Functional Interaction of an Arginine Conserved in the Sixteen Amino Acid Insertion Module of *Escherichia coli* Methionyl-tRNA Formyltransferase with Determinants for Formylation in the Initiator tRNA†

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ABSTRACT: Formylation of initiator methionyl-tRNA by methionyl-tRNA formyltransferase (MTF) is important for initiation of protein synthesis in eubacteria. The determinants for formylation are clustered mostly in the acceptor stem of the initiator tRNA. Previous studies suggested that a 16 amino acid insertion loop, present in all eubacterial MTF's (residues 34−49 in the *E. coli* enzyme), plays an important role in specific recognition of the initiator tRNA. Here, we have analyzed the effect of site-specific mutations of amino acids within this region. We show that an invariant arginine at position 42 within the loop plays a very important role both in the steps of substrate binding and in catalysis. The kinetic parameters of the R42K and R42L mutant enzymes using acceptor stem mutant initiator tRNAs as substrates suggest that arginine 42 makes functional contacts with the determinants at the 3:70 and possibly also the 2:71 base pairs in the acceptor stem of the initiator tRNA. The kinetic parameters of the G41R/R42L double mutant enzyme are essentially the same as those of R42L mutant, suggesting that the requirement for arginine at position 42 cannot be fulfilled by an arginine at position 41. Along with other data, this result suggests that the insertion loop, which is normally unstructured and flexible, adopts a defined conformation upon binding to the tRNA.

Initiation of protein synthesis in eubacteria and in the eukaryotic organelles, mitochondria and chloroplasts, occurs with formyl-methionine (*1*, *2*). A specialized initiator tRNA, $tRNA^{fMet}$, with unique sequence and structural features, is involved in this process (*3*). Formylation of the methionine attached to the 3′ end of this tRNA is catalyzed by methionyl-tRNA formyltransferase (MTF,[1] EC 2.1.2.9; 10-formyltetrahydrofolate:L-methionyl-tRNA *N*-formyltransferase). The formylation reaction is highly specific; the enzyme formylates the initiator methionyl-tRNA and no other tRNA. The enzyme has in addition a preference for the amino acid methionine (*4*−*9*). Previous studies have shown that the most important sequence and structural elements crucial for formylation of the *E. coli* initiator tRNA are clustered in the acceptor stem (Figure 1; references *10*−*12*). The absence of a base pair between positions 1 and 72, a unique feature common to all eubacterial initiator tRNAs (*13*), is a critical determinant in the acceptor stem. Mutation of A72 to G72, which generates a C1:G72 base pair, results in strong negative effects in formylation both in vivo and in vitro (*10*−*12*, *14*). Coupling of this mutation with the A73 to G73 mutation at the discriminator position makes the resulting G72G73 mutant tRNA an even worse substrate for MTF.

The crystal structure of *E. coli* MTF is known (15). The enzyme consists of two domains that are connected by a linker region. The N-terminal domain is structurally homologous to glycinamide ribonucleotide formyltransferase (GARF), which, like MTF, uses N^{10}-formyltetrahydrofolate (FTHF) as a formyl group donor in formylation reactions (*15*−*17*). One of the notable differences between MTF and GARF sequences is the insertion in MTF of a 16 amino acid sequence in the loop region between the second β-strand and the second α-helix from the N-terminus in contrast to a 3 amino acid loop in GARF (Figure 2). This 16 amino acid insertion sequence is found in MTF from all eubacteria

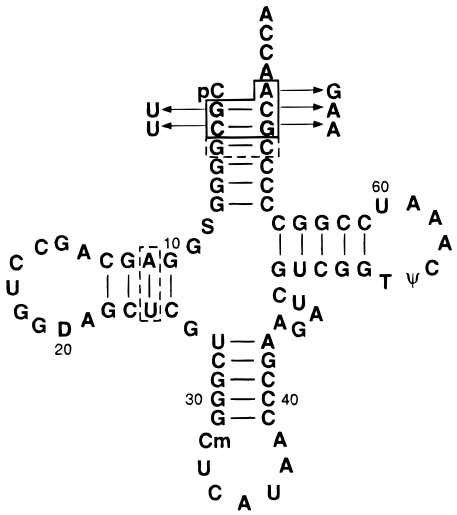

FIGURE 1: Cloverleaf structure of *E. coli* initiator $tRNA_2^{fMet}$. Arrows indicate the mutant tRNAs used in this study. Nucleotides playing a major role in formylation are boxed by solid lines whereas those playing a somewhat minor role are boxed by dashed lines.

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[1] Abbreviations: MTF, methionyl-tRNA formyltransferase; GARF, glycinamide ribonucleotide formyltransferase; MetRS methionyl-tRNA synthetase.

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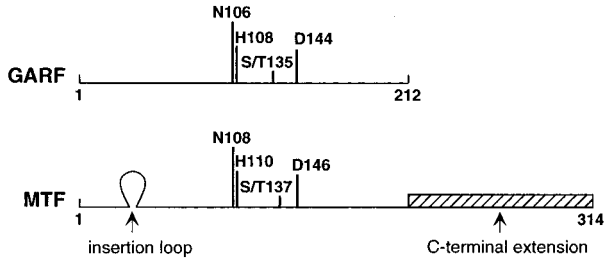

FIGURE 2: Schematic alignment of GARF and MTF sequences from various sources. The amino acid numbering of MTF begins with serine found at the N-terminus of the *E. coli* protein. The amino acids Asn, His, Ser/Thr, and Asp thought to be involved in catalysis in GARF (18) and also found in MTF are indicated. Arrows indicate sites of insertion in MTF compared to GARF which are thought to be important in tRNA recognition.

whose sequences are known to date, and it contains many highly conserved amino acid residues (*8*).

