The tRNA Recognition Mechanism of Folate/FAD-dependent tRNA Methyltransferase (TrmFO)*§

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Background: RNA modification enzymes select specific RNAs as substrates.

Results: A novel assay for folate-dependent tRNA methyltransferase (TrmFO) was developed that clarified positive and negative determinants of TrmFO.

Conclusion: TrmFO recognizes a T-arm structure including the U54U55C56 sequence and G53-C61 base pair; A38 prevents incorrect methylation of U32.

Significance: Studying how proteins recognize RNA is crucial for understanding RNA maturation processes.

The conserved U54 in tRNA is often modified to 5-methyluridine (m5U) and forms a reverse Hoogsteen base pair with A58 that stabilizes the L-shaped tRNA structure. In Gram-positive and some Gram-negative eubacteria, m5U54 is produced by folate/FAD-dependent tRNA (m5U54) methyltransferase (TrmFO). TrmFO utilizes nitrogen(10)-methylene tetrahydrofolate (CH2THF) as a methyl donor. We previously reported an in vitro TrmFO assay system, in which unstable [14C]CH2THF was supplied from [14C]serine and tetrahydrofolate by serine hydroxymethyltransferase. In the current study, we have improved the TrmFO assay system by optimization of enzyme and substrate concentrations and introduction of a filter assay system. Using this assay, we have focused on the tRNA recognition mechanism of TrmFO. 42 tRNA mutant variants were prepared, and experiments with truncated tRNA and microhelix RNAs revealed that the minimum requirement of TrmFO exists in the T-arm structure. The positive determinants for TrmFO were found to be the U54U55C56 sequence and G53-C61 base pair. The gel mobility shift assay and fluorescence quenching showed that the affinity of TrmFO for tRNA in the initial binding process is weak. Furthermore, we inhibited TrmFO with truncated tRNA and microhelix RNAs revealed that the G53-C61 Watson-Crick base pair in the T-stem enhances the hydrophobic interaction, and stabilizes the L-shaped tRNA structure (13–15).

More than 100 modified nucleosides have been found in various RNA species, with methylation being one of the most common chemical modifications in RNA (1–3). In particular, tRNA contains abundant methylated nucleotides, which stabilize the L-shaped tRNA structure and improve molecular recognition (1–3). One such modification involves a conserved uridine at position 54 (U54) in the T-loop of tRNA, which is often modified to 5-methyluridine (m5U; ribothymidine), a modification that has been found in tRNAs from eukaryotes, eubacteria, and some archaea (Pyrococcus abyssi (4, 5)) (1–3). In some thermophilic eubacteria, such as Thermus thermophilus (6–9) and Aquifex aeolicus (10), the m5U54 is further modified to 5-methyl-2-thiouridine (m5s2U54). At least four protein components are required for sulfur-transfer reaction in 2-thiouridylation (9). Furthermore, a hyperthermophilic archaeon, Pyrococcus furiosus, has an m5sU modification in tRNA (11), although the position of this modification has not been determined as 54. Moreover, 2-O-methyl-5-methyluridine at position 54 (m5Um54) has been found in some eukaryotic tRNAs (e.g. in human tRNA15s) (12). These m5U54 modifications and their derivatives form a reverse Hoogsteen base pair with the conserved A58 (or derivatives such as 1-methyladenosine at position 58 (m1A58) in the L-shaped tRNA structure (13, 14). This m5U54-A58 tertiary base pair stacks with the conserved G53-C61 Watson-Crick base pair in the T-stem, enhances the hydrophobic interaction, and stabilizes the L-shaped tRNA structure (13–15).

The formation of m5U54 is catalyzed by tRNA (m5U54) methyltransferases (tRNA (uracil-5’)-methyltransferase; EC 2.1.1.35) (16). The enzymes can be divided into two types according to their methyl group donors: S-adenosyl-L-methionine (AdoMet)-dependent methyltransferases and folate/FAD-dependent methyltransferases. AdoMet-dependent tRNA (m5U54) methyltransferases are also involved in sulfur-transfer reaction in 2-thiouridylation (9).

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This article contains supplemental Figs. 1 and 2.

The abbreviations used are: AdoMet, S-adenosyl-L-methionine; THF, tetrahydrofolate; CH2THF, N5,N10-methylene tetrahydrofolate; SHMT, serine hydroxymethyltransferase; MB, methylene blue; nt, nucleotide.

2 The abbreviations used are: AdoMet, S-adenosyl-L-methionine; THF, tetrahydrofolate; CH2THF, N5,N10-methylene tetrahydrofolate; SHMT, serine hydroxymethyltransferase; MB, methylene blue; nt, nucleotide.
methyltransferases are present in eubacteria, some archaea, and eukaryotes (4, 5, 16). *Escherichia coli* AdoMet-dependent tRNA (m5U54) methyltransferase (classical name, RNA uridine methyltransferase) is one of the best-studied tRNA modification enzymes. The enzymatic activity was initially detected in crude *E. coli* cell extract, after which several purification procedures were developed (17–19). The gene encoding the enzyme was identified as *trmA* (20). An overexpression system for TrmA in *E. coli* was developed, and the purification procedures were devised (21). The studies on recombinant TrmA have significantly contributed to the tRNA modification enzyme field.

The studies on recombinant TrmA have significantly contributed to the tRNA modification enzyme field. TrmA catalyzes methyl transfer to a 17-mer T-arm-like microhelix RNA and recognizes the conserved nucleotides U54 and C56 (22, 23). Therefore, this enzyme catalyzes in vitro methyl transfer to part of 16S rRNA (24). In the reaction, TrmA forms a covalent bond complex with substrate RNA (25, 26), and methyl transfer takes place by a single displacement mechanism (27).

