A Thermotable tRNA (Guanosine-2′-)-methyltransferase from Thermus thermophilus HB27 and the Effect of Ribose Methylation on the Conformational Stability of tRNA*

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An S-adenosylmethionine-dependent tRNA (guanosine-2′)-methyltransferase (EC 2.1.1.34) was purified to the homogeneous state (2,400-fold) from a cell-free extract of an extreme thermophile, Thermus thermophilus HB27. The enzyme was highly resistant to heat as reported for other enzymes from thermophilic organisms. The enzyme is monomeric and its molecular weight was estimated to be about 20,000. The Km values for S-adenosylmethionine and for Escherichia coli tRNA were determined to be 0.47 μM and 10 nM, respectively, while the K for a competitive inhibitor S-adenosylhomocysteine, was 1.67 μM. When yeast tRNA was methylated with the purified Gm-methyltransferase, a stoichiometric amount of methyl group was incorporated into the invariant guanosine at position 18 in the D-loop. Yeast tRNA and E. coli tRNA, which were quantitatively methylated with the enzyme, were very similar to the native tRNAs with regard to amino acid acceptor activity and melting temperature, but were more resistant to RNase T1 and RNase A digestions than the corresponding native tRNAs.

It is widely accepted that the modification of tRNA is a post-transcriptional event, and that the reaction is catalyzed with highly site-specific enzymes (1). In tRNA of extreme thermophiles, Thermus thermophilus HB8 and T. thermophilus HB27, there exists a unique set of modified nucleosides, 2-thioribothymidine, 1-methyladenosine, and 2′-O-methylguanosine, and among these nucleosides, S-T is most responsible for the thermostability of the thermophile tRNAs (2-6). In the case of T. thermophilus HB27, it was found that the contents of Gm, m′A, and S-T increased significantly by raising the growth temperature of the bacterial cells (6). This suggests certain functional roles of Gm and m′A in the thermophile tRNA at high temperatures.

In order to elucidate the mechanisms of biosynthesis of these modified nucleosides and their roles in the function of tRNA, especially at high temperatures, we studied the enzymes concerned with the formation of these nucleosides. As reported previously (6), S-adenosylmethionine-dependent tRNA (guanosine-2′)-methyltransferase (EC 2.1.1.34) and tRNA (adenine-1′)-methyltransferase (EC 2.1.3.6) were isolated from the cell-free extract of T. thermophilus HB27 and it was found that Gm-methyltransferase catalyzed the specific methylation of the invariant G18 in the D-loop of tRNA (6) (the number of residues conforms to the proposed rule on the basis of the numbering of yeast tRNA (7)). The ribose methylation of G18 in the D-loop is one of the most invariant modifications found in both prokaryotic and eukaryotic tRNAs (8). However, there has been little study on the enzymes responsible for the ribose methylation. Since the enzymes from thermophiles are known to be generally more resistant to heat and protein denaturing reagents than the corresponding enzymes from mesophilic organisms (9, 10), it is worthwhile to use the extreme thermophiles as the enzyme source.

In this paper, we report on the purification and properties, as well as the site specificity, of S-adenosylmethionine-dependent tRNA (guanosine-2′)-methyltransferase and tRNA (adenine-1′)-methyltransferase activity as well as a Gm-methyltransferase activity were detected, when unfractionated Escherichia coli tRNA was used as the substrate (6). These methyltransferases could be easily separated by DEAE-cellulose column chromatography, since m′A-methyltransferase, but not Gm-methyltransferase, was adsorbed on the DEAE-cellulose column at the neutral pH (6). On the other hand, the Gm-methyltransferase was strongly adsorbed on the CM-Sephadex C-50 or phosphocellulose column. Most of the protein was not retained on the CM-Sephadex C-50 column, and the Gm-methyltransferase activity was eluted with 0.2-0.25 M KCl. The final step of purification was performed

MATERIALS AND METHODS

RESULTS

Purification of Gm-methyltransferase from T. thermophilus HB27 Cells—In a cell-free extract of T. thermophilus HB27, a m′A-methyltransferase activity as well as a Gm-methyltransferase activity were detected, when unfractionated Escherichia coli tRNA was used as the substrate (6). These methyltransferases could be easily separated by DEAE-cellulose column chromatography, since m′A-methyltransferase, but not Gm-methyltransferase, was adsorbed on the DEAE-cellulose column at the neutral pH (6). On the other hand, the Gm-methyltransferase was strongly adsorbed on the CM-Sephadex C-50 or phosphocellulose column. Most of the protein was not retained on the CM-Sephadex C-50 column, and the Gm-methyltransferase activity was eluted with 0.2-0.25 M KCl. The final step of purification was performed

