutation had strong effects on k_{off} of the full-length deacylated tRNAs (Figure 3A).

50S E-site mutation C2394A restores tight binding of certain tRNAs to ribosomes harboring G1338U

The effects of ΔA76 in G1338U ribosomes could be related to a reduced occupancy of the P/E site. To further investigate relationship between P/E-site binding and k_{off}, we compared dissociation rates of 6 different tRNA species (tRNA^{Val}, tRNA^{Glu}, tRNA^{Met}, tRNA^{Phe}, tRNA^{Tyr} and tRNA^{Arg}) from ribosomes harboring various 30S P-site mutations in the absence and presence of C2394A (Figure 4; Supplementary Table 2). Mutation C2394A of 23S rRNA targets the 50S E site and is predicted to shift the P/P ⇆ P/E equilibrium to the left (10,18,19,28,36). k_{off} of tRNAs from the P site of ribosomes containing the wild-type 30S subunit was virtually unchanged by C2394A. k_{off} of *N*-acetyl-Val-tRNA^{Val} (AcVal-tRNA^{Val}) and *N*-acetyl-Tyr-tRNA^{Tyr} (AcTyr-tRNA^{Tyr}) was also unaffected by C2394A (Figure 4A, E). In the presence of the 30S P-site mutations, k_{off} of tRNA^{Val} and tRNA^{Glu} was strongly decreased by C2394A (Figure 4A, B). C2394A modestly decreased k_{off} of tRNA^{Met} and tRNA^{Phe}, and tRNA^{Tyr} in G1338U ribosomes but did not decrease k_{off} of tRNA^{Arg} in any case tested (Figure 4A–F).

To quantify the uniformity among k_{off} of different elongator tRNAs, we calculated the variance (σ^2) of the natural logarithm of k_{off} (see Material and methods section). A uniform set of rate constants should give $\sigma^2 \approx 0.02$ based on the experimental uncertainties of the rates. It was evident that whereas A790G, m^{2}G966U, and G1338U all increase σ^2 in ribosomes with control 50S