These studies influenced later research of other tRNA modification enzymes. For example, the local RNA structure recognition is commonly observed in tRNA (Y55) synthase (28), tRNA guanine transglycosidase (29), tRNA (Gm18) methyltransferase (30, 31), tRNA (m1G37) methyltransferase (32), tRNA (m7G46) methyltransferase (33), tRNA (i6A37) isopentenyltransferase (34, 35), and others. The single displacement methyl transfer mechanism is common to at least tRNA (m2G26/m2G26/m2G27) methyltransferase (36, 37) and tRNA (Gm18) methyltransferase (38, 39). Furthermore, the eukaryotic enzyme was identified from *Saccharomyces cerevisiae* as Trm2 (39–41). Recently, it has been reported that some archaea have a TrmA-type enzyme (4, 5). A crystallographic study (42) and bioinformatics research (5) suggest that TrmA has a Class I AdoMet-dependent methyltransferase fold, which is the typical catalytic domain of methyltransferases (43). Moreover, several rRNA (m5U) methyltransferases have sequence and structural similarities to TrmA, suggesting that these TrmA family enzymes are derived from a common ancestral protein (5).

In contrast, folate/FAD-dependent tRNA (m5U54) methyltransferases have been less well explored. The enzymatic activity was found in crude cell extract of *Enterococcus faecalis* (classical name, *Streptococcus faecalis*), and m5U formation without AdoMet was confirmed (44). The enzyme, which contains FAD and requires N5,N10-methylene tetrahydrofolate (CH2THF) as a methyl group donor, was purified (45). However, for a long time, the gene encoding the enzyme remained unidentified until Urbanovicius et al. (46) biochemically identified the gene, namely *trmFO*, from *B. subtilis*. Their study suggested that TrmFO is found widely among Gram-positive and some Gram-negative bacteria, including *T. thermophilus* (46). In our previous study, we devised an *in vitro* assay system for TrmFO activity and solved the crystal structures of free, tetrahydrofolate (THF)-bound, and glutathione-bound forms of *T. thermophilus* TrmFO (47). During the course of this study, FAD redox states of *B. subtilis* TrmFO were reported (48). Furthermore, it has also been reported that *B. subtilis* TrmFO forms a covalent bond complex with tRNA (48, 49). In the current study, we focused on the tRNA recognition mechanisms of *T. thermophilus* TrmFO.

**EXPERIMENTAL PROCEDURES**

**Materials**—[U-14C]Serine (1.85 GBq/mmol) was purchased from Morabek. [3-3H]Serine (892 GBq/mmol) was obtained from PerkinElmer Life Sciences. THF was bought from Sigma. DER1 filter (code number 3658-323) was a product of Whatman. Q-Sepharose Fast Flow was purchased from GE Healthcare. Microhelix RNAs were synthesized by Hokkaido System Science. Other chemical reagents were of analytical grade.

**Preparation of Enzymes**—The expression systems and purification procedures for *T. thermophilus* TrmFO and serine hydroxymethyltransferase (SHMT) were described in the supplemental material of our previous report (47). The expression systems and purification procedures of tRNA (Gm18) methyltransferase (TrmH), tRNA (m7G46) methyltransferase (TrmI), tRNA (W55) methyltransferase (TruB), and tRNA (m1A58) methyltransferase (Trml) were described in our previous reports (33, 50–52).

**Measurement of [14C]CH2THF Formation Activity by SHMT**—Formation of [14C]glycine from [14C]serine by SHMT was monitored using thin layer chromatography. The same amounts of [14C]CH2THF and [14C]glycine are produced by the SHMT reaction. The reaction mixture (50 μl: 0.2 μM SHMT, 10 μM THF, and 100 μM [14C]serine in buffer A (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 6 mM 2-mercaptoethanol, and 50 mM KCl)) was incubated for appropriate times (0–60 min) at 60 °C. Non-radioisotope-labeled serine and glycine were added to the sample (final concentrations, 500 μM each), and then the mixtures were frozen in liquid nitrogen. The sample (1 μl each) was spotted onto a cellulose thin layer plate (Merck; TLC cellulose, F, catalog number 1.05565.0001) and separated by solvent (phenol-saturated with water, 28% ammonia water (99:1)). The [14C]serine and [14C]glycine were monitored with a Fuji Photo Film BAS 2000 imaging analyzer. The standard markers (non-radioisotope labeled serine and glycine) were detected by a ninhydrin reaction.

**Measurements of TrmFO Methylation Activity**—Transfer RNA transcripts were prepared by T7 RNA polymerase as described previously (32, 50, 51). The transcripts were purified by Q-Sepharose Fast Flow column chromatography and 10% polyacrylamide gel (7 M urea) electrophoresis (PAGE). In order to optimize the assay conditions, we tested various concentrations of enzymes and substrates, as described under “Results.” Data in Figs. 3, 4, 7, and 8 were obtained as follows. To visualize the methyl group acceptance activity, we employed a gel assay system. The reaction mixtures (50 μl: 0.4 μM SHMT, 5.1 μM tRNA transcript, 5 μM THF, 3 mM NADPH, and 50 μM [14C]serine in buffer A) were preincubated for 10 min at 60 °C. The methyl transfer reaction was started by the addition of TrmFO (final concentration, 0.25 μM). The mixtures were incubated for 30 min at 60 °C. The reaction was stopped by the addition of 50 μl of phenol/chloroform (1:1). The RNA was recovered by ethanol precipitation and then separated by 10% PAGE (7 M urea). The gel was stained with methylene blue (MB) for detection of RNA and dried. The incorporation of the
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[14C]methyl group into the transcripts was quantified using a Fuji Photo Film BAS2000 imaging analyzer system. For the kinetic assay, [3H]serine was used instead of [14C]serine. The final concentration of serine was adjusted to 55 μM by the addition of non-radioisotope-labeled serine. The incubation was performed for 10 min at 60 °C. Aliquots (25 μl) were spotted onto a DE81 filter. The filter was washed with 250 mM Na2HPO4 five times at room temperature. The methyl group incorporation was measured by a liquid scintillation counter.