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§ The abbreviations used are: s-T, 2-thioribothymidine; m′A, 1-methyladenosine; Gm, 2′-O-methylguanosine; Gm-methyltransferase, tRNA (guanosine-2′)-methyltransferase; m′A-methyltransferase, tRNA (adenine-1′)-methyltransferase; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; tRNA, tRNA methylated at 2′-OH of G18; tRNA, tRNA methylated at N1 of A58.

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by affinity column chromatography using AdoHcy-Sepharose 4B (Fig. 4 in Miniprint).

The Gm-methyltransferase was tightly bound to the column, and attempts to elute the enzyme were unsuccessful under conditions such as high salt concentrations (2 M KCl or 2 M CaCl₂) or low pH (pH 4.0). Even 2.25 mM AdoHcy failed to elute the enzyme activity from the column. Considering that the enzymes from *T. thermophilus* are generally resistant to protein denaturants (9, 10), the elution of the enzyme was carried out using the buffer containing 6 M urea. The purified enzyme still retained 93% of its original activity after incubation in 6 M urea solution at 4 °C for 4 h.

Table I summarizes the typical purification of the Gm-methyltransferase. The recovery of the Gm-methyltransferase activity was calculated on the basis of methyltransferase activity in each fraction using yeast tRNA³⁷ as the substrate. Since the partially purified m' A-methyltransferase failed to methylate tRNAs containing m' A at position 58, such as *T. thermophilus* HB8 tRNA³⁷ and yeast tRNA³⁷, only the Gm-methyltransferase activity can be detected in the extract of *T. thermophilus* HB27 when yeast tRNA³⁷ is used as a substrate.

**Purity and Molecular Weight of the Gm-methyltransferase**—When the purified preparation of Gm-methyltransferase was subjected to polyacrylamide gel electrophoresis at pH 4.3, a single protein band was observed. The purified enzyme also gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5 in Miniprint). From this electrophoresis, the molecular weight of the enzyme was estimated to be 21,000, by comparing the relative mobility of the enzyme with those of standard proteins (Fig. 6 in Miniprint).

For determination of the molecular weight of the enzyme under nondenaturing conditions, the purified enzyme was subjected to analytical gel filtration on a column of Sephacryl S-200 superfine (Fig. 7 in Miniprint). The molecular weight of the enzyme was calculated to be 20,000.

**Thermal Stability of Gm-methyltransferase**—This enzyme was resistant to heat, as with other enzymes obtained from *T. thermophilus* (2). In the presence of 10% glycerol at pH 7.5, the enzyme retained 90% of its activity even after a 20-min incubation at 80 °C; the activity decreased rapidly at 90 °C (Fig. 8 in Miniprint).

**Enzymatic Properties of Gm-methyltransferase**—The pH optimum for the enzyme was found to be pH 7.2-7.5. As shown in Table II, magnesium ion and spermine stimulated the enzyme activity 3 and 8 times, respectively, at their optimal concentrations, being about 5 mM in each case.

The results of kinetic analysis are also summarized in Table II. Double reciprocal plots of initial rate data at varying concentrations of AdoMet (0.35, 0.44, 0.88, 1.76 μM) and fixed concentration of *E. coli* tRNA³⁷ (1/UV versus 1/(AdoMet)) yield a series of straight lines intersecting at a point in the left of the vertical axis and above the horizontal axis. Secondary plots of the slope and intercept gave the *Kₘ* of 10 nM for tRNA³⁷. By varying the *E. coli* tRNA³⁷ concentration (10, 18, 27, 53 nM) at a fixed concentration of AdoMet, the *Kₘ* for AdoMet was estimated to be 0.47 μM.

AdoHcy, a product inhibitor, strongly inhibited the Gm-methyltransferase activity. The initial rates of enzyme reaction in the presence of AdoHcy (0, 0.69, 1.38, 2.75 μM) were determined against four concentrations of AdoMet (0.34, 0.56, 0.85, 1.69 μM) at fixed *E. coli* tRNA³⁷ level (132 nM). Double reciprocal plots of 1/V versus 1/(AdoMet) gave a series of intersecting lines on the vertical axis, indicating competitive inhibition. From a replot of the slope, the *Kₙ* for AdoHcy was calculated to be 1.67 μM.

