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Role of ribothymidine in the thermal stability of transfer RNA as monitored by proton magnetic resonance

Pari Davanloo[+], Mathias Sprinzl[+*], Kimitsuna Watanabe[†], Martin Albani[**] and Helga Kersten[**]

[+]Max-Planck-Inst. für experimentelle Med., Abt. Chem., Hermann-Rein-Str. 3, D-3400 Göttingen, GFR, [†]Mitsubishi-Kasei Inst. Life Sci., 11 Minamiooya, Machida-shi, Tokyo-to, 194, Japan, and [**]Inst. Physiol. Chem., Univ. Erlangen-Nürnberg, Wasserturmstr. 5, D-8520 Erlangen, GFR

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ABSTRACT

In order to elucidate the functional role of the modified uridines at position 54 of tRNA, the 270 MHz high-field proton NMR spectra of methionine tRNAs from E.coli, from a mutant thereof, and from T. thermophilus, containing ribothymidine, uridine and 2-thioribothymidine, respectively, have been measured as a function of temperature. A comparison of the NMR melting profiles of the minor nucleosides from these tRNAs shows that the melting temperature of the uridine containing tRNA is 6°C lower than that of the wild type tRNA whereas that of the 2-thioribothymidine tRNA is 7°C higher than that of the wild type tRNA. These results, therefore, demonstrate that these modifications serve for stabilization of the tertiary structure of tRNA.

INTRODUCTION

An interesting feature of tRNA structure is the occurence of minor nucleosides which are located at specific positions of the sequence[1,2]. Their functional role in tRNA is not yet understood. Among the minor nucleosides ribothymidine (T) and pseudouridine (Ψ) which are present in all procaryotic tRNAs at positions 54 and 55, are of interest because they are probably involved in the binding of tRNA to ribosome[3]. However, Ofengand et al.[4] have shown that Escherichia coli $tRNA_1^{Val}$ in which ribothymidine and pseudouridine are replaced by 5-fluorouridine can function in aminoacylation and in *in vitro* protein synthesis as effectively as the native tRNA. While the E. coli mutant tRNA in which the ribothymidine is completely replaced by uridine behaves identically to the wild type tRNA with regard to *in vitro* aminoacylation[5] and poly U-directed synthesis of polyphenylalanine[5,6], there is a distinct advantage of cells having ribothymidine in their tRNA over cells lacking ribothymidine under *in vivo* conditions[7]. Furthermore, the replacement of uridine in position 54 by ribothymidine in some eukaryotic tRNAs significantly influences their efficiency in *in vitro* protein synthesis[8,9].

Recent investigations on the tRNAs isolated from an extreme thermophilic

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bacterium, Thermus thermophilus HB8, containing 2-thioribothymidine in position 54, have demonstrated[10,11] that there is a linear correlation between the thermal stability of tRNA and the modification at position 54.

In the experiments reported in this communication we have attempted to elucidate the functional significance of residue 54 regarding its involvement in the stabilization of tRNA structure. For this purpose we chose E. coli $tRNA_1^{fMet}$(T54), an E. coli $tRNA_1^{fMet}$(U54) from the strain Trm^- in which ribothymidine is completely replaced by uridine, and T. thermophilus $tRNA^{fMet}$ (s^2T54) [abbreviated as $tRNA^{fMet}$(T54), $tRNA^{fMet}$(U54), $tRNA^{fMet}$(s^2T54)] and studied the proton NMR of the minor nucleosides in the high-field spectral region[12,13]. The NMR melting temperatures (T_m^{NMR}) of the minor nucleosides are related to the melting temperatures as measured by an optical method (T_m^{OM}) since both techniques reflect unstacking of bases. By the NMR technique, however, it is possible to measure directly the structural changes in the position where the monitored residue is located. Hence we have compared the T_m^{NMR} of the minor nucleosides in these tRNAs. Our results show that the modification of uridine at position 54 of tRNA is an important contribution to the thermal stability of the tRNA tertiary structure.

