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Effect of a 2-Methylthio-*N*6-Isopentenyladenosine Deficiency on Peptidyl-tRNA Release in *Escherichia coli*

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We examined the effect of *miaA*, a mutation conferring a deficiency in 2-methylthio-*N*6-isopentenyladenosine in tRNA, on patterns of peptidyl-tRNA accumulation in *Escherichia coli* strains deficient in peptidyl-tRNA hydrolase activity. A specific reduction in peptidyl-tRNA accumulation was seen for tRNAs which normally contain the 2-methylthio-*N*6-isopentenyladenosine modification. These results provide new evidence in support of the ribosome editor model, which links peptidyl-tRNA release to mistranslation events.

The isolation of a temperature-sensitive mutation in an *Escherichia coli* gene coding for peptidyl-tRNA hydrolase (*pth*) was reported by Atherly and Menninger in 1972 (1). This enzyme specifically cleaves peptides from cytoplasmic peptidyl-tRNA. The *pth* mutation is conditionally lethal (16). This implies that peptidyl-tRNA hydrolase is an essential enzyme in *E. coli*. Yet, in the classical description of protein synthesis, there is no step where cytoplasmic peptidyl-tRNA is generated. In addition, the release of a nonfunctional, half-finished peptide from the ribosome seems extremely wasteful.

These considerations led Menninger (11) to propose that the release of peptidyl-tRNA is associated with a proofreading mechanism, the so-called ribosome editor. In this model, the premature release of peptidyl-tRNA is the result of an active editing mechanism which recognizes and specifically releases noncognate tRNA molecules that have escaped proofreading in the acceptor (A) site (23). Recent evidence suggests that peptidyl-tRNA release is associated with the translocation step (15).

The ribosome editor hypothesis leads to a very precise prediction. The vast majority of, if not all, tRNA molecules released as peptidyl-tRNA should be noncognate; i.e., they should have been released in the process of mistranslating a codon. However, testing this precise prediction directly has proven difficult (2, 3).

Here we describe a new approach to test the ribosome editor hypothesis. We previously studied the effect of *miaA*, a mutation which causes a deficiency in 2-methylthio-*N*6-isopentenyladenosine in tRNA, on the readthrough and suppression of nonsense mutations in *E. coli* (21). This modification in 2-methylthio-*N*6-isopentenyladenosine, hereafter called isopentenyladenosine, occurs 3′ adjacent to the anticodon in all tRNAs that read codons beginning with uracil (17). Those tRNAs which normally contain the isopentenyladenosine modification will be referred to as *miaA* tRNAs, and those which do not will be referred to as non-*miaA* tRNAs. Normal protein synthesis and growth are barely affected in an *E. coli* mutant containing the *miaA* mutation, but there is a drastic effect on the ability of *miaA* tRNAs to misread and suppress nonsense mutations (21). This is understandable, because a misreading tRNA must rely more heavily on tRNA modifications such as the isopentenyladenosine modification for stabilization in the A site of a programmed ribosome.

The *miaA* mutation can be used to tag genetically a subpopulation of tRNAs. Based on our previous results, introduction of the *miaA* allele should specifically reduce the ability of *miaA* tRNAs to misread other codons but leave all other aspects of protein synthesis more or less unaltered. Here we report how the *miaA* mutation influenced patterns of peptidyl-tRNA release. Our approach had the advantage that the ribosome and therefore the ribosome editor remained unaltered throughout these experiments.