**Stoichiometry of Methylating Reaction toward tRNA**—Yeast tRNA³⁷ was rapidly methylated and approximately 1 mol of methyl group was incorporated into 1 mol of tRNA³⁷ (Fig. 9 in Miniprint). Even on adding again the same amounts of enzyme and S-adenosyl-[methyl-³⁷C]methionine initially added to the reaction mixture after 90 min of incubation, only a small increase could be observed in the incorporation. This suggests that the methylation is limited to a single guanosine residue in the tRNA. *E. coli* tRNA³⁷ was a less efficient substrate than yeast tRNA³⁷ (Fig. 9 in Miniprint). The methyl group was hardly incorporated into *T. thermophilus* HB8 tRNA³⁷, since the tRNA³⁷ had the Gm18 in situ.

**Determination of the Site in Yeast tRNA³⁷ Methylated with Gm-methyltransferase**—The [³⁷C]-methylated tRNA³⁷ was prepared using S-adenosyl-[methyl-³⁷C]methionine (see "Materials and Methods" in Miniprint), and completely di-
gested with RNase A under the conditions described by RajBhandary et al. (24). The digests were fractionated on a DEAE-Sephadex A-25 column in the presence of 7 M urea (25). The incorporated methyl group was detected in the largest oligonucleotide with 81% recovery of the radioactivity (Fig. 10 in Miniprint). The fragment must be the modified GGGAGAGCp considering the nucleotide sequence of yeast tRNA\(^{\text{Glu}}\) shown in Fig. 1. A portion of the radioactive fractions was digested with snake venom phosphodiesterase and E. coli alkaline phosphomonoesterase. The nucleoside composition of the fragment was determined to be G, Gm, A, and C in the molar ratio of 4.3:0.9:5.1:7.1:0, and most of the radioactivity was found in the position of Gm (Fig. 11 in Miniprint). This indicates that one G of the octanucleotide GGGAGAGCp is ribose-methylated. The residual portion of the radioactive fractions was digested with Nuclease P1. Most of the radioactivity co-chromatographed with Gm but not with pGm (data not shown). Therefore, the [methyl-\(^{14}C\)]Gm must be located at the 5'-end of the octanucleotide, which is the position corresponding to position 18 from the 5'-end of yeast tRNA\(^{\text{Glu}}\) (Fig. 1).

Read-off Sequencing Analysis of the Methylated Yeast tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\) and methylated tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\) (see “Materials and Methods” in Miniprint), was partially digested with RNase T1 in the presence of either MgCl\(_2\) or EDTA. Fig. 2 shows partial digestion patterns on 20% polyacrylamide gel electrophoresis in the presence of 7 M urea. In the absence of Mg\(^{2+}\), extensive digestion occurred at 4 °C in both the native and methylated tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\). On the other hand, in the presence of 10 mM MgCl\(_2\), tRNA was considerably resistant to RNase digestion. It appears that G18, G19, and G20 are the most susceptible sites for RNase T1 digestion, as reported by Wrede et al. (16). G3, G4, and G20 are also cleaved under the present conditions; however, m'G10 and G15 are scarcely attacked by RNase T1.

For methylated tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\), the band corresponding to G18 is absent not only on the electrophoretogram of the partial digestion products with RNase T1 but also on alkaline digestion. This confirms that the Gm residue is, in fact, located at position 18 of the methylated yeast tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\), since at the position where 2'-O-methyl residue is present, no hydrolysis should occur either with RNase T1 nor with alkali. Except for Gm18, the initial cleavage sites of the methylated tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\) with RNase T1 were guanosine residues G19 and G20 in the D-loop.

**Effect of Ribose Methylation of G18 on the Conformational Stability of tRNA**—By using the purified methylated yeast tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\) and E. coli tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\), we determined the conformational stability of these modified tRNAs with respect to (i) amino acid acceptor activity, (ii) melting temperature and (iii) susceptibility to RNases. The results are summarized in Table III. Methylated tRNAs accepted almost the same amount of amino acid as did unmodified tRNAs, showing that the tRNA samples retained fully the biochemical activity after the modification.