Using a mutant initiator tRNA defective in formylation because of the G72G73 change, we have isolated and characterized suppressor mutations in the *E. coli* MTF, which specifically compensate for the formylation defect of this tRNA (*8*). Interestingly, the site of suppressor mutation is located within the 16 amino acid insertion sequence. This result supports the notion that the 16 amino acid insertion sequence in MTF plays an important role in the specific recognition of the determinants in the acceptor stem of the tRNA.

This paper reports on the effect of site-specific mutagenesis of the highly conserved amino acid residues in and around the 16 amino acid insertion sequence in MTF. The results indicate that an invariant arginine (Arg42) within the loop is very important for enzyme activity. Kinetic analyses were performed using G72, U2:A71, and U3:A70 mutant initiator tRNAs as substrates in formylation by the wild-type and R42K and R42L mutant enzymes. The results suggest that Arg42 makes functional contacts with the determinants in the acceptor stem of the initiator tRNA.

MATERIALS AND METHODS

Chemicals, Enzymes, and Radioisotopes. Folinic acid was obtained from Sigma; Talon-Sepharose was from Clontech. All the restriction enzymes and DNA-modifying enzymes were obtained either from New England Biolabs or from Boehringer Mannheim. Oligonucleotide primers used for DNA sequencing and site-specific mutagenesis were custom-synthesized by Genosys Biotechnologies. DNA sequencing kit Sequenase (Version 2.0) was from Amersham. [^{35}S]-Methionine (specific activity = 1175 Ci/mmol) was purchased from NEN−DuPont. All other routinely used chemicals were of the highest purity grade available.

Site-Specific Mutagenesis of the Gene for MTF and Purification of Mutant MTF Proteins. The plasmid pQE16 FMTp, expressing the wild-type MTF as a C-terminal 6 × His fusion protein, has been described (*8*). This was used as a template for the construction of mutants by Quik Change mutagenesis with *Pfu* DNA polymerase (Stratagene). The wild-type and mutant MTFs were expressed in *E. coli* JM109 and purified by using Talon affinity resin as described (*8*). The purified proteins were stored in 20 mM imidazole, 150 mM KCl, 10 mM β-mercaptoethanol, and 50% glycerol at −20 °C with no detectable loss in activity. The purity of the MTF preparation was assessed by SDS/polyacrylamide gel electrophoresis. Protein was estimated by the dye binding assay using IgG as standard (*19*).

Purification of MetRS. MetRS was also expressed and purified as a C-terminal 6 × His fusion protein using the Talon affinity resin. For this purpose, the plasmid pACMS3 (*20*, *21*) containing the MetRS gene was amplified by polymerase chain reaction using *Pfu* DNA polymerase and the following two primers: 5′GACTCCATGGCTCAAGTC-GCGAAG3′ and 5′GGGAGATCTTTTCACCTGATGAC-CCGG3′ containing the recognition sites for the restriction enzymes *Nco*I and *Bgl*II, respectively. The incorporation of the *Nco*I site in the primer results in a change of the second codon of MetRS from ACT (Thr) to GCT (Ala). The PCR product was digested with *Nco*I+*Bgl*II and cloned into the expression vector pQE60 (Amp^R, Qiagen) to generate pQE60MRS5. This plasmid overexpresses MetRS with 6 × His fused at the C-terminus.

Construction of Plasmids Expressing Wild-Type and Mutant tRNAs and Their Purification. pUC19 plasmid containing the wild-type $tRNA_2^{fMet}$ gene and pBR322 plasmid containing the G72 mutant tRNA gene have been described (*22*, *23*). The constructs expressing the U2:A71 and U3:A70 mutant tRNAs were generated as follows: M13mp8RF/trnfMU2:A71 (*24*) was digested with *Pst*I+*Eco*RI, and the small fragment (∼450 bp) was cloned into the respective sites of plasmid pTZ19R. The tRNA sequence was confirmed by sequencing the resulting plasmid, pTZ19R/trnfMU2:A71. Similarly, pTZ19R/trnfMU3:A70 (*24*) was constructed by digesting M13mp8/trnfMU1/U3:A70 with *Pst*I+*Eco*RI and cloning the small fragment into pTZ19R. Using this plasmid as a template, U1 was changed back to C1 by site-specific mutagenesis to generate pTZ19R/trnfMU3:A70, and the sequence of the mutant tRNA gene was confirmed by DNA sequencing. The resulting plasmids pTZ19R/trnfMU2:A71 and pTZ19R/trnfMU3:A70 were used to prepare the corresponding mutant tRNAs. The wild-type and mutant tRNAs were expressed from the respective plasmids in *E. coli* B and purified by electrophoresis of total tRNA on a 15% native polyacrylamide gel (*23*, *24*). The purity of tRNAs, assessed by aminoacylation assays, was >95%.