MATERIALS AND METHODS

(a) tRNA Samples

T. thermophilus $tRNA^{fMet}$ was purified as described elsewhere[14]. Its acceptor activity was 1615 pmol methionine/A_{260} unit tRNA. E.coli $tRNA_1^{fMet}$ was isolated from bulk E. coli MRE 600 tRNA obtained from Boehringer Mannheim, GFR, using standard purification procedure[15]. E. coli $tRNA_1^{fMet}$ was further purified by chromatography on an RPC 5 column[16]. It accepted 1450 pmol methionine/A_{260} unit tRNA. E. coli Trm^- mutant $tRNA^{fMet}$ was purified by the same procedure from bulk E. coli Trm^- tRNA and had an acceptor activity of 1500 pmol methionine/A_{260} unit tRNA. The mutant strain (a kind gift of Dr. Björk) was grown essentially as described[17]. The nucleoside composition of all tRNA species was determined by a chromatographic method described by Uziel et al.[18] and corresponded to the sequence data.

(b) Sample Preparations and NMR Measurements

The NMR samples were prepared by extensive dialysis first against 10 mM EDTA (ethylenediaminetetraacetic acid) in 100 mM KCl, 10 mM potassium phosphate pH 6.6 and then several times against 10 mM $MgCl_2$, 100 mM KCl, and

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10 mM potassium phosphate pH 6.6. The samples (0.3 ml) were then lyophilized, dissolved in 99 % D_2O (Merck, Darmstadt, GFR), lyophilized again, redissolved in 0.3 ml of 99.9 % D_2O and transferred to a wilmed 5 mm NMR tube.

The proton NMR spectra were measured on a Bruker WH270 MHz spectrometer operating in fourier transform mode (90° pulse, 2.7 s acquisition time, 5 s pulse delay and quadrature phase detection). Temperature was measured before and after each run using the chemical shift difference between the methylene and hydroxyl protons of ethylene glycol[19]. The field was locked to D_2O in the solvent. Chemical shifts are reported with respect to DSS (2,2-dimethyl-2-silapentane-5-sulfonate).

RESULTS

E. coli $tRNA_1^{fMet}$(T54) contains (besides 4-thiouridine) four minor nucleosides: 5,6-dihydrouridine (D20), 2'-O-methylcytidine (Cm32), N^7-methylguanosine (m^7G46) and 5-methyluridine (T54) (Fig. 1). Hence, there are four reporter groups which can be studied in the high-field proton NMR region: in the dihydrouridine loop, anticodon loop, variable loop and T-Ψ-C loop. The 270 MHz proton NMR spectrum of this tRNA molecule in the presence of Mg^{2+} measured at 45°C is compared with that of $tRNA^{fMet}$(U54) in Fig. 2. The assignment of the resonances is shown above the peaks. They are based on the spectra of yeast $tRNA^{Phe}$ and the spectra of the free minor nucleosides[12]. The resonances

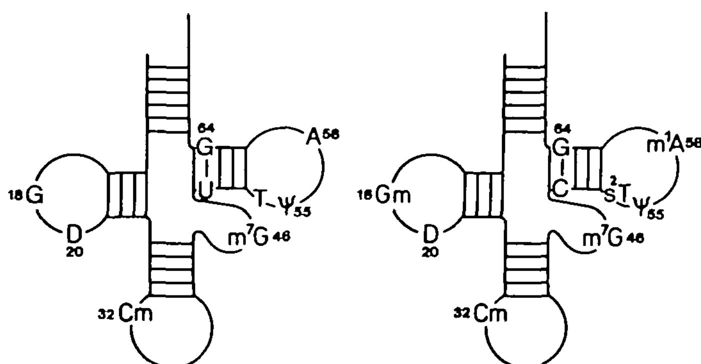

Fig. 1:

The minor nucleosides and their location in the cloverleaf structures of E. coli $tRNA_1^{fMet}$(T54) (left) and T. thermophilus $tRNA^{fMet}$(s^2T54) (right). In addition, the bases which are different in two tRNAs are indicated. In E. coli Trm^- $tRNA^{fMet}$ the T54 is replaced quantitatively by U54.

