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Striking effects of coupling mutations in the acceptor stem on recognition of tRNAs by *Escherichia coli* Met-tRNA synthetase and Met-tRNA transformylase

(initiator tRNA/tRNA–protein interactions/peptidyl-tRNA hydrolase)

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ABSTRACT **We measured kinetic parameters *in vitro* and directly analyzed aminoacylation and formylation levels *in vivo* to study recognition of *Escherichia coli* initiator tRNA mutants by *E. coli* Met-tRNA synthetase and Met-tRNA transformylase. We show that, in addition to the anticodon sequence, mutations in the "discriminator" base A73 also affect aminoacylation. An A73 → U change has a small effect, but a change to G73 or C73 significantly lowers V_{max}/K_m^{app} for *in vitro* aminoacylation and leads to appreciable accumulation of uncharged tRNA *in vivo*. Significantly, coupling of the G73 mutation with G72, a neighboring-base mutation, results in a tRNA essentially uncharged *in vivo*. Coupling of C73 and U73 mutations with G72 does not have such an effect. Elements crucial for Met-tRNA transformylase recognition of tRNAs are located at the end of the acceptor stem. These elements include a weak base pair or a mismatch between nucleotides (nt) 1 and 72 and base pairs 2·71 and 3·70. The natures of nt 1 and 72 are less important than the fact that they do not form a strong Watson–Crick base pair. Interestingly, the negative effect of a C·G base pair between nt 1 and 72 is suppressed by mutation of the neighboring nucleotide A73 to either C73 or U73. The presence of C73 or U73 could destabilize the C1·G72 base pair at the end of an RNA helix. Thus, in some tRNAs, the discriminator base could affect stability of the base pair between nt 1 and 72 and thereby the structure of tRNA at the end of the acceptor stem.**

As part of structure–function relationship studies of *Escherichia coli* initiator methionine tRNA (Fig. 1), we previously described mutagenesis of the $tRNA^{fMet}$ gene and presented functional studies of the mutant tRNAs *in vitro* and *in vivo*. These studies showed that (*i*) the (GGG)·(CCC) sequence conserved in the anticodon stem of initiator tRNAs was important in targeting the tRNA to the ribosomal P site (1), (*ii*) the C1×A72 mismatch at the end of the acceptor stem was important in preventing the tRNA from binding to the elongation factor EF-Tu and, thereby, from functioning in the elongation step of protein synthesis (2, 3), and (*iii*) the sequence and/or structural features important for recognition of tRNA by Met-tRNA transformylase were mostly clustered at the end of the acceptor stem (4).

We are also studying recognition of the initiator tRNA by *E. coli* Met-tRNA synthetase (MetRS). Schulman and coworkers (5) have concluded that the anticodon sequence of methionine tRNA contains the major determinants for recognition by *E. coli* MetRS. This conclusion was based on the findings that (*i*) mutations in the anticodon nucleotides greatly affected the kinetic parameters in aminoacylation (6, 7) and (*ii*) transfer of methionine anticodon CAU to *E. coli*

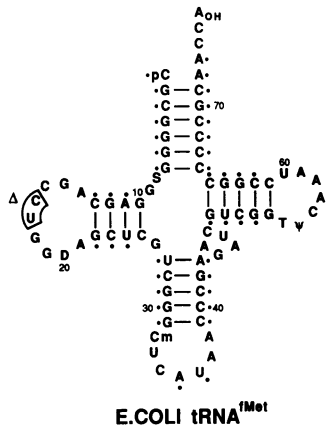

FIG. 1. Sequence of *E. coli* $tRNA_2^{fMet}$ in cloverleaf form; sites of mutation are indicated by dots (substitution) or a triangle (deletion).

$tRNA^{Val}$ (8), $tRNA_1^{Ile}$ (9), and $tRNA_2^{Ile}$ (10) conferred methionine identity on them *in vitro*. The mutant initiator tRNAs with changes throughout the molecule that we have generated (Fig. 1) provide a complementary approach (5, 11, 12) for studying recognition of tRNAs *in vitro* by *E. coli* MetRS. In addition, our recently developed method to separate uncharged, aminoacylated, and formylaminoacylated forms of tRNA from each other allows direct analysis of the effect of each mutation on aminoacylation and formylation *in vivo* (13). We report the results of such studies.

