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Thiolation of transfer RNA in *Escherichia coli* varies with growth rate

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ABSTRACT

We have used an affinity electrophoresis assay which when combined with Northern hybridization techniques permits us to estimate the degree of thiolation of individual tRNA species in *Escherichia coli*. We observe that the levels of 4-thio 2′(3′)-uridine (4-thioU) in many but not all tRNAs varies dramatically at different bacterial growth rates: Five tRNAs are completely thiolated at all growth rates, while another eight tRNAs are incompletely thiolated and the fraction of the unthiolated form of these tRNA species increases as the growth rates increase. Transfer RNA_2^{Glu} contains 4-thioU as well as (methylamino)methyl-2-thio uridine ($mnm^5$2-thioU). The level of $mnm^5$2-thioU of $tRNA_2^{Glu}$ is invariant with growth rate. Surprisingly, none of the thirteen tRNA species that we have studied is completely unmodified in all growth media. In particular, at the slowest growth rates every tRNA class that we have studied contains a form that has 4-thioU residues.

INTRODUCTION

Transfer RNAs contain modified nucleosides that are usually created at nucleotide positions in the single stranded regions of tRNA precursor forms (1). Such modifications are generally not essential for cell viability. Instead, they may be required for the fine tuning of tRNA function in translation or in other functions of tRNA beside translation. For example, modifications localized in the anticodon loop such as the (methylamino)methyl-2-thio uridine ($mnm^5$2-thioU) at position 34 and the 2-methylthio-N6-isopentenyl-adenosine (m2-thio i^6A) at position 37, have been shown to be involved in modulating codon recognition during translation (2–4). However, modifications that are localized outside the anticodon loop such as 4-thio 2′(3′)-uridine (4-thioU) at position 8 (rarely at 9) and 5-methyl-2-thio-uridine ($m^5$2-thioU) at position 54 are normally less important for translation but they have been associated with photoprotection (5) as well as with the maintenance of the structural integrity of tRNAs at high temperatures (6). Finally, physiological stress such as starvation for amino acids influences the proportion of modification of tRNA (7, 8). Likewise, changes in the degree of tRNA modification may occur when bacteria switch from one growth phase to another (9, 10).

We have used novel assays to determine the effects of growth rates on the level of two thionucleosides of thirteen tRNA species in *Escherichia coli*. One of these is the major thionucleoside, 4-thioU, that has been detected in eubacterial and mitochondrial tRNAs at position 8 (rarely at 9). We find that its occurrence varies dramatically depending on growth rates for some tRNA species but that it is invariant in other tRNAs. In general we observe that unthiolated tRNAs accumulate at higher growth rates. In addition, we have studied a less common thionucleoside, $mnm^5$2-thioU, which has only been detected in eubacteria. We observe that its occurrence at position 34 of $tRNA_2^{Glu}$ is invariant at different growth rates. The data suggest that the different enzyme systems responsible for generating these two thionucleosides are independently regulated at different growth rates.

MATERIALS and METHODS

Bacterial strains and growth medium

The *E.coli* laboratory wild-type strain W1485 (11) was used to determine the levels of thiolation. The 4-thioU-deficient strain, PM2 (nuv^-) (5), was kindly provided by Alain Favre. Cells were cultivated in M9 medium (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl) supplemented with vitamin B1 (0.01 mM), $FeCl_3$ (0.03 mM), $CaCl_2$ (0.1 mM), $MgSO_4$ (1 mM) and various carbon sources: acetate 0.4%; succinate 0.4%; or glucose 0.4%. The rich medium consists of the M9 supplemented medium plus 0.4% glucose, 20 amino acids, purines and pyrimidines as described by Neidhardt et al. (12). Cells were grown aerobically at 37°C with vigorous shaking in a water-bath and maintained in a steady state of balanced growth for several generations by serial dilutions of cultures. Cultures (40 ml) were then harvested on ice at OD_{450} close to 0.8, centrifuged and resuspended in a washing buffer at pH 7.4 (25 mM Tris-HCl, 60 mM KCl, 10 mM $MgCl_2$ and 20% sucrose). Finally, the washed cells were centrifuged.