MATERIALS AND METHODS

Strain MF100 [F^+ *lacZ* (UAG) *pth* Δ(*gal-bio*)] was obtained from J. R. Menninger. Strain TRPX [*trpR lacZ118* (UAA) *miaA* Tn*10*] was obtained from C. Yanofsky. It contains a Tn*10* transposon 5 to 10% cotransducible with *miaA*. Strains TRPX and DEV15 [KL16 *lacZ* (UGA)] have been described previously (21). We previously showed that the presence of the *miaA* mutation can be detected as a phenotype of reduced readthrough at UGA codons (21). This observation was exploited to introduce *miaA* into MF100 as follows. First, MF100 was transduced with bacteriophage P1 grown on DEV15 to select Lac^+ recombinants on minimal-lactose plates. Readthrough at the *lacZ* (UGA) codon in this strain is 4.5%, resulting in sufficient β-galactosidase activity to grow on minimal lactose (21). MF100 derivatives containing the *lacZ* (UGA) allele were distinguished from MF100 Lac^+ recombinants (which could arise from intragenic recombination between the two *lacZ* alleles) by assays for β-galactosidase specific activity (22). An MF100 *lacZ* (UGA) derivative was retained and named DEV22. The presence of the *lacZ* (UGA) allele was further confirmed by showing that it was suppressible by *sup-9*, a UGA suppressor, as previously described (21). DEV22 was then transduced with P1 grown on strain TRPX to select tetracycline-resistant recombinants. Of 40 recombinants, 2 to 4 were expected to acquire the *miaA* mutation by cotransduction (21). The *miaA* mutation reduces readthrough at this *lacZ* (UGA) allele 60-fold (21). A double mutant containing *lacZ* (UGA) and *miaA* mutations therefore can no longer grow on minimal lactose and is easily distinguished from its *lacZ* (UGA) $miaA^+$ counterpart. MF100 *lacZ* (UGA) *miaA* derivatives were obtained at the expected frequency, and one of them was designated DEV23. The absence of the isopentenyladenosine

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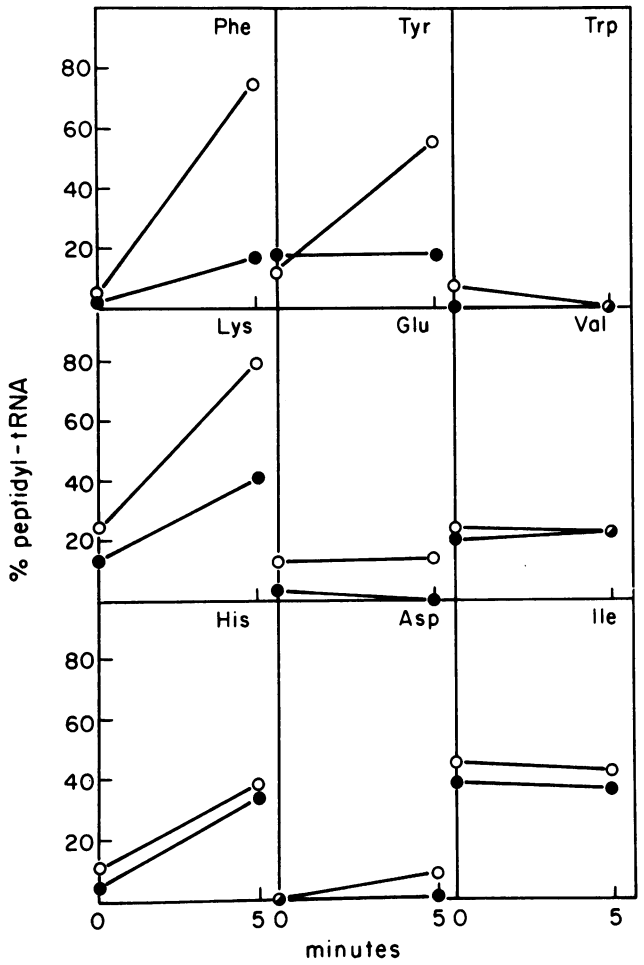

FIG. 1. Peptidyl-tRNA accumulation at 40°C in strains DEV24 (*miaA*$^+$) (○) and DEV25 (*miaA*) (●). tRNAs for Phe, Tyr, and Trp are *miaA* tRNAs; tRNAs for Lys, Glu, Val, His, Asp, and Ile are non-*miaA* tRNAs. Each point represents the average value from two to four independent tRNA preparations. Experimental error was always within 10%. See the text for details.

modification was also confirmed (data not shown) by thin-layer chromatography, by using ^{35}S labeling as previously described (4). Finally, for reasons extraneous to this project, we transduced DEV23 and MF100 with P1 grown on strain KL16 (Hfr *thi-1 relA1 spoT1*) (21), and we selected Lac$^+$ recombinants. The resulting strains were MF100 Lac$^+$ (designated DEV24) and MF100 Lac$^+$ *miaA* (designated DEV25).