Gel Mobility Shift Assay—The gel mobility shift assay was performed as described in a previous report (31). Purified protein and 0.05 A260 units of T. thermophilus tRNA^Phe transcript were incubated in 20 μl of buffer B (50 mM Tris base, 50 mM acetic acid, and 5 mM Mg(OAc)2) at 4 °C for 20 min. 4 μl of loading solution (0.25% bromphenol blue and 30% glycerol) was prepared with 1× buffer B. Electrophoresis was performed at 4 °C for 1 h at 100 V. To detect protein, the gel was stained with Coomassie Brilliant Blue. Methylation blue was used for RNA detection.

Fluorescence Measurement—Fluorescence measurement was performed with a fluorescence spectrophotometer (F-2500, Hitachi) at 25 °C. The excitation wavelength was 295 nm. Fluorescence intensity at 320 nm was monitored. The methyl transfer reaction utilizes SHMT to produce [14C]CH2THF, which is not commercially available. Then the [14C]CH2THF is consumed in the system that is produced by the SHMT reaction. To measure the kinetic parameters for electron donors and tRNA, excess amounts of methyl donor ([14C]CH3THF) are required. The [14C]-methylated RNA is separated by 10% PAGE (7 M urea) and is detected by autoradiography. This system is relatively convenient and semiquantitative. However, there are two problems for the study of kinetics with this assay. The first problem is that the usable ranges of TrmFO and substrate concentrations are narrow. In simple terms, the rate-limiting factor of the methyl transfer reaction may be the concentration of [14C]CH2THF that is produced by the SHMT reaction. To measure the kinetic parameters for electron donors and tRNA, excess amounts of methyl donor ([14C]CH3THF) are required. The second problem is that the gel assay system is not suitable for the many samples required for a kinetic study. To overcome these problems, we have devised a new in vitro TrmFO assay system.

To supply an excess amount of [14C]CH3THF to the reaction mixture, a sufficient amount of THF needs to be added as the source of [14C]CH3THF. However, excess amounts of THF inhibit the TrmFO reaction, because THF is an analog of [14C]CH3THF. In order to determine the optimal THF concen-
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tration, we performed the methyl transfer assay with various concentrations of THF. Supplemental Fig. 1 (top) shows one example of these pilot experiments; in this case, >10 μM THF inhibited the TrmFO (0.8 μM) reaction. After these pilot experiments, we determined the practical concentrations of THF and TrmFO to be 5 and 0.25 μM, respectively. Next, we compared the electron donors (supplemental Fig. 1 (bottom)). This experiment revealed that NADPH is superior to NADH as an electron donor, although TrmFO can use both NADPH and NADH. Furthermore, we confirmed that 3 mM NADPH is enough for the TrmFO reaction (supplemental Fig. 1). In the presence of 50 μM [14C]serine, 5 μM THF, 3 mM NADPH, 5.1 μM tRNA^Phe^ transcript, and 0.25 μM TrmFO, we optimized the SHMT concentration. Around 0.4 μM SHMT gave the maximum velocity of TrmFO methyl transfer reaction (data not shown). Under these optimized conditions (50 μM [14C]serine, 5 μM THF, 3 mM NADPH, 5.1 μM tRNA^Phe^ transcript, 0.4 μM SHMT, and 0.25 μM TrmFO), the TrmFO reaction maintained linearity for 10 min at 60 °C (data not shown). Long incubations of more than 20 min caused the reverse reaction of SHMT with glycine produced. This could be visualized by monitoring of [14C]serine and [14C]glycine in the reaction mixture (Fig. 2A).

After the 10-min period, sufficient [14C]serine remained in the reaction mixture. In contrast, after the 20-min period, half of the [14C]serine was consumed, and the same amount of [14C]glycine was produced. According to the progress of the TrmFO methyl transfer reaction, [14C]CH₂THF was consumed, and the equilibrium between [14C]serine and [14C]glycine shifted to the [14C]glycine side. After 30 min, more than 90% of [14C]serine was converted to [14C]glycine. Thus, we concluded that the initial velocity of TrmFO methylation can be measured for 10 min under the optimized conditions.

The second problem, involving the time and effort needed for analyzing multiple samples, was solved by employing a filter assay system instead of PAGE. We initially tested Whatman 3MM paper and washing with 5% trichloroacetic acid. However, this conventional method was not good because a considerable amount of [14C]CH₂THF remained on the filter. The best system was found to be the combination of Whatman DE81 paper and washing with 250 mM sodium phosphate buffer (data not shown). This filter assay system enabled us to use [3H]serine instead of [14C]serine. The detection limit of methylated RNA was dramatically improved because of the difference of specific activities between [3H]serine (892 GBq/mmol) and [14C]serine (1.85 GBq/mmol).