RNA preparation and electrophoretic fractionation

The preparation of the crude RNA has previously been described by Emilsson and Kurland (13). Here, cells (6 mg dry weight) were resuspended in the washing buffer without sucrose but containing lysozyme (4 mg/ml) instead. The cells were lysed by freezing in a dry ice-ethanol mixture and thawing at 45°C. Finally

the lysed cells (200 μl) were incubated in a detergent-phenol solution (400 μl) at pH 7.4 (25 mM Tris-HCl, 60 mM KCl, 10 mM $MgCl_2$, 0.6% Brij58, 0.2% Na-deoxycholate, 0.02% sodium dodecyl sulfate and 60 μl phenol) and the RNA extracted by adding phenol-chloroform (600 μl). This RNA preparation yields crude RNA with > 85% recovery (13). The amount of (bulk) tRNA in the lysate (600μl) that derives from cells growing at moderate rates (1.1 cell doublings per hour) can be estimated from our previous data (13, 14) to be roughly 110 μg; or 2.5 μg of individual tRNA that is moderately abundant. We used freshly grown cells for the preparation of the RNA lysates which were used directly in the polyacrylamide gel electrophoresis (PAGE) system, described below. This was done to prevent the loss of nucleoside modifications which can occur during prolonged handling of the samples and which is caused by atmospheric oxidation.

The ligand, (N-Acryloylamino)phenyl mercuric Chloride (APM), was synthesized, characterized and stored as described by Igloi (15). APM was cast with the 10% PAGE gels containing 7M urea to yield a final concentration of 50 μM − 100 μM. Gels ($15\times18\times0.1cm^3$) were prerun for 6 hours in TBE buffer at pH 8.3 (TBE is 100 mM Tris base, 100 mM boric acid, 2 mM EDTA). The amount of the RNA lysate sample was adjusted so that approximately 80 ng of single tRNA species was loaded in the PAGE system. Here, the unfractionated tRNA of roughly 2 − 10 μg (bulk) tRNA was diluted in 30% formamide containing 0.25% xylene cyanol and applied to the gels. Electrophoresis was then carried out at 120 volts for 24 hrs in the TBE buffer at room temperature.

Chemical modification of thionucleosides with CNBr was done according to Saneyoshi and Nishimura (16).

Northern hybridization

The proportion (percent) of thiolated tRNA was determined by Northern hybridization as described by Emilsson and Kurland (13) but with some modifications of that method. The sulfur linkage formed between the retarded tRNA fraction and the APM ligand was disrupted by incubating the gel in 0.2 M β-mercaptoethanol for 1 hour prior to blotting. The gel was then blotted onto a Hybond-N^+ filter in a Trans-blot cell for 5 hrs at 300 mA in 25 mM Na phosphate buffer at pH 6.5. The filters were prehybridized and hybridized as previously described by Emilsson and Kurland (13, 14). The labelled bands were excised from the filter and the radioactivity was measured in a liquid scintillation counter by standard techniques. Since we probe identical tRNAs in different fractions in a single blotting experiment, the proportional level of thiolation can simply be measured as a ratio of the radioactivity in the retarded fraction to the radioactivity of total hybridization in that lane.

The tRNA specific probes for the leucine, glycine, proline and glutamate tRNA isoacceptors were synthetic oligodeoxyribonucleotides with chain lengths between 18 and 22 nucleotides that are complementary to the TF stem, the variable loop plus the anticodon stem and loop for smaller tRNAs. The specificity of the probes has been documented earlier (13, 14). The following oligonucleotides (5′ to 3′) were used; Gly1: CCCTCGTATAGAGCTTGGGAA; Gly2: CCCGCATCATCAGCTTGGAAGGC; Gly3: CTCGCGACCCCGACCTTGGCAAG; Pro1: CCTCCGACCCCTTCGTCCCG; Pro2: CCTCCGACCCACTGGTCCCAAA; Pro3: CCTCCGACCCCCGACACCCCAT; Leu1: CCCCCACGTCCGTAAGGACA; Leu2: CCCGTAAGCCCTATTGGGCA; Leu3: CACCTTGCGGCGCCAGAA; Leu4: CCGGCACGTATTTCTACGG; Leu5: CCCGCACAGCGCGAACGCCG; Glu2: CCCCTGTTTACCGCCGTG; Phe: TAGCTCAGTCGGTAGAGCA.

The $tRNA^{Phe}$ probe is complementary to the 5′ half of the tRNA molecule and some nonspecific hybridzation occurs when it is used. This problem was solved by washing the hybridized filter with 2×SSC buffer at pH 7.0 (SSC is 150 mM NaCl, 15 mM Na citrate) plus 0.5% SDS (sodium dodecyl sulfate) at 60°C as described previously (14). The probes were end-labelled by incubating 30 − 50 pmol DNA, [γ-^{32}P]ATP and 10 units of T4 kinase in a standard kinase buffer at 37°C for 1 hour. The detection limit of the hybridization method is less than 1 ng of pure tRNA species. The determinations of the proportional tRNA levels were reproducible well within a 10% standard deviation for all species and subfractions studied.