Peptidyl-tRNA was prepared as described by Menninger (10). Briefly, growth of cells was stopped by the addition of trichloroacetic acid and Brij 58. After centrifugation, the pellet was suspended in buffer, mixed with an equal volume of equilibrated phenol, and sonicated. After ethanol precipitation, aminoacyl-tRNA was stripped of its amino acids by $CuSO_4$ treatment, and the resulting mixture of stripped aminoacyl-tRNA and peptidyl-tRNA was recovered by ethanol precipitation.

Partially purified tRNA synthetases, prepared as previously described (4), were freed of any residual peptidyl-tRNA hydrolase activity by passage over a DEAE-cellulose column (14). tRNA-charging experiments were performed as previously described (4), by using ^{14}C-amino acids purchased from Amersham Corp. Partially purified peptidyl-tRNA hydrolase was prepared by the method of Menninger et al. (14) from strain LL (4).

RESULTS

To test the effect of the *miaA* mutation on patterns of peptidyl-tRNA release, we prepared tRNA from strains DEV24 and DEV25 grown at 30°C and from identical cultures shifted to 40°C for various times, as described by Menninger (10). It is worth mentioning that the procedure of Menninger for preparation of tRNA must be followed exactly (for details, see Materials and Methods). Other purifications of tRNA, such as the procedure of Zubay (24), do not allow detection of peptidyl-tRNA. The reason for this is not clear. Accumulation of peptidyl-tRNA is measured by a subtraction assay (10). Peptidyl-tRNA cannot be charged in vitro by its corresponding aminoacyl-tRNA synthetase. However, the peptide on peptidyl-tRNA can be cleaved by partially purified peptidyl-tRNA hydrolase obtained from a *pth*$^+$ strain (14). tRNA charging, in the presence and absence of active peptidyl-tRNA hydrolase, measures total tRNA available for charging and the nonblocked fraction of tRNA available for charging, respectively. From these data, the fraction of peptidyl-tRNA was calculated as follows: (total tRNA − nonblocked tRNA)/total tRNA.

The choice of labeled amino acids used was governed by the following considerations. First, amino acids for *miaA* tRNAs include phenylalanine, leucine, serine, tyrosine, cysteine, and tryptophan. Leucine and serine were not tested because their corresponding tRNAs include both *miaA* and non-*miaA* tRNAs. Cysteine was not tested because it reportedly accumulates very slowly as peptidyl-tRNA (12). This left us with phenylalanine, tyrosine, and tryptophan to test *miaA* tRNAs. Second, the choice of amino acids for non-*miaA* tRNAs was arbitrary and based purely on availability. Of 14 possible amino acids, the following 6 were tested: lysine, glutamic acid, valine, histidine, aspartic acid, and isoleucine.

Results are shown in Fig. 1. Pilot experiments using tRNA^{Lys} and tRNA^{Phe} showed that the rate of peptidyl-tRNA accumulation was linear during the first 5 min at 40°C but leveled off by 10 min in strains containing *miaA* (at 50% for tRNA^{Lys} and at 22% for tRNA^{Phe}). This observation is in contrast to *miaA*$^+$ strains, in which accumulation either continues for at least 15 min or levels off at about 90% peptidyl-tRNA (12). Data points for other amino acids were limited to tRNA preparations after a 0- and 5-min incubation at 40°C.

Regarding strain DEV24 (*pth miaA*$^+$), our data showed clear evidence of peptidyl-tRNA accumulation for tRNA^{Phe}, tRNA^{Tyr}, tRNA^{Lys}, and tRNA^{His}. The small differences observed for other tRNAs were within experimental error and not meaningful. These results are in qualitative agreement with those of Menninger (12), who showed that different tRNAs accumulate at different rates. The precise pattern of accumulation is, however, strain dependent (2, 3). Therefore, an exact correspondence between our results and those of Menninger was neither expected nor found. Accumulation of peptidyl-tRNA under our conditions was already significant at zero time for several amino acids; this can be explained by the fact that, at 30°C, peptidyl-tRNA hydrolase from the *pth*-containing mutant has only 2 to 4% of the activity observed in a wild-type strain (16).