Thus, we successively improved the in vitro TrmFO assay system for kinetic studies. In fact, we could determine the apparent kinetic parameters for T. thermophilus tRNA^Phe^ transcript (Fig. 2B) and electron donors (Fig. 2C). To our surprise, both $K_m$ values for tRNA and electron donors were relatively

FIGURE 2. A, conversion of [14C]serine to [14C]glycine by SHMT. [14C]Serine and [14C]glycine were separated by thin layer chromatography and monitored by autoradiogram. The left panel shows spots corresponding to serine and glycine markers visualized by ninhydrin reaction. The right panel is the autoradiogram. The lane marked — SHMT is the negative control, in which SHMT was not added into the reaction mixture. B, determination of kinetic parameters of TrmFO for T. thermophilus tRNA^Phe^ transcript by Lineweaver-Burk plot. Initial velocities of TrmFO methylation were measured for 10 min at 60 °C with various concentrations of tRNA^Phe^ transcript under the optimized conditions (50 μM [14C]serine, 0.4 μM SHMT, 0.25 μM TrmFO, 5 μM THF, and 3 mM NADPH). C, determination of kinetic parameters of TrmFO for electron donors by Lineweaver-Burk plot. Initial velocities of TrmFO methylation were measured for 10 min at 60 °C with various concentrations of NADPH (or NADH) under the optimized conditions (50 μM [14C]serine, 0.4 μM SHMT, 0.25 μM TrmFO, 5 μM THF, and 5.1 μM tRNA^Phe^ transcript). Error bars, S.E.
large when compared with those of other tRNA methyltransferases and oxidoreductases. This may be caused by the existence of SHMT. In general, CH$_2$THF is unstable and scarcely exists in a living cell. In thermophilic bacterial cells, CH$_2$THF may be directly passed from SHMT to TrmFO. Although the formation of a complex between SHMT and TrmFO was not observed by the gel filtration column chromatography (data not shown), SHMT may have a weak affinity for TrmFO. This interaction hinders binding of tRNA and electron donors to TrmFO and is reflected in the relatively large $K_m$ values. Furthermore, during the course of this study, Hamdane et al. (49) proposed a very interesting hypothetical mechanism for formation of a TrmFO-tRNA complex based on the locations of catalytic and tRNA-binding cysteine residues. According to their theory, TrmFO monomer binds to tRNA, and then a second TrmFO binds to the complex. If correct, complicated reaction intermediates should be considered. Therefore, it should be mentioned that the kinetic parameters in this paper are apparent values. However, these values give clues to allow consideration of the interaction between TrmFO and tRNA.

**Methyl Group Acceptance Activities of Truncated tRNA Molecules**—To address the recognition sites in tRNA, we prepared nine truncated *T. thermophilus* tRNAPhe transcripts (Fig. 3). The methyl group acceptance activities of these truncated tRNAPhe transcripts using TrmFO were visualized using the previous gel assay system. The transcripts (5.1 $\mu$m) were incubated with 0.2 $\mu$m TrmFO, 0.4 $\mu$m SHMT, 5 $\mu$m THF, 3 mm NADPH, and 50 $\mu$m $[^{14}$C]serine at 60 °C for 30 min and then analyzed by 10% PAGE (7 M urea). Thus, the data in Fig. 3 do not represent the initial velocities. Furthermore, the initial velocities for microhelix RNAs (I and J; see Table 1) were slow. To visualize the methyl group acceptance activity of microhelix RNAs, the concentrations of TrmFO and RNA were increased to 0.4 and 12.0 $\mu$m, respectively, and the imaging plate was exposed for longer durations. The kinetic parameters determined using $[^{3}$H]serine are summarized in Table 1. The gel was stained with MB to visualize the RNA molecules (Fig. 3, left), and then $[^{14}$C]methyl group incorporation was monitored by autoradiography (Fig. 3, right). As shown in Fig. 3, the methyl group acceptance activity of tRNAPhe full-length (wild type) was clearly observed. Similarly, truncated tRNA molecules showed methyl group acceptance activity (Fig. 3, B–G). In

**TABLE 1**

<table>
<thead>
<tr>
<th>Transcript Feature</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>Relative $V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> Full-length</td>
<td>1.1</td>
<td>0.1</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td><strong>B</strong> Corresponding to nucleotides 44–76</td>
<td>2.6 ± 0.4</td>
<td>2.0 ± 1.0</td>
<td>15</td>
</tr>
<tr>
<td><strong>C</strong> Deletions of aminoacyl stem and D-arm</td>
<td>1.9 ± 0.6</td>
<td>2.5 ± 0.5</td>
<td>20</td>
</tr>
<tr>
<td><strong>D</strong> Corresponding to nucleotides 18–76</td>
<td>4.5 ± 0.5</td>
<td>4.5 ± 1.5</td>
<td>20</td>
</tr>
<tr>
<td><strong>E</strong> Deletions of D-arm</td>
<td>1.5 ± 0.5</td>
<td>6.0 ± 0.0</td>
<td>70</td>
</tr>
<tr>
<td><strong>F</strong> Deletions of aminoacyl stem</td>
<td>1.3 ± 0.3</td>
<td>3.0 ± 0.0</td>
<td>35</td>
</tr>
<tr>
<td><strong>G</strong> Deletions of anticodon arm</td>
<td>1.2 ± 0.2</td>
<td>6.5 ± 0.5</td>
<td>90</td>
</tr>
<tr>
<td><strong>H</strong> Deletions of T-arm</td>
<td>1.5 ± 0.5</td>
<td>6.0 ± 0.0</td>
<td>70</td>
</tr>
<tr>
<td><strong>I</strong> 19-nt T-arm</td>
<td>&gt;0.3</td>
<td>&lt;0.7</td>
<td>&lt;40</td>
</tr>
<tr>
<td><strong>J</strong> 22-nt T-arm</td>
<td>&gt;0.1</td>
<td>&lt;0.5</td>
<td>&lt;80</td>
</tr>
</tbody>
</table>

*ND, methyl group incorporation was not detectable.
$^a$ The parameters for I and J were not correctly measured due to low $V_{max}$ values.
contrast, truncated tRNA\(^{\text{Phe}}\), in which the T-arm was deleted, had completely lost methyl group acceptance activity (Fig. 3H). Thus, the minimum requirement of TrmFO seems to exist in the T-arm structure of tRNA. To confirm this idea, we prepared synthetic microhelix RNAs (Fig. 3, I and J). The 19-nt T-arm mimics the T-arm structure of \textit{E. coli} tRNA\(^{\text{Phe}}\) and was previously used for the crystallization of \textit{E. coli} TrmA and RNA complex (42). The 22-nt T-arm has an artificial sequence to reinforce the stem structure. As shown in Fig. 3, I and J, both microhelix RNAs were clearly methylated, revealing that the minimum requirement(s) of TrmFO exists in the T-arm structure. Although the kinetic parameters for microhelix RNAs were not measured correctly due to the low methyl transfer activity, the tendency to small \(K_m\) values was confirmed. The other part of tRNA (e.g. the D-arm in the L-shaped tRNA) may hinder the initial binding process.