Comparisons of the melting profiles of native yeast tRNA\(^{\text{Glu}}\), and yeast tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\) in the presence and absence of 10 mM MgCl\(_2\) were made (Fig. 12 in Miniprint). Both tRNAs show the same hyperchromicity at 260 nm. It is intriguing that no significant difference could be observed in the melting profiles between native and modified tRNAs in either the presence or absence of Mg\(^{2+}\). Similar results were obtained with the comparison of melting profiles of E. coli tRNA\(^{\text{Met}}\), yeast tRNA\(^{\text{Met}}\), and tRNA\(^{\text{Met, Escherichia coli}}\), although the melting temperatures of these tRNA\(^{\text{Met}}\)s in the presence of Mg\(^{2+}\) are 5.5-6 °C lower than that of T. thermophilus tRNA\(^{\text{Met}}\)

Fig. 3 shows the time course of increase in absorbance at 260 nm for native and methylated yeast tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\) (tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\), caused by RNase T1, and RNase A digests in the presence of 10 mM Mg\(^{2+}\). The initial rate of increase in absorbance at 260 nm of tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\) by digestion with RNase T1 was much lower than that of native tRNA\(^{\text{Glu}}\). In the case of RNase A digestion, the difference in the initial rates can also be seen between native and modified tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\), although this difference is smaller than that of RNase T1. Digestion is reasonable, since methylation occurs at G18, which is just the cleavage site of RNase T1 but not that of RNase A. When excess of RNase T1 or RNase A was added to the reaction mixtures, both native and methylated tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\) showed the same hyperchromicity change. This precludes the possibility that the methylated tRNA takes on a special conformation.

**Fig. 1.** Clover-leaf structure of yeast tRNA\(^{\text{Glu}}\) with an arrow to indicate the site methylated with Gm-methyltransferase.

**Fig. 2.** Partial digestion patterns of 5'-\(^{32}P\)-labeled yeast tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\) and methylated tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\). Left, native yeast tRNA\(^{\text{Glu}}\); right, methylated yeast tRNA\(^{\text{Glu}}\)\(^{(\text{mcm})}\). Lane 1, no enzyme; Lane 2, RNase T1 digestion in the presence of EDTA; Lane 3, RNase T1 digestion in the presence of 10 mM MgCl\(_2\); Lane 4, alkaline hydrolysis. Gel electrophoresis was performed on a 20% polyacrylamide gel (40 × 20 × 0.05 cm) containing 7 M urea at 900 V for 4.5 h.
The modified tRNA was extracted with 88% (v/v) phenol. The recovered tRNA was further methylated with the purified Gm-units of tRNA is equal to 1.66 nmol.

**FIG. 3. Susceptibilities of yeast tRNA" and tRNA" to RNase T, and RNase A in the presence of 10 mM MgCl₂.** Increase in absorbance at 260 nm accompanied by RNase digestion, as described in Fig. 3. For details, see the legend to Fig. 3.

The number of methyl groups/mol of tRNA was estimated from the radio-activity of ¹⁴C incorporated into tRNA assuming that 1.0 nmol/A₂₆₀ unit. The reaction mixture contained 0.01 M KCl, 0.2 A₂₆₀ unit of tRNA, 6.25 units of RNase T, or 3 μg of RNase A or without RNases in a total volume of 0.4 ml. The arrow indicates the addition of RNase T or RNase A to start the reaction. I, native tRNA"; II, tRNA"-Gm, E, no enzyme.

Table III summarizes the relative susceptibilities of modified yeast tRNA" and E. coli tRNA" to RNase T, and RNase A in the presence of 10 mM MgCl₂. Increase in absorbance at 260 nm accompanied by RNase digestion of tRNA was recorded on a Gilford spectrophotometer model 2400-S equipped with autothermoprogrammer model 2527. The rate of temperature increase was set at 0.5 °C/min.

The melting profiles of tRNAs were measured in 0.01 M KCl, 0.2 A₂₆₀ unit of tRNA, 6.25 units of RNase T, or 3 μg of RNase A, and compared them with those of the native tRNAs and T. thermophilus HB8 tRNA" (6). Thus, it can be concluded that Gm-methyltransferase could be stoichiometrically methylated with the purified Gm-methyltransferase and purified by gel electrophoresis on a 10% polyacrylamide gel.