Enzyme Assays and Measurement of Kinetic Parameters in Formylation of tRNA. The assay for formylation used a two-step reaction. First, the substrate tRNA was quantitatively aminoacylated using [^{35}S]methionine and MetRS. For the aminoacylation step, the incubation mixture (10 μL) contained 20 mM imidazole hydrochloride (pH 7.5), 0.1 mM Na_2EDTA, 2 mM ATP, 150 mM NH_4Cl, 10 mM $MgCl_2$, 10 μg/mL BSA, 100 μM [^{35}S]methionine (specific activity = 8000−10 000 cpm/pmol), varying amounts of tRNA (0.3−18.0 μM), and excess of purified MetRS. The incubation was for 15 min at 37 °C. Following aminoacylation, 2 μL of N^{10}-formyltetrahydrofolate (final concentration = 0.3 mM) and 3 μL of appropriately diluted MTF (0.012−2.73 ng; depending on the specific activity of the MTF preparation and the substrate tRNA used in formylation assay) were added for determination of the initial rate of formylation. The reaction was allowed to proceed for 5 min at 37 °C and terminated by the addition of 15 μL of 0.36 M $CuSO_4$ in 1.1 M Tris-HCl (pH 7.3) and incubated further for 5 min. Acid-precipitable radioactivity was measured as described

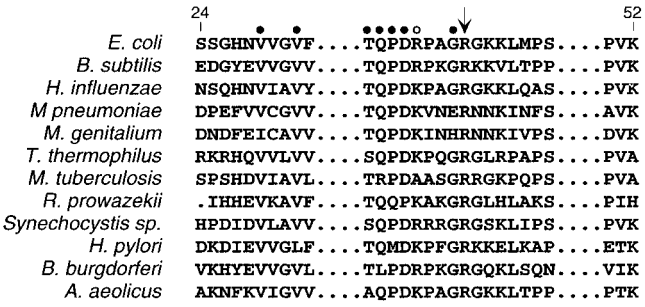

FIGURE 3: Alignment of MTF sequences from eubacteria highlighting the insertion of a 16 amino acid sequence. Closed circles indicate the amino acids that are conserved in 9 out of 12 sequences. The open circle indicates a basic amino acid conserved in 11 out of 12 sequences. The downward arrow indicates an invariant arginine residue. The data base accession numbers for the MTF sequences are P23882 (*E. coli*), P94463 (*B. subtilus*), P44787 (*H. influenzae*), P75235 (*M. pneumoniae*), P47605 (*M. genitalium*), P43523 (*T. thermophilus*), Z80108 (*M. tuberculosis*), P50932 (*R. prowazekii*), D64001 (*Synechocystis sp.*), P56461 (*H. pylori*), AE001119 (*B. burgdorferi*), and AE000774 (*A. aeolicus*).

before (*25*). Control experiments showed that the aminoacyl ester linkage in the aminoacyl-RNA was completely stable during the course of the formylation reaction. One unit of MTF activity is defined as the amount of enzyme required to formylate 1 pmol of methionyl-tRNA per minute at 37 °C. Specific activity of MTF is defined as units of enzyme per milligram of protein. Values of K_m and V_{max} were determined form Lineweaver−Burk double-reciprocal plots.

RESULTS

Previous work led to the identification of position 41 in MTF as the site of a suppressor mutation (*8*). The G41 to R and G41 to K mutations increased the rates of formylation of the U35A36/G72G73 mutant initiator tRNA by factors of 26−27-fold. These mutations are within the 16 amino acid sequence (residues 34−49 in *E. coli* MTF), which is present in all MTFs and which is highly conserved. Figure 3 shows the extent of sequence conservation around this region. Amino acids corresponding to positions 29, 32, 34, 35, 36, 37, and 41 in *E. coli* MTF are conserved in 9 out of 12 sequences (closed circles), position 38 is either an arginine or a lysine in 11 out of 12 sequences (open circle), and position 42 (↓) has an invariant arginine. The residues at these positions were targeted individually for mutagenesis, and the mutant enzymes were purified as 6 × His fusion proteins. The proteins thus obtained are essentially homogeneous (Figure 4). Control experiments showed that the wild-type *E. coli* MTF without the six histidine residues does not bind to the Talon-Sepharose column. Therefore, the mutant enzymes are free of any endogenous wild-type MTF.

Specific Activity of Mutant Proteins. As a quick screen for the effect of mutations on activities of the mutant MTFs, the specific activities of the mutant enzymes were measured under the conditions of both "high" substrate concentration (Met-tRNA$_2^{fMet}$ = 5 μM, ∼9 × K_m, ref *8*) and "low" substrate concentrations (Met-tRNA$_2^{fMet}$ = 0.5 μM, ∼1 × K_m). The results are presented in the form of a bar diagram in Figure 5, with the specific activity of the wild-type enzyme being set at 100%. Problems in the sensitivity of the assay compared to the background radioactivity did not allow the use of a lower concentration of the tRNA substrate.

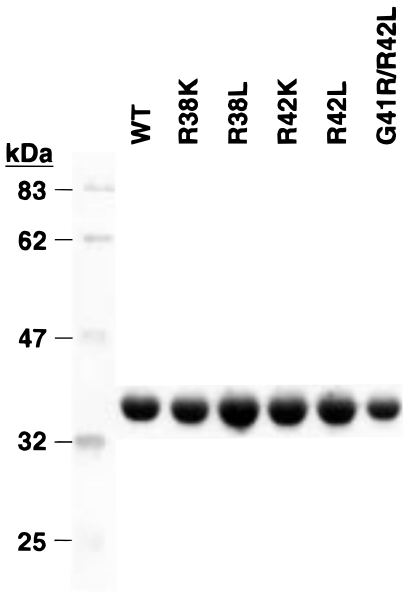

FIGURE 4: SDS/12% polyacrylamide gel electrophoresis of purified wild-type MTF and some of the mutant MTFs used in this study.

