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Two $tRNA_1^{Ile}$ Species from an Extreme Thermophile, *Thermus thermophilus* HB8: Effect of 2-Thiolation of Ribothymidine on the Thermostability of tRNA†

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ABSTRACT: From *Thermus thermophilus* HB8 grown at 65 °C, two major $tRNA^{Ile}$ species have been purified by column chromatography and polyacrylamide gel electrophoresis. The nucleotide sequence of one of these two $tRNA_1^{Ile}$ species ($tRNA_{1a}^{Ile}$) has been determined to be pGGGCGAUUAGCUCAGCUGmGUDA-GAGCGCACGCCUGAUt^{6}AAGCGUGAGm^{7}GUCGGUGGs^{2}TΨCAm^{1}AGUCCACCAUCGCCCA-CCA_{OH}. The nucleotide sequence of the other species ($tRNA_{1b}^{Ile}$) is found to be the same as that of $tRNA_{1a}^{Ile}$ except for the modification in position 54; $tRNA_{1a}^{Ile}$ has s^{2}T(54) while $tRNA_{1b}^{Ile}$ has T(54). The melting temperature of $tRNA_{1a}^{Ile}$ is as high as 86.2 °C while that of $tRNA_{1b}^{Ile}$ is 83.3 °C. The single replacement of an oxygen atom (2-carbonyl oxygen) of T(54) by a sulfur atom significantly contributes to the thermostability of the $tRNA_{1a}^{Ile}$ species. In addition, the methylation of G(18) and A(58) possibly contributes to the thermostability of *T. thermophilus* $tRNA_{1a}^{Ile}$ and $tRNA_{1b}^{Ile}$ species.

The organism *Thermus thermophilus* HB8 is an extremely thermophilic bacterium that grows in the temperature range 50–85 °C. Several tRNA species have been purified from *T. thermophilus* HB8 and found to be more thermostable than the corresponding tRNA species from *Escherichia coli* (Oshima et al., 1976; Watanabe et al., 1976a, 1980; Davanloo et al., 1979). Among those tRNA species from *T. thermophilus* HB8, $tRNA_{f1}^{Met}$ and $tRNA_{f2}^{Met}$ have been sequenced and found to include 2-thioribothymidine (s^{2}T)[1] and 1-methyladenosine (m^{1}A) in the TΨC loop and 2′-*O*-methylguanosine (Gm) in the D loop (Watanabe et al., 1979a). These modified nucleosides have also been found in other tRNA species from extreme thermophiles (Watanabe et al., 1980). Among these three modifications, 2-thiolation of ribothymidine (T) in the TΨC loop has been anticipated to be largely responsible for the thermostability of extreme thermophile tRNA species (Oshima et al., 1976; Watanabe et al., 1976a,b, 1979a, 1980, 1983; Davanloo et al., 1979). In order to examine the contribution of the 2-thiolation itself for the thermostability, however, it is necessary to obtain a pair of tRNA species with the same nucleotide sequence except for the modification in position 54 (T or s^{2}T) [the numbering is in accord with Gauss & Sprinzl (1984)].

We have now succeeded in the isolation of such a pair of tRNA species (major $tRNA^{Ile}$ species) from *T. thermophilus* HB8; one species ($tRNA_{1a}^{Ile}$) has s^{2}T(54) while the other species ($tRNA_{1b}^{Ile}$) has T(54). These two tRNA species also

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[1] Abbreviations: acp^{3}U, 3-(3-amino-3-carboxypropyl)uridine; D, dihydrouridine; Gm, 2′-*O*-methylguanosine; m^{1}A, 1-methyladenosine; m^{7}G, 7-methylguanosine; Ψ, pseudouridine; s^{2}T, 2-thioribothymidine; s^{4}U, 4-thiouridine; T, ribothymidine; t^{6}A, *N*^{6}-(threoninocarbonyl)adenosine; HPLC, high-performance liquid chromatography; IleRS, isoleucyl-tRNA synthetase; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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have Gm(18) and $m^1A(58)$ in common. The melting temperature of $tRNA_{1a}^{Ile}$ is found to be 3 °C higher than that of $tRNA_{1b}^{Ile}$. Thus, the single replacement of an oxygen atom (2-carbonyl oxygen) of T(54) by a sulfur atom significantly enhances the thermostability of extreme thermophile tRNA.

