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RNA recognition mechanism of eukaryote tRNA (m^7G46) methyltransferase (Trm8–Trm82 complex)

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Abstract **Yeast tRNA (m^7G46) methyltransferase contains two protein subunits (Trm8 and Trm82). To address the RNA recognition mechanism of the Trm8–Trm82 complex, we investigated methyl acceptance activities of eight truncated yeast $tRNA^{Phe}$ transcripts. Both the D-stem and T-stem structures were required for efficient methyl-transfer. To clarify the role of the D-stem structure, we tested four mutant transcripts, in which tertiary base pairs were disrupted. The tertiary base pairs were important but not essential for the methyl-transfer to yeast $tRNA^{Phe}$ transcript, suggesting that these base pairs support the induced fit of the G46 base into the catalytic pocket.**

Keywords: RNA modification; tRNA methyltransferase; 7-Methylguanine; Trm8–Trm82; TrmB; Cell-free protein synthesis

1. Introduction

To date, more than 80 modified nucleosides have been found in tRNA [1–4]. All of these modified nucleosides are produced post-transcriptionally by specific tRNA modification enzymes [5]. N^7-Methylguanosine at position 46 (m^7G46) of tRNA is one of the most commonly modified nucleosides: it is widely found in eukaryotes and bacteria as well as some Archaea [3,4]. The m^7G46 is located in the variable region in tRNA and forms a tertiary base pair with the C13–G22 base pair [6,7].

The m^7G46 modification is produced by tRNA (m^7G46) methyltransferase [tRNA (guanine-N^7-)-methyltransferase, EC 2. 1. 1. 33] [5]. This enzyme activity was first detected in a cell extract of *Escherichia coli* [8] and has been purified more than 1000 fold [9]. The enzyme activity has also been purified from *Salmonella typhimurium* [10] and *Thermus flavus* [11]. Furthermore, the tRNA m^7G46 modification activity has been detected in crude extracts from higher eukaryotes [12–14]. Recently, it has been reported that yeast tRNA (m^7G46) methyltransferase is composed of two protein subunits (Trm8 and Trm82) and their genes have been identified [15]. More recently, bacterial genes have also been identified as *trmB* (classical name, *yggH*) from *E. coli* [16] and *Aquifex aeolicus* [17]. A gene disruption mutant of yeast [15] or *E. coli* [16] has revealed that the tRNA (m^7G46) methyltransferase activity is not essential for cell viability. However, it has been reported that a yeast double mutant strain lacking both *trm8* and *trm4* genes showed rapid degradation of $tRNA^{Val}$ [18]. Thus, the m^7G46 modification in yeast contributes to the stability of tRNA in conjunction with the other modified nucleotide(s) around the variable region in tRNA. Moreover, recently, we have reported that a gene involved in m^7G modification of tRNA is required for infection by the phytopathogenic fungus *Colletotrichum lagenarium* [19].

Although eukaryote tRNA (m^7G46) methyltransferases contain two protein subunits (Trm8 and Trm82 in yeast [15]; METTL1 and WDR4 in human [15,20]), the enzymes from eubacteria are composed of only TrmB protein [16,17,21]. TrmB has motifs conserved in *S*-adenosyl-L-methionine (AdoMet) dependent methyltransferases, however, the amino acid residues involved in the catalytic center of mRNA Cap m^7G methyltransferase (Abd1) are not found, suggesting that the reaction mechanism of TrmB differs from that of Abd1 [16]. Recently, a site-directed mutagenesis study based on bioinformatics elucidated amino acid residues required for substrate binding and methyl-transfer [22]. More recently, the crystal structure of *Bacillus subtilis* TrmB has revealed that eubacterial TrmB is a unique Rossmann fold containing methyltransferase (namely, Class I fold [23]) [21].

In previous work, we reported about the tRNA recognition mechanism of *A. aeolicus* TrmB [17]. *A. aeolicus* is a hyperthermophilic eubacterium, which grows at close to 95 °C [24]. The *A. aeolicus* enzyme has an extra C-terminal portion and a shortened N-terminus as compared to its mesophilic counterpart from *E. coli* [16] or *B. subtilis* [21]. Our previous study revealed that the most important recognition sites of the *A. aeolicus* enzyme are included in the T-arm structure of tRNA [17], suggesting that *A. aeolicus* TrmB accesses the G46 residue from the T-stem side. This eubacterial enzyme is composed of only a TrmB subunit [17]. In contrast, the yeast enzyme is a heterodimer composed of two protein subunits (Trm8 and Trm82) [15]. In this report, we demonstrate differences in tRNA recognition mechanisms between eubacterial and eukaryote tRNA (m^7G46) methyltransferases.