The mutation of amino acids 29, 32, 34, 35, 36, 37, and 50 to the amino acids as indicated had less than 2-fold effects on the specific activity of the enzyme at a tRNA concentration of 5 μM (Figure 5A). Even at lower substrate concentration the specific activity was not significantly affected by these mutations (Figure 5B). Thus, mutations at these positions have minimal effects on the activity of the enzyme. The effect of mutations of Gly41 to Arg and Lys has been described before. Mutations to Asp, Gln, and Leu also had minimal effects with, at most, a 3-fold drop in activity with the D41 enzyme. Further studies with these mutants are described below under a separate section. With residue 38, while mutation of Arg38 to Lys had essentially no effect, mutation to Leu lowered the specific activity by up to 12-fold. The most significant effects of mutation are seen in the case of the invariant Arg at position 42. At higher substrate concentration, mutation to Lys and Gln lowered the specific activity by about 5- and 10-fold respectively, whereas mutation to Leu had the most severe effect. The same trend is seen at the lower substrate concentrations for these mutations, with the activity of the L42 enzyme being barely detectable. The activity of the L42 enzyme was measurable only when the mutant enzyme used in assays was >300 times that of the wild-type enzyme (∼12 pM). These results suggest that amino acids at positions 38 and 42 are important for formylation. Consequently, the enzymes with mutations at these positions were selected for more detailed studies, including the determination of kinetic parameters in formylation of Met-tRNA$_2^{fMet}$.

Steady-State Kinetic Parameters in Formylation of Wild-Type Initiator tRNA. The kinetic parameters in formylation of the wild-type tRNA by the wild-type and mutant enzymes are compared in Table 1. The results are as follows:

1. Residue 38. The R38 to K mutation had a small effect on K_m (2.2-fold increase) and virtually no effect on k_{cat}, leading to a decrease in k_{cat}/K_m of less than 2-fold. In contrast, the R38 to L mutation resulted in a decrease in k_{cat}/K_m of approximately 28-fold due to a 6-fold increase in K_m and about a 5-fold decrease in k_{cat}. These results suggest that a positively charged amino acid, R or K, at position 38

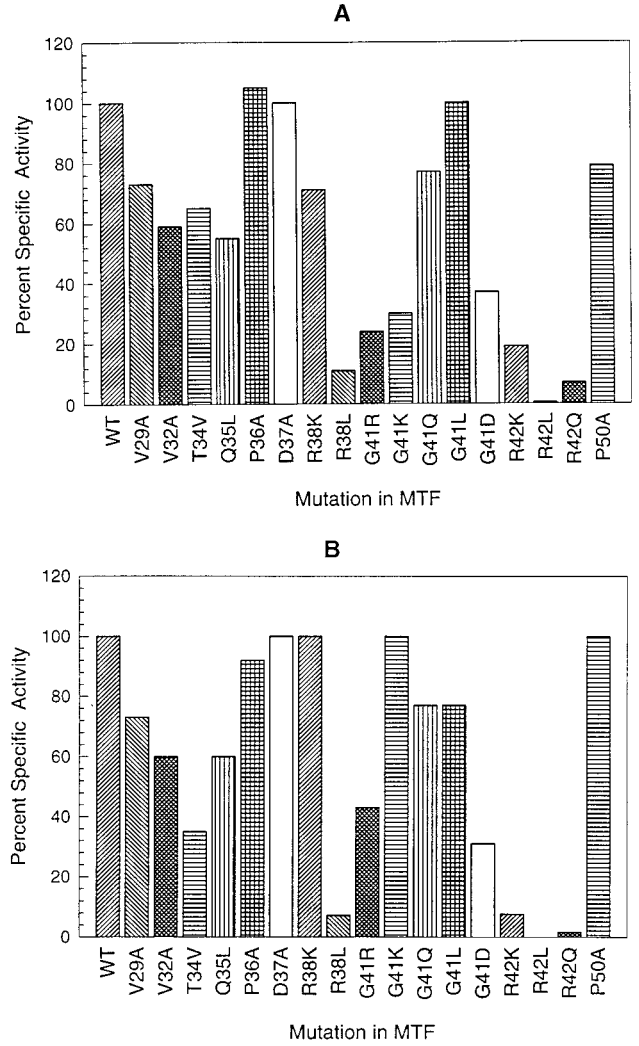

FIGURE 5: Specific activities of mutant MTFs measured at (A) high concentration ($10 \times K_m$) and (B) low concentration ($1 \times K_m$) of the initiator tRNA substrate presented in the form of a bar diagram with the specific activity of the wild-type enzyme fixed at 100%. The specific activities correspond to the initial rates observed per unit amount of the enzyme.

contributes to enzyme activity. This is consistent with the presence of either of these amino acids at this position in most of the known MTF sequences, with the exception of the enzyme from *M. tuberculosis* (Figure 3).