MATERIALS AND METHODS

tRNA Mutants. These are named according to the base changes. Some of the initiator tRNA mutants have been described (1, 2, 4). Additional mutants C72, G1·C72, A1, T72, A1·T72, C73, T73, G73, G72C73, G72T73, and ΔC17T17.1 were generated as before. An *E. coli* $tRNA^{Phe}$ mutant, C32C34T36, was assembled by ligation of four synthetic oligonucleotides.

***E. coli* Strains.** *E. coli* CA274 (*lacZam trpEam*) and AA7852 (*pth*^{ts}) carrying a temperature-sensitive mutation in peptidyl-tRNA hydrolase (14) have been described (15). *E. coli* B 105 was obtained from G. W. Walker, Massachusetts Institute of Technology.

Mutant $tRNA_2^{fMet}$ genes were cloned into either pBR322 or pTZ19R (pUC derivative) and used to transform *E. coli* CA274, B 105, or AA7852. In some cases, transformants also contained another plasmid (pACYC based; ref. 16), which did (+) or did not (−) contain the gene for *E. coli* MetRS (Figs. 2–5).

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Abbreviations: nt, nucleotide(s); MetRS, Met-tRNA synthetase; GlnRS, Gln-tRNA synthetase.

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The mutant $tRNA^{Phe}$ gene was put under control of a T7 promoter in a pUC119-based vector obtained from L. H. Schulman, Albert Einstein College of Medicine (8). tRNA was made by *in vitro* transcription with T7 RNA polymerase (17).

Isolation of Mutant tRNAs. These isolations were as described (1, 2). The mutants analyzed in Tables 2 and 4 were expressed in *E. coli* B 105, which lacks the $tRNA_2^{fMet}$ species (N.M. and U.L.R., unpublished work).

Enzymes. A purified preparation of the truncated form of *E. coli* MetRS from P. Rosevear (University of Texas) was used. An *E. coli* S100 preparation freed of nucleic acids was the source for Met-tRNA transformylase. T7 RNA polymerase was from United States Biochemical.

Detection of Mutant tRNAs by RNA-Blot Hybridizations. This procedure was done as described (13). The deoxyribooligonucleotide probes were labeled at the 5′ end with ^{32}P. Probes complementary to wild-type tRNA were used to detect mutant tRNAs with changes in the acceptor stem, TψC stem, or deletion in the D loop. Because the mutant tRNAs are overproduced significantly, background signal from hybridization of these probes to wild-type endogenous tRNA is negligible (see Fig. 2*A*, lane 10). Probes used to detect other mutant tRNAs are complementary to the specific mutants. A probe complementary to nucleotides (nt) 2–36 of *E. coli* $tRNA^{Tyr}$ has been described (13).

Uncharged tRNA, Met-tRNA, and fMet-tRNA used as markers in Figs. 2–5 were generated as before, except that MetRS was used instead of Gln-tRNA synthetase (GlnRS) (13). A, B, and C indicate, respectively, the approximate location of uncharged tRNA, fMet-tRNA, and Met-tRNA.

RESULTS

Kinetics of Aminoacylation of Mutant tRNAs *in Vitro*. Mutations in the D stem, anticodon stem, TψC stem, and in the acceptor stem have small or no effect on the relative V_{max}/K_m^{app} in the aminoacylation reaction (Table 1). Mutations at nt 1 and 72 also have virtually no effect (<2-fold). Mutations in the second and third base pairs have small effects, depending on the nature of the base-pair change. These mutations also seem to affect aminoacylation only slightly *in vivo* (see below).