### Table III

| tRNA          | Content of methyl group | Amino acid acceptor activity | Measuring temperature | RNase T, digestion | RNase A digestion |
|---------------|-------------------------|-----------------------------|----------------------|-------------------|-------------------|
|               | mmol/mol of tRNA"       | mmol/A₂₆₀ unit              | °C                   | ΔA₉₀/90 min × 10⁻³ | %                 | ΔA₉₀/90 min × 10⁻³ | %                 |
| Yeast tRNA"   |                         |                             |                      |                   |                   |                   |
| tRNA"         | Gm18 (1.0)              | 1.58                        | 76.0                 | 4.7 (100)         | 3.3 (100)         |
| E. coli tRNA" | Gm18 (1.1)              | 1.62                        | 76.0                 | 1.2 (25.5)        | 2.2 (66.6)        |
| T. thermophilus| Gm18 (0.97), mA" (1.0) | 1.67                        | 83.5                 | 0.75 (62.5)       | 2.7 (74.3)        |

* The melting profiles of tRNAs in 0.01 M Tris-HCl (pH 7.5), 0.01 M MgCl₂, 0.2 mM KCl were recorded by a Gilford spectrophotometer model 2400-S equipped with autothermoprogrammer model 2527. The rate of temperature increase was set at 0.5 °C/min.

**FIG. 5 in Miniprint.**

The molecular weight of the enzyme was estimated to be 21,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 20,000 by gel filtration on Sephacryl S-200 column, suggesting that the enzyme is a monomeric protein in solution. The Gm-methyltransferase was completely inhibited with AdoHcy-Sepharose 4B was used for the final purification. In this chromatography, the usual elution conditions using high salts, pH 3.5, or even AdoHcy (2.25 mM) were of no effect. Thus, the elution buffer containing 6 M urea was used, because it is known that the enzyme from T. thermophilus is generally resistant to protein denaturants (10, 11).

The melting temperatures of tRNAs were measured in 0.01 M KCl, 0.2 A₂₆₀ unit of tRNA, 6.25 units of RNase T, or 3 μg of RNase A, and compared them with those of the native tRNAs and T. thermophilus HB8 tRNA" (6). Thus, it can be concluded that Gm-methyltransferase is very effective for the purification (the enzyme is 6-fold purified by this step) and in spite of nonspecific elution conditions, the enzyme preparation after this step appeared to be homogeneous, judging from polyacrylamide gel electrophoresis (see Fig. 5 in Miniprint).

In the present study, we confirmed that the methylation site in tRNA with this enzyme is G18 in the D-loop by using the purified enzyme preparation and yeast tRNA" as a substrate (see Fig. 1). This conclusion had been suggested by a previous experiment using the partially purified enzyme and E. coli tRNA" (6). Thus, it can be concluded that Gm-methyltransferase from T. thermophilus HB27 catalyzes the specific methylation of G18 in the "invariant" GG sequence of the D-loop in tRNA.

By using this purified Gm-methyltransferase, yeast tRNA" and E. coli tRNA" could be stoichiometrically methylated (Table III). E. coli tRNA" could be further methylated with partially purified mA-methyltransferase resulting in double-methylated tRNA and tRNA (Table III). The charging activity of the methylated tRNAs thus obtained were almost the same as that of unmodified tRNAs, showing that in vitro modification at G18 and A58 does not affect the conformation of tRNA so much.

It is intriguing that not only the melting temperatures (Table III) but also the melting profiles of yeast tRNA" did not cleave with RNases or that the tRNA has already nicked in the structure, making it insensitive to RNase digestion.

In this paper, we describe the purification of tRNA (guanosine-2'-)-methyltransferase from T. thermophilus HB27 to 2400-fold by four purification steps. An affinity chromatography of AdoHcy-Sepharose 4B was used for the final purification. In this chromatography, the usual elution conditions using high salts, pH 3.5, or even AdoHcy (2.25 mM) were of no effect. Thus, the elution buffer containing 6 M urea was used, because it is known that the enzyme from T. thermophilus is generally resistant to protein denaturants (10, 11).

The melting temperatures of tRNAs were measured in 0.01 M KCl, 0.2 A₂₆₀ unit of tRNA, 6.25 units of RNase T, or 3 μg of RNase A, and compared them with those of the native tRNAs and T. thermophilus HB8 tRNA" (6). Thus, it can be concluded that Gm-methyltransferase is very effective for the purification (the enzyme is 6-fold purified by this step) and in spite of nonspecific elution conditions, the enzyme preparation after this step appeared to be homogeneous, judging from polyacrylamide gel electrophoresis (see Fig. 5 in Miniprint).