In vitro assays

The aminoacylation reactions were performed in polymix (17) containing; 5 mM Mg acetate, 0.5 mM $CaCl_2$, 0.5 mM KCl, 5 mM NH_4Cl, 8 mM putresine, 1 mM spermidine, 5 mM K phosphate, 1 mM DTE, 2 mM GTP, 2 mM ATP, 0.3 μg Myokinase, 5 μg Pyruvatekinase, 20 mM phosphoenolpyruvate and finally 7 μM of elongation factor Tu (EF-Tu) to stabilize the charged tRNA. We measured the kinetic parameters v_{max} (pmoles tRNA charged per sec) and K_M (μM) for purified leucine, glycine and phenylalanine accepting tRNA species. The tRNAs were identified and purified as column fractions (13, 14). The purity of an isoacceptor tRNA fraction was determined by crossreacting it with oligonucleotides specific for the other tRNA isoacceptors, showing > 90% purity for each identified fraction. We also note that small impurities of tRNAs non-cognate for a particular aminoacyl-tRNA synthetase do not interfere with the charging

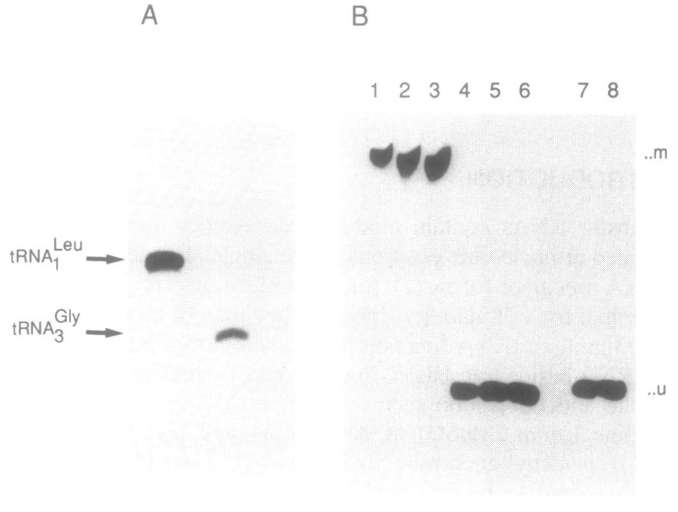

Figure 1. Fractionation of tRNA species In panel **A**, the crude RNA was fractionated in a standard 10% PAGE system containing 7M urea; then the gel was blotted on a filter and hybridized with oligonucleotide probes specific for $tRNA_1^{Leu}$ and $tRNA_3^{Gly}$. In panel **B** total RNA was titrated and fractionated in the 10% affinity PAGE system containing 100μM APM. The RNA samples derived from the wild type strain (W1485) in lanes 1, 2, and 3 were not incubated with CNBr while those in lanes 4, 5, 6 were incubated with CNBr. The filter was then hybridized with a probe specific for $tRNA_5^{Leu}$. RNA that derived from the 4-thioU less strain (PM2) was loaded in lane 7 while lane 8 had the PM2 derived RNA that was incubated with CNBr prior to electrophoresis. The position of modified tRNA containing 4-thioU is indicated by an 'm' and the unmodified is indicated by an 'u'.

reactions since they have low affinity for the enzyme. The tRNAs were obtained from either the wild-type strain (W1485) or from the 4-thioU-deficient strain (PM2). The two differently modified tRNAs were titrated from 0.4 μM to 8 μM at a limiting concentration of the aminoacyl-tRNA synthetase in the presence of > 100 μM of the cognate ^{3}H-labelled amino acid.

The association rates (k_{cat}/K_M) of ternary complexes with phe-tRNA^{Phe} (+ or − 4-thioU) to ribosomes were measured in an optimized poly(U)-translation system (17, 18). Here the amount of phe-tRNA^{Phe} is measured in the assay and since only aminoacylated tRNA binds elongation factor Tu (EF-Tu) under these conditions small impurities of deacylated tRNA do not interfere. The precision of the aminoacylation and translation assays is better than 5%.

Chemicals and Enzymes

[γ-^{32}P]ATP used for labelling, ^{3}H-labelled amino acids and the Hybond-N$^+$ filter were purchased from Amersham. The polynucleotide kinase that was used to label the oligonucleotide probes was from Pharmacia. All components used for the RNA fractionation and electrophoresis were of ultrapure grade and were purchased from IBI (International Biotechnologies, Inc.). The chemicals used for the synthesis of the mercury (II) ligand (APM) were purchased from Fluka Chemie.