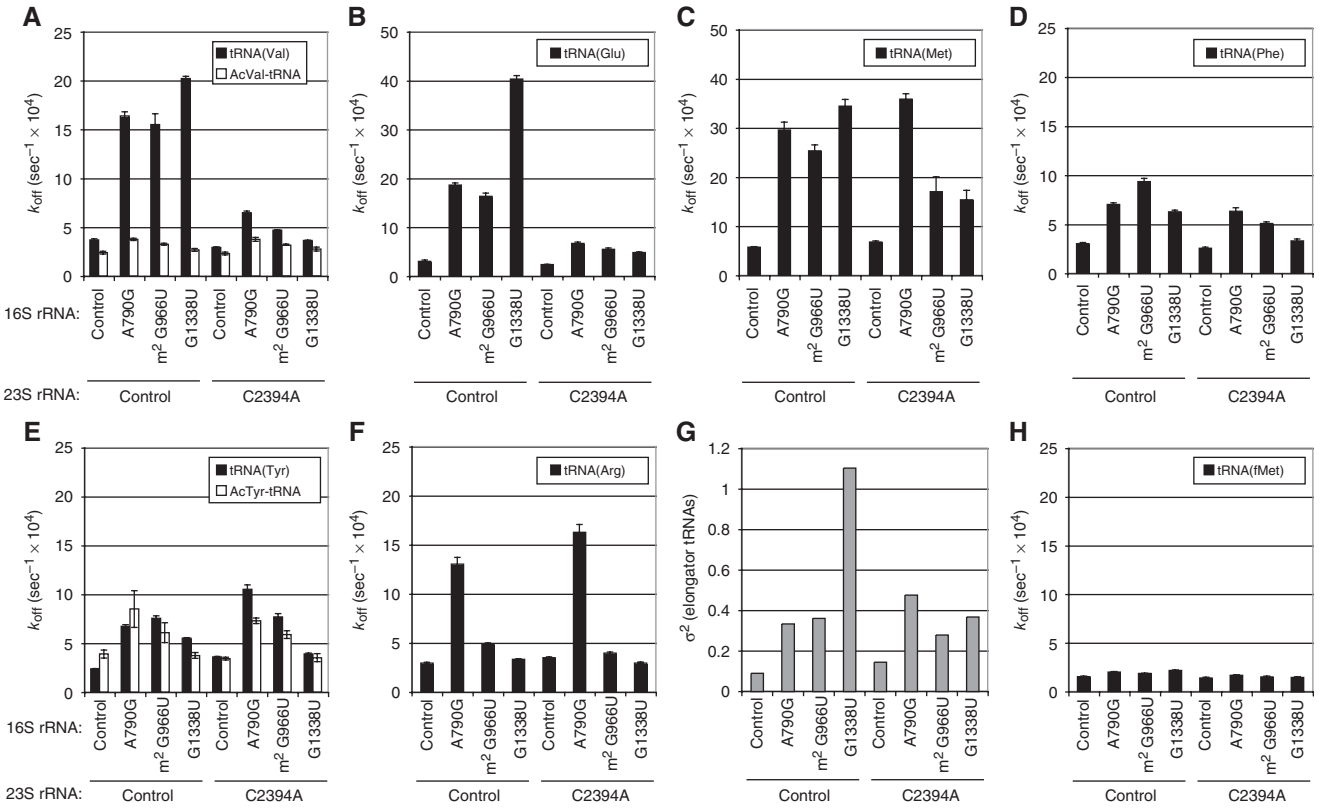

Figure 4. Effects of E-site mutation C2394A on dissociation of tRNA from the P site. k_{off} of tRNA^{Val} and AcVal-tRNA^{Val} (**A**), tRNA^{Glu} (**B**), tRNA^{Met} (**C**), tRNA^{Phe} (**D**), tRNA^{Tyr} and AcTyr-tRNA^{Tyr} (**E**), and tRNA^{Arg} (**F**) from the P site was measured in ribosomes harboring rRNA mutations (as indicated). Note that the scale of the *y*-axis in (B and C) is larger than in the other analogous panels. Data represent the mean ± SEM from three independent experiments. (**G**) Variance among elongator tRNA species. Variance was calculated from the natural logarithm of k_{off} of six deacylated tRNAs in (A–F). (**H**) k_{off} of initiator tRNA^{fMet} from the P site.

subunits, introduction of C2394A greatly decreased σ^2 (i.e. increased uniformity) in ribosomes harboring G1338U (Figure 4G).

k_{off} of initiator tRNA^{fMet} is unaffected by mutations of the 30S P site

Initiator tRNA^{fMet} is the only tRNA that enters the P site directly (i.e. without first passing through the A site). Initiator tRNA^{fMet} has distinct structural features such as a mismatch between bases C1 and A72 and three consecutive G-C base pairs in the anticodon stem. The C1×A72 mismatch is important for formylation of the methionyl group, which allows IF2 recognition and prevents EF-Tu interaction (38). The three G–C base pairs in the anticodon stem are necessary for efficient initiation, presumably due to their ability to stabilize fMet-tRNA^{fMet} in the 30S P site (23,38–40). To investigate how these structural differences affect the binding of tRNA to the 70S ribosome, we measured k_{off} of initiator tRNA^{fMet} in the presence of the 30S P-site mutations (Figure 4H; Supplementary Table 2). Surprisingly, none of the ribosomal mutations affected k_{off} of tRNA^{fMet} in the P site. This phenomenon seems specific for tRNA^{fMet}, because k_{off} of tRNA^{Met}, like other elongator tRNAs, was affected by these mutations (Figure 4C). The fact that tRNA^{fMet} and tRNA^{Met} possess the same anticodon suggests that the differential effects observed are due to properties of the tRNA body.