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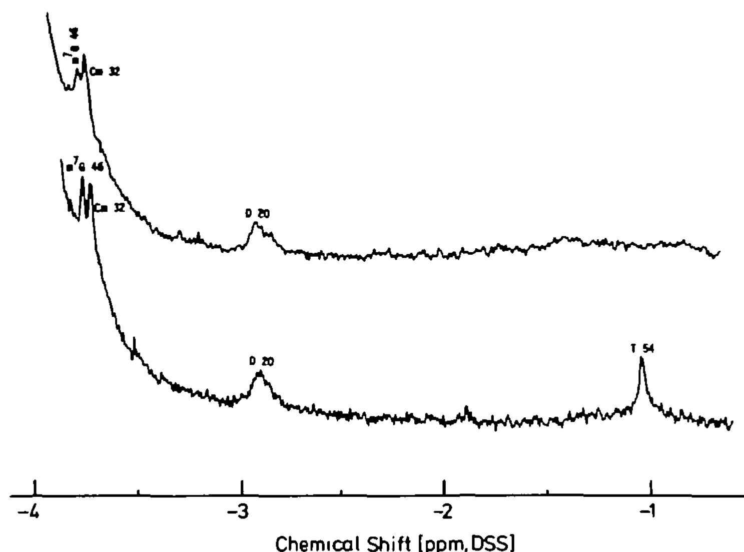

Fig. 2:

270 MHz proton NMR spectra, in the high-field region, of 0.51 mM E. coli $tRNA^{fMet}$(T54) (lower spectrum) and 0.51 mM E. coli Trm^{-} $tRNA^{fMet}$(U54) (upper spectrum) at 45°C. The solution conditions are 10 mM Mg^{2+}, 100 mM KCl, and 10 mM potassium phosphate at pH 6.6 in D_2O.

of the nucleosides T54, D20 and m^7G46 exhibit a sharp transition in a cooperative manner with T_m^{NMR} values around 82°C for all three resonances (Fig. 3a). The nucleoside Cm32 is strongly stacked in the anticodon loop and experiences an upfield shift of 0.29 ppm through the temperature range of 30° to 95°C. The nucleoside m^7G46 undergoes a downfield shift of 0.19 ppm upon unfolding. This value is in good agreement with the calculated ring current shielding[12] (0.21) for the m^7G from its neighbouring nucleosides in yeast $tRNA^{Phe}$.

The high-field NMR spectrum of the $tRNA^{fMet}$(U54) is the same as that of the wild type $tRNA^{fMet}$(T54) except that the resonance belonging to T54 is not present (Fig. 2). The nucleosides D20 and m^7G46 in $tRNA^{fMet}$(U54) show a temperature transition with T_m^{NMR} of about 76°C, 6°C lower than for $tRNA^{fMet}$ (T54) (Fig. 3b). The methyl resonance from the nucleoside m^7G in ribothymidine-deficient $tRNA^{fMet}$ also exhibits a broader temperature transition.

In T. thermophilus $tRNA^{fMet}$(s^2T54) the minor nucleosides occur in the dihydrouridine loop (Gm18, D20), in the anticodon loop (Cm32), in the variable loop (m^7G46) and in the T-Ψ-C loop (s^2T54, m^1A58) (Fig. 1). The 270 MHz

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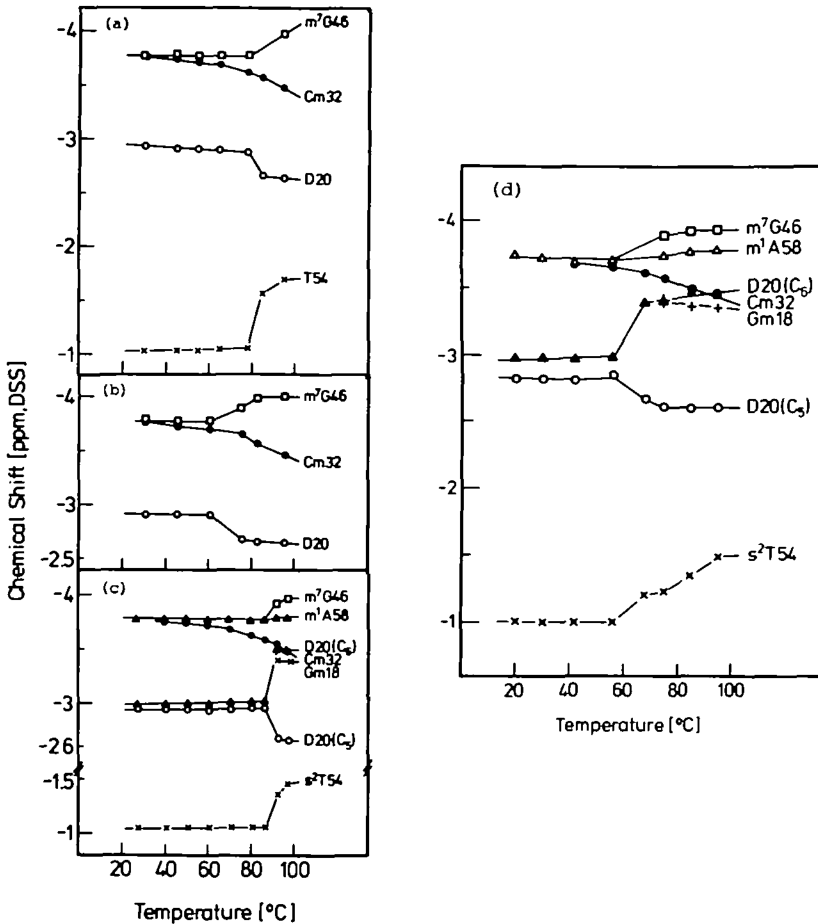