RESULTS

The fractionation method we employ to separate thiolated tRNA species from nonthiolated species is based on the affinity of mercury (II) for sulfhydryl groups. The reagent APM contains a mercury (II) ligand and it is present in the PAGE gel during the electrophoretic fractionation of the tRNA's (15). In the presence of APM the electrophoretic mobility of a thiolated component will be retarded relative to its nonthiolated homologue. The identity of the homologous tRNA species is established by hybridization with specific oligonucleotide probes as we described previously (13). A special requirement of the present technique is that the blotting reaction is carried out with the gels after they have been treated with β-mercaptoethanol. By measuring the amounts of retarded and nonretarded tRNA that are hybridized with a particular probe we determine the proportion of a tRNA species that is thiolated (Figures 1, 2 and 3).

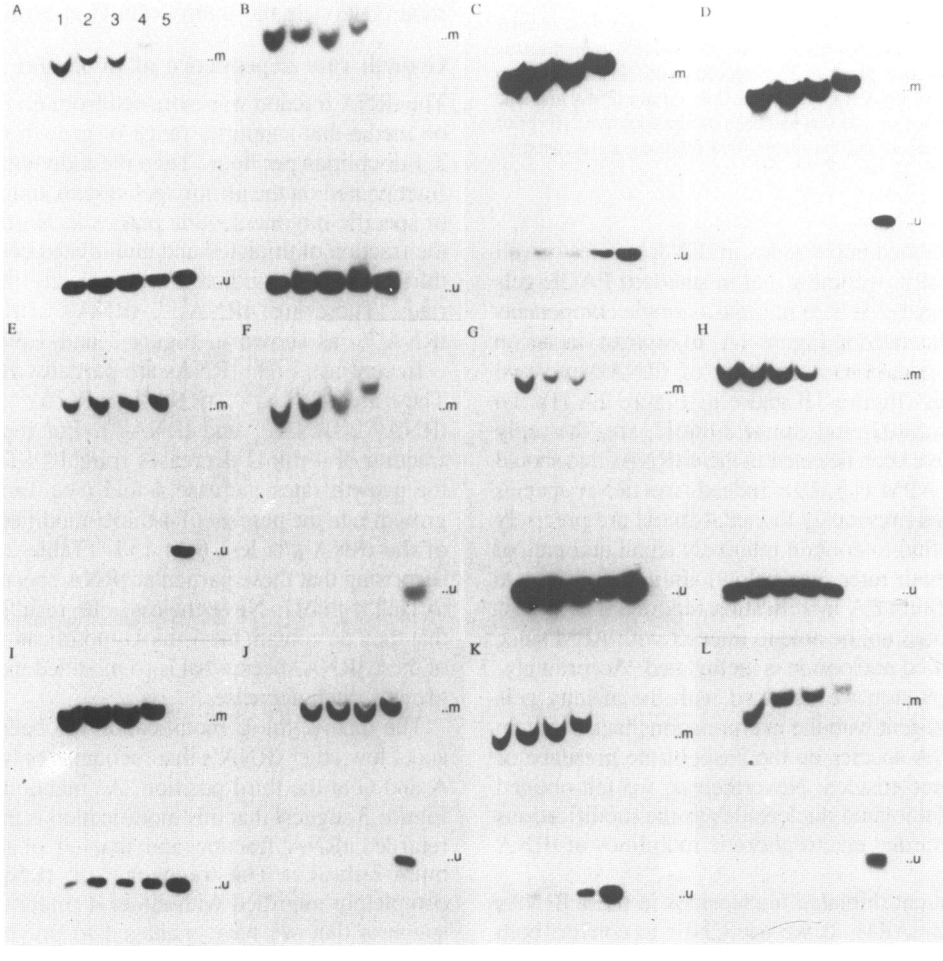

Figure 2. Twelve tRNA species that were fractionated in 10% affinity PAGE gels containing 50μM APM and hybridized with their respective oligonucleotide probes. Lanes 1, 2, 3 and 4 in each panel refer to RNA derived from the wild type strain (W1485) grown at 0.5 cell doublings per hour (dbgls/hr) , 0.8 dbgls/hr, 1.1 dbgls/hr and 2.1 dbgls/hr, respectively. Lane 5 refers to the RNA derived from the 4-thioU deficient strain (PM2). The retarded, modified tRNA is indicated by 'm' and the unmodified by 'u'. The probes applied in samples in panel **A** through panel **L** were specific for tRNA$_1^{Leu}$ (**A**), tRNA$_2^{Leu}$ (**B**), tRNA$_3^{Leu}$ (**C**), tRNA$_4^{Leu}$ (**D**), tRNA$_5^{Leu}$ (**E**), tRNA$_1^{Gly}$ (**F**), tRNA$_2^{Gly}$ (**G**), tRNA$_3^{Gly}$ (**H**), tRNA$_1^{Pro}$ (**I**), tRNA$_2^{Pro}$ (**J**), tRNA$_3^{Pro}$ (**K**) and tRNA^{Phe}(**L**).