During the course of this study, it has been reported that \textit{B. subtilis} TrmFO methylates a 31-mer mini-RNA, which includes the T-arm sequence of \textit{B. subtilis} tRNA\(^{\text{Asp}}\) (49). The results from our current study are in good agreement with these observations.

**Methyl Acceptance Activities of T-arm Mutant Transcripts**—These results prompted us to investigate the effects of mutations in the T-arm, and we therefore prepared 17 mutant transcripts (Fig. 4A). Because U55, C56, and A58 bases are conserved in the T-arm of all bacterial tRNAs, these nucleotides were substituted by other nucleotides (Fig. 4, U55C, C56U, A58G, and A58U). As shown in Fig. 4A, the variants U55C and C56U completely lost methyl group acceptance activity, demonstrating that U55 and C56 are absolutely required for TrmFO recognition. The results also demonstrated that the methyl group acceptance activities of A58G and A58U variants were clearly decreased. Kinetic studies revealed that the \(K_m\) values for the A58G and A58U variants were not significantly changed. Thus, the substitution of A58 does not have an effect on the initial binding process between TrmFO and tRNA and the releasing process of methylated tRNA from the complex. A58 forms a reverse Hoogsteen base pair with U54, the target uridine (13, 14). With the introduction of U54 into the expected catalytic pocket of TrmFO (47), the U54-A58 tertiary base pair should be disrupted. Therefore, the substitution of A58 mainly influences the \(V_{\text{max}}\) value. The other nucleotides (G57, U59, and U60) in the T-loop were individually substituted as shown in Fig. 4 (G57C, G57A, U59C, U60C, and U60G). These variants have methyl group acceptance activities comparable with that of the wild-type transcript (Fig. 4 and Table 1), suggesting that G57, U59, and U60 do not form a tertiary base pair. These nucleotides (G57, U59, and U60) may have normal L-shaped tRNA structure because G57, U59, and U60 do not form a tertiary base pair. Therefore, these variants seem to bind to TrmFO like the wild-type tRNA in the initial binding process. After the initial binding process, tRNA (at least the T-arm) should change structure to introduce U54 into the catalytic pocket. These nucleotides (G57, U59, and U60) may have
an indirect effect on this structural change process (or prestructural change process). The mutations of these nucleotides may perturb the local structure of the T-arm, and this perturbation may enhance the exchange of tRNA from the TrmFO-tRNA complex at high tRNA concentrations. The kinetic parameters of these variants in Table 2 were calculated from the velocities at low tRNA concentrations (\(<2 \mu M\)), although the kinetics are not approximated by the Michaelis-Menten equation.

Because the G53-C61 base pair in the T-stem is conserved in almost all tRNAs (1–3), we prepared eight variants in which the G53-C61 base pair was substituted by the other base pair or was disrupted (Fig. 4B). As shown in Fig. 4, these mutations produced complete loss of methyl group acceptance activity, showing that the G53-C61 base pair works as the essential positive determinant for TrmFO. Taking these results together, we conclude that the U54U55C56 sequence and the G53-C61 base pair are absolutely required for TrmFO recognition.

### TABLE 2

<table>
<thead>
<tr>
<th>Transcript</th>
<th>(K_m) (\muM)</th>
<th>(V_{max}) nmol mg(^{-1}) h(^{-1})</th>
<th>Relative (V_{max}/K_m) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.1 ± 0.1</td>
<td>6.5 ± 0.5</td>
<td>100</td>
</tr>
<tr>
<td>U55C</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C56U</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G57A</td>
<td>2.1 ± 0.4</td>
<td>12.0 ± 2.0</td>
<td>100</td>
</tr>
<tr>
<td>G57C</td>
<td>(&gt;5.0)(^b)</td>
<td>(&lt;28.0)</td>
<td>(&lt;100)</td>
</tr>
<tr>
<td>A58G</td>
<td>2.2 ± 0.8</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>A58U</td>
<td>1.3 ± 0.5</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>U59C</td>
<td>(&gt;2.0)(^b)</td>
<td>(&lt;11.0)</td>
<td>(&lt;90)</td>
</tr>
<tr>
<td>U60C</td>
<td>(&gt;1.0)(^b)</td>
<td>(&lt;7.0)</td>
<td>(&lt;120)</td>
</tr>
<tr>
<td>U60G</td>
<td>(&gt;2.0)(^b)</td>
<td>(&lt;11.0)</td>
<td>(&lt;90)</td>
</tr>
<tr>
<td>G53C/C61G</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G53A/C61U</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G53A/C61A</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G53A</td>
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<td>C61U</td>
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<td>Disruption of T-stem (DT-stem)</td>
<td>ND</td>
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\(^a\) ND, methyl group incorporation was not detectable.

\(^b\) Values in parentheses were calculated from data of low substrate concentrations.

FIGURE 5. A, gel mobility shift assay. The gel was stained with Coomassie Brilliant Blue for detection of protein and then stained with methylene blue for detection of RNA. No shift band corresponding to a complex of TrmFO and tRNA was observed. B, fluorescence quenching by tRNA\(^{Phe}\) transcript and tRNA\(^{Phe}\) without the T-arm. Fluorescence derived from tryptophan residues in TrmFO was measured in the presence of various concentrations of tRNA\(^{Phe}\) transcript (circles) and tRNA\(^{Phe}\) without the T-arm (triangles).