Fig. 3:

The chemical shifts of the methyl and methylene proton resonances from the minor nucleosides in: (a) 0.51 mM E. coli $tRNA_1^{fMet}$ (T54); (b) 0.51 mM E. coli Trm^- $tRNA^{fMet}$ (U54); (c) 0.57 mM T. thermophilus $tRNA^{fMet}$ (s^2T54) (all in the presence of 100 mM KCl, 10 mM $MgCl_2$ and 10 mM potassium phosphate buffer pH 6.6 in D_2O); (d) 0.57 mM T. thermophilus $tRNA^{fMet}$ (s^2T54) (in the presence of 200 mM KCl, no Mg^{2+}, and 10 mM potassium phosphate, at pH 6.6 in D_2O) as a function of temperature.

spectra of $tRNA^{fMet}$(s^2T54) were taken at various temperatures (Fig. 4) and the assignment of each signal was based on the spectra of yeast $tRNA^{Phe}$ [12,13] and E. coli $tRNA_1^{fMet}$ (Figs. 2, 3a). All resonances in the spectrum of $tRNA^{fMet}$(s^2T54) are well separated. The resonance at 1.04 ppm is assigned to

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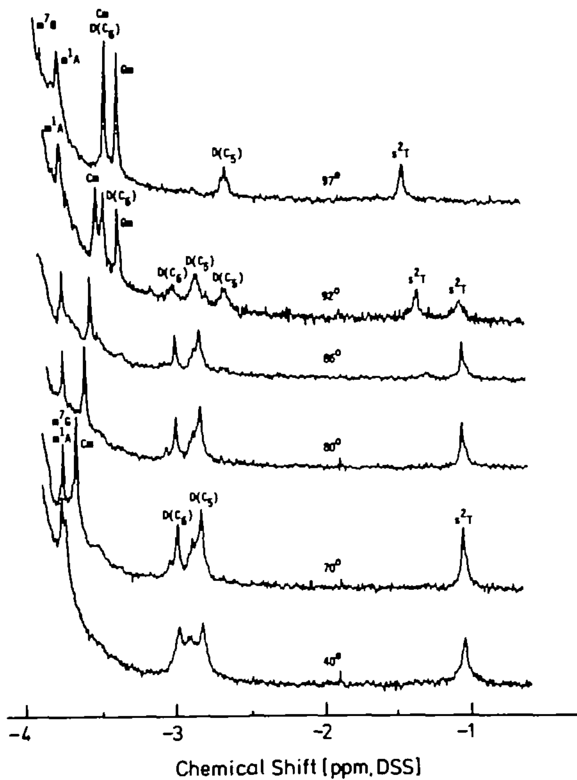

Fig. 4:

270 MHz proton NMR spectra, in the high-field region, of 0.57 mM T. thermophilus $tRNA^{fMet}$ (s^2T54) at various temperatures. The solution conditions are indicated in the legend to Fig. 2.

the 5-methyl group of s^2T54. This peak starts to shift at 86°C and at 97°C is at 1.44 ppm. The T_m^{NMR} of this transition is at 89°C. The C_5 and C_6 methylene protons of D20 are at 2.84 and 3.0 ppm, respectively. On melting of tRNA the D20(C_5) and D20(C_6) resonances move to 2.64 and 3.46 ppm, respectively, with a narrow cooperative transition T_m^{NMR} of 89°C (Fig. 3c). The Gm18 resonance is only visible when tRNA starts to melt, i.e. above 86°C. Since the Gm18 residue is involved in a hydrogen-bond pair with the C56 residue according to the three-dimensional structure[20] of $tRNA^{Phe}$ it is plausible that the methyl resonance from this residue at lower temperature has been broadened or superimposed with other peaks and therefore is not visible.