Materials and Methods

Materials. Uniformly ^{14}C-labeled amino acids were obtained from New England Nuclear. DEAE-Sephadex A-50 and Sepharose 4B were purchased from Pharmacia Fine Chemicals, and BD-cellulose was from Boehringer Mannheim. Isoleucyl-tRNA synthetase (IleRS) (EC 6.1.1.5) was purified from *T. thermophilus* HB8 as described previously (Kohda et al., 1984). Bovine pancreatic ribonuclease (EC 3.1.27.5) and *E. coli* phosphomonoesterase (EC 3.1.3.1) were obtained from Sigma, and snake venom phosphodiesterase I (EC 3.1.4.1) was from Worthington Biochemicals.

Purification of $tRNA_1^{Ile}$ Species. Crude tRNA was prepared, by the procedure of Zubay (Zubay, 1962), from cells of *T. thermophilus* HB8 grown at 65 °C. Fractionation of tRNA was performed by chromatography on columns of DEAE-Sephadex A-50 (Nishimura et al., 1967; Yoshida et al., 1971), BD-cellulose (Gillam et al., 1967), and Sepharose 4B with a reverse salt gradient (Holmes et al., 1975). A BD-cellulose column was equilibrated with 0.35 M NaCl in 0.02 M sodium acetate buffer (pH 6.0), and elution of tRNA was performed with a linear gradient of NaCl (from 0.36 to 1.5 M) in the same buffer. The aminoacylation of tRNA was assayed as reported before (Nishimura et al., 1967; Watanabe et al., 1976a; Kohda et al., 1984). Two-dimensional polyacrylamide gel electrophoresis (Ikemura & Dahlberg, 1973) was performed for preparing $tRNA_1^{Ile}$ species with a polyacrylamide gel 2 mm thick and 40 cm long. After the first electrophoresis (500 V, 7 h) of a 10% polyacrylamide gel, the only band detectable by UV absorption was cut out from the gel and embedded at the top of a 20% polyacrylamide gel for succeeding electrophoresis. After the second electrophoresis at 500 V for 20 h, each of two tRNA bands was cut out from the gel and homogenized with a solution containing 0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1% sodium dodecyl sulfate, and 0.1 mM EDTA. The extracted tRNA species were precipitated by the addition of 2.5 volumes of ethanol.

Characterization of $tRNA_1^{Ile}$ Species. The base compositions of tRNA species were analyzed by high-performance liquid chromatography (HPLC) with an M&S Pack C_{18} column (4.6 mm × 15 cm) on a Waters HPLC system. Isocratic elution was performed with 0.05 M $NH_4H_2PO_4$ (pH 5.1) containing 5% (v/v) methanol at a flow rate of 0.8 mL/min. The 400-MHz ^1H NMR spectra of *T. thermophilus* $tRNA_1^{Ile}$ species in ^2H_2O solution containing 0.1 M NaCl and 0.01 M $MgCl_2$ at 25 °C were recorded with a Bruker WM-400 spectrometer.

Determination of Nucleotide Sequences of $tRNA_1^{Ile}$ Species. The sequences of $tRNA_1^{Ile}$ species were determined by the postlabeling method (Kuchino et al., 1981; Nishimura & Kuchino, 1983). tRNA species were treated with formamide, and the 5′ termini of degradation fragments were labeled with ^{32}P. The labeled fragments were digested by nuclease P_1. The 5′-^{32}P-labeled mononucleotides thus prepared were identified by two-dimensional thin-layer chromatography (5 cm × 5 cm) with solvent system I [first dimension, isobutyric acid/concentrated ammonia/water (50:1:29 v/v/v); second dimension, 2-propanol/concentrated HCl/water (70:15:15 v/v/v)]. Furthermore, for positions 52–56 of tRNA species, the identification of 5′-^{32}P-labeled nucleotides was further confirmed by two-dimensional thin-layer chromatography (10 cm × 10 cm) with solvent system I and with solvent system II [first dimension, isobutyric acid/concentrated ammonia/water (66:1:33); second dimension, 100 mL of 0.1 M sodium phosphate buffer (pH 6.8) containing 2 mL of 1-propanol and 60 g of ammonium sulfate] (Nishimura & Kuchino, 1983; Godson, 1983).