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2. Materials and methods

2.1. Materials

[Methyl-^{14}C]-AdoMet (1.95 GBq/mmol) and [methyl-^{3}H]-AdoMet (2.47 TBq/mmol) were purchased from ICN. Cold AdoMet was obtained from Sigma. DE52 was a product of Whatman. Ni-NTA superflow resin came from Qiagen. Sephadex G-25 was from Pharmacia. DNA oligomers were bought from Invitrogen, and T7 RNA polymerase was from Toyobo, Japan. Other chemical reagents were of analytical grade.

2.2. Preparation of yeast tRNA (m^7G46) methyltransferase

Yeast Trm8–Trm82 heterodimer was synthesized by a wheat germ in vitro translation system [25–28]. Because there was a 6 × His tag sequence expressed at the N-terminus of Trm82, the synthesized enzyme could be purified by Ni-NTA superflow column chromatography and DE52 column chromatography [28]. The Trm8–Trm82 heterodimer fractions were assessed by 12.5% SDS–polyacrylamide gel electrophoresis, combined, and dialyzed against buffer A (50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 50 mM KCl). Glycerol was added to the sample to a final concentration 50% and stored at −30 °C until required.

2.3. Measurement of enzyme activity

A standard assay for enzyme purification was performed according to a previously reported method with slight modifications [17]. Briefly, 0.1 μg protein, 0.1 A260unit yeast tRNA^{Phe} transcript, and 37 μM ^{14}C-AdoMet were incubated for 30 min at 30 °C and then a filter assay performed. Yeast tRNA^{Phe} transcripts were prepared according to our previous reports [17,29]. Before the assay, the transcripts were annealed in the buffer (50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, and 100 mM NaCl) by cooling from 70 °C to 40 °C. CD spectra were measured with a JASCO spectropolarimeter J-820 equipped with a JASCO PTC-423L thermo-controller. Cuvettes with a 1 mm path length were used. Each sample in the annealing buffer was pre-incubated at 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or 70 °C for 15 min and then the spectrum was recorded from 300 to 200 nm at each temperature. The scan was repeated three times. In order to visualize the methyl-transfer reaction, we used 10% PAGE (7 M urea) and an imaging analyzer system. Briefly, tRNA (0.3 A260 units) was incubated with 300 ng of the purified heterodimer and 38 μM [methyl-^{14}C]-AdoMet for 10 min at 30 °C in 30 μl of buffer A, extracted with phenol, collected by ethanol precipitation, and then loaded onto a 10% polyacrylamide gel (7 M urea). The gel was stained with methylene blue and dried. The incorporation of ^{14}C-methyl groups into tRNA was monitored with a Fuji Photo Film BAS2000 imaging analyzer. Apparent kinetic parameters, K_m and V_{max}, were determined by a Lineweaver–Burk plot of the methyl-transfer reaction as measured by a [methyl-^{3}H]-AdoMet filter assay: 1 μg of the protein, 38 μM AdoMet and various concentrations of the transcript in 50 μl of buffer B were incubated for 5 min at 30 °C.

3. Results

3.1. Preparation of yeast tRNA (m^7G46) methyltransferase

Yeast tRNA (m^7G46) methyltransferase (Trm8–Trm82 heterodimer) was successfully synthesized by a wheat germ cell-free protein synthesis system and purified as shown in Fig. 1. The molecular ratio of the Trm8 and Trm82 proteins in this sample was determined by measuring the band intensities in the gel stained with Coomasie brilliant blue and found to be 0.94:1. The enzyme assay using yeast tRNA^{Phe} transcript and ^{14}C-AdoMet clearly showed that this fraction contained a strong methyl-transfer activity (data not shown, see Fig. 2). Kinetic parameters with yeast tRNA^{Phe} transcript at 30 °C were determined by a filter assay using ^{3}H-AdoMet. The values derived from this assay for K_m and V_{max} were 1.5 μM and 1.4 μmol mg^{-1} h^{-1}, respectively (Table 1).