In the spectrum of E. coli $tRNA_1^{fMet}$ lacking m^1A58 modification, a reson-

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ance could be observed at 3.79 ppm at low temperature which was assigned to m^7G46. This peak moved to 3.98 ppm at the melting temperature (Figs. 2,3a). Hence, we suggest that in the $tRNA^{fMet}(s^2T54)$ which contains the nucleosides m^7G46 and m^1A58, the resonances from these nucleosides are superimposed at low temperature at 3.79 ppm. Then, at the melting temperature, the m^7G46 moves to 3.97 ppm which indicates a downfield shift of 0.18 ppm upon unfolding. This observed shielding value of 0.18 ppm for m^7G46 of $tRNA^{fMet}(s^2T54)$ is slightly lower than that observed for m^7G46 (0.22 ppm) in yeast $tRNA^{Phe}$. In the crystal structure[20] of yeast $tRNA^{Phe}$, the m^7G46 is stacked between the A21 on one side and the A9 on the other. By analogy, the stacking interaction for the m^7G46 of $tRNA^{fMet}(s^2T54)$ should be between the A21 and G9. The differences in the stacking interaction between the nucleosides m^7G and A9 in the yeast $tRNA^{Phe}$ as compared to the stacking of m^7G and G9 stacking in the $tRNA^{fMet}(s^2T54)$ may explain the slight difference in the shielding value of m^7G that we have observed in these two tRNAs. Saleminк et al.[21] have also shown by NMR in the low field spectral region that the m^7G46·G23·C13 triple interaction in E. coli $tRNA_1^{fMet}$ occurred at about 1.1 ppm lower field than in yeast $tRNA^{Phe}$ and they have attributed this chemical shift difference to structural variation in the two tRNAs.

The temperature dependences of the chemical shifts of the minor nucleosides in $tRNA^{fMet}(s^2T54)$ in the absence of Mg^{2+} were also measured (Fig. 3d). In contrast to the experiment performed in the presence of Mg^{2+} containing buffer (Fig. 3c), the melting profile for the s^2T54 now exhibits two transitions. The first transition takes place between 55°C and 75°C with T_m^{NMR} = 62°C, in which the 5-methyl resonance moves from 1.0 ppm to 1.2 ppm. The second transition occurs between 75°C and 97°C in which s^2T moves further to 1.5 ppm. The resonances at 1.2 and 1.5 ppm represent the nucleoside s^2T54 in the environments where the tertiary structure and the T-Ψ-C stem, respectively, have melted. The C_5 and C_6 methylene resonances of D20 melt in a single cooperative transition at the same T_m^{NMR} (62°C) as the first melting transition of s^2T54. The m^7G46 resonance shows a broad transition with T_m^{NMR} of 68°C. All T_m values are compiled in Table 1.

Our observation that the thermal transition of D20(C_5,C_6) resonances and the first thermal transition of s^2T54 take place at the same temperature might reflect the simultaneous melting of the dihydrouridine stem and the tertiary interactions. This result is consistent with the temperature jump melting study of E. coli $tRNA_1^{fMet}$, reported by Crothers et al.[22], in which the dihydrouridine stem helix melts when the tertiary structure unfolds.

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Table 1:

Comparison of the NMR melting transition of the minor nucleosides in E. coli $tRNA^{fMet}$(T54), E. coli Trm⁻ $tRNA^{fMet}$(U54) and T. thermophilus $tRNA^{fMet}$(s^2T54)

$tRNAs^{fMet}$	T_m^{NMR} (°C)			T_m^{OM} (°C)
	m^7G46	D20 (C_5,C_6)	Nucleoside 54	
E. coli (T54) with Mg^{2+}	82[a]	82[a]	(T54) 82[a]	83[a]
E. coli Trm⁻ (U54) with Mg^{2+}	76[a]	76[a]	(U54) can not be seen in NMR	not determined
T. thermophilus (s^2T54) with Mg^{2+}	89[a]	89[a]	(s^2T54) 89[a]	88[c]
T. thermophilus (s^2T54) without Mg^{2+}	68[b]	62[b]	(s^2T54) first transition 62[b]	74.3[d]

The T_m^{OM} data are taken from the reports by Oshima et al.[23] and Watanabe et al.[14]. The buffer systems used are: (a) 100 mM KCl, 10 mM Mg^{2+}, and 10 mM potassium phosphate at pH 6.6 in D_2O; (b) 200 mM KCl, no Mg^{2+}, and 10 mM potassium phosphate at pH 6.6 in D_2O; (c) 10 mM Mg^{2+}, 200 mM NaCl, 10 mM Tris-HCl at pH 7.5; (d) 200 mM NaCl and 10 mM Tris-HCl at pH 7.5.