2. Residue 42. R42 to L mutation resulted in an ~1367-fold decrease in k_{cat}/K_m due to significant effects on both K_m (~17-fold increase) and k_{cat} (~79-fold decrease). R42 to K mutation resulted in an ~23-fold decrease in k_{cat}/K_m, the effect being almost entirely due to an increase (~16-fold) in the K_m of the enzyme for the tRNA, whereas the R42 to Q mutation resulted in an approximately 226-fold decrease in k_{cat}/K_m. The K_m and k_{cat} values for these mutant enzymes revealed clear patterns in that (a) all the mutant enzymes K42, Q42, and L42 showed a 16−17-fold increase in K_m and (b) k_{cat} was dependent on the nature of the side chain of the substituted amino acid. The k_{cat} values ranged in the order WT ~ K42 > Q42 > L42. These results suggest an important role for R42 both in the steps of tRNA substrate binding and in catalysis.

3. Residues 41 and 42. As shown above, mutation of R42 to L has a very strong negative effect on the activity of MTF whereas mutation of the neighboring G41 to R has only a small effect (*8*). Therefore, we have studied the properties of the G41R/R42L double mutant to ask whether Arg at position 41 can compensate for the strong negative effect of mutation of the Arg42 to Leu. The results (Table 1) show that the G41R/R42L double mutant is as poor as the R42L single mutant.

Table 1: Kinetic Parameters in Formylation of Wild Type Initiator tRNA Using Wild-Type and Mutant MTFs[a]

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	$(k_{cat}/K_m)_{wt\ MTF}/(k_{cat}/K_m)_{mutant\ MTF}$
wt	0.54 ± 0.11	39.12 ± 9.06	72.44	−
R38K	1.21 ± 0.50	46.31 ± 5.59	38.27	1.9
R38L	3.24 ± 1.00	8.26 ± 1.97	2.55	28.4
R42K	8.66 ± 1.40	27.66 ± 10.71	3.19	22.7
R42Q	9.60 ± 0.30	3.10 ± 0.11	0.32	226.3
R42L	9.00 ± 2.02	0.48 ± 0.11	0.053	1366.8
G41R R42L	8.34 ± 0.12	0.448 ± 0.03	0.0526	1377.2

[a] Kinetic parameters listed are the average of four to six separate measurements. tRNA concentrations used varied from 0.2 to 2.0 μM for wild-type MTF and the mutant at position 38, and from 0.5 to 6.0 μM for the MTF mutant at position 42 and for the G41R/R42L double mutant. The enzyme concentrations varied from 0.025 to 5.68 nM depending on the mutant.

Steady-State Kinetic Parameters in Formylation of Mutant Initiator tRNAs Using Wild-Type MTF and Mutant MTFs at Position 42. The determinants for formylation in the initiator tRNA are clustered mostly in the acceptor stem (Figure 1). A critical determinant is the C1 × A72 mismatch at the end of the acceptor stem. Mutation of A72 to G72, which generates a C1:G72 base pair, and mutations of the neighboring G2:C71 and C3:G70 base pairs to U2:A71 and U3:A70, respectively, each have strong negative effects on formylation (*10*−*12*). To assess whether Arg42 is interacting with any of the determinants for formylation, we studied the effect of R42 to K and R42 to L mutations on the kinetic parameters in formylation of these mutant tRNAs and compared them to those in formylation of the wild-type initiator tRNA by these enzymes. Specifically, we were interested in seeing whether the detrimental effect of the R42 to K and R42 to L mutations in formylation of the wild-type initiator tRNA would persist in formylation of these mutant tRNAs. The results are shown in Table 2.

G72 Mutant tRNA. This tRNA is a poor substrate for the wild-type MTF and has an ~280-fold decrease in k_{cat}/K_m. Compared to the wild-type enzyme, the k_{cat}/K_m with this tRNA is down by a factor of 34-fold for the R42K mutant and 508-fold for the R42L mutant (Table 2, last column). These values are comparable to the corresponding decreases in k_{cat}/K_m for these enzymes with the wild-type initiator tRNA as substrate. Thus, the R42 to K and R42 to L mutations still have as detrimental an effect on the kinetic parameters in formylation of the G72 mutant initiator tRNA as formylation of the wild-type initiator tRNA.

U2:A71 Mutant tRNA. This tRNA is a poor substrate for wild-type MTF and has an ~42-fold decrease in k_{cat}/K_m. With the R42K mutant, the K_m for the tRNA is similar to that of wild-type MTF whereas the k_{cat} is lower by a factor of 4. With the R42L mutant, the K_m is slightly higher (~2.5-fold), and k_{cat} is substantially lower (~55-fold). However, compared to the wild-type MTF, the relative values of k_{cat}/K_m are down only by factors of 6- and 141-fold for the R42K

Table 2: Kinetic Parameters in Formylation of the G72, U2:A71, and U3:A70 Mutant Initiator tRNAs Using Wild-Type and R42K and R42L Mutant MTFs[a]

tRNA	enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	$(k_{cat}/K_m)_{wt\ MTF}/(k_{cat}/K_m)_{mutant\ MTF}$
wt	wt	0.54 ± 0.11	39.12 ± 9.06	72.44	–
	R42K	8.66 ± 1.40	27.66 ± 10.71	3.19	22.7
	R42L	9.00 ± 2.02	0.48 ± 0.11	0.053	1366.8
G72	wt	12.29 ± 1.64	3.19 ± 0.53	0.259	–
	R42K	54.74 ± 10.58	0.42 ± 0.13	0.0077	33.6
	R42L	129.3 ± 11.68	0.066 ± 0.021	0.00051	507.8
U2:A71	wt	5.97 ± 1.82	10.34 ± 3.67	1.73	–
	R42K	8.87 ± 2.31	2.58 ± 1.12	0.29	6.0
	R42L	15.10 ± 4.89	0.186 ± 0.09	0.0123	140.6
U3:A70	wt	12.50 ± 4.40	8.31 ± 3.90	0.66	–
	R42K	13.65 ± 2.46	22.27 ± 5.64	1.63	0.4
	R42L	14.04 ± 2.34	0.66 ± 0.13	0.047	14.0