The molecular weight of the enzyme was estimated to be 21,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 20,000 by gel filtration on Sephacryl S-200 column, suggesting that the enzyme is a monomeric protein in solution. The Gm-methyltransferase activity was enhanced from three to several times with either Mg²⁺ or spermine and inhibited with AdoHcy, the reaction product. These features are similar to those for other tRNA methyltransferases so far reported, such as mA-methyltransferase, tRNA (N'-guanine)- and tRNA (guanine-1)-methyltransferases from rat liver or other organisms (1, 26, 27).

In the present study, we confirmed that the methylation site in tRNA with this enzyme is G18 in the D-loop by using the purified enzyme preparation and yeast tRNA" as a substrate (see Fig. 1). This conclusion had been suggested by a previous experiment using the partially purified enzyme and E. coli tRNA" (6). Thus, it can be concluded that Gm-methyltransferase from T. thermophilus HB27 catalyzes the specific methylation of G18 in the "invariant" GG sequence of the D-loop in tRNA.

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(Fig. 12) and E. coli tRNA$_{28}$ and tRNA$_{28}$A hardly differed from those of the corresponding native tRNAs. This clearly precludes the possibility that in addition to s'T54, either Gm18 or m$'58$ is also responsible for the thermostability of the thermophile tRNAs as has been speculated (3, 4).

Agris et al. (28) have already reported that the 2'-O-methyl nucleosides have little effect on the melting profile of tRNA, using unfractionated tRNAs from Bacillus steatheromor- lus, a moderately thermophilic bacteria; the tRNAs extracted from the cells grown at 70 °C contained 2'-O-methyl nucleosides three times as much as those from the cells grown at 50 °C, nevertheless both tRNAs showed nearly the same melting profiles. The present study, which is consistent with their observation, further clarified the fact through use of purified tRNA and the site-specific tRNA-methyltransferase.

Agris et al. proposed that Gm18 serves to suppress the susceptibility to RNase T$_1$ digestion, since the Gm residue cannot be cleaved with the RNase (28, 29). Wrede et al. (16) reported that the initial cleavage sites of yeast tRNA$_{28}$ with RNase T$_1$, in the presence of Mg$^{2+}$, are G18, G19, and G20 in the D-loop. In addition, G18 and G19 are involved in the tertiary hydrogen-bonding between the D-loop and the T-loop (30, 31). The digestion rate shown in Fig. 3 may be interpreted as follows. Once these sites (G18, G19, and G20) are attacked by the enzyme, resulting in a rapid increase in the absorbance (Curve II in Fig. 3A).

In the case of the RNase A digestion, there exists no such time lag in the absorbance increase (Fig. 3B), as observed for RNase T$_1$ digestion. However, significant differences in susceptibilities toward RNase A is observed between native tRNA$_{28}$ and tRNA$_{28}$.

A similar tendency was observed for E. coli tRNA$_{28}$, tRNA$_{28}$, and tRNA$_{28}$A, but with less efficiency. In this case, it is evident that the methylation of G18 decreases the susceptibility to 62 and 74% for the RNase T$_1$ and RNase A digests, respectively. Further methylation of A58 again decreases the susceptibility toward both RNases to exactly the same level as that of Thermophilus tRNA$_{28}$.

The susceptibility of certain residues toward RNases may depend on the local fluctuation surrounding the residues, which may be suppressed to some extent by the presence of the methylated nucleosides. Such a fluctuation may be independent of the melting temperature of the polynucleotide chain as a whole, as Englander et al. (32) proposed on the basis of the hydrogen-exchange study.

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Thermophile tRNA (Guanosine-2’-)methyltransferase

**Materials**

- Thermophile cells were cultured at 38°C in the medium described previously (12, 13). Harvested at the late log phase and stored at -20°C until use. 
- All enzymes and nucleic acids were purchased from Sigma. 
- Adenosine 3’-monophosphate was supplied by Dr. T. Kinbara, the National Cancer Center Research Institute, Tokyo. 
- Thermophile tRNA (Guanosine-2’)-methyltransferase (EC 2.1.1.66) was purchased as a crude preparation from Yakult Honsha Co. Ltd.