FIGURE 6. Inhibition experiments with non-substrate RNA. Methylation of the wild-type tRNA\(^{Phe}\) transcript by TrmFO was inhibited by methylated tRNA\(^{Phe}\) (A), tRNA\(^{Phe}\) without the T-arm (B), and the tRNA\(^{Phe}\) U54C variant (C). The inhibition caused by methylated tRNA\(^{Phe}\) was weak, suggesting that this tRNA is excluded before the structural change process or in the early stage of the structural change process.

The Affinity of TrmFO for RNA in the Initial Binding Process Is Weak, and Positive Determinants in the T-arm Are Probably Required for the Structural Change Process—The apparent \(K_m\) value for tRNA is considerably large, and several tRNA variants show high concentration inhibition, suggesting that the affinity of TrmFO for RNA in the initial binding is weak. To address this issue, we performed a gel mobility shift assay, which has been...
previously used for binding assays of several modification enzymes (10, 31, 39). As shown in Fig. 5A, no shift band corresponding to the complex of TrmFO and tRNAPhe transcript was observed under the tested condition, although TrmFO can methylate this tRNA transcript. We tried the gel mobility shift assay under several conditions (e.g. in the presence or absence of SHMT, NADPH, and/or folate analogues). However, we did not detect a shift band derived from the complex formation under any of these conditions (data not shown). These results are in line with the idea that TrmFO has weak affinity for tRNA in the initial binding process.

If TrmFO repeatedly binds and releases RNA in a short time during the initial binding, the weak affinity could be rationally explained. If this idea is correct, TrmFO would show affinity for non-substrate RNAs. To confirm this idea, we performed fluorescence quenching experiments (Fig. 5B). T. thermophilus TrmFO has six tryptophan residues. Fortunately, the addition of tRNAPhe transcript (circles in Fig. 5B) caused a decrease in fluorescence derived from tryptophan residues similar to the wild-type tRNAPhe transcript, demonstrating that TrmFO interacts with non-substrate RNA with the same affinity as for substrate RNA. To clarify whether or not the selection of substrate RNA takes place in the initial binding process, we performed an inhibition assay (Fig. 6). We prepared three types of non-substrate tRNA (methylated tRNAPhe, tRNAPhe without the T-arm, and the tRNAPhe U54C variant). As shown in Fig. 6A, the methylated tRNA did not strongly inhibit the methyl transfer reaction. This result suggests that the methyl group of m5U54 in the methylated tRNA causes steric hindrance with CH2THF in the catalytic pocket. Thus, the methylated tRNA seems to be released after the initial binding process. The weak affinity for tRNA in the initial binding process has an advantage for release of the methylated tRNA. Furthermore, because the methylated tRNA is more abundant as compared with the non-methylated precursor tRNA, this mechanism is rational for effective methylation in a living cell. The tRNA without the T-arm inhibited the methyl transfer reaction strongly as compared with methylated tRNA (Fig. 6B). This result suggests that tRNA without the T-arm is released during the structural change process. Thus, positive determinants in the T-arm mainly function during the structural change process. The tRNAPhe U54C variant strongly inhibited...
the methyl transfer reaction (Fig. 6C), suggesting that the US4C variant progresses the structural change process.

Taking these results together, we concluded that TrmFO does not exclude non-substrate RNA in the initial binding process and that affinity for RNA in the initial binding process is weak. Our results suggest that the positive determinants in the T-arm structure are probably required for progression of the structural change process.

TrmFO Can Distinguish the T-arm from the Anticodon Arm in T. thermophilus tRNAPro—The above conclusion prompted us to ask one question, namely how does TrmFO distinguish the T-arm from the anticodon arm? In general, the nucleotide sequence of the T-arm is partially similar to that of the anticodon arm, although the T-arm and anticodon arm conformations are completely different (54). T. thermophilus tRNAPro has a distinct anticodon arm, in which the essential recognition sequences of TrmFO can be found (Fig. 7A). If TrmFO simply recognizes the nucleotide sequences in the stem and loop structure, TrmFO should methylate both U54 and U32 in tRNAPro. Fortunately, the RNA sequence of T. thermophilus tRNAPro has been reported (55); U32, which corresponds to U54, is known to be unmodified (Fig. 7A). That is to say, this natural modification pattern suggests that TrmFO can distinguish the T-arm from the anticodon arm in T. thermophilus tRNAPro.

Initially, we assumed that the three-dimensional core structure in tRNAPro prevents the incorrect methylation of U32. Thus, we considered steric hindrance by the three-dimensional core. To investigate this idea, we prepared eight tRNAPro variants (Fig. 7B). As shown in Fig. 7C, precursor tRNAPro transcript was clearly methylated. When U54 in the precursor was replaced by C, the methyl group acceptance activity was completely lost (Fig. 7C, lane 2), showing that the methylation site is only U54. When the T-arm was deleted or both D- and T-arms were deleted, U32 was not methylated (Fig. 7C, lanes 3 and 4). Furthermore, when both G18-U55 and G19-C56 tertiary base pairs were disrupted, only U54 was methylated (Fig. 7C, lanes 5 and 6). Moreover, the substitution of the U54-U55 sequence by a C54-C55 sequence brought about the loss of methyl group acceptance activity, showing that the methylation of U32 had not occurred (Fig. 7C, lane 7). Although we could experimentally show that TrmFO is able to distinguish the T-arm from the anticodon arm, these results revealed that our initial idea was incorrect. Thus, the three-dimensional core of tRNAPro does not work as the negative determinant for TrmFO.