The effect of the *miaA* mutation was most clear-cut for tRNA^{Phe} and tRNA^{Tyr}. There was a drastic reduction in peptidyl-tRNA accumulation for these tRNAs. The accumulation of tRNA^{Trp} was negligible in the *miaA*$^+$ genetic

background; thus, any decrease due to the *miaA* mutation would be undetectable. The possibility that there was a substantial accumulation of $tRNA^{Trp}$ as peptidyl-tRNA after longer incubation at 40°C was not investigated.

The effect of the *miaA* mutation on accumulation of non-*miaA* tRNAs was negligible within experimental error except for a slight effect on the fast-accumulating $tRNA^{Lys}$. The reason for this is not clear. In any case, the effect on $tRNA^{Lys}$ (about twofold) was significantly smaller than the effect on $tRNA^{Phe}$ (4.6-fold) and on $tRNA^{Tyr}$, in which peptidyl-tRNA accumulation was abolished.

The observation that peptidyl-tRNA accumulation ceased more quickly in *miaA*-containing strains than in wild-type strains prompted us to check the effect of the *miaA* mutation on the survival of peptidyl-tRNA-hydrolase-deficient cells after incubation at 40°C. Results are shown in Fig. 2. Strain DEV25 (*pth miaA*) died two to three times faster than did strain DEV24 (*pth* $miaA^+$) during the first 20 min at 40°C. The difference was small but reproducible. The most significant difference was perhaps that strain DEV25 started to die right away, whereas there was very little killing of strain DEV24 in the first 10 min. Survival fell to about 2% and then remained constant for longer incubations in both strains (data not shown). These observations are in full agreement with the results of Menninger (13).

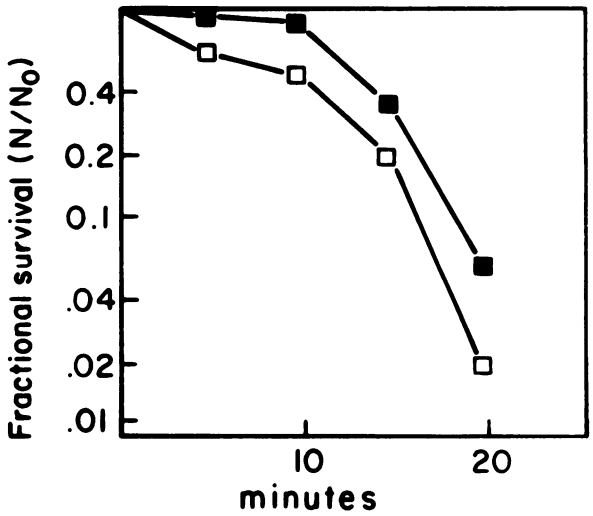

FIG. 2. Survival of strains DEV24 ($miaA^+$) (■) and DEV25 (*miaA*) (□) at 40°C. N_0, Number of viable cells at time zero.

DISCUSSION

The results from Fig. 1 lead to two important conclusions. First, we can infer that most peptidyl-tRNA release, observed for *miaA* tRNAs in the wild-type strain, was due to a process which was strongly dependent on the isopentenyladenosine group. This follows from the fact that the loss of this group leads to reduced peptidyl-tRNA accumulation for *miaA* tRNAs. Two possibilities can be considered: (i) that this process is misreading, i.e., that the *miaA* mutation exerts its effect primarily on noncognate tRNAs; or (ii) that this process is normal translation, i.e., that the *miaA* mutation exerts its effect primarily on cognate tRNAs.

We favor the first hypothesis, which supports the ribosome editor model. We have already shown that the *miaA* mutation drastically reduces the ability of $tRNA^{Trp}$ to misread UGA codons (21) without having a significant effect on normal protein synthesis. Evidence from triplet binding experiments (5, 21) suggests that the isopentenyladenosine group stabilizes the tRNA-ribosome interaction in the A site of the programmed ribosome. Therefore, it is expected that the number of noncognate *miaA* tRNAs which escape proofreading in the A site will be reduced in a *miaA* genetic background, because proofreading mechanisms operating in the ribosomal A site rely on differences in dissociation rates between cognate and noncognate tRNAs (23).