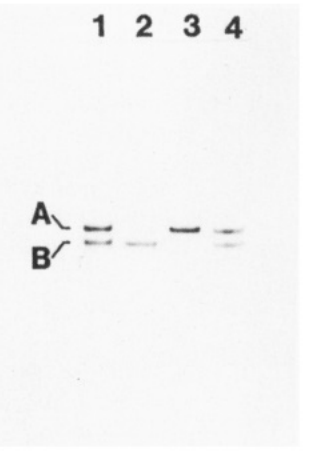

FIGURE 1: The 20% polyacrylamide gel electrophoresis of $tRNA_1^{Ile}$ species: (lane 1) $tRNA_1^{Ile}$ preparation as purified by column chromatography; (lanes 2 and 3) $tRNA_{1b}^{Ile}$ and $tRNA_{1a}^{Ile}$ species as isolated by preparative polyacrylamide gel electrophoresis, where band A is due to the $tRNA_{1a}^{Ile}$ species [with $s^2T(54)$] and band B to the $tRNA_{1b}^{Ile}$ species [with T(54)]; (lane 4) the mixture of $tRNA_{1a}^{Ile}$ and $tRNA_{1b}^{Ile}$ species.

Measurement of Melting Temperatures of $tRNA_1^{Ile}$ Species. For measurement of melting temperature, each tRNA species as extracted from the gel (0.8–1.0 A_{260} unit) was dialyzed 3 times, each against 1 L of a solution of 0.01 M Tris-HCl (pH 7.5) containing 0.2 M NaCl and 0.01 M magnesium acetate at 4 °C. tRNA species as prepared by this procedure have been found to be in their native tertiary structures (Watanabe et al., 1981) and are adequate to the measurements of melting temperatures. Melting profiles were monitored by the hyperchromicitiy at 260 nm, using a Gilford spectrometer (Model 250) with an autothermoprogrammer (Model 2527). The rate of increase in temperature was set at 0.5 °C/min.

Results

Separation of Two $tRNA_1^{Ile}$ Species. After purification by a series of column chromatography, 1 A_{260} unit of $tRNA_1^{Ile}$ preparation accepted 1.7 nmol of L-isoleucine, as expected for a highly pure preparation. By analysis with 20% polyacrylamide gel electrophoresis, this $tRNA_1^{Ile}$ preparation was found to contain two components (lane 1 in Figure 1). These two components were then separated from each other by two-dimensional gel electrophoresis on a large scale and were extracted from the gel. Thus, the two subspecies of $tRNA_1^{Ile}$ were clearly isolated from each other, as shown in Figure 1 (lanes 2 and 3). Hereafter, the $tRNA_1^{Ile}$ species with the lower mobility in the gel is denoted as $tRNA_{1a}^{Ile}$, while the other species with the higher mobility is denoted as $tRNA_{1b}^{Ile}$. These two $tRNA_1^{Ile}$ species are much more abundant than other $tRNA^{Ile}$ species in the *T. thermophilus* cell, as found from the chromatographic patterns in the course of purification (data not shown).

Characterization of Two $tRNA_1^{Ile}$ Species. These two $tRNA_1^{Ile}$ species as extracted from the gel were in fact confirmed to be specific to isoleucine; both $tRNA_1^{Ile}$ species were charged with L-isoleucine by *T. thermophilus* IleRS to the same extent. After digestion with pancreatic ribonuclease, snake venom phosphodiesterase I, and *E. coli* phosphomono-

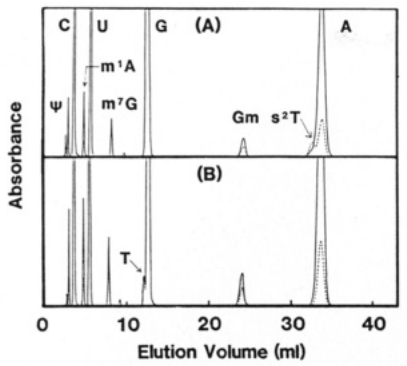

FIGURE 2: High-performance liquid chromatography of nucleosides of $tRNA_{1a}^{Ile}$ species [with $s^2T(54)$] (A) and $tRNA_{1b}^{Ile}$ species [with T(54)] (B). Detector sensitivity was 0.02 absorbance unit at full scale; wavelengths were 254 (···) and 280 nm (—); flow rate was 0.8 mL/min; solvent was 0.05 M $NH_4H_2PO_4$ (pH 5.1) with 5% (v/v) methanol.