Table 1. Relative specificity constants of aminoacylation of mutant initiator tRNAs by *E. coli* MetRS

	Location on tRNA	Relative V_{max}/K_m^{app}
WT $tRNA_2^{fMet}$		1.0
Mutant		
T1	Acceptor stem	1.6*
G72	Acceptor stem	1.9*
T1G72	Acceptor stem	1.0*
T1/T2·A71	Acceptor stem	3.8*
T2·A71/G72	Acceptor stem	4.5*
T1/T3·A70	Acceptor stem	1.2*
T1/A3·T70	Acceptor stem	1.2
G3·C70	Acceptor stem	5.8
C4·G69	Acceptor stem	1.9
C5·G68	Acceptor stem	2.3
C6·G67	Acceptor stem	1.0
C7·G66	Acceptor stem	0.6
C11·G24	Dihydrouridine stem	1.0
(C11C12A13) ·(A22G23G24)	Dihydrouridine stem	0.7
C52·G62	TψC stem	3.2
(A49G50A51C52) ·(G62T63C64T65)	TψC stem	3.5
(C27A28T29C30A31) ·(T39G40A41T42G43)	Anticodon stem	3.2
T35A36	Anticodon loop	>2000

WT, wild type.
*Results are described in ref. 18. Relative V_{max}/K_m^{app} is the ratio of V_{max}/K_m^{app} of $tRNA_{f2}^{Met}$ to the V_{max}/K_m^{app} of each mutant tRNA.

Table 2. Kinetic parameters in aminoacylation of tRNAs with mutations at nt 72 and 73 by *E. coli* MetRS

tRNA	V_{max}, arbitrary units	K_m^{app}, μM	Relative V_{max}/K_m^{app}
$tRNA_2^{fMet}$	298.5	13.4	1.0
T73	293.3	32.1	2.4
C73	42.1	63.3	33.5
G73	49.9	65.1	29.1
G72	398.8	41.0	2.3
G72T73	232.6	30.8	2.9
G72C73	6.7	11.8	39.2
G72G73	1.5	31.3	475

Mutations that strongly affect aminoacylation are located in the anticodon (refs. 5–8, Table 1) and in the discriminator base (Table 2). Mutations in the anticodon sequence make the tRNA an extremely poor substrate for MetRS. In addition, transfer of the methionine tRNA anticodon CAU to *E. coli* $tRNA^{Phe}$ results in a tRNA that is now an excellent substrate for *E. coli* MetRS (Table 3). These results agree with the conclusions of Schulman (5) that the anticodon sequence contains the major determinants for recognition of tRNAs by *E. coli* MetRS.

The effect of mutations in the discriminator base 73 (19) depends on the nature of the mutation: A73 → U has only a small effect, whereas change to C73 or G73 lowers V_{max} by a factor of ≈six to seven and increases K_m by a factor of ≈five, decreasing V_{max}/K_m overall by a factor of ≈30 compared with wild-type tRNA. The order of activity of the mutants resembles that described (20) for the same changes introduced into tRNA with *in vitro* replacement techniques (21), although the effects here are more appreciable. More importantly, the G73 and C73 mutations also affect aminoacylation of the mutant tRNAs *in vivo* (see below).

Coupling of the G73 and G72 mutations has an even more striking effect on aminoacylation. The V_{max}/K_m^{app} for the G72G73 mutant is down by a factor of ≈475 compared with ≈29 for the G73 mutant alone (Table 2). Coupling of T73 or C73 mutation with G72 does not have such an effect.

Aminoacylation Levels of Mutant tRNAs *in Vivo*. Analysis of aminoacylation and formylation levels of tRNAs *in vivo* (13) essentially agrees with the kinetic parameters obtained *in vitro*. Changing 3 of the 4 base pairs (bp) in the D stem, deleting nt C17 and U17.1 in the D loop (22), and changing all 5 bp in the anticodon stem and 4 of the 5 bp in the TψC stem have no effect on aminoacylation or formylation of the mutant tRNAs *in vivo* (data not shown). These nucleotides are probably not important recognition elements for MetRS or Met-tRNA transformylase. Also, even though these mutant tRNAs are overproduced, they are all essentially quantitatively aminoacylated and formylated *in vivo*. Thus, neither MetRS nor Met-tRNA transformylase is ordinarily present in limiting amounts in *E. coli*.