50S P-site mutation G2252U increases k_{off} of Ac-aa-tRNA, but not deacylated tRNA

We also measured k_{off} of tRNAs from ribosomes harboring mutation G2252U in 23S rRNA. G2252 forms a Watson–Crick base pair with C74 of tRNA in the 50S P site, and thus this mutation is predicted to shift the P/P $\leftrightarrows$ P/E equilibrium to the right (3,8,18,19,28,41). We found that dissociation of tRNA^{Val} and tRNA^{Tyr} was unaffected by G2252U, whereas that of AcVal-tRNA^{Val} and AcTyr-tRNA^{Tyr} was accelerated by the mutation (Figure 5; Supplementary Table 2). These data are consistent with the idea that deacylated tRNA can readily occupy either the P/P or P/E site, whereas peptidyl-tRNA is largely restricted to the P/P site.

Effects of 50S mutations C2394A and G2252U on k_{on} of tRNA

To test if the 50S mutations affect k_{on}, we measured association of tRNA^{Val} and tRNA^{Tyr} with ribosomes harboring C2394A or G2252U (Figure 6). C2394A inhibited association of tRNA^{Val} and tRNA^{Tyr} with the ribosome by 110-fold and 16-fold, respectively, suggesting that the

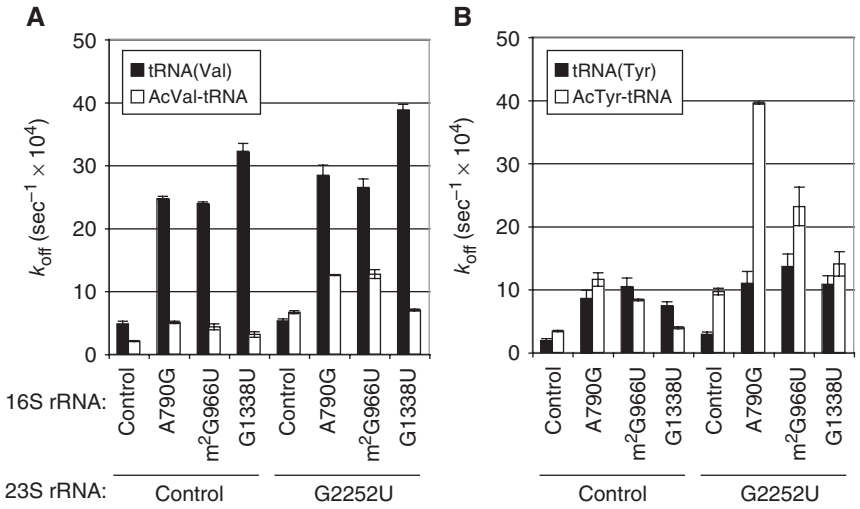

Figure 5. Effects of 50S P-site mutation G2252U on k_{off}. k_{off} of tRNA^{Val} and AcVal-tRNA^{Val} (**A**), and tRNA^{Tyr} and AcTyr-tRNA^{Tyr} (**B**) from the P site was measured in ribosomes harboring rRNA mutations (as indicated). Data represent the mean ± SEM from three independent experiments.