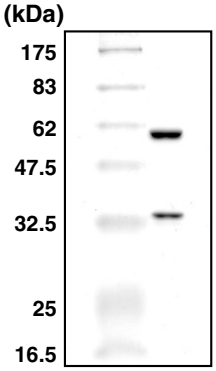

Fig. 1. 12.5% SDS–PAGE analysis of the purified Trm8–Trm82 complex. Four micrograms of the purified Trm8–Trm82 complex was analyzed by 12.5% SDS–PAGE. The gel was stained with Coomasie brilliant blue.

3.2. Methyl acceptance activities of truncated tRNA molecules

To address the recognition sites in tRNA, we prepared eight truncated yeast tRNA^{Phe} transcripts (Fig. 2), because three-dimensional structure of yeast tRNA^{Phe} was established [6,7]. The methyl acceptance activities of these truncated transcripts by *A. aeolicus* tRNA (m^7G46) methyltransferase were previously investigated: the transcripts F, G, and H in Fig 2 were well methylated and transcripts C, E and I were slowly methylated [17]. After the annealing, we measured CD-spectra of the transcripts at various temperatures (15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C) to check the conformation. For example, transcript C had a clear peak (260–280 nm) derived from high order structures at 15–40 °C (data not shown). The transcripts were incubated with 0.5 μg enzyme and ^{14}C-AdoMet at 15 or 30 °C for 10 min, and then were analyzed by 10% polyacrylamide gel (7 M urea) electrophoresis: the gel was stained with methylene blue to visualize the RNA molecules (Fig. 2 lower left panel) and then ^{14}C-methyl group incorporation monitored by autoradiography (Fig. 2 lower right panel). Because the results at 15 °C showed the same tendencies of the results at 30 °C, Fig. 2 shows only the results at 30 °C. As shown in Fig. 2A, methyl-transfer to full-length yeast tRNA^{Phe} was clearly detected, consistent with the result of the filter assay. When truncated RNA corresponding to positions from 34 to 48 (Fig. 2B) was tested, methyl-transfer was not observed. This result demonstrates that Trm8–Trm82 does not simply recognize the variable region sequence. When the aminoacyl stem and D-arm were deleted (Fig. 2C), methyl acceptance activity was not observed. Further, deletions of the T-arm and the D-arm (Fig. 2D) gave the same result. Thus, the essential recognition site(s) seems to include in the aminoacyl-stem, D-arm, and/or T-arm. To our surprise, deletion of the nucleotides from 1 to 18 gave complete loss of methyl acceptance activity (Fig. 2E). In our previous study, this transcript was well methylated by *A. aeolicus* TrmB [17]. Thus, this result demonstrates the importance of the D-arm structure in RNA recognition by Trm8–Trm82. In fact, deletion of the D-arm (Fig. 2F) resulted in complete loss of methyl acceptance activity. In contrast, deletion of the aminoacyl-stem (Fig. 2G) or anticodon-arm (Fig. 2H) did not produce such a severe effect, although the methyl acceptance activities were decreased. These results show that one of the essential sites exists in the D-arm structure. Kinetic parameters for these