With the acceptor stem mutants also, the tRNAs are found mostly as Met-tRNA or fMet-tRNA *in vivo* (Fig. 2*A*). Thus,

Table 3. Kinetic parameters in aminoacylation of *E. coli* $tRNA^{Met}$ (elongator species) and T7 RNA polymerase transcript of an *E. coli* $tRNA^{Phe}$ mutant carrying C32C34T36 mutations in the anticodon

tRNA	V_{max}, arbitrary units	K_m, μM	V_{max}/K_m^{app}
$tRNA^{Met}$	20	5	4
$tRNA^{Phe}$ (mutant) with CAU anticodon	10	5	2

the aminoacylation step is not affected in these mutants. An exception is the T1 mutant, which contains a significant fraction of the tRNA in the uncharged form (faster band in lane 3 of Fig. 2*A*). As discussed (13), the most likely reason for this result is that the T1 mutant tRNA is not only a good substrate for Met-tRNA transformylase, which converts it to fMet-tRNA, but is now also a substrate for peptidyl-tRNA hydrolase (24), which hydrolyzes the fMet-tRNA to formylmethionine and tRNA (25). The accumulation of uncharged T1 mutant tRNA suggests that peptidyl-tRNA hydrolase hydrolyzes fMet-tRNA almost as fast as MetRS aminoacylates the released tRNA. This conclusion is supported by the fact that all of the T1 mutant is present as fMet-tRNA in cells that are overproducing MetRS (Fig. 2*B*, lane 3) or which have a temperature-sensitive mutation (14) in peptidyl-tRNA hydrolase (Fig. 2*B*, lanes 4 and 5).

Small amounts of uncharged tRNA are found *in vivo* for several other acceptor stem mutants. As for the T1 mutant, this result could be because some of these mutants are partly formylated *in vivo* and are substrates for peptidyl-tRNA hydrolase. Alternatively, even for tRNAs containing the CAU anticodon, aminoacylation *in vivo* may be somewhat sensitive to changes in the acceptor stem. Martinis and Schimmel (26) have shown recently that minihelix and microhelix variants of acceptor stem of $tRNA^{Met}$ can be aminoacylated *in vitro* with methionine, although at an extremely slow rate, and that mutations in the acceptor stem sequence abolish this aminoacylation.