[a] Kinetic parameters listed are the average of four or more experiments. The tRNA and MTF concentrations used were as follows. For the G72 tRNA, the tRNA concentrations varied from 1.2 to 12 μM and MTF, from 1.25 to 569 nM depending on the mutant; for the U2:A71 tRNA, the tRNA concentrations varied from 0.6 to 6 μM and MTF, from 1.24 to 22.75 nM; and for the U3:A70 tRNA, the tRNA concentrations varied from 0.9 to 9 μM and MTF, from 0.125 to 4.5 nM.

Table 3: Ratio of Specificity Constants in Formylation of the Wild-Type and Mutant Initiator tRNAs by the Wild-Type and Mutant MTFs[a]

	$(k_{cat}/K_m)_{wt\ tRNA}/(k_{cat}/K_m)_{mutant\ tRNA}$		
enzyme	G72 tRNA	U2:A71 tRNA	U3:A70 tRNA
wt	280	42	109
	−(3.47)	−(2.30)	−(2.89)
R42K	414	11	1.95
	−(3.71)	−(1.48)	−(0.41)
R42L	104	4.3	1.12
	−(2.86)	−(0.9)	−(0.07)

[a] Numbers in parentheses are the differences in the free energy of transition state formation ($\Delta\Delta G^{\circ\ddagger}$) based on $\Delta\Delta G^{\circ\ddagger} = -RT\ 1n\ [(k_{cat}/K_m)_{wt\ tRNA}/(k_{cat}/K_m)_{mutant\ tRNA}]$.

Table 4: Kinetic Parameters in Formylation of Wild-Type Initiator tRNA Using Wild-Type MTF and Mutants at Position 41 of MTF[a]

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	$(k_{cat}/K_m)_{wt\ MTF}/(k_{cat}/K_m)_{mutant\ MTF}$
wt	0.54 ± 0.11	39.12 ± 9.06	72.44	–
G41R	0.66 ± 0.16	11.40 ± 0.16	17.27	4.2
G41K	1.02 ± 0.03	14.60 ± 0.06	14.31	5.1
G41L	$1.62 \pm .0.03$	31.5 ± 2.01	19.44	3.7
G41D	3.00 ± 0.30	33.0 ± 0.50	11.00	6.6

[a] Kinetic parameters listed are the average of four or more experiments. The tRNA concentrations used varied from 0.3 to 3 μM, and the enzyme concentration, depending on the mutant, was 0.025−0.1 nM.

and R42L mutants, respectively. These results suggest that the effects on K_m and k_{cat} seen for the R42K and R42L mutants using the wild-type initiator tRNA as substrate (Table 2, last column) are less severe with the U2:A71 mutant tRNA.

U3:A70 Mutant tRNA. This tRNA is a poor substrate for wild-type MTF and has an ~109-fold decrease in k_{cat}/K_m. Interestingly, with both the R42K and R42L mutant enzymes, there is no further appreciable increase in K_m, and the k_{cat} for the R42K mutant is, in fact, slightly higher (~2.7-fold) than that for the wild-type MTF. Even for the R42L mutant, relative to wild-type MTF, the k_{cat}/K_m is down only by a factor of about 14 compared to 1367 for the wild type initiator tRNA (Table 2, last column). Thus, with the U3:A70 mutant tRNA as substrate, there are only small effects of the R42 to K and R42 to L mutations compared to the large effects seen with the wild-type initiator tRNA as substrate.

Table 3 presents some of the above data in a format which allows a direct comparison of the effect of R42 to K and R42 to L mutations in MTF on the ratio of specificity constants (k_{cat}/K_m) for the wild-type and mutant initiator tRNAs. Also shown are the free energy differences ($\Delta\Delta G^{\circ\ddagger}$) (*26*−*28*) corresponding to the ratios of the k_{cat}/K_m [$\Delta\Delta G^{\circ\ddagger} = -RT1n\ [(k_{cat}/K_m)_{wt\ tRNA}/(k_{cat}/K_m)_{mutant\ tRNA})]$. With wild-type MTF, the G72 mutation results in an ~3.5 kcal/mol difference in the free energy of transition state formation between the wild-type and the G72 mutant initiator tRNA, and this difference mostly persists with the R42K and R42L mutant MTFs. In contrast, the 2.9 kcal/mol difference in the free energy of transition state formation between the wild-type and the U3:A70 mutant initiator tRNA is down to 0.41 kcal/mol with the R42K mutant and to 0.07 kcal/mol with the R42L mutant. Thus, with the U3:A70 mutant initiator tRNA as substrate, the R42K and R42L mutants are not much worse than the wild-type enzyme. These results suggest that R42 of MTF interacts functionally with the C3:G70 base pair in the wild-type initiator tRNA. The results with the U2:A71 mutant initiator tRNA suggest that R42 also makes some functional contact with the G2:C71 base pair.