**Methods**

- **Activity chromatography (Section 2)**: The pooled fractions (40 ml) of CM-Sepharose 4B chromatography were directly applied to a column of Sephacyr S-400 (1.0 x 40 cm) previously equilibrated with a buffer consisting of 0.01 M Tris-CL (pH 7.5), 0.1 M NaCl, and 0.1% N,N,N-trimethyl-1-propanol. The column was washed with 20 ml of the starting buffer and then washed with a buffer consisting of 0.01 M Tris-CL (pH 7.5), 0.1 M NaCl, and 0.1% N,N,N-trimethyl-1-propanol, and then again washed with the starting buffer. The enzyme was eluted at 40 ml of the starting buffer containing 40% ethanol. Each fraction (40-50) was directly dialyzed against the standard buffer containing 50% glycerol and stored at -20°C.

- **Preparation of tRNA methylated with the (G)-methyltransferase**: For determination of the ratio of methylated in yeast tRNA20, 4.4 units of yeast tRNA20 were methylated with 1.0 ml of Thermophile (G)-methyltransferase (0.1 U/ml) and 1.0 ml of purified guanosine-2’-methyltransferase in 0.2 ml at 37°C under the standard assay conditions described above. After 45 min of incubation, 1.0 ml of the control guanosine-2’-methyltransferase in 50 ml was added and the reaction mixture in incubated for an additional 60 min. The modified tRNA was extracted with 88% (v/v) phenol and the aqueous layer was washed twice with chloroform. 

- **Preparation of S-100 labeled tRNA and the partial purification of tRNAe**: T7-32P-labeled yeast tRNA20 was methylated with E. coli (G)-methyltransferase and subsequent purification with 3% SDS polyacrylamide gel as described by Kuroiwa et al. (17).

- **Preparation of S-100 labeled tRNA20**: The labeled tRNA was prepared by electrophoresis in the 3% polyacrylamide-urea system (20). The tRNA was divided into aliquots and stored at -80°C.

**Other experimental procedures**

- **Activated Sepharose 4B**: The pooled fractions (40 ml) of CM-Sepharose 4B chromatography was directly applied to a column of Sephacyr S-400 (1.0 x 40 cm) as described by Kuroiwa et al. (18).

**Notes**: The purification yield was determined by the method of Kuroiwa et al. (19). The elution from the CM-Sepharose 4B column was monitored by the guanine content.

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RESULTS

Figure 4. The Acrylo-Sepharose 4B column chromatography of Gm-methyltransferase from T. thermophilus HB87. The active fractions of a CM-Sepharose C-50 column chromatography (Fraction III) was applied to a column of Acrylo-Sepharose 4B as described in "Materials and Methods". The column was washed with the starting buffer containing 2 M KCl (instead of 0.25 M KCl (from fraction No.15) as depicted by the first arrow and then washed with the starting buffer again. The second arrow indicates the application of the buffer containing 4 M urea for elution of the enzyme. The fraction size from fraction No.1 to No. 15 was 7.5 ml and that from fraction No.16 to No.46 was 4 ml. Absorbance at 280 nm: 100 radioactivity of $^{14}$C-methyl group incorporated into E. coli tRNA by 16 ul of each fraction.

Figure 5. SDS-polyacrylamide gel electrophoresis of Gm-methyltransferase fractions. A: approximately 30 µg of the enzyme preparation after step III in Table I; B: 3 µg of the enzyme after step IV. The samples were dissolved in 0.01 M sodium phosphate (pH 7.2), 0.1% SDS, 1% 2-mercaptoethanol and 25% glycerol and heated in a boiling bath for 5 min. The electrophoresis (5 W/gel) was carried out using 7.5% polyacrylamide gels with Coomassie Brilliant Blue R-250 as a tracking dye.

Figure 6. Molecular weight estimation of purified Gm-methyltransferase (Fraction IV) by SDS-polyacrylamide gel electrophoresis. The protein standards were: subunits of RNA polymerase B from T. thermophilus HB8 (1) 63,180,000, (2) 43,140,000, (3) 140,000, (4) 140,000, (5) 139,000, (6) horse heart cytochrome c (12,400). The arrow indicates the relative mobility of Gm-methyltransferase.