The A38 in the Anticodon Arm Works as a Negative Determinant for TrmFO Recognition—Next, we considered whether a negative determinant might exist in the anticodon loop. To address this issue, we designed four tRNAPro US4C variants in which the anticodon arm sequence of tRNAPro US4C was substituted by the T-arm sequence (Fig. 8A–D). The variants (A–D) contained C54 to abolish U54 methylation. We additionally prepared four tRNAPro variants in which U32 was substituted by C to determine the methylation site (E–H). The methyl group acceptance activities of these variants were tested by the gel assay (lower panels). The gel was stained with MB (left) and then subject to autoradiography (right). WT, the positive control, in which the wild-type tRNAPro transcript was used as the substrate.

![FIGURE 8. Replacement of anticodon loop sequence by the T-loop sequence in tRNAPro.](image)

Methyltransferase reaction by A38—In the T-arm, the methylation site U54 forms a reverse Hoogsteen base pair with A58, and the U54-U58 tertiary base pair stacks with the conserved G53-C61 base pair (Fig. 9A). In contrast, U32 in the anticodon arm of the tRNAPro transcript probably forms a Watson-Crick base pair with A38, and the U32-A38 base pair stacks between the G31-C39 and U33-G37 base pairs (Fig. 9B, left). The substitution of A38 by U causes a structural change of this anticodon arm and results in the emergence of a structural equilibrium between the T-arm-like and open loop structures (Fig. 9B, middle and right). This raises the possibility of the anticodon arm being recognized by TrmFO. Thus, the A38 sequence in T. thermophilus tRNAPro would prevent incorrect U32 methylation by
TrmFO. To investigate this hypothetical mechanism, we prepared three tRNAPro variants (Fig. 9). In these variants, G36 and A38 were substituted by A and U, respectively. These substitutions form a U54-A36 tertiary base pair and disrupt the U32-A38 Watson-Crick base pair, and the structural equilibrium in Fig. 9B is shifted to the T-loop-like structure side. In agreement with the hypothesis, the methyl group acceptance activities of these variants were dramatically improved (Fig. 9C). Because these variants do not have U32, the methylation site is U32. These results support the hypothesis in Fig. 9B. The substitution of G36 by A increased the velocity of incorrect U32 methylation by TrmFO. U32 and mutated A36 probably form a reverse Hoogsteen base pair, as seen with U54-A58 in the T-arm. This result suggests that coexistence of U32 and A36 in a single tRNA is to be avoided in order to prevent incorrect U32 methylation.

Effects of Other tRNA Modifications on TrmFO Methylation—In our previous publications (51, 52), we reported the existence of a network formed between modified nucleotides and modification enzymes in T. thermophilus tRNA modification. Briefly, the existence of the m^7G46 modification enhances the velocities of Gm18 and m^1G37 formations, in vivo and in vitro (51). In contrast, the existence of the Ψ55 modification decreases the velocity of Gm18, s^2U54, and m^1A58 formations in vivo (52). In these studies, we showed that the deletion of the m^7G46 or Ψ55 modification in native tRNA^Phe does not affect the m^5U54 content (51, 52). Furthermore, we demonstrated that the m^3U54 modification does not have any effect on the
velocities of m7G46 and Ψ55 formations in vitro (51, 52). However, during these studies, the in vitro TrmFO assay system had not been optimized. Therefore, we have investigated whether the other tRNA modifications have an effect on the TrmFO activity. We prepared four tRNA modification enzymes, tRNA (Gm18) methyltransferase (TrmH) (56), tRNA (m7G46) methyltransferase (TrmB) (57), tRNA (Ψ55) synthase (TruB) (58), and tRNA (m1A58) methyltransferase (TrmI) (59) (Fig. 10A). T. thermophilus tRNA_Phe transcript was individually modified by these enzymes for 3 h at 55 °C and then recovered by phenol/chloroform treatment and ethanol precipitation. The contents of Gm18, m7G46, and m1A58 modification per tRNA molecule were determined by measuring the methylation of transcripts by the enzymes using [14C]AdoMet and were calculated to be 0.86, 0.81, and 0.89, respectively. The content of Ψ55 modification per tRNA molecule was determined to be 0.9–1.00 by HPLC nucleoside analysis (data not shown). As shown in Fig. 10B, Gm18 and m7G46 modifications did not have a significant effect on the velocity of m5U54 formation as catalyzed by TrmFO. In the previous studies (51, 52), the m7G46 and Ψ55 modifications were seen not have an effect on in vivo m5U54 formation. In the case of the m7G46 modification, the current in vitro result is in good agreement with our previous in vivo result, whereas our in vitro experiment showed that the Ψ55 modification slightly accelerates the TrmFO reaction (Fig. 10B). In contrast, the m1A58 modification produced by TrmI clearly accelerated the initial velocity of TrmFO methylation; this effect was confirmed by the repeated experiments (Fig. 10B). This observed effect is probably caused by the reinforcement of the U54-A58 tertiary base pair. The positive determinants (U54U55C56 sequence and G53-C61 base pair) are shown in Fig. 11A. These positive determinants are conserved in all T. thermophilus tRNAs. The positive determinants, U54, U55 (Ψ55 in Fig. 11B), and C56 nucleotides, could be placed on the TrmFO surface. In contrast, the positive determinant, G53-C61 base pair was embedded in the T-arm structure. Because the variant, which has a G53-U61 base pair, was not methylated (Fig. 4), TrmFO can distinguish the G53-C61 and G53-U61 base pairs. In general, cytidine can be discriminated from uridine by the 4-amino group. Therefore, the G53-C61 base pair recognition by TrmFO may...
be not mediated by the direct recognition of bases but by ribose-phosphate backbone recognition. As described above, positive determinants in the T-arm probably function in the structural change process. To clarify the precise mechanism of G53-C61 recognition, further study will be required.