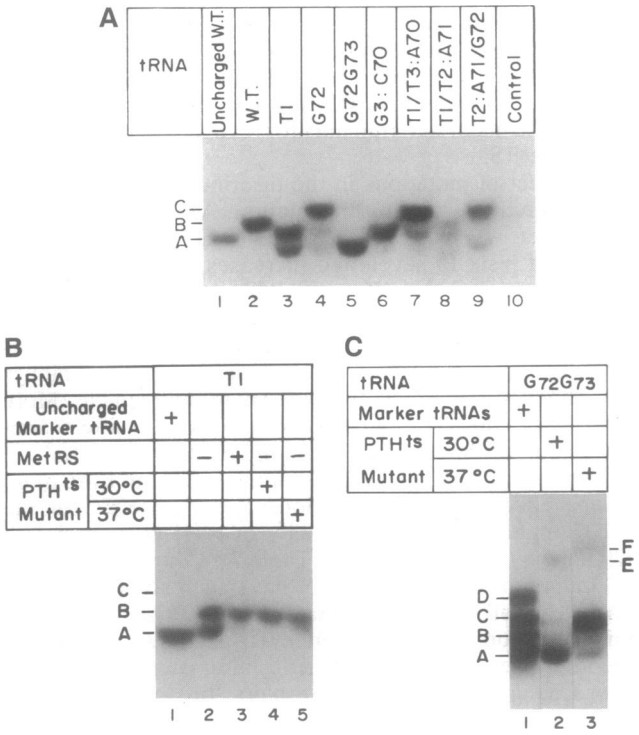

FIG. 2. RNA blot analysis of tRNA from transformants carrying the various mutant $tRNA_2^{fMet}$ genes, as indicated with a probe complementary to $tRNA_2^{fMet}$. A, B, and C indicate locations of uncharged tRNA, fMet-tRNA, and Met-tRNA corresponding to wild-type tRNA, respectively. (*A*) Acceptor stem mutants. Lane 1, uncharged wild-type (W.T.) tRNA used as marker; lane 10, tRNA isolated from cells transformed with pBR322. (*B*) The T1 mutant expressed in *E. coli* CA274 (lanes 2 and 3) and *E. coli* AA7852 [temperature-sensitive mutant of peptidyl-tRNA hydrolase (PTH^{ts}), lanes 4 and 5]. An overnight culture of AA7852 transformants was diluted 30-fold into fresh medium and grown for 3 hr at either 30°C (lane 4) or 37°C (lane 5) before isolation of tRNA. (*C*) The G72G73 mutant expressed in *E. coli* AA7852 (pth^{ts}), which is overproducing *E. coli* MetRS. This blot was also probed with an oligonucleotide complementary to $tRNA^{Tyr}$. Lane 1 contains markers of uncharged wild-type $tRNA^{fMet}$ (A), fMet-tRNA (B), and Met-tRNA (C). D, E, and F indicate locations of uncharged $tRNA^{Tyr}$, Tyr-$tRNA^{Tyr}$, and peptidyl-$tRNA^{Tyr}$, respectively. The peptidyl-$tRNA^{Tyr}$ migrates slower than Tyr-$tRNA^{Tyr}$ and is smeared in the hybridization pattern, indicating heterogeneity in size and charge of peptides attached to $tRNA^{Tyr}$. Virtually all of the $tRNA^{Tyr}$ species is present as peptidyl-tRNA in the AA7852 (pth^{ts}) transformants grown at 37°C (23).

The ≈475-fold decrease in V_{max}/K_m^{app} seen *in vitro* for the G72G73 mutant is also reflected in the extremely low steady-state levels of aminoacylation *in vivo* (Fig. 2*A*, lane 5). Either the tRNA is not aminoacylated to a significant extent or the rate of its aminoacylation is much slower than rate of its use, possibly as an elongator methionine tRNA (2, 3). The latter possibility is supported by a finding in cells carrying a temperature-sensitive mutation in peptidyl-tRNA hydrolase (14) and overproducing MetRS: when protein synthesis is blocked (23) by shifting cells to nonpermissive temperature, the G72G73 mutant now accumulates mostly as aminoacyl-tRNA (Fig. 2*C*, lane 3). That this mutant tRNA accumulates as Met-tRNA and not as fMet-tRNA also confirms the earlier conclusion that the G72G73 mutant tRNA is a very poor substrate for Met-tRNA transformylase (4).

Formylation of Mutant tRNAs *in Vivo*. Kinetic parameters in formylation of mutant tRNAs indicated previously that the key determinants for recognition by Met-tRNA transformylase are clustered at the end of the acceptor stem (4, 38). Results of *in vivo* analyses confirm and extend these conclusions. Mutations in D stem, anticodon stem, TψC stem, and deletion of 2 nt in the D loop have no effect on formylation of the mutant tRNAs *in vivo* (data not shown). However, mutations in the acceptor stem cause strong effects. At nt 1 and 72, the G72 mutant with a "strong" C1·G72 bp is formylated very poorly, whereas the T1 mutant with a "weak" U1·A72 bp is formylated well (Fig. 2*A*). Mutations in the second base pair, G2·C71 → U2·A71, or in the third base pair, C3·G70 → U3·A70 result in accumulation of mutant tRNAs, mostly as aminoacyl-tRNA. Mutation of C3·G70 → G3·C70 produces a tRNA found mostly in the formylated form (Fig. 2*A*, lane 6). These results are essentially identical to those for the corresponding acceptor stem mutants that carry an additional mutation in the anticodon sequence from CAU to CUA (13). However, in contrast to the report (13) that the tRNAs were aminoacylated with glutamine due to the anticodon sequence change (27) and aminoacylation of the mutant tRNAs was partial in most cases, in the current work most mutant tRNAs are aminoacylated almost quantitatively with methionine. This fact removes any ambiguity in interpretation of the previous results due to possible effect of the amino acid attached to the mutant tRNA on formylation (28) or problems in sensitivity of detection of the formylated tRNA.