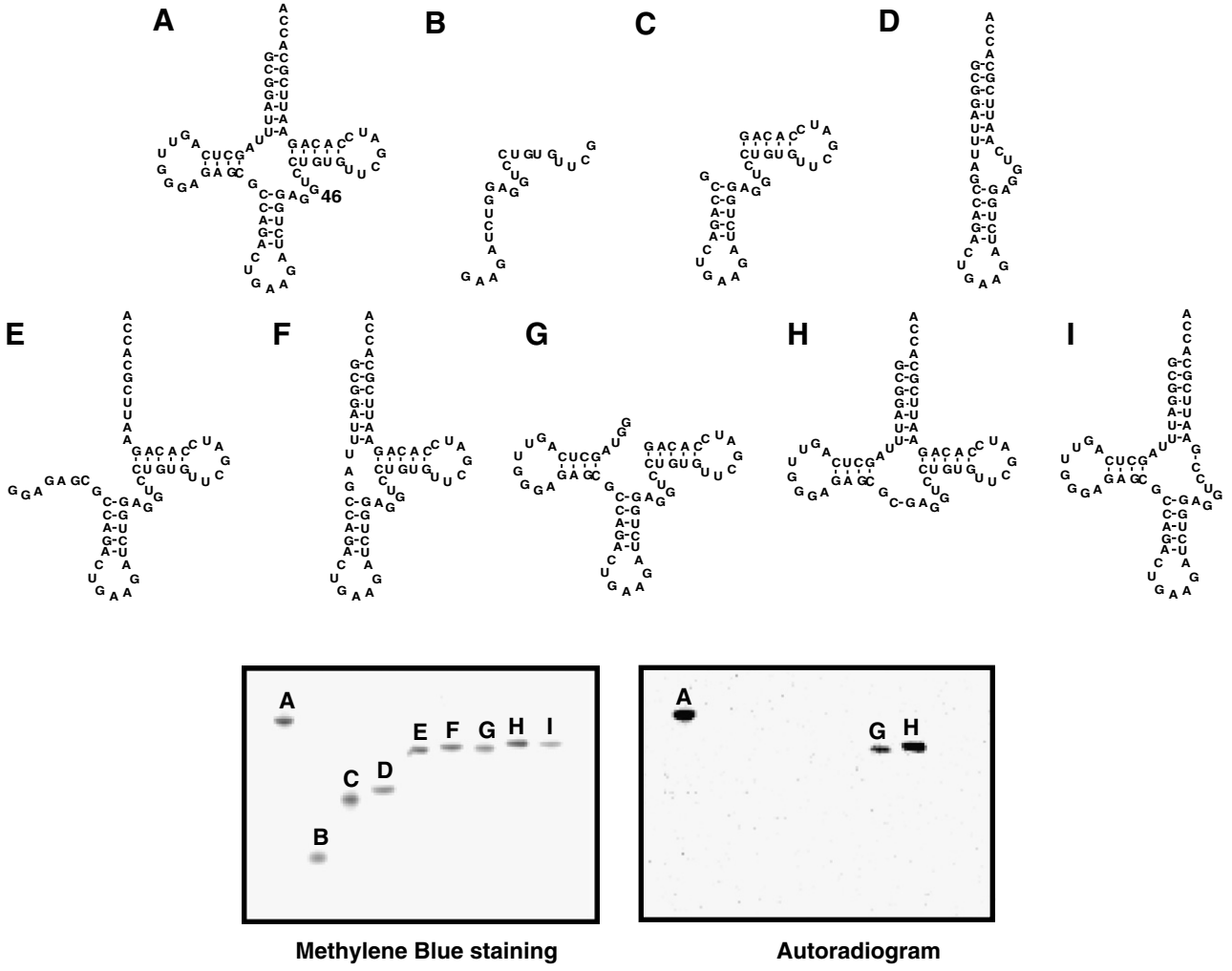

Fig. 2. Methyl-transfer assay of truncated yeast $tRNA^{Phe}$ transcripts. Full-length yeast $tRNA^{Phe}$ transcript (A) and truncated tRNA molecules (B–I) are shown as clover-leaf models. The position 46 is shown in the full-length transcript. Lower panels show the result of the gel assay. After the methyl-transfer reaction, the transcripts were analyzed by 10% polyacrylamide gel (7 M urea) electrophoresis. The gel was stained with methylene blue (left panel) and then incorporation of ^{14}C-methyl groups was monitored by autoradiography (right panel). The bands of transcripts are shown by the transcript names (A–I).

Table 1
Kinetic parameters for $tRNA^{Phe}$ variants

Transcript	Features	K_m (μM)	V_{max} (μmol mg^{-1} h^{-1})	Relative V_{max}/K_m (%)
A	Full length	1.5 ± 0.04	1.4 ± 0.10	100
B	Corresponding to nucleotides (34-48)		Not detectable	
C	Deletions of aminoacyl-stem and D-arm		Not detectable	
D	Deletions of T-arm and D-arm		Not detectable	
E	Deletions of nucleotides (1–18)		Not detectable	
F	Deletion of D-arm		Not detectable	
G	Deletion of aminoacyl-stem	3.7 ± 0.46	0.63 ± 0.20	19
H	Deletion of anticodon-arm	1.4 ± 0.08	0.85 ± 0.03	66
I	Deletion of T-arm		Not detectable	
J	Disruption of C13–G22–G46 tertiary base pair	0.50 ± 0.05	0.24 ± 0.10	52
K	Disruption of D-arm structure		Not detectable	
L	Disruption of T-arm structure		Not detectable	
M	Disruption of tertiary base pairs between D- and T-arms	0.35 ± 0.05	0.11 ± 0.03	33
N	Substitution of U47 by A	1.26 ± 0.47	0.28 ± 0.08	24
O	Substitution of C48 by A	0.25 ± 0.01	0.04 ±0.01	17