**DISCUSSION**

In this study, we have focused on tRNA recognition by folate/FAD-dependent tRNA methyltransferase, TrmFO. In general, the activity measurement of folate-dependent methyltransferase is not so easy, because $^{14}$CCH$_2$THF is not commercially available. In the early study, $^{14}$CCH$_2$THF was chemically synthesized from THF and $^{14}$Cformaldehyde (44). Because this method is difficult for most biochemical researchers, TrmFO remained a relatively unstudied enzyme. Urbonavicius et al. (46, 60) detected TrmFO activity by 32P-externally labeled tRNA. Although this system is convenient, measurement of velocities is not so easy. Therefore, we initially altered our *in vitro* TrmFO assay system, which we had previously reported (47). We optimized the concentrations of enzymes and substrates and determined the practical ranges. In addition, a filter assay system was introduced instead of the gel assay system. These alterations enabled us to measure the kinetic parameters of TrmFO for electron donors and tRNA transcripts, as shown in Fig. 2. Our assay system may be applicable to the assay of other folate/FAD-dependent tRNA methyltransferases (e.g. the MnmE-MnmG (previous name, GidA) complex, which is involved in the first steps (nm5U34 and cmnm5U34 syntheses) of mmn5U34 formation) (for a recent review, see Ref. 61). The MnmE-MnmG complex changes the pathway by supply of substrates (ammonium ion and glycine) and is regulated by GTP (61). Therefore, the construction of an *in vitro* MnmE-MnmG complex assay system is likely to be more difficult than for TrmFO.

Our current kinetic study showed that NADPH is superior to NADH as an electron donor for TrmFO (Fig. 2). In contrast, it has been reported that MnmG (GidA) uses only NADH as an electron donor (62). Thus, although both folate/FAD-dependent tRNA methyltransferases (TrmFO and MnmG) have a similar FAD-binding domain (47, 62, 63), the binding modes of electron donors are completely different. To understand this difference, we compared the structures of TrmFO and MnmG (data not shown). Because the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD. In contrast, the isoalloxazine ring of FAD in MnmG is embedded in the protein (62, 63), the phosphate group of NADPH may cause steric hindrance to gain access to the FAD.
microhelix RNA complex revealed that TrmA mainly contacts the U54 base and ribose-phosphate backbone of the T-arm (42). In contrast, TrmFO recognizes the conserved nucleotide sequences (U54U55C56 and G53-C61 base pair) in the T-arm. The positive determinants for TrmFO are rather similar to those for tRNA (Ψ55) synthase (TruB) (28) and the 2-thiouridylation complex for s2U54 (7). TruB recognizes the U54U55 sequence and A58 (28), whereas the 2-thiouridylation complex recognizes the U54U55C56 sequence and A58 (7). In comparison, TrmFO has an absolute requirement for the recognition of the G53-C61 base pair while recognizing A58 (U54-A58 tertiary base pair) weakly.

During the course of study, Hamdane et al. (49) reported that B. subtilis TrmFO forms a covalent complex with tRNA via Cys-226. According to their report (49), we also attempted to detect the T. thermophilus TrmFO-tRNA complex; however, we were unable to observe the formation of the covalent complex by electrophoresis, in which the gel was stained with methylene blue and Coomassie Brilliant Blue (data not shown). The stability of the covalent complex would therefore seem to be quite different between B. subtilis and T. thermophilus TrmFO enzymes. The striking similarity of the amino acid sequences of both enzymes suggests that these TrmFO enzymes have the same catalytic mechanism (46). In previous work, we proposed the other cysteine (Cys-51 in T. thermophilus TrmFO, which corresponds to Cys-53 in B. subtilis TrmFO) to be the site of covalent bond formation based on the crystal structure (47). In contrast, Hamdane et al. experimentally verified the covalent bond formation site as Cys-226 in B. subtilis TrmFO (49). Therefore, the covalent bond formation site in our previous hypothetical mechanism should be modified to fit in with the proposal of Hamdane et al. (49). If Cys-226 in B. subtilis TrmFO is the covalent bond formation site, TrmFO may form a dimer structure during the enzymatic reaction (49). To clarify this issue, further study will be necessary.

The formation of a covalent intermediate between substrate RNA and enzyme was also observed in the TrmA reaction; TrmA forms a covalent bond complex not only with tRNA but also with rRNA (24, 64). The formation of a complex between E. coli TrmA and 16S rRNA is essential for cell viability (64). It has been reported that B. subtilis trmFO gene disruption strains can survive (46). However, the enzyme (at least B. subtilis TrmFO) has the potential to form a covalent bond with other RNA species as well as tRNA because the RNA recognition mechanism of TrmFO is relatively simple, as described in this paper.

In previous works (51, 52), we reported the existence of a network between modified nucleotides and modification enzymes in T. thermophilus tRNA modification. In the current study, we confirmed that the U54 modification catalyzed by TrmFO is not significantly influenced by the presence of the Gm18 and m2G46 modifications, whereas the Ψ55 modification may slightly contribute to m1U54 formation. In contrast, the m1A58 modification has a clear positive effect on TrmFO activity. In the previous study, we found that 10–30% of U54 in tRNA^Phe from cells cultured at 55 °C is unmodified U (or s^U) (52). In contrast, U54 in tRNA^Phe from cells cultured at 70 °C is nearly fully modified to m1U54 (or m5s^U54) (52). Therefore, in vivo TrmFO activity seems to be mainly regulated by culture temperatures. At high temperatures (>70 °C), the m1U54 methylation catalyzed by TrmFO, the m1A58 methylation catalyzed by TrmA, and the s2U54 sulfur transfer reaction catalyzed by the 2-thiouridylation complex seem to have a synergistic effect on m5s^2U54 formation. To clarify the role of the m1A54 modification on the other modifications, extensive analysis of a T. thermophilus trmFO gene disruption strain is necessary.

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