Figure 7. Analytical gel filtration of Gm-methyltransferase on a column of Sephacryl S-200 superfine. One milliliter of sample containing approximately 30 µg of the purified enzyme (Fraction IV) was applied on a column of Sephacryl S-200 superfine (1.5 x 120 cm) previously equilibrated with the standard buffer. 1.0-ml fractions were collected at 4°C and the flow rate of 5.4 ml/hr. The elution position of the enzyme was determined by a methyl-transferase assay under standard assay conditions. Arrows indicate elution position of five following standard substances used for calibration of the column: Blue dextran (V0), bovine serum albumin (I), ovalbumin (II), cymotryptapogen A (III) and horse heart cytochrome c (IV). The Gm-methyltransferase was eluted as a single peak immediately after cymotryptapogen A had been eluted. The inset figure shows the calibration curve for molecular weight estimation obtained from the elution positions of four standard proteins as described above. RV is the average distribution constant calculated according to the equation of RV = (Ve/Vo)/(V/Vo), where V0, Ve and V represent void volume, elution volume and total volume respectively. The value of RV of Gm-methyltransferase is indicated by an arrow.

Figure 8. Heat stability of purified Gm-methyltransferase. Thirty microliters of purified enzyme (82 µg/ml) in the standard buffer containing 10% glycerol were incubated at the indicated temperatures for 10 min and cooled in an ice-bath for 2 min. The remaining activity was then measured with 10 µl of the enzyme solution under the standard assay conditions at 37°C. The inset figure indicates the time-dependent heat stability of the enzyme at 70°C and 90°C.

Figure 9. Extent of methylation of various tRNA$^{\text{fmet}}$s by Gm-methyltransferase. The assay was carried out under standard assay conditions at 37°C in 500 µl of the reaction mixture containing 0.6 µl of enzyme and yeast tRNA$^{\text{fmet}}$(→→), E. coli tRNA$^{\text{fmet}}$(→→) and T. thermophilus HB8 tRNA$^{\text{fmet}}$(→→). At indicated times, 50 µl of the solution were withdrawn and the radioactivity of $^{14}$C-methyl group incorporated into tRNA was measured. In the case of the methylation of yeast tRNA$^{\text{fmet}}$, the arrow indicates further addition of the same amount of enzyme and S-adenosyl-$^{14}$C-methionine initially added. The concentration of tRNA was calculated assuming that 1 $\mu$g is equal to 1.66 pmol.
Thermophile tRNA (Guanosine-2’-) methyltransferase

Figure 10. DEAE-Sephadex A-25 column chromatography of an RNase A digest of [14C]methylated yeast tRNA. Ten A260 units of [14C]methylated tRNA were prepared as described in “Materials and Methods” were digested with RNase A. The digest was applied to a column of DEAE-Sephadex A-25 (0.3X90 cm) equilibrated with 0.02 M Tris-HCl (pH 7.5) containing 7 M urea and 0.14 M NaCl. Oligonucleotides were eluted with a linear gradient of 0.14 M NaCl to 0.7 M NaCl in 7 M urea-0.02 M Tris-HCl (pH 7.5) buffer. Fractions of 1.0 ml of effluent were collected at flow rate of 7.6 ml/h. ——, absorbance at 260 nm; —-—, radioactivity.

Figure 11. Nucleoside analysis of the radioactive oligonucleotide separated from the RNase A digest of [14C]methylated yeast tRNA by high pressure liquid chromatography. Fractions in the radioactive region of DEAE-Sephadex A-25 column chromatography in the presence of 7 M urea (figure 10) were pooled, desalted and digested with snake venom phosphodiesterase and E. coli alkaline phosphomonoesterase. The resulting nucleosides were analyzed on a reversed-phase column of YBondapak C18 (4x300 mm) at room temperature. Solvent, 0.05 M NH4H2PO4 (pH 5.1) with 5% (v/v) methanol; flow rate, 1.0 ml/min; detector range, 0.02 absorbance unit full scale at 254 nm. Fractions (1.0 ml) were collected and radioactivity present in each fraction was determined by liquid scintillation counting method (histogram).

Figure 12. Melting profiles of native yeast tRNA and yeast tRNA methylated at G18. The melting profiles of native yeast tRNA and those of yeast tRNA methylated at G18 as described in “Materials and Methods”, are compared in both the presence and absence of 10 mM MgCl2. The solvent was 0.01 M Tris-HCl (pH 7.5), 0.2 M NaCl. Recording of melting profiles was carried out using a Gilford spectrophotometer model 2400-S equipped with auto-thermoprogrammer model 2500. The rate of increase in temperature was set at 0.5°C per min. Native yeast tRNA (●); yeast tRNA (■).
A thermostable tRNA (guanosine-2’)-methyltransferase from Thermus thermophilus HB27 and the effect of ribose methylation on the conformational stability of tRNA.
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