"Not detectable" means that methyl-transfer was not observed in the experiments shown in Figs. 2 and 3.

truncated RNA molecules were measured by filter assay with ^3H-AdoMet (Table 1). In addition, the deletion of T-arm structure (Fig. 2I) gave a complete loss of the methyl acceptance activity. Thus, the T-arm structure is one of the essential

recognition sites of Trm8–Trm82. This feature is shared by *A. aeolicus* TrmB [17]. These experimental results suggest that the D-arm structure as well as the T-arm structure plays a key role in tRNA recognition by Trm8–Trm82. Thus, the recognition requirements of yeast Trm8–Trm82 for the G46 base are stricter than those of *A.aeolicus* TrmB.

3.3. Methyl acceptance activities of D-arm mutant transcripts

These results prompted us to investigate the effects of mutations in the D- or T-arm. We therefore prepared four mutant transcripts (Fig. 3). In the L-shaped tRNA structure, the m^7G46 base forms a tertiary base pair with the C13–G22 base pair in the D-stem. When this C13–G22 base pair was disrupted, methyl acceptance activity was considerably decreased but not lost (Fig. 3J). Thus, the C13–G22 base pair is important but not essential for the recognition by Trm8–Trm82. When the D-stem structure was disrupted (Fig. 3K), methyl acceptance activity was hardly detectable. Thus, the D-stem structure is very important and is a semi-essential recognition site of Trm8–Trm82. When the T-stem structure was disrupted, methyl acceptance activity was completely lost (Fig. 3L), demonstrating that the T-stem structure is essential for recognition. We also disrupted the interaction between the T- and D-loops (Fig. 3M). The methyl acceptance activity of this mutant transcript was severely decreased, suggesting that the locations of the D-arm and the T-arm in tRNA affect RNA recognition by Trm8–Trm82, although these positions are not essential. Kinetic parameter analysis revealed these decreases in the methyl acceptance activities toward the mutant transcripts to be mainly caused by decreases in V_{max} values (Table 1).

3.4. The m^7G46 modification in native yeast tRNA molecules

In order to consider native m^7G46 modification patterns, we retrieved all yeast tRNA sequence data from the databases [3,4,30]. The m^7G46 modification in yeast is only found in tRNA species, which have an N44N45G46pyrimidine (Py) 47Py48 sequence in the variable region. In the sequence, Py47 and Py48 are often modified to D47 and m^5C48, respectively. Only one exception is $tRNA^{Ala}$, which has an A44G45G46D47C48 sequence: G46 is not modified to m^7G46. This tRNA has a unique base pair, U13–G22, in the D-stem. The U13–G22 base pair in $tRNA^{Ala}$ probably separates G22 from G46, and the tertiary base pair between G22–G46 seems to be disrupted or changed. We prepared $tRNA^{Ala}$ transcript and analyzed its methyl acceptance activity. The $tRNA^{Ala}$ transcript was not methylated in the tested conditions including a long incubation (24 h). This result coincides with native $tRNA^{Ala}$ modification and is in line with the importance of the C13–G22–G46 tertiary base pair, however it could not be explained only by the tertiary base pair, because yeast $tRNA^{Phe}$ mutant transcript J was methylated (Fig. 3). This discrepancy suggests existence of negative determinant in the $tRNA^{Ala}$ sequence, which does not exist in yeast