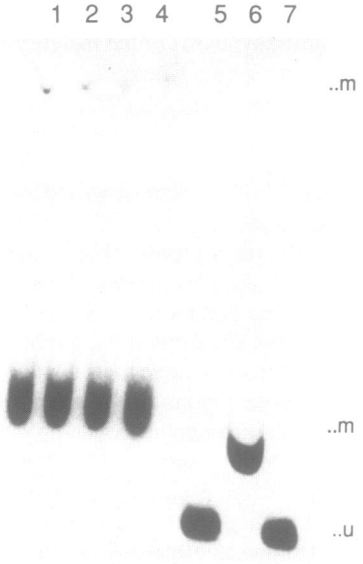

Figure 3. Northern hybridization of $tRNA_2^{Glu}$. The RNA was fractionated in an affinity PAGE gel containing 100μM APM. Lane 1 to 4 refer to RNA derived from the wild type strain (W1485) growing at the same rates as described in lanes 1 to 4 in Figure 2. Lane 5 shows RNA derived from the W1485 strain that was growing at 0.5 doublings per hour and incubated with CNBr prior to electrophoresis. Lane 6 shows RNA from the 4-thioU less strain (PM2) and lane 7 shows the PM2 derived RNA sample that had been incubated with CNBr prior to electrophoresis. The modified and the unmodified fractions are denoted by 'm' and 'u', respectively.

The presence of thiolated nucleosides in tRNA species is well established (19). Initially, we knew that in standard PAGE gels the tRNA species we analyzed here migrate as single components (13, 14); this is also illustrated in Figure 1A. In contrast, inclusion of APM in the gels occasions a splitting of tRNAs into two electrophoretic species (Figure 1B and e.g. Figure 2A,H). To this we add that 4-thioU and $mnm^5$2-thioU are the only thionucleosides that have been detected in these tRNAs that should be able to react with APM (15, 19). Indeed, the tRNA species that have been reported previously to lack 4-thioU are precisely those tRNAs that we find to contain relatively small amounts of 4-thioU at higher growth rates (see below). In addition we are persuaded that the m2-thio i^6A modification reported in $tRNA_5^{Leu}$ at position 37 (20) should not be able to interact with APM since the sulfur in this modified nucleoside is methylated. Accordingly, the patterns of fractionation we obtained with the affinity gels were superficially consistent with the interpretation that the system was fractionating tRNA species on the basis of the presence or absence of thiolated nucleosides. Nevertheless, we felt obliged to confirm the role of thiolated nucleosides as the modifications responsible for the retarded electrophoretic mobilities of tRNA species.

There are two different thiolated nucleosides in these tRNAs that can be retarded by APM. If we use CNBr to convert both the 4-thioU and the 2-thioU groups to U via a thiocyanate intermediate (16), tRNA species that originally had either one or both of these modifications will migrate in the PAGE system unhindered by APM. In contrast, tRNA from a mutant (PM2) that lacks 4-thioU can be used to identify the tRNA species that normally have 2-thioU because in the absence of the 4-thio derivative the treatment with CNBr will only influence the migration of tRNA species that contain 2-thioU. The experiments shown in Figures 1B, 2 and 3 illustrate these effects.

The data in Figure 1B show that the CNBr-treated tRNA and the tRNA derived from the 4-thioU deficient strain migrate in the same way. This implies that the thionucleoside in $tRNA_5^{Leu}$ which interacts with the APM containing gel is indeed 4-thioU. It has been suggested recently that the nucleoside at position 34 in $tRNA_5^{Leu}$ is 5-carboxymethylaminomethyl-uridine ($cmnm^5U$) (21) and that it is not as suggested earlier a 2-thioU derivative (22). Our results are in accordance with the more recent identification since we see no evidence of a retardation in the affinity gel of this tRNA species after CNBr treatment of tRNA from the 4-thioU deficient strain (see Figure 1B).