Effect of Mutations at Position 41 on Formylation of Wild-Type and the G72 Mutant Initiator tRNA. Gly41 is present in 10 out of 12 MTFs of known sequence. Previous analysis of the R41 and K41 mutants showed that the k_{cat}/K_m values of these enzymes are 4−5-fold lower than that of the wild-type enzyme with the wild-type initiator tRNA as substrate. This conserved Gly has been further mutated to Asp and Leu, and the effects of these mutations on the kinetic parameters in formylation of the wild-type initiator tRNA have been studied. The results (Table 4) show only small effects on the K_m and k_{cat}, resulting in, at most, a 6.6-fold decrease in k_{cat}/K_m for the Asp41 enzyme. Thus, although Gly41 might be required for optimal enzyme activity, several other amino acids can be tolerated at this position.

We showed previously that the presence of a positive charge at position 41 in the G41R and G41K enzymes resulted in a 26−27-fold increase in the rates of formylation

Table 5: Kinetic Parameters in Formylation of the G72 Mutant of Initiator tRNA Using Wild-Type MTF and Mutants at Position 41 of MTF[a]

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	$(k_{cat}/K_m)_{wt\ MTF}/(k_{cat}/K_m)_{mutant\ MTF}$
wt	12.29 ± 1.64	3.19 ± 0.53	0.26	–
G41R	1.08 ± 0.22	2.30 ± 0.38	2.13	0.12
G41K	1.43 ± 0.35	2.02 ± 0.78	1.41	0.18
G41L	$7.41 \pm .1.11$	0.17 ± 0.06	0.023	11.4

[a] Kinetic parameters listed are the average of four or more experiments. The tRNA concentrations varied from 0.5 to 5 μM, and the enzyme concentration, depending on the mutant, was 1.25−6.6 nM.

of the U35A36/G72G73 mutant initiator tRNA (*8*). This tRNA is aminoacylated with glutamine because of the U35A36 anticodon sequence mutation (*22*, *29*). Control experiments using the U35A36 mutant initiator tRNA as substrate showed that the G41R and G41K enzymes compensated specifically for the strong negative effect of the G72G73 mutations in the acceptor stem of the tRNA (*8*). In the current work, we have asked whether the G41R and G41K enzymes will also compensate for the strong negative effect of the G72 mutation alone (*10*−*12*). In contrast to the U35A36/G72G73 mutant initiator tRNA, the G72 mutant tRNA is aminoacylated with methionine. The results are shown in Table 5. The G41R and G41K enzymes show an increase in k_{cat}/K_m in formylation of the G72 mutant tRNA by factors of about 8- and 5-fold, respectively, relative to that of the wild-type enzyme. This is in contrast to formylation of the wild-type initiator tRNA, where these same enzymes show a 4−5-fold decrease in k_{cat}/K_m. The effects are almost entirely due to an increase in affinity of these enzymes (decrease in K_m) for the G72 mutant initiator tRNA. The G41L mutant, which does not have a positive charge at position 41, does not show a similar increase in k_{cat}/K_m. These results show that the negative effect of the G72 mutation in the initiator tRNA in formylation can be partially compensated for by the presence of a positive charge at position 41 in the enzyme.

DISCUSSION

Analysis of the site-specific mutants of MTF described here provides strong support to previous suggestions that the 16 amino acid insertion sequence in the loop region between the second β-strand and the second α-helix of MTF plays an important role in the specific recognition of the determinants for formylation in the tRNA. Specifically, we have shown that amino acids 38 and 42 within the insertion sequence are important for function of *E. coli* MTF. At position 38, mutation of Arg to Lys had essentially no effect whereas mutation to Leu lowered the k_{cat}/K_m by about 30-fold, suggesting that a positively charged amino acid is important. At position 42, mutation of the invariant Arg to Lys results in an increase in the K_m of the tRNA by about 16-fold with little effect on k_{cat}, whereas mutation to Leu resulted in a similar increase in K_m and also a lowering of k_{cat} by about 80-fold (Table 1). Assuming that there are no major effects of these mutations on the overall structure of the enzyme, these results suggest that Arg42 is very important for the function of the enzyme. Part of the side chain of Arg42 contributes to the formation of the Michaelis complex whereas the positive charge of arginine contributes toward stabilization of the transition state. The 1367-fold difference in k_{cat}/K_m between the Arg42 and Leu42 enzymes corresponds to a difference in the free energy of stabilization of the transition state of about 4.4 kcal/mol (*26*). Independently, Blanquet and co-workers have also noted the importance of the amino acids at positions 38 and 42 of MTF based on mutations of these amino acids to alanine residues (E. Schmitt, M. Panvert, S. Blanquet, and Y. Mechulan, personal communication).

The determinants for formylation in the initiator tRNA are clustered mostly at one end of the acceptor stem. The key requirements are a weak base pair or no base pair between nucleotides 1 and 72, a G2:C71 base pair, and a C3:G70 base pair. To assess whether Arg42 is interacting with these determinants in the tRNA, we investigated whether the detrimental effect of R42 to K or R42 to L mutations in formylation of the wild-type initiator tRNA would also persist in formylation of the mutant initiator tRNAs. We found that the effect of R42 to K or to L mutations on the kinetic parameters in formylation is essentially the same with the G72 mutant as with the wild-type initiator tRNA. In contrast, the effect of the same mutations in MTF is less severe with the U2:A71 mutant tRNA and much less severe with the U3:A70 mutant tRNA as substrate (Tables 2 and 3). In fact, with the U3:A70 mutant tRNA as substrate, the R42K enzyme is better than the wild-type enzyme. The most straightforward interpretation of these results is that there is functional interaction between Arg42 of MTF and the C3:G70 and to some extent the G2:C71 base pairs (*30*, *31*) in the tRNA. It is also possible that Arg42 interacts with the ribose phosphate backbone in this region of the tRNA and that the backbone conformation is dependent upon the nature of the base pairs.