The above results with the T1 and G72 mutants suggest that Met-tRNA transformylase prefers a tRNA substrate that has either a "weak" base pair or a mismatch between nt 1 and 72 and that the nature of nucleotides at these positions may not be too critical. To test this hypothesis, we generated mutant tRNAs with either U×G, C×C, A×A, or C×U mismatches and either a G·C or a A·U bp between nt 1 and 72. Mutant tRNAs carrying mismatches between nt 1 and 72 are present predominantly as fMet-tRNA *in vivo* (Fig. 3, lanes 2, 4, 10, and 12). In contrast, the G1·C72 mutant with a strong G·C bp is found mostly as Met-tRNA (lane 6). The A1·T72 mutant with an A·U bp is found mostly as a mixture of uncharged tRNA and fMet-tRNA (lane 8). The finding that a small but significant fraction of the A1·T72 mutant tRNA is found as Met-tRNA suggests that the tRNA with an A1·U72 bp is a poorer substrate for Met-tRNA transformylase than the mutant tRNA with a U1·A72 bp. This result confirms the results of *in vitro* formylation in which the A1·T72 mutant has a V_{max}/K_m^{app} lower by a factor of ≈13 compared with wild-type tRNA (data not

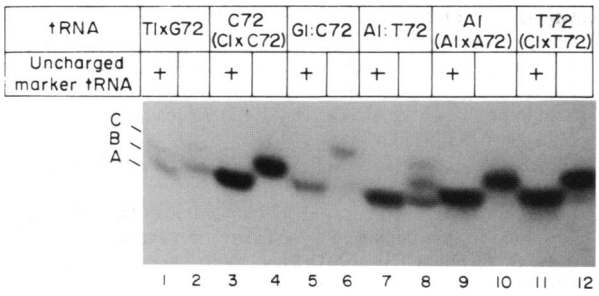

FIG. 3. RNA blot analysis of tRNA from transformants carrying $tRNA_2^{fMet}$ genes with mutations affecting the first base pair in the acceptor stem. Lanes 1, 3, 5, 7, 9, and 11 contain uncharged tRNA corresponding to the mutants. The nature of mismatch between nt 1 and 72 is indicated within parentheses. See legend for Fig. 2.

shown). Most eukaryotic initiator tRNAs, which have an A1·U72 bp, can be formylated quantitatively by the *E. coli* Met-tRNA transformylase (29–31). However, the V_{max}/K_m^{app} for these tRNAs is lower by a factor of 50–100 compared with *E. coli* $tRNA_2^{fMet}$ (data not shown).

The finding that a significant fraction of the A1·T72 mutant tRNA is uncharged *in vivo* (Fig. 3, lane 8) indicates that this mutant tRNA is also a substrate for peptidyl-tRNA hydrolase (25). Consequently, as for the T1 mutant, the mutant fMet-tRNA is hydrolyzed to formylmethionine and tRNA, increasing steady-state levels of uncharged tRNA.

Effect of Mutations in the Discriminator Base on Formylation. Mutations in the discriminator base alone to G73, C73, or U73 have essentially no effect on formylation *in vivo* (Fig. 4); there is no accumulation of Met-tRNA. Therefore the discriminator base A73 probably does not play a major role in recognition by Met-tRNA transformylase. This conclusion explains recent results of Schulman and coworkers (32) showing that mutants of *E. coli* initiator tRNA carrying the tryptophan anticodon CCA and G73 in the discriminator position can initiate protein synthesis in *E. coli*.