Transfer RNA_2^{Glu} is thought to contain $mnm^5$2-thioU as well as 4-thioU (19). The former thionucleoside has been shown to interact less tightly with APM than does 4-thioU (15). We note that in Figure 3 the major U shaped bands from wild type $tRNA_2^{Glu}$ appear to migrate faster than the minor, strongly interacting $tRNA_2^{Glu}$. However, the mobility of the same tRNA species from the 4-thioU deficient strain, which had been treated with CNBr migrates faster still (see Figure 3). These observations suggest that there is an additional thionucleoside in $tRNA_2^{Glu}$ that interacts more weakly than 4-thioU. We conclude that the second modification is the $mnm^5$2-thioU at position 34.

Growth rate dependence of thiolation

The tRNA fraction was extracted from *E.coli* that were cultivated on media that support a range of growth rates between 0.5 and 2.1 doublings per hour. Then the individual tRNA species were fractionated on the affinity gel system and identified with the aid of specific oligonucleotide probes in Northern blots to estimate the fraction of thiolated and unthiolated components. Five of the thirteen tRNAs studied are completely thiolated at all growth rates. These are: $tRNA_4^{Leu}$, $tRNA_5^{Leu}$, $tRNA_1^{Gly}$, $tRNA_2^{Pro}$ and $tRNA^{Phe}$, as shown in Figure 2 and Table 2.

In contrast, eight tRNAs are partially modified with 4-thioU. They are, $tRNA_1^{Leu}$, $tRNA_2^{Leu}$, $tRNA_3^{Leu}$, $tRNA_2^{Gly}$, $tRNA_3^{Gly}$, $tRNA_1^{Pro}$, $tRNA_3^{Pro}$ and $tRNA_2^{Glu}$. For these tRNA species the fraction of 4-thioU decreases roughly 4-fold on the average as the growth rates increase 4-fold (see Table 2). At the highest growth rate the percent of 4-thioU modified components for four of the tRNA's is less than 15% (Table 2). Therefore it is not surprising that these particular tRNA species have been reported to lack 4-thioU. Nevertheless, our results clearly demonstrate that they do contain the 4-thioU modification and that the percent of these tRNA species that is so modified increases as the bacterial growth rates decrease.

The $mnm^5$2-thioU modification has been detected in $tRNA_2^{Glu}$ and a few other tRNA's that recognize only codons in a box with A and G at the third position. As mentioned above, the data in Figure 3 suggest that this modification is the cause of the weakly retarded tRNA fraction and that all of the $tRNA_2^{Glu}$ contains $mnm^5$2-thioU. The recovery of tRNA species that are completely modified with either 4-thioU or with $mnm^5$2-thioU suggests that we have managed to prevent significant loss of thionucleosides that otherwise might have occurred during the preparation and fractionation of our samples.

Thiolation kinetics

We have shown previously that tRNA species in *E.coli* accumulate at different rates that depend on the bacterial growth

Table 1. Identity of the thirteen tRNA species and their accumulation rates.

tRNA (a, b)	Anticodon (5′–3′)	4-thioU data (c)	Accumulation rates ($\times 10^9$) (d) at different growth rates (doublings per hour) 0.5/hr	0.8/hr	1.1/hr	2.0/hr
Leu1 (4)	CAG	–	16	27	38	96
Leu2 (1)	GAG	–	6	7	8	9
Leu3 (1)	UAG		1	2	3	9
Leu4 (1)	CAA	+	8	8	9	9
Leu5 (1)	UAA	+	10	9	9	9
Gly1 (1)	CCC	+	6	6	6	7
Gly2 (1)	UCC	–	5	9	14	35
Gly3 (4)	GCC	–	17	27	42	116
Pro1 (1)	CGG		8	15	23	64
Pro2 (1)	UGG		7	7	6	7
Pro3 (1)	GGG		8	9	10	13
Glu2 (4)	UUC	–	9	14	23	70
Phe (2)	GAA	+	14	21	22	31

(a,b) The tRNA and/or tDNA sequences derived from Sprinzl et al. (19) and Komine et al. (23). Values in parenthesis correspond to the number of tRNA genes.
(c) The presence of 4-thioU data in the indicated tRNA species as reported by Sprinzl et al. (19). Here tRNA containing the modification is indicated by '+' and those lacking 4-thioU are indicated by '–'.
(d) The accumulation rates are expressed as number of tRNAs accumulating per cell mass and minute as described previously (13, 14).