esterase, the nucleotide compositions of the two $tRNA_1^{Ile}$ species were analyzed by HPLC. As shown in Figure 2, $tRNA_{1a}^{Ile}$ contains s^2T, pseudouridine (Ψ), m^1A, Gm, and 7-methylguanosine (m^7G) as modified nucleosides (Figure 2A), while $tRNA_{1b}^{Ile}$ contains T, Ψ, m^1A, Gm, and m^7G (Figure 2B). In addition to these modified nucleosides, dihydrouridine (D) and N^6-(threoninocarbonyl)adenosine (t^6A) were detected by ^1H NMR measurement. The chemical shifts of the proton resonances of modified nucleosides as located in $tRNA_1^{Ile}$ species will be reported elsewhere.

Nucleotide Sequences of Two $tRNA_1^{Ile}$ Species. The nucleotide sequences of $tRNA_{1a}^{Ile}$ and $tRNA_{1b}^{Ile}$ species were analyzed by the postlabeling method (Kuchino et al., 1981; Nishimura & Kuchino, 1983). $tRNA_{1a}^{Ile}$ and $tRNA_{1b}^{Ile}$ were found to have the same sequences except for the modification in position 54. The 5′-labeled modified nucleotides from position 54 were unequivocally identified to be s^2T for $tRNA_{1a}^{Ile}$ and T for $tRNA_{1b}^{Ile}$, by two-dimensional thin-layer chromatography with solvent system I (Figure 3A) and with solvent system II (Figure 3B). The complete nucleotide sequences of these $tRNA_1^{Ile}$ species are arranged in the cloverleaf form as shown in Figure 4. We have also obtained the $tRNA_1^{Ile}$ species with 4-thiouridine [$s^4U(8)$] from another lot of *T. thermophilus* cells.

Melting Temperatures of Two $tRNA_1^{Ile}$ Species. The melting profiles of $tRNA_{1a}^{Ile}$ and $tRNA_{1b}^{Ile}$ species, in a solution of 0.01 M Tris-HCl (pH 7.5), 0.2 M NaCl, and 0.01 M magnesium acetate, were monitored by the hyperchromicity (H) at 260 nm (Figure 5). The melting temperatures of these tRNA species were obtained from the peaks of the finite differences ($\Delta H/\Delta T$) of the melting profiles (Figure 5). From the results for three different lots of tRNA preparations, the melting temperatures of $tRNA_{1a}^{Ile}$ with $s^2T(54)$ and $tRNA_{1b}^{Ile}$ with T(54) were determined to be 86.2 and 83.3 °C, respectively, within the accuracy of 0.2 °C.

Discussion

Nucleotide Sequence of $tRNA_1^{Ile}$ Species from T. thermophilus. The nucleotide sequences of *T. thermophilus* $tRNA_1^{Ile}$ species (Figure 4) are quite similar to that of *E. coli* major $tRNA^{Ile}$ ($tRNA_1^{Ile}$) with the same anticodon GAU (Yarus & Barell, 1971). Except for differences in modifications, the sequence homology is as high as 84% for *T. thermophilus* $tRNA_1^{Ile}$ species and *E. coli* $tRNA_1^{Ile}$; out of 77 nucleotides in the sequences of $tRNA_1^{Ile}$ species, nine nucleotides in the acceptor stem, two in the anticodon stem, and one in the D loop are different between *T. thermophilus* and *E. coli*. On the other hand, the sequence homology is 75% for *T. thermophilus*