The ribosome editor is postulated to operate only on those noncognate tRNAs which have escaped A site proofreading. If the effect of the *miaA* mutation is primarily on noncognate tRNAs (our first hypothesis), many fewer noncognate *miaA* tRNAs should be encountered by the ribosome editor in a strain containing *miaA* as compared with a wild-type ($miaA^+$) strain. As a result, there should be an overall decrease in *miaA* tRNAs being released by the ribosome editor in a *miaA* background. Our results fully support this idea.

Whether the effect of the *miaA* mutation is primarily expressed in the ribosomal A site during proofreading or later during editing warrants further discussion. Since overall accumulation of *miaA* tRNAs as peptidyl-tRNA is reduced in a *miaA* genetic background, there are only two possible models. (i) There is less substrate to be released, i.e., the model advocated here. In this case, it does not matter, for the arguments presented, whether the ribosome editor releases the remaining substrate with equal or different efficiency. (ii) The number of noncognate *miaA* tRNAs escaping A site proofreading is unchanged, but the ribosome editor works less efficiently on *miaA* tRNAs lacking the isopentenyladenosine group. The latter model is untenable, since it would predict increased error rates for *miaA* tRNAs, while in fact the opposite effect has been demonstrated, at least with respect to readthrough at nonsense codons (21).

The second hypothesis, i.e., that the isopentenyladenosine group primarily exerts its effect on normal translation, seems to us less plausible, but it is not inconsistent with available data. In this case, the reduced accumulation of *miaA* peptidyl-tRNAs could be explained only by saying that the presence of the isopentenyladenosine group causes cognate *miaA* tRNAs to accumulate as peptidyl-tRNA.

In either case, the fact that the *miaA* mutation caused an overall decrease in *miaA* peptidyl-tRNA release also suggests that the isopentenyladenosine group does not enhance the general stability of cognate translocations of *miaA* tRNAs, since cognate translocations greatly outnumber noncognate translocations (23).

Within the framework of the ribosome editor model, we can also rationalize why double-mutant DEV25 dies two to three times faster at 40°C than does single-mutant DEV24. There is only a 10-fold excess of tRNAs per ribosome in the cell (7). After a temperature shift to 40°C, peptidyl-tRNA accumulation begins, and less and less tRNA remains available for continued protein synthesis. There is ample evidence that depletion of a tRNA species leads to increased misreading of its corresponding codon(s) (6, 8, 9, 18–20). Misreading could keep protein synthesis going for a while at the expense of increased error rates. But according to the ribosome editor hypothesis, increased error rates will lead to an increase in peptidyl-tRNA release. This positive feedback would lead to depletion of other tRNA species, followed by increased misreading of their corresponding codons, and so forth. Such a model is supported by the observation of Menninger (10) that an initial rate of leucine peptidyl-tRNA

release of 1/2,600 peptide bonds formed rose to 1/90 peptide bonds formed as incubation at 40°C continued.

This model can also explain why certain peptidyl-tRNA species accumulate faster than others after a shift to 40°C. Rapidly accumulating species would be those most prone to misreading noncognate codons, even under normal circumstances, whereas slowly accumulating species would be those that misread only codons whose corresponding cognate tRNA is practically depleted.