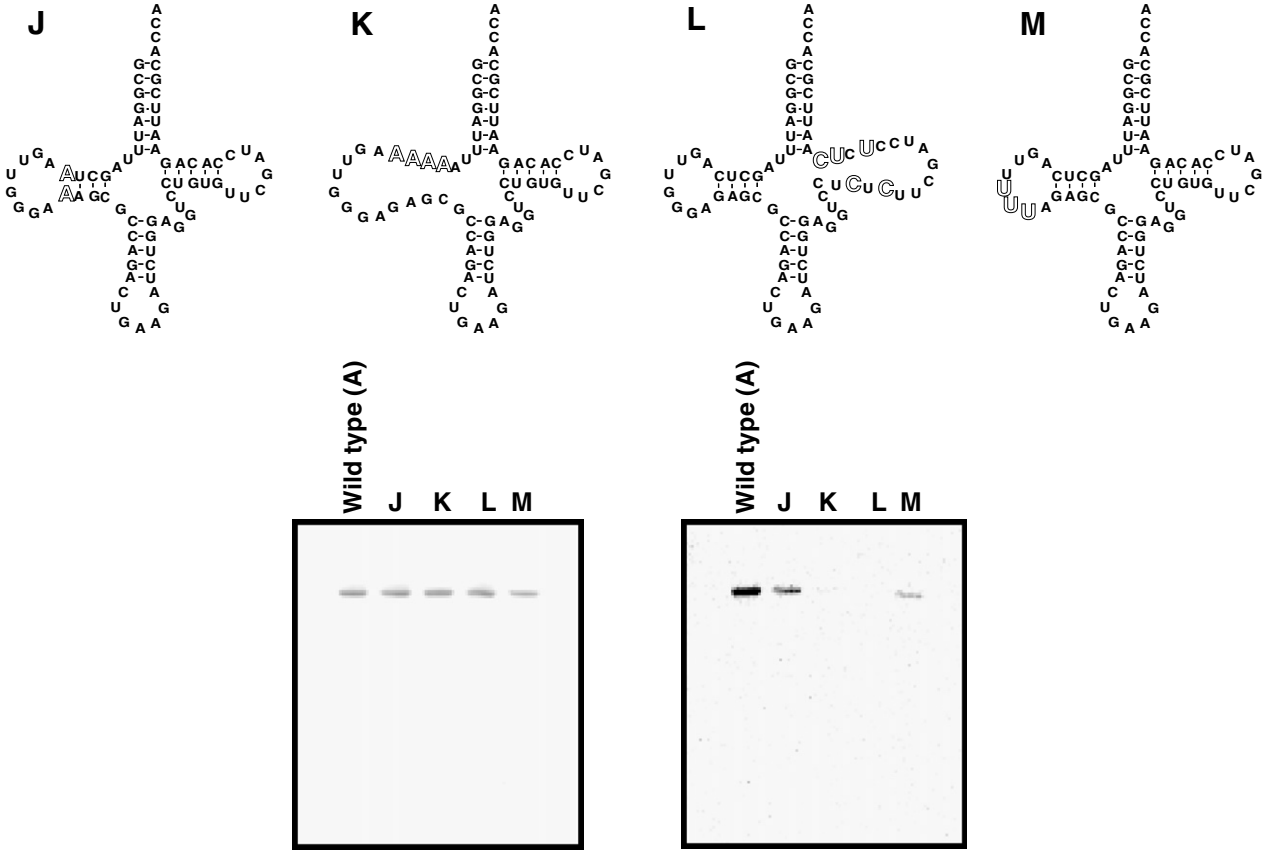

Fig. 3. Methyl-transfer assay of mutant yeast $tRNA^{Phe}$ transcripts. The mutation sites are highlighted as closed letters. The methyl group incorporations were monitored by the same method in Fig. 2. The lane labeled "Wild-type (A)" is the wild-type yeast $tRNA^{Phe}$ transcript shown in Fig. 2A. The lanes (J–M) correspond to the transcripts (J–M).