Table 2. The degree of tRNA thiolation as a function of growth rate (doublings per hour).

tRNA (a)	% 4-thioU at different growth rates (b) 0.5/hr	0.8/hr	1.1/hr	2.1/hr	Thiolation rate ($\times 10^9$) (c) 0.5/hr	0.8/hr	1.1/hr	2.1/hr
Leu1major	60	34	28	7	10	10	11	7
Leu2	52	35	31	28	3	3	3	2
Leu3	98	94	91	85	1	2	3	8
Leu4	100	100	100	100	8	8	9	9
Leu5	100	100	100	100	10	9	9	9
Gly1	100	100	100	100	6	6	6	7
Gly2	35	15	15	3	2	2	2	1
Gly3major	70	60	50	15	12	16	21	17
Pro1major	85	76	74	40	7	11	17	26
Pro2	100	100	100	100	7	7	6	7
Pro3	93	89	87	70	7	8	9	9
Glu2	12	10	5	4	1	1	1	3
Phe	100	100	100	100	14	21	22	31

(a) Transfer RNA_1^{Leu}, $tRNA_3^{Gly}$ and $tRNA_1^{Pro}$ are the major tRNAs within the respective tRNA family.
(b) The fraction (percent) of 4-thioU of a tRNA population is measured as the radioactivity of the retarded tRNA subfraction to that of the total hybridized tRNA in the lane.
(c) Thiolation rate expressed as 4-thioU molecules accumulating per cell mass and minute.

Table 3. The kinetic parameters of the aminoacylation reaction with tRNA containing 4-thioU (+) or lacking 4-thioU (–).

tRNA (substrate) (a)	($\times 10^{-6}$) $K_M(+)$	$K_M(-)$	$v_{max}(+)$	$v_{max}(-)$
Leu1	3.9	4.1	2.7	2.6
Leu2	4.1	4.5	2.2	2.6
Leu4, 5	6.7	7.0	2.6	2.7
Gly1, 2	8.3	8.1	6.2	6.1
Gly3	4.6	4.7	3.6	4.0
Phe	3.3	3.3	1.4	1.4

(a) The tRNA species were purified by column fractionation and the identity and purity of each species was established both by aminoacylation reactions as well as by Northern hybridization with specific oligonucleotide probes (13, 14).

Table 4. The translation kinetics of tRNA containing and lacking 4-thioU.

tRNA	EF-Tu titrations k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	EF-G cycle k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
phe (+)	6.9	0.36×10^8	42.6	0.58×10^8
phe (–)	6.2	0.33×10^8	43.0	0.60×10^8

(a) The tRNAPhe either contains (+) or lacks (–) 4-thioU.
(b) The elongation factor Tu (EF-Tu) titrations were carried out as described previously (18) for a polyU-primed, *In vitro* translation system.
(c) The elongation factor G (EF-G) titrations were carried out as described previously (18) for a poly U-primed, *in vitro* translation system.

rates (13, 14). The accumulation rates for the thirteen tRNAs are summarized in Table 1; they are presented as tRNA accumulated per bacterial mass per unit time (13, 14).

When we compare the tRNA accumulation rates and the degree of thiolation for a given tRNA, the following correlations are obtained: First the tRNAs that are completely modified with 4-thioU are those that accumulate at nearly constant rates at different growth rates. These are $tRNA_4^{Leu}$, $tRNA_5^{Leu}$, $tRNA_1^{Gly}$, $tRNA_2^{Pro}$ and $tRNA^{Phe}$(see Tables 1 and 2). In contrast, the tRNAs incompletely modified with 4-thioU are those that accumulate at rates that accelerate significantly with increasing growth rates; these are: $tRNA_1^{Leu}$, $tRNA_3^{Leu}$, $tRNA_2^{Gly}$, $tRNA_3^{Gly}$, $tRNA_1^{Pro}$, $tRNA_3^{Pro}$ and $tRNA_2^{Glu}$ (see Tables 1 and 2). Indeed,

we note that as the accumulation rates increase, the fraction of the tRNA species that contain 4-thioU decreases sharply.

These correlations can be analyzed in more detail. The tRNAs that are cognate to major codons within their amino acid family, such as $tRNA_1^{Leu}$, $tRNA_3^{Gly}$ and $tRNA_1^{Pro}$ increase between 6- and 8-fold in their rates of accumulation. In parallel, the fraction of these tRNA species that have 4-thioU decreases between 7- and 2-fold within the same range of growth rates. The cellular levels of $tRNA_1^{Leu}$ and $tRNA_3^{Gly}$ are always higher than the expression levels of $tRNA_1^{Pro}$. This may explain why $tRNA_1^{Pro}$ has proportionally higher levels of 4-thioU than $tRNA_1^{Leu}$ and $tRNA_3^{Gly}$ do, even though its rate of accumulation accelerates slightly faster than the other tRNA's. Similarly, the accumulation rate for $tRNA_3^{Leu}$ accelerates as the growth rates increase but this tRNA is always a relatively rare species (13). We believe that this is the reason that $tRNA_3^{Leu}$ has relatively high levels of 4-thioU with the lowest level close to 85% at the highest growth rates (see Tables 1 and 2). Accordingly, we suggest that the degree of thiolation is for tRNA species with 4-thioU nucleosides inversely related to the amounts of that tRNA in the bacterium at any particular growth rate. The only exception to this correlation that we have found so far is $tRNA_2^{Leu}$. This species is only partially thiolated (see Table 2) but it accumulates at roughly constant rates at all growth states (Table 1). We have no explanation for the exceptional behaviour of this tRNA.