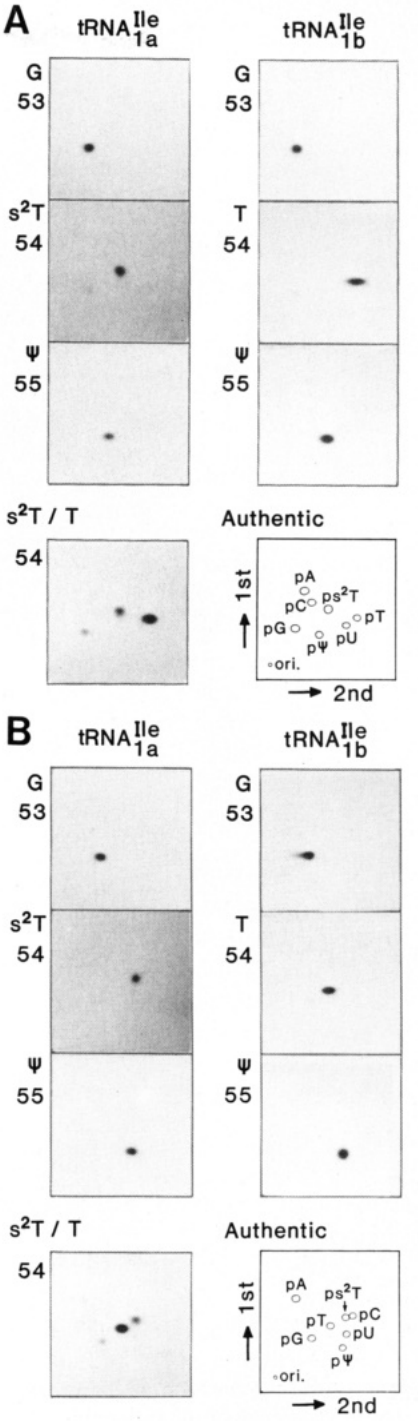

FIGURE 3: Two-dimensional thin-layer chromatography of 5′-^{32}P-labeled mononucleotides from positions 53–55 of *T. thermophilus* $tRNA_{1a}^{Ile}$ and $tRNA_{1b}^{Ile}$ species, with solvent systems I (A) and II (B) (see Materials and Methods). The mononucleotide preparations from position 54 of the two tRNA species were cochromatographed [marked as s^2T/T], clearly showing the mobility difference between ps^2T and pT. For comparison, the chromatographic patterns of authentic mononucleotides are also presented [marked as authentic].

$tRNA_1^{Ile}$ and *E. coli* minor $tRNA^{Ile}$ species ($tRNA_2^{Ile}$) with the anticodon N*AU (N*: modified nucleoside yet unidentified) (Kuchino et al., 1980).

Modified Nucleosides in T. thermophilus $tRNA_1^{Ile}$. *T. thermophilus* $tRNA_1^{Ile}$ species have seven modified nucleosides including Gm(18), D(21), $t^6A(37)$, $m^7G(46)$, $s^2T(54)$ [or T(54)], $\Psi(55)$, and $m^1A(58)$. In comparison, the *E. coli* $tRNA_1^{Ile}$ species (Yarus & Barell, 1971) has D(21), $t^6A(37)$, $m^7G(46)$, and T(54) but not Gm(18), $s^2T(54)$, and $m^1A(58)$. $tRNA_f^{Met}$ from *T. thermophilus* HB8 also has Gm(18), s^2T-

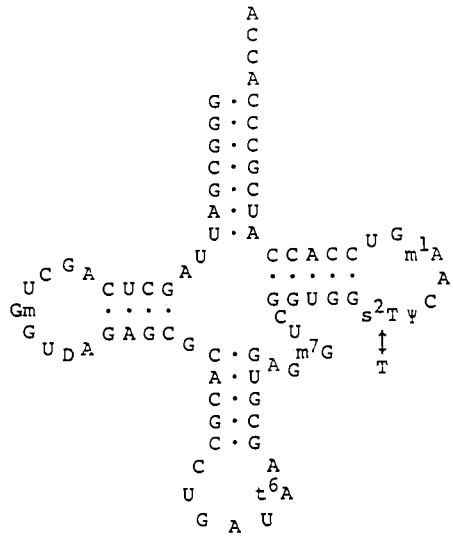

FIGURE 4: Nucleotide sequences of *T. thermophilus* $tRNA_{1a}^{Ile}$ species [with $s^2T(54)$] and $tRNA_{1b}^{Ile}$ species [with T(54)] in the cloverleaf form. In the case of $tRNA_1^{Ile}$ species from another lot of cells, U(8) is modified to $s^4U(8)$.