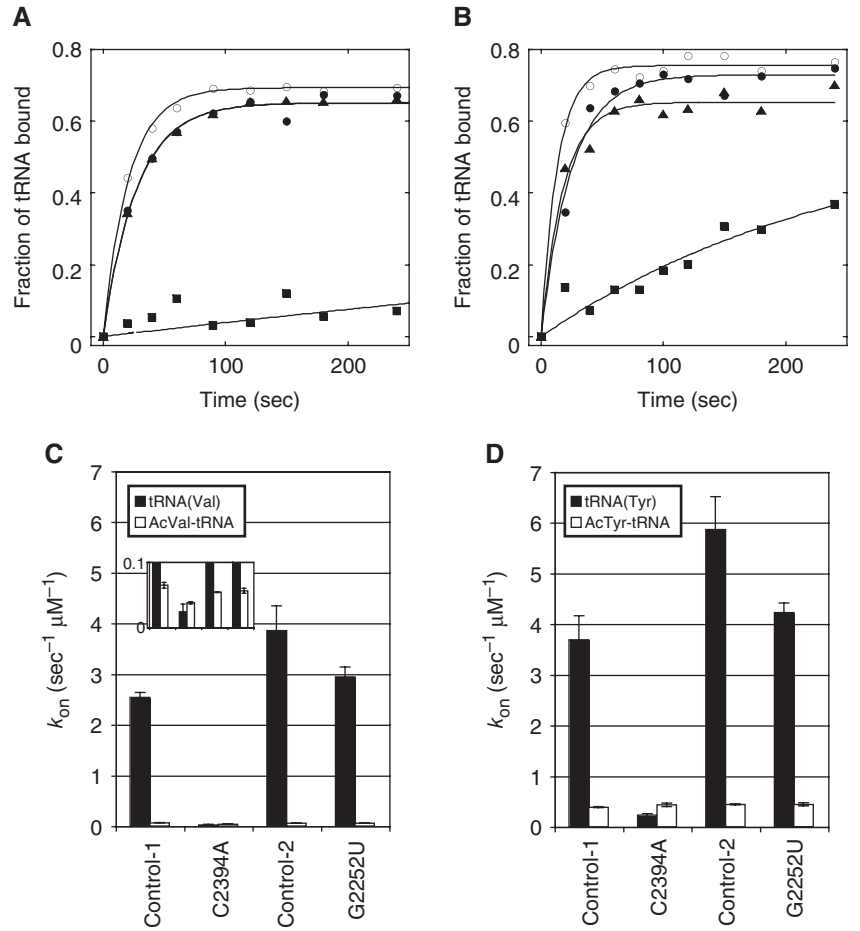

Figure 6. Effects of 50S mutations C2394A and G2252U on k_{on}. (**A** and **B**) Examples of experiments measuring the association rate (k_{on}) of tRNA^{Val} (A) or tRNA^{Tyr} (B) with ribosomes harboring C2394A (filled square) or G2252U (filled triangle) at 14 nM. Appropriate controls (control-1 for C2394A, filled circle; control-2 for G2252U, open circle) are also included. (**C** and **D**) k_{on} of tRNA^{Val} (C) or tRNA^{Tyr} (D) in the deacyl or *N*-acetyl-aminoacyl form (as indicated) to ribosomes harboring the 50S mutations. Data represent the mean ± SEM from three independent experiments.

50S E site is critical for association of deacylated tRNA. We then measured k_{on} of AcVal-tRNA^{Val} and AcTyr-tRNA^{Tyr} with the same set of ribosomes. It was found that the *N*-acetyl-aminoacyl form of both tRNA species binds to control ribosomes much more slowly (>40-fold and >9-fold for tRNA^{Val} and tRNA^{Tyr}, respectively) than deacylated tRNAs (Figure 6C and D). The overall k_{on} of Ac-aa-tRNA was virtually unaffected by the 50S mutations and similar to k_{on} of deacylated tRNA with ribosomes harboring C2394A, suggesting that tRNA can bind to ribosomes, albeit at a relatively slow rate, via a pathway in which the 50S E site is not involved.

DISCUSSION

In this study, we compare the effects of a number of ribosomal mutations on tRNA binding. Several mutations (A790G, m^{2}G966U, G1338U, C795G and S9Δ113–117) destabilize elongator tRNAs in the P site of the 70S ribosome. The degree to which a given ribosomal mutation affects tRNA binding depends on the species of tRNA. For example, A790G generally increases k_{off} of all elongator tRNA species tested (tRNA^{Val}, tRNA^{Glu}, tRNA^{Met}, tRNA^{Phe}, tRNA^{Tyr} and tRNA^{Arg}), while m^{2}G966U and G1338U are unable to destabilize tRNA^{Arg}. The relative effects of the 30S mutations vary significantly among tRNA species (e.g. tRNA^{Val} and tRNA^{Glu} are destabilized most by G1338U, tRNA^{Phe} and tRNA^{Tyr} are destabilized most by m^{2}G966U, and tRNA^{Arg} is destabilized most by A790G). These data support the idea that multiple contacts in the 30S subunit idiosyncratically tune tRNA binding to the P site (12).