A similar study of MTF mutants at position 41 shows that the G72 mutant tRNA, which is as poor a substrate for the R42L enzyme as the wild-type initiator tRNA, is a better substrate for the G41K and G41R mutant enzymes. Compared to the wild-type initiator tRNA, the k_{cat}/K_m in formylation of the G72 mutant tRNA is up by factors of 27 and 35, respectively, for the G41K and G41R mutants (Tables 4 and 5). As pointed out previously (*8*), these results suggest that amino acid 41 comes close to base pair 1:72 and somehow facilitates melting of this base pair (*32*). Thus, the combined results of the kinetic parameters in formylation of the wild-type and mutant initiator tRNA by the wild-type and mutant MTFs suggest that amino acid 41 in the insertion loop comes close to base pair 1:72 and amino acid 42 comes close to base pairs 2:71 and 3:70. These finding provide strong support to previous suggestions (*8*, *15*) that amino acids in the insertion loop play an important role in recognition of the determinants for formylation in the acceptor stem of the initiator tRNA. It is possible that in addition to these amino acid side chains, the peptide backbone of the insertion loop also interacts with the tRNA.

The crystal structure of MTF and proteolytic susceptibility of the insertion loop indicated that amino acids 40−45 in the loop were unstructured and flexible (*8*, *15*). We have, therefore, examined whether the very poor activity of the R42L mutant could be rescued by mutation of the preceding Gly41 to an Arg. We find that the G41R/R42L double mutant is as inactive as the R42L single mutant (Table 1). Thus, although the crystal structure and the protease sensitiv-

ity of the insertion loop indicate that this loop has a flexible structure, the requirement for Arg at position 42 cannot be fulfilled by an Arg at position 41. This suggests that the insertion loop adopts a defined conformation upon binding to the tRNA (*8*) and supports the idea of the side chain of Arg42 making specific contacts with some of the key determinants for formylation in the initiator tRNA in an induced fit mechanism (*33*).

Arg is one of the most versatile of the amino acids in terms of its potential for formation of hydrogen bonds and electrostatic bonds. It has a unique arrangement of a positively charged side chain and an array of five potential hydrogen bond donors (*34*). Consequently, Arg often plays an important role in the specific recognition of many RNAs and DNAs by proteins (*35*−*37*). For example, in the HIV-1 Tat protein−TAR RNA interactions, a single Arg donates a pair of hydrogen bonds to N^7 and O^6 of guanine in the major groove and one each to two nearby phosphates (*35*). Similarly, in the U1A protein−U1RNA complex, Arg52 plays a critical role by forming hydrogen bonds with N^7 and O^6 of G16 and N^1 of A6 (*38*). In the structure of the *E. coli* glutaminyl-tRNA synthetase−$tRNA^{Gln}$ complex, Arg133 forms hydrogen bonds with two phosphate oxygens of G73 and C74 and with the 2′-OH of ribose (*39*). A combination of interactions along any of these lines is possible between Arg42 of MTF and the G2:C71 and C3:G70 base pairs and/or the neighboring phosphates and riboses. It should be noted that while the major grooves of RNA helices are usually deep and narrow and not readily accessible (*40*), the major grooves of base pairs at the ends of RNA helices or adjacent to looped regions are accessible (*41*, *42*). Furthermore, eubacterial initiator tRNAs do not have a Watson−Crick base pair between nucleotides 1 and 72 at the end of the acceptor stem. Consequently, the major grooves of base pairs 2:71 and 3:70 are likely to be accessible for hydrogen bond formation. In addition to Arg42, the 16 amino acid insertion sequence contains on an average 3 other basic amino acids in MTF from different sources. The overall positive charge density in this region of MTF could provide additional electrostatic binding energy, which helps orient the Arg42 for its specific interactions (*34*). Alternatively, these positively charged residues, in particular the Arg38, which we have shown to be important for MTF function, could facilitate the formylation reaction by stabilizing a form of tRNA acceptor stem structure, in which the 3′-nucleotide is folded back toward the tRNA 5′-end (*43*, *44*).

Besides eubacterial MTFs, the sequences of two mitochondrial MTFs are known. The yeast mitochondrial MTF has 32 amino acids in the insertion loop compared to 16 in eubacterial MTFs and contains an Arg at the position corresponding to Arg42 in eubacterial MTFs. In contrast, the recently sequenced bovine mitochondrial MTF does not have an Arg at the corresponding position (*45*). However, the bovine mitochondrial MTF is different from *E. coli* MTF in that it shows much less discrimination (9-fold) between the initiator and elongator species of *E. coli* methionine tRNAs. It is not known whether there is a correlation between the absence of Arg42 in bovine mitochondrial MTF and its reduced ability to discriminate between initiator and elongator species of methionine tRNAs. It is worth noting that unlike other protein synthesis systems including fungal and plant mitochondria (*13*, *46*), mammalian mitochondria contain a single species of methionine tRNA (*47*), which is thought to be used both for initiation as formylmethionyl-tRNA and for elongation as methionyl-tRNA. Consequently, only a fraction of the mammalian mitochondrial Met-tRNA is formylated and used for initiation. The inability to discriminate between the initiator and elongator species of methionine tRNA is, therefore, an important property of bovine mitochondrial MTF.

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