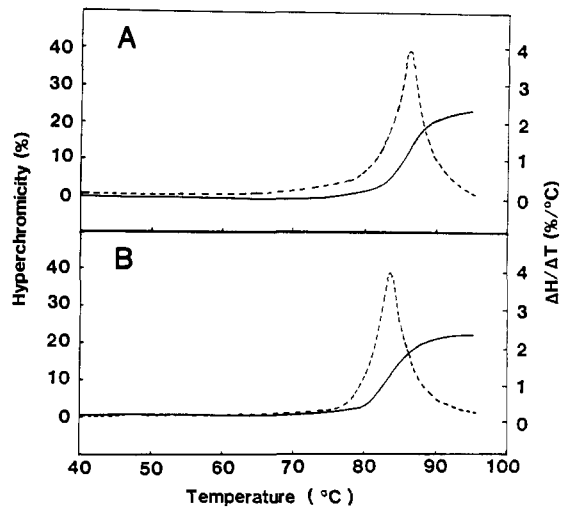

FIGURE 5: Melting profiles as monitored by the hyperchromicity (%) at 260 nm [H] (—) and the finite difference curves of the melting profiles [$\Delta H/\Delta T$] (– –): (A) $tRNA_{1a}^{Ile}$ species [with $s^2T(54)$]; (B) $tRNA_{1b}^{Ile}$ species [with T(54)].

(54), and $m^1A(58)$ (Watanabe et al., 1979a). On the other hand, the *E. coli* $tRNA_1^{Ile}$ species has 3-(3-amino-3-carboxypropyl)uridine (acp^3U) in position 47, whereas the *T. thermophilus* $tRNA_1^{Ile}$ species has the unmodified U in this position. Similarly, it has been found, by HPLC analyses of nucleotide compositions, that $tRNA^{Phe}$ from *E. coli* has acp^3U while $tRNA^{Phe}$ from *T. thermophilus* HB8 does not (Watanabe et al., 1980).

2-Thiolation of T(54) Significantly Enhances the Thermostability of $tRNA_1^{Ile}$. The melting temperature of *T. thermophilus* $tRNA_{1a}^{Ile}$ with $s^2T(54)$ is now found to be higher by 3 °C than that of $tRNA_{1b}^{Ile}$ with T(54). Just this 2-thiolation of ribothymidine clearly enhances the thermostability of *T. thermophilus* $tRNA_1^{Ile}$. How can such a single replacement of an oxygen atom (2-carbonyl oxygen) by a sulfur atom enhance the thermostability of tRNA molecule?

We have already found, by ^1H NMR analyses, that the ribose moiety of s^2T predominantly takes the C3′-endo form, whereas that of T is in the equilibrium of nearly equal fractions of the C2′-endo form and the C3′-endo form. The enthalpy difference between the C2′-endo form and C3′-endo form is

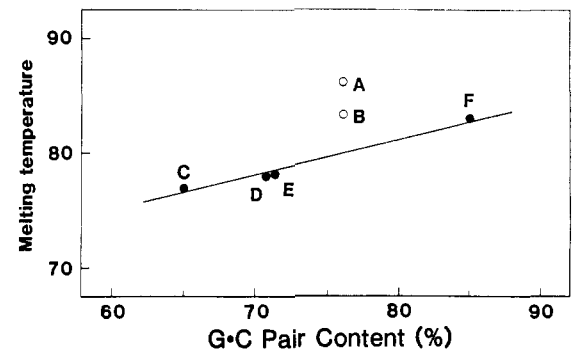

FIGURE 6: Relation between melting temperatures and G·C pair contents of tRNA species, including *T. thermophilus* $tRNA_{1a}^{Ile}$ (A) and $tRNA_{1b}^{Ile}$ (B) and *E. coli* $tRNA_m^{Met}$ (C), $tRNA^{Tyr}$ (D), $tRNA_1^{Ile}$ (E), and $tRNA_f^{Met}$ (F). The values of the melting temperatures of *E. coli* tRNA species are taken from Watanabe et al. (1976a).

much larger in s^2T (1.0 kcal/mol) than in T (0.2 kcal/mol) (Watanabe et al., 1979b). We have also found that such a conformational "rigidity" of s^2T [almost exclusively in the C3′-endo form] is due to the steric interaction between the bulky 2-thiocarbonyl group of the base and the 2′-OH group of the ribose ring. This type of steric effect of the 2-thiocarbonyl group is primarily responsible for the extraordinary thermostability of polyribonucleotides having 2-thiopyrimidine nucleosides (Yamamoto et al., 1983). This suggests that the thermostability of tRNA species from *T. thermophilus* is enhanced by the modification of T to s^2T primarily because of the rigidity of the $s^2T(54)$ residue itself (Yokoyama et al., 1979, 1981; Watanabe et al., 1979b; Yamamoto et al., 1983). Now, the enhanced thermostability of $tRNA_{1a}^{Ile}$ [with $s^2T(54)$] in comparison to that of $tRNA_{1b}^{Ile}$ [with T] as found in the present study is certainly ascribed to the steric effect of the 2-thiocarbonyl group in s^2T.