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LITERATURE CITED

1. **Atherly, A. G., and J. R. Menninger.** 1972. Mutant *Escherichia coli* strain with temperature sensitive peptidyl-transfer RNA hydrolase. Nature (London) New Biol. **240**:245–246.
2. **Caplan, A. B., and J. R. Menninger.** 1979. Tests of the ribosomal editing hypothesis: amino acid starvation differentially enhances the dissociation of peptidyl-tRNA from the ribosome. J. Mol. Biol. **134**:621–637.
3. **Caplan, A. B., and J. R. Menninger.** 1984. Dissociation of peptidyl-tRNA from ribosomes is perturbed by streptomycin and by *strA* mutations. Mol. Gen. Genet. **194**:534–538.
4. **Elseviers, D., L. A. Petrullo, and P. J. Gallagher.** 1984. Novel *E. coli* mutants deficient in biosynthesis of 5-methylaminomethyl-2-thiouridine. Nucleic Acids Res. **12**:3521–3534.
5. **Gefter, M. L., and R. L. Russell.** 1969. Role of modifications in tyrosine transfer RNA: a modified base affecting ribosome binding. J. Mol. Biol. **39**:145–157.
6. **Goldman, E.** 1982. Effect of rate-limiting elongation on bacteriophage MS2 RNA-directed protein synthesis in extracts of *Escherichia coli*. J. Mol. Biol. **158**:619–636.
7. **Ingraham, J. L., O. Maaløe, and F. C. Neidhardt.** 1983. Growth of the bacterial cell, p. 3. Sinauer Assoc., Inc., Sunderland, Mass.
8. **Johnston, T. C., P. T. Borgia, and J. Parker.** 1984. Codon specificity of starvation induced misreading. Mol. Gen. Genet. **195**:459–465.
9. **Koontz, S. W., H. Jakubowski, and E. Goldman.** 1983. Control of RNA and protein synthesis by the concentration of Trp-tRNA^{Trp} in *Escherichia coli* infected with bacteriophage MS2. J. Mol. Biol. **168**:747–763.
10. **Menninger, J. R.** 1976. Peptidyl transfer RNA dissociates during protein synthesis from ribosomes of *Escherichia coli*. J. Biol. Chem. **251**:3392–3398.
11. **Menninger, J. R.** 1977. Ribosome editing and the error catastrophe hypothesis of cellular ageing. Mech. Ageing. Dev. **6**:131–142.
12. **Menninger, J. R.** 1978. The accumulation as peptidyl-transfer RNA of isoaccepting transfer RNA families in *Escherichia coli* with temperature sensitive peptidyl-transfer RNA hydrolase. J. Biol. Chem. **253**:6808–6813.
13. **Menninger, J. R.** 1979. Accumulation of peptidyl tRNA is lethal to *Escherichia coli*. J. Bacteriol. **137**:694–696.
14. **Menninger, J. R., M. C. Mulholland, and W. S. Stirewalt.** 1970. Peptidyl-tRNA hydrolase and protein chain termination. Biochim. Biophys. Acta **217**:496–511.
15. **Menninger, J. R., and D. P. Otto.** 1982. Erythromycin, carbomycin, and spiramycin inhibit protein synthesis by stimulating the dissociation of peptidyl-tRNA from ribosomes. Antimicrob. Agents Chemother. **21**:811–818.
16. **Menninger, J. R., C. Walker, P. F. Tan, and A. G. Atherly.** 1973. Studies on the metabolic role of peptidyl-tRNA hydrolase. Mol. Gen. Genet. **121**:307–324.
17. **Nishimura, S.** 1972. Minor components in transfer RNA: their characterization, location and function. Prog. Nucleic Acid Res. Mol. Biol. **12**:49–85.
18. **Parker, J., T. C. Johnston, and P. T. Borgia.** 1980. Mistranslation in cells infected with bacteriophage MS2: direct evidence for Lys for Asn substitution. Mol. Gen. Genet. **180**:275–281.
19. **Parker, J., T. C. Johnston, P. T. Borgia, G. Holtz, E. Remaut, and W. Fiers.** 1983. Codon usage and mistranslation. *In vivo* basal level misreading of the MS2 coat protein message. J. Biol. Chem. **258**:10007–10012.
20. **Parker, J., J. W. Pollard, J. D. Friesen, and C. P. Stanners.** 1978. Stuttering: high-level mistranslation in animal and bacterial cells. Proc. Natl. Acad. Sci. USA **75**:1091–1095.
21. **Petrullo, L. A., P. J. Gallagher, and D. Elseviers.** 1983. The role of 2-methylthio-N6-isopentenyladenosine in readthrough and suppression of nonsense codons in *Escherichia coli*. Mol. Gen. Genet. **190**:289–294.
22. **Strigini, P., and E. Brickman.** 1973. Analysis of specific misreading in *Escherichia coli*. J. Mol. Biol. **75**:659–672.
23. **Yarus, M., and R. C. Thompson.** 1983. Precision of protein biosynthesis, p. 23–63. *In* J. R. Beckwith, J. Davies, and J. A. Gallant (ed.), Gene function in prokaryotes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Zubay, G.** 1962. The isolation and fractionation of soluble ribonucleic acid. J. Mol. Biol. **4**:347–356.