The G72 mutant is a very poor substrate for Met-tRNA transformylase; V_{max}/K_m^{app} value is lower by a factor of 495 compared with the wild-type tRNA (4). However, coupling of the G72 mutation with either C73 or T73 mutation results in tRNAs that are much better substrates for Met-tRNA transformylase *in vitro* and *in vivo*. The V_{max}/K_m^{app} values for the G72C73 and G72T73 mutants decrease by a factor of only ≈3.7-fold compared with wild-type tRNA (Table 4). *In vivo* also, although the G72 mutant is present predominantly as Met-tRNA, the G72C73 and G72T73 mutants are present predominantly as fMet-tRNA (Fig. 5). With the G72G73 mutant, most of the tRNA is found in the uncharged form. The small amount of this mutant tRNA that is aminoacylated

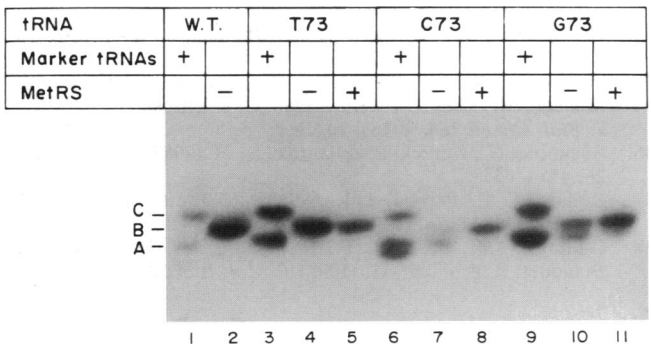

FIG. 4. RNA blot analysis of tRNA from transformants carrying $tRNA_2^{fMet}$ genes with mutations in the discriminator base. Lanes 1, 3, 6, and 9 contain a mixture of uncharged and charged tRNA (Met-tRNA) corresponding to the various mutants. See legend for Fig. 2.

Table 4. Kinetic parameters in formylation of mutant tRNAs by *E. coli* Met-tRNA transformylase

tRNA	Relative V	K_m^{app}, μM	Relative V_{max}/K_m^{app}
$tRNA_2^{fMet}$	1.4	7.3	1.0
G72C73	0.76	14.6	3.7
G72T73	0.79	15.1	3.7

with methionine in cells overproducing MetRS stays as Met-tRNA and is not formylated to fMet-tRNA. This result combined with those of Fig. 2*C*, lane 3, suggests that although C73 and T73 mutations can suppress the effect of having a strong base pair at the end of the acceptor stem helix, the G73 mutation cannot.

DISCUSSION

Recognition by MetRS. Results of our studies on MetRS recognition *in vitro* and *in vivo* support the conclusion that the anticodon sequence is a major determinant for MetRS recognition (5). Thus, there is good correlation between the results from two complementary approaches: (*i*) showing that mutations at many sites in the tRNA (Fig. 1), except in the anticodon and the discriminator base, have no effect on MetRS recognition (Table 1) and (*ii*) showing that mutations in the anticodon severely affect aminoacylation and introduction of methionine anticodon to valine (8), two species of isoleucine (9, 10), and phenylalanine tRNA (Table 3)—all four of which contain A73 in the discriminator position—confers methionine identity on them.

The discriminator base also plays a role in aminoacylation in that mutation of A73 → C or G affects aminoacylation kinetics *in vitro* (Table 2) and aminoacylation levels *in vivo* (Fig. 4). Because A73 → U has little effect and A73 and U73 have no functional groups in common, the detrimental effect of the C73 or G73 change is more likely from negative interactions and/or an altered structure than from loss of a direct contact between MetRS and the discriminator base.

Effect of the G73 mutation is strikingly exacerbated by an additional mutation of the neighboring nucleotide. Coupling of the G73 mutation with G72, which in itself has a minor effect on MetRS recognition, produces a mutant tRNA (G72G73) that is an extremely poor substrate for MetRS *in vitro* (Table 2) and *in vivo* (Fig. 2*A*, lane 5). This result is mostly due to an effect on V_{max}. Perhaps the G72G73 mutant tRNA adopts a structure that cannot be easily altered to make the tRNA CCA end fit into the catalytic pocket of MetRS.