DISCUSSION

The methyl and methylene resonances from the minor nucleosides T, s^2T, D and m^7G in E. coli $tRNA_1^{fMet}$(T54), E. coli Trm⁻ $tRNA^{fMet}$(U54) and T. thermophilus $tRNA^{fMet}$(s^2T54) experience large chemical shifts upon unfolding of tRNA. Hence they are sensitive probes for analysis of structural differences which affect the stability of the tertiary structure of tRNAs. A comparison of T_m^{NMR} values of the minor nucleosides of $tRNA_1^{fMet}$(T54) and $tRNA^{fMet}$(U54) which differ only in the methylation of nucleoside 54 in T-Ψ-C loop, reveals that the tRNA structure is less stable in methyl deficient $tRNA^{fMet}$(U54), the T_m^{NMR} value being 6°C lower than that for wild type E. coli $tRNA^{fMet}$(T54). On the other hand the nucleoside T54 in $tRNA^{fMet}$(T54) exhibits a 7°C lower

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transition than the nucleoside s^2T54 in T. thermophilus $tRNA^{fMet}$(s^2T54). Therefore this observation implies that the modification of the T54 to s^2T54 is an important contributing factor to the high thermal stability of the T. thermophilus tRNAs. However, beside the s^2T54 modification, the modification of G18 to Gm18 in T. thermophilus $tRNA^{fMet}$ has also to be considered as a factor contributing to thermal stability, since this nucleoside is also expected to participate in the tertiary interaction. Taking into account that except for the U50·G64 pair in E. coli $tRNA_1^{fMet}$ which is replaced by the C50·G64 pair in T. thermophilus, the basepairing schemes in both tRNAs are the same (Fig. 1), the significant difference between the termal stabilities of the two tRNAs must be interpreted in terms of the tertiary interactions. In the three dimensional structure[20] of yeast $tRNA^{Phe}$ the dihydrouridine loop and T-Ψ-C loop are connected through G19·C56 and G18·Ψ55 pairs. The T54 forms a hydrogen bonded pair with m^1A58 which is sandwiched between the base-pairs Ψ55 · G18 and G53·C61. Since the three nucleosides U54, G18 and A58 are directly involved in the stabilization of tertiary structure of the tRNA molecule, it is therefore conceivable that the modifications of these nucleosides change their preferred stacking interaction, and might be responsible for the increased thermal stability of the tertiary structure.

It has been reported[24] that the 2-thio modification of uridine increases the stacking properties of these bases in poly(s^2U) and is therefore a major contributing factor to high thermal stability of poly(s^2U) as compared to poly(U). It is plausible that a modification of T54 to s^2T54 will have a similar effect i.e. it will increase the stacking interactions and stabilize the tertiary structure of tRNA. Similarly the modification of G18 to Gm18 may affect the strength of stacking interactions. It was reported by Maelicke et al.[25] that the Gm34 residue occurring in the anticodon of $tRNA^{Phe}$ from yeast increases the stability of the stack in which the anticodon is involved.

Finally, pairs of Trm^- mutant and wild type $tRNA^{fMet}$ from E. coli which differ only in U54 methylation are suitable for a comparative study of the function of ribothymidine 54 in tRNAs. Our results clearly imply that the presence of T54 increases the thermal stability of E. coli $tRNA^{fMet}$ over the Trm^- mutant tRNA which lacks the modification of the nucleoside. In the case of T. thermophilus and E. coli $tRNA^{fMet}$, having three minor nucleoside differences (Gm, s^2T and m^1A), our results indicate that the s^2T as well as the Gm and m^1A might be important in increasing the thermal stability of T. thermophilus over E. coli $tRNA^{fMet}$.

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DEFINITION

1 A_{260} unit is the amount of material which, dissolved in 1 ml aqueous solution, has an absorption of 1.0 using 1-cm-path-length cell. The numbering of nucleoside residues in the tRNA sequence is according to Sprinzl et al.[2].

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