Other Factors Affecting Thermostability of $tRNA_1^{Ile}$. The G·C pair content is one of the basic factors that affect the thermostability of tRNA. A linear relation has been found between the melting temperatures and G·C pair contents (in stems) of mesophile tRNA species (the solid line in Figure 6) (Watanabe et al., 1976a); if an A·U pair is replaced by a G·C pair, the G·C pair content is increased by about 5%, and the melting temperature of tRNA will be raised by about 1 °C.

From the solid line in Figure 6, the melting temperature of *T. thermophilus* $tRNA_1^{Ile}$ species may be estimated as 80 °C, if no modifications characteristic of extreme thermophiles are present. Actually, however, even in the case of *T. thermophilus* $tRNA_{1b}^{Ile}$ with T(54), the observed melting temperature (83.3 °C) is higher by about 3 °C than the expected value of 80 °C. This suggests that, for the case of *T. thermophilus* $tRNA_{1a}^{Ile}$ and $tRNA_{1b}^{Ile}$ species, the thermostability is enhanced by the presence of modified nucleosides, namely, Gm(18) and $m^1A(58)$, which have also been found in other *T. thermophilus* tRNA species.

By contrast, the melting temperatures of *E. coli* $tRNA_f^{Met}$ and yeast $tRNA^{Phe}$ are not raised by the in vitro methylation of G(18) and A(58), although the residues around these sites of methylation become more resistant to the ribonuclease digestion probably because of some stabilization in the local structure (Kumagai et al., 1982). However, in $tRNA_1^{Ile}$ species from *T. thermophilus*, the numbers of nucleotides on both sides of the Gm(18)-G(19) sequence in the D loop are different from those of *E. coli* $tRNA_f^{Met}$ and yeast $tRNA^{Phe}$. Such differences in the D loop structure have been expected to affect the conformation of the G(18)-G(19) moiety as associated with

the A(58)-bearing TΨC loop (Rich et al., 1980). The methylation in positions 18 and 58 possibly contributes to the local structural stability, thus enhancing the overall thermostability in the case of $tRNA_1^{Ile}$ species from *T. thermophilus* HB8.

Biological Significance of 2-Thiolation of T(54). In the present study, we have succeeded in the isolation of $tRNA_{1a}^{Ile}$ [with $s^2T(54)$] and $tRNA_{1b}^{Ile}$ [with T(54)] in nearly equal amounts from *T. thermophilus* HB8 as grown at 65 °C. The extent of thiolation in unfractionated tRNA as prepared from whole cells or from polysomes has been found to increase as the growth temperature of *T. thermophilus* HB8 is raised (Watanabe et al., 1976b, 1983, 1984). On the other hand, *T. thermophilus* IleRS charges *T. thermophilus* $tRNA_{1b}^{Ile}$ and *E. coli* $tRNA_1^{Ile}$ [with T(54)] as well as *T. thermophilus* $tRNA_{1a}^{Ile}$ (data not shown). Similarly, glutamyl-tRNA synthetase from *T. thermophilus* HB8 can charge $tRNA^{Glu}$ from *E. coli* as well as $tRNA^{Glu}$ from *T. thermophilus* (Hara-Yokoyama et al., 1984). Accordingly, without any loss in aminoacylation of tRNA, the *T. thermophilus* HB8 cell can adapt its tRNA species to a wide range of environment temperatures simply by adjusting the extent of 2-thiolation of T(54).

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Registry No. RNA (*Thermus thermophilus* strain HB8 isoleucine-specific transfer 1a), 97950-88-4; RNA (*Thermus thermophilus* strain HB8 isoleucine-specific transfer 1b), 97950-89-5; ribothymidine, 1463-10-1; 2-thioribothymidine, 32738-09-3.

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