$tRNA^{Phe}$ transcript. Furthermore, in yeast tRNA species, $tRNA^{Pro}1$, $tRNA^{Pro}2$, $tRNA^{Val}1$ and $tRNA^{Val}2$ have an m^7G46 modification and unique nucleotides, Ψ13 and U22, in the D-arm. The tertiary base pair, C13–G22–G46, does not exist in these tRNA species, although it may be replaced by a Ψ13–U22–G46 tertiary base pair. We prepared the $tRNA^{Val}1$ transcript and tested its methyl acceptance activity. This transcript was methylated very slowly during a long incubation (24 h), however we could not measure the initial velocity correctly: the initial velocity was estimated to be below 0.05% of that to yeast $tRNA^{Phe}$ transcript. This result strongly suggests that the other factor(s) such as modified nucleotide(s) in $tRNA^{Val}1$ precursor, another protein and/or RNA is involved in $tRNA^{Val}1$ recognition by Trm8–Trm82. The Ψ13 modification may be required for efficient methylation by Trm8–Trm82, because this modification occurs in $tRNA^{Pro}1$, $tRNA^{Pro}2$, $tRNA^{Val}1$ and $tRNA^{Val}2$. Moreover, to clarify whether the Py47 and Py48 are essential for Trm8–Trm82 recognition or not, we made two mutant yeast $tRNA^{Phe}$ transcripts, which were substituted U47 or C48 by A. The U47A mutant transcript was well methylated (Table 1). On the other hand, the C48A mutant transcript was decreased its methyl acceptance activity through decrease of V_{max} value (Table 1). Because the substitution of C48 by A disrupts the G15–C48 tertiary base pair, this structural perturbation of the variable region seems to cause the decrease of the methyl acceptance activity. Thus, we confirmed that the Py47 is not important and the Py48 is required for efficient methylation.

4. Discussion

In this study, we have demonstrated that the major recognition sites of Trm8–Trm82 are the D- and T-stem structures. The G46–C13–G22 tertiary base pair is not essential but important for efficient methyl-transfer. The G18–U55 and G19–C56 tertiary base pairs are not essential, however disruption of the T- and D-loop interaction causes a severe decrease in methyl acceptance activity. Because these disruptions of the tertiary base pairs perturb the distance and angle of the G46 base from the T-stem, Trm8–Trm82 would seem to recognize the G46 base from the T-stem side. This feature is shared with the *A. aeolicus* TrmB [17]. In the current study, the importance of the D-stem structure in the recognition mechanism of the Trm8–Trm82 was unexpected. In the mechanism, the base pair at position 13 and 22 seems to play a key role. Recently, Alexandrov et al. have reported the systematic double deletion mutants of tRNA modification enzyme genes in yeast [18]. In their study, *trm4–trm8*, *pus7–trm8* and *dus3–trm8* double deletion mutants showed rapid degradations of $tRNA^{Val}1$: *trm4*, *pus7* and *dus3* genes are encoded tRNA (m^5C34, 40, 48, 49) methyltransferase [31], tRNA (Ψ13, 35) synthase [32], and tRNA (D47) synthase [33], respectively. Their results clearly demonstrate that the modified nucleotides around the variable region contribute the in vivo stability of tRNA. In the current study, requirement of the other factor(s) for efficient methylation of the $tRNA^{Val}1$ was suggested. To clarify these issue, further studies, especially elucidations of relationship between modified nucleotide(s) and recognition mechanism by modification enzyme(s), are necessary. Taking the experimental results together, we concluded that Trm8–Trm82 has stricter requirements for the recognition of the G46 base than *A. aeolicus* TrmB. The measurement of kinetic parameters revealed that the decreases in methyl acceptance activities were mainly caused by decreases in V_{max} values, suggesting that the enzyme captures the truncated or mutated tRNA molecule in the same manner as a full-length wild type transcript, but that the induced fit of the G46 base into the catalytic pocket is slow down.

There is a clear structural difference between eukaryotic and bacterial tRNA (m^7G46) methyltransferase: the eukaryotic enzyme is a heterodimer [15], while bacterial enzyme is a monomer [16] or homodimer [21]. In the case of the yeast enzyme, amino acid sequence analysis strongly suggests that the Trm8 protein functions as a catalytic subunit [15]. However, the role of the Trm82 subunit is still unclear. The tRNA docking model reported in the *B. subtilis* TrmB crystal study suggests that the one TrmB protein captures one tRNA molecule without support from the other subunit [21]. As described above, because yeast Trm8–Trm82 has stricter recognition requirements for the tRNA molecule than bacterial TrmB, the tRNA contact regions in Trm8–Trm82 seem to be increased. Thus, a part of the Trm82 subunit may be in contact with the tRNA molecule. To clarify these issues, more detailed studies are necessary.

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