Deacylated tRNA spontaneously oscillates between the P/P and P/E sites of the ribosome. Because no other intermediate binding states have been observed in the absence of factors, it is reasonable to assume that tRNAs dissociate from either the P/P or P/E site in our experiments (Figure 7). Since the P/P $\leftrightarrows$ P/E transition occurs much more rapidly (1–5 s^{-1}; 1,3) than dissociation of tRNA (0.0002–0.005 s^{-1}), the observed dissociation rate (k_{off}) expressed as a function of the rate and equilibrium constants shown in Figure 7 is

$$k_{off} = \frac{k_{offP/P} + k_{offP/E} \bullet K_{P/E}}{1 + K_{P/E}}$$

According to this model, the observed k_{off} is more strongly influenced by $k_{offP/P}$ or $k_{offP/E}$ when the P/P $\leftrightarrows$ P/E equilibrium is shifted to the left or right, respectively. Mutation C2394A, which decreases $K_{P/E}$ (*i.e.*, shifts the P/P $\leftrightarrows$ P/E equilibrium to the left), decreases k_{off} of tRNA^{Val} from the P site (Figure 4A). Mutation G2252U, which increases $K_{P/E}$, has little effect on k_{off} (Figure 5A). These data suggest that dissociation via the E site is the primary dissociation pathway of tRNA^{Val}. Consistent with this idea, addition of E-site tRNA prevents dissociation of tRNA^{Val} from the P site when spontaneous reverse translocation is inhibited by tetracycline (42). tRNA^{Glu}, like tRNA^{Val}, also appears to dissociate mostly from the P/E site because C2394A inhibits dissociation of tRNA^{Glu}. In contrast, tRNA^{Met}, tRNA^{Phe}, tRNA^{Tyr} and tRNA^{Arg} may dissociate from the P/P and P/E sites at similar rates because C2394A has little or no effect on k_{off} of these tRNAs. Unlike deacylated tRNAs, acylated tRNAs are believed to be largely restricted to the P/P site (2,3,34,35). The 50S P-site mutation G2252U increases k_{off} of AcVal-tRNA^{Val} and AcTyr-tRNA^{Tyr}, an effect that requires the acyl group in both cases. Because C2394A does not affect k_{off} of these acylated tRNAs, it is likely that they dissociate primarily from the P/P site.

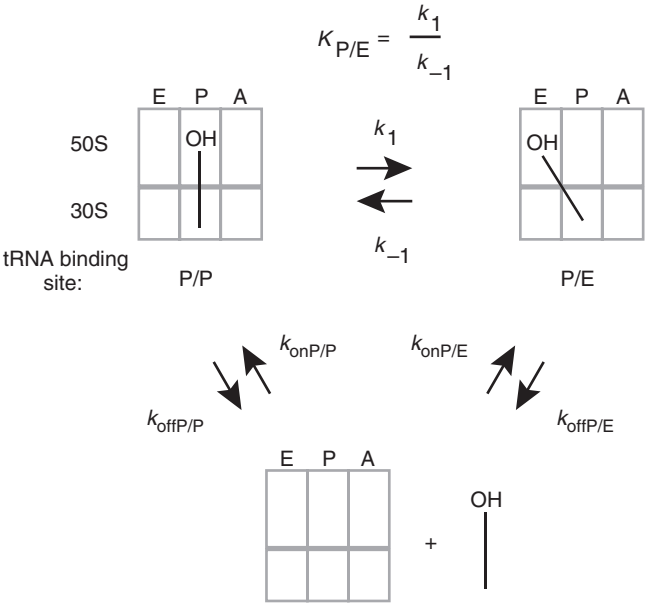

Figure 7. A model for dissociation and association pathways of P-tRNA.

Association of tRNA^{Val} and tRNA^{Tyr} with the ribosome is strongly inhibited by C2394A, suggesting that the 50S E site is involved in the association pathway of deacylated tRNA. Because acylation of these tRNAs also inhibits association to a similar degree, we conclude that affinity for the 50S E site is critical for rapid non-enzymatic association of tRNA with the P site *in vitro*, in agreement with an earlier study (43). That deacylated tRNA passes through the E site for P-site dissociation and association is consistent with the previous suggestion that the E site provides a labile intermediate site between the P site and the solvent (5,13).