The results of aminoacylation *in vivo* parallel the kinetic parameters for aminoacylation *in vitro*. An important exception is the T1 mutant, which is a good substrate for MetRS *in vitro* (Table 1) but is substantially uncharged *in vivo* (Fig. 2*B*); this is because the T1 mutant with a U1·A72 bp is now a

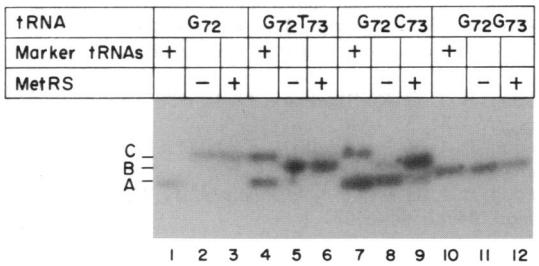

FIG. 5. RNA blot analysis of tRNAs from transformants carrying $tRNA_2^{fMet}$ genes with mutations at nt 72 coupled to the discriminator base. Lanes 1 and 10 contain uncharged tRNA, and lanes 4 and 7 contain a mixture of uncharged tRNA and Met-tRNA corresponding to the various mutants. See legend for Fig. 2.

substrate for peptidyl-tRNA hydrolase. This effect of the T1 mutation on aminoacylation levels highlights another crucial role *in vivo* of the mismatch between nt 1 and 72 conserved in all eubacterial initiator tRNAs. In addition to preventing the tRNA from binding to elongation factor-Tu (33, 34) and thereby sequestering the tRNA for use in initiation (2, 15), the mismatch maintains high steady-state levels of fMet-tRNA *in vivo* by preventing hydrolysis by peptidyl-tRNA hydrolase. The mismatch may also be important for recognition of the tRNA by Met-tRNA transformylase (see below). Thus, a single structural feature conserved in all eubacterial initiator tRNAs could play a crucial role in specifying three of their distinctive properties.

Recognition by Met-tRNA Transformylase. The key determinants for Met-tRNA transformylase recognition of tRNAs include a weak base pair or a mismatch between nt 1 and 72, a G·C bp between nt 2 and 71 and a C·G (or less preferably G·C) bp between nt 3 and 70. tRNAs carrying the wild-type C×A mismatch or the mutant U×G, C×C, A×A, or C×U mismatches are essentially fully formylated *in vivo*, whereas tRNAs carrying strong base pairs, such as C1·G72 or G1·C72, are found mostly as Met-tRNA (Fig. 3). Significantly, the severe effect on formylation of having a C1·G72 bp can be compensated for by a secondary change of the neighboring base A → C or U (Table 4, Fig. 5). A likely explanation of this result is that the C1·G72 bp, which is at the end of an RNA helix and may, therefore, have a tendency to "breathe," is stabilized by stacking (35) of the neighboring base A73 on top of the C1·G72 bp. Change of A73 to pyrimidine bases, such as C73 or U73, could destabilize the C1·G72 bp by loss of this stacking interaction (36). Perhaps the C1·G72 bp is destabilized only upon aminoacylation of the tRNA or upon binding of the aminoacyl-tRNA to Met-tRNA transformylase. For example, in the crystal structure of *E. coli* GlnRS–$tRNA^{Gln}$ complex, the U1·A72 bp is disrupted upon enzyme binding (37).

Initiator tRNAs in mitochondria of Paramecium and Tetrahymena contain a G1·C72 bp (22); these represent the only two exceptions to the finding that initiator tRNAs have either no Watson–Crick base pair or a weak base pair between nt 1 and 72. Interestingly, in these cases the discriminator base is cytosine or uracil. Thus, although little is known about Met-tRNA transformylase in mitochondria, a weak or a disrupted base pair between nt 1 and 72 could be a feature necessary for formylation of all eubacterial, chloroplast, and mitochondrial initiator tRNAs.

We dedicate this paper to the memory of our friend and colleague Dr. LaDonne H. Schulman. She inspired us and many others with the elegance of her science and with her courage, cheerfulness, and concern for others even at a time of extreme personal adversity.

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