It is possible to calculate from the fraction of each tRNA species that contains 4-thioU and from the specific accumulation rate of that tRNA (13, 14), the rate of thiolation for each tRNA species as the number of tRNAs thiolated per cell mass per unit time (Table 2). In addition, we can use these figures to calculate the total rate of thiolation per cell mass per unit time at different growth rates. We find that the average rate of thiolation expressed as 4-thioU molecules per cell mass and unit time to be at 0.5 cell doublings per hour (dblgs/hr) = 7×10^9; at 0.8 dblgs/hr = 8×10^9; at 1.1 dblgs/hr = 9×10^9; at 2.1 dblgs/hr = 10×10^9. Thus, the accumulation rate of 4-thioU in tRNA is very nearly constant over a 4-fold range of growth rates. Furthermore, this nearly constant rate is very similar to the accumulation rate of a minor tRNA (see Table 1). In contrast, the accumulation rates of major tRNA isoacceptor species increase as much as 8-fold in the same growth rate range (see Table 1).

Influence of 4-thioU on translation

It is possible to determine the influence of the 4-thioU modification on the kinetics of the aminoacylation reaction by titrating with a particular tRNA from normal as well as from 4-thioU deficient (PM2) cells, under identical aminoacyl-tRNA synthetase limiting conditions. We have in this way measured the maximum turnover rate (v_{max}) and the concentration of tRNA at which half the v_{max} is obtained (K_M) for thiolated and unthiolated tRNA species. As shown in Table 3 there are no significant differences in these parameters between tRNA that contains 4-thioU and the same tRNA that lacks the modified nucleoside.

We also did titration experiments using $tRNA^{Phe}$ that either contained or lacked 4-thioU in a poly(U)-primed translation system as described by Ehrenberg et al. (18). Here, we could determine the influence of the lack of 4-thioU on the different steps in the tRNA cycle in translation. Neither the EF-Tu cycle nor the EF-G cycle was affected by the lack of this modification (see Table 4).

DISCUSSION

Our data show that growth rates affect the distribution of the thionucleoside 4-thioU in bacterial tRNA populations. The level of 4-thioU of some tRNA species decreases sharply as growth rates increase, while others are completely modified with 4-thioU at all growth rates. In particular, tRNA species which accumulate at rates that accelerate with increasing growth rates are only partially thiolated. Conversely, tRNAs that accumulate at nearly constant rates are almost always completely thiolated. In general, tRNAs that are expressed at low levels contain higher levels of 4-thioU than tRNAs expressed at high levels. Finally, there seems to be a near constant total rate of modification of tRNA by 4-thioU per mass of cell at all the growth rates that we studied.

Only a small fraction of $tRNA_2^{Glu}$ is modified with 4-thioU but virtually all of this tRNA contains either $mnm^5$2-thioU or 2-thioU at all growth rates. The uncertainty arises from the fact that we can not exclude the possibility that there is an incomplete methylation of the 2-thioU group. Thus, we only detect the presence of the sulfur in tRNA and thiolation precedes the methylation of this modified nucleoside (1). Nevertheless, it is clear from the data obtained for $tRNA_2^{Glu}$ that the expression of these two thionucleosides are regulated independently.

We compared eight tRNA species containing and lacking the 4-thioU group with respect to their kinetic characteristics in their respective aminoacylation reactions. In addition, we studied the kinetics of poly(Phe) synthesis mediated by $tRNA^{Phe}$ containing and lacking 4-thioU. We have failed to detect any functional differences in translation between tRNA containing the 4-thioU modification and tRNA lacking this nucleoside. Furthermore, the growth rate characteristics of the PM2 strain which lacks 4-thioU are not different from that of the parental strain which utilizes this modification (5). Altogether, these observations persuade us that the role of 4-thioU is not to be found in translation. Indeed, it has been suggested the principle function of 4-thioU is in photoprotection of bacteria (5). It is still a mystery why the relative amounts of this modification in the tRNA should be lower at fast growth rates than at slow growth rates.

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