For deacylated tRNA^{Val} and tRNA^{Phe}, the 30S mutations increase k_{off} to various extents depending on the mutation. But when these same tRNAs are acylated, only a narrow range of k_{off} values (near 2.5×10^{-4} s^{-1}) is observed among all the mutant ribosomes (Figure 2B; Supplementary Table 1). A similar narrow range of k_{off} values is seen when dissociation of tRNA^{fMet} from the set of mutant ribosomes is measured (Figure 4H; Supplementary Table 2). It is as though the effects of the 30S mutations are masked in some way when the *N*-acetyl-aminoacyl groups are present or when tRNA^{fMet} is used. A similar phenomenon has been observed for the A site, where substantial variation in

k_{off} among a set of tRNA derivatives was revealed only when mRNA-tRNA or mRNA-rRNA interactions were weakened (44). It was proposed that there exists a stability threshold for A-tRNA, such that k_{off} values below $0.5 \times 10^{-4}\,s^{-1}$ are not observed regardless of how many tRNA-ribosome contacts are optimized. This stability threshold may explain how uniform binding of the various natural tRNA substrates is achieved. The molecular basis of this stability threshold remains unclear but presumably involves a conformational change in the ribosomal complex that facilitates A-tRNA release. The rate of this conformational change may set and thereby reflect the minimum k_{off} threshold. Based on our data, we propose that a stability threshold also exists for P-tRNA. Whether a common molecular mechanism sets the thresholds for both A-tRNA and P-tRNA remains to be determined, but the fact that these k_{off} thresholds are similar is consistent with this possibility.

The 50S E-site mutation C2394A generally stabilizes tRNA in G1338U ribosomes and thereby increases the uniformity of tRNA binding. The simplest interpretation of these data is that G1338U makes tRNA in the P/E site particularly vulnerable to dissociation. Chemical protection experiments have shown that the movement of tRNA from the P/P to P/E site changes the reactivity of only two nucleotides in 16S rRNA: A702 becomes hyper-reactive and G1338 becomes further protected (2). Increased protection of G1338 suggests an altered interaction of this residue with the anticodon stem of tRNA in the P/E site. X-ray structures of ribosomes containing P/P-tRNA reveal that the loop containing G1338 and A1339, together with the 790 loop, form a 'gate' between the P and E sites that appears to lock the ASL in the P site (18–20,45). Cryo-EM studies indicate that movement of tRNA into the P/E site promotes a large-scale conformational change termed ratchet-like subunit rotation (RSR; 7,46–51). The RSR includes a rotation of the 1338–1339 loop and an opening of the 'gate' (52). We propose that this rearrangement buries the base of G1338 deeper into the minor groove of the anticodon stem of tRNA, which would explain the enhanced protection of G1338 by P/E-tRNA (2). Because the RSR conformation opens the 'gate', G1338 may be more critical in RSR ribosomes for preventing premature tRNA release.

Initiator $tRNA^{fMet}$ binds tightly to the P site of 70S ribosomes, and the 30S mutations analyzed here have no appreciable effect on k_{off} (Figure 4H). The robust nature of $tRNA^{fMet}$ binding could be conferred by the unique features of $tRNA^{fMet}$, such as the C1×A72 mismatch or the three consecutive G–C base pairs in the anticodon stem (38). However, neither C1U nor A72G (either of which allows the 1–72 base pair to form) increased k_{off} of $tRNA^{fMet}$ in the P site (data not shown). Furthermore, converting the anticodon stem of $tRNA^{fMet}$ into that of $tRNA^{Glu}$ had only a marginal effect on k_{off} from 70S ribosomes (<2-fold; data not shown). This latter observation was quite unexpected, since these 3 consecutive G–C base pairs serve to stabilize initiator tRNA in the P site of in the 30S subunit (39,40). One explanation is that the combination of these elements and the unique conformation that $tRNA^{fMet}$ tends to adopt (53) contribute to its stable binding. Further experiments will be necessary to sort out which features of $tRNA^{fMet}$ contribute to its robust interaction with the 70S ribosome.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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