Transfer RNA (m^7G46) methyltransferase catalyzes the methyl transfer from S-adenosylmethionine to N^7 atom of the guanine 46 residue in tRNA. Analysis of the *Aquifex aeolicus* genome revealed one candidate open reading frame, aq065, encoding this gene. The aq065 protein was expressed in *Escherichia coli* and purified to homogeneity on 15% SDS-polyacrylamide gel electrophoresis. Although the overall amino acid sequence of the aq065 protein differs considerably from that of the Trm8 from E. coli, the purified aq065 protein possessed a tRNA (m^7G46) methyltransferase activity. The modified nucleoside and its location were determined by liquid chromatography-mass spectroscopy. To clarify the RNA recognition mechanism of the enzyme, we investigated the methyl transfer activity to 28 variants of yeast tRNA^{Phe} and *E. coli* tRNA^{Thr}. It was confirmed that 5'-leader and 3'-terminator RNAs of tRNA precursor are not required for the methyl transfer. We found that the enzyme specificity was critically dependent on the size of the variable loop. Experiments using truncated variants showed that the variable loop sequence inserted between two stems is recognized as a substrate, and the most important recognition site is contained within the T stem. These results indicate that the L-shaped tRNA structure is not required for methyl acceptance activity. It was also found that nucleotide substitutions around G46 in three-dimensional core decrease the activity.

Transfer RNAs have now been shown to contain >80 modified nucleosides (1–3). All of the modified nucleosides of tRNA are generated post transcriptionally by specific tRNA modification enzymes (3–6). The substrate recognition is an important feature of these enzymes. Transfer RNA modification enzymes must distinguish specific tRNAs from the other tRNA. Transfer RNA modification enzymes (3–6) are composed of two protein subunits (Trm8 and Trm82), and the enzyme specificity was critically dependent on the size of the variable loop. Experiments using truncated variants showed that the variable loop sequence inserted between two stems is recognized as a substrate, and the most important recognition site is contained within the T stem. These results indicate that the L-shaped tRNA structure is not required for methyl acceptance activity. It was also found that nucleotide substitutions around G46 in three-dimensional core decrease the activity.

Transfer RNA modification enzymes to be elucidated (24–30). Furthermore, crystal structure studies of some enzymes have begun to elucidate the interaction between DNA and the protein (31–35).

N^7-Methylguanosine at position 46 (m^7G46) of tRNA is widely found in eukaryotes and bacteria as well as some Archaea. This modification is found in almost class I tRNAs that have the semi-conserved G46 residue (1–3). In addition, there are a limited number of examples where the m^7G modification is found at other positions, for example, G34 in mitochondria tRNA^{Sue} from starfish (36) or squid (37) and G36 in chloroplast tRNA^{Leu} (38). The m^7G46 modification is catalyzed by tRNA (m^7G46) methyltransferase (tRNA (guanine-N^7)-methyltransferase, EC 2.1.1.33). This enzymatic activity was first detected in a cell extract of *Escherichia coli* (39) and then purified more than 1000-fold (40). The enzyme activity has also been purified from *Salmonella typhimurium* (41) and *Thermus flavus* (42). Furthermore, the tRNA m^7G46 modification activity has been detected in the crude extracts from higher eukaryotes (i.e. *Xenopus laevis* (43), human (44), and plant (45)). Recently, it has been reported that yeast tRNA (m^7G46) methyltransferase is composed of two protein subunits (Trm18 and Trm82), and their genes have been identified (46). Furthermore, an *E. coli* gene encoding a tRNA (m^7G46) methyltransferase activity (*yggH*) has been reported (47). We independently identified *yggH* as a responsible gene for m^7G46 modification in *E. coli* by using a systematic gene disruption system. We searched for *yggH* homologs in several genomes of thermophilic organisms, since proteins from these sources are generally more stable than their mesophilic counterparts (15, 23, 49–51). After some preliminary trials we selected *Aquifex aeolicus*, a hyper-thermophilic eubacterium, as the target species. *A. aeolicus*, which was isolated from a hot spring in Yellowstone National Park, can grow at nearly 95 °C (52). The 16S rRNA gene of *A. aeolicus* has been analyzed from the perspective of molecular evolution, and it was suggested that this bacterium is the earliest diverging eubacterium (53). The complete genome sequence of this organism was determined in 1998 (54). Characterization of the *yggH* homolog from *A. aeolicus* should help clarify the molecular evolution of m^7G46 modification in tRNA.

In this paper, we report that *A. aeolicus* open reading frame aq065, which shares relatively poor homology with *E. coli* *yggH*, encodes a tRNA (m^7G46) methyltransferase. We have studied the substrate RNA recognition mechanism of the enzyme.
**EXPERIMENTAL PROCEDURES**

**Materials**—[Methyl-\(^{14}\)C]AdoMet (1.95 GBq/nmol) and [methyl-\(^{3}\)H]AdoMet (2.47 TBq/nmol) were purchased from ICN. Cold AdoMet was obtained from Sigma. DE52 was a product of Whatman. CM-Toyopearl 650M came from Tosoh, Japan. DNA oligomers were bought from Invitrogen, and T7 RNA polymerase was from Toyobo, Japan. Toyopearl 650M came from Tosoh, Japan. DNA oligomers were bought from Invitrogen, and T7 RNA polymerase was from Toyobo, Japan. Other chemical reagents were of analytical grade.

**Construction of A. aeolicus aq065 Protein Expression System in E. coli**—The aq065 gene was amplified by PCR from A. aeolicus genomic DNA using following primers: AYGGHN, 5'-GGG GCA TAT GCT CTG C-3', AYGGHC, 5'-GGG GCT ACA TAT GCT CTG C-3'. The recognition sites of restriction enzymes NdeI and SalI are underlined. The amplified DNA was cloned into pET30a expression vector (Novagen) utilizing the restriction enzyme sites. The resulting expression construct, pET30a-AAYGGH, was introduced into E. coli BL21(DE3)-Codonplus-RIL strain (Strategene) for expression.

**Expression and Purification of the aq065 Protein**—The expression of the aq065 protein in E. coli cells was carried out according to the manufacturer's manual (Novagen). After the isopropyl 1-thio-\(-\beta\)-galactopyranoside induction, the cells were collected by centrifugation at 6500 \(x\) g for 20 min, snap-frozen in liquid nitrogen, and stored at \(-80^\circ\)C until required. The cells (5 g) were suspended in 25 ml of the buffer A containing 200 mM KCl. The enzyme was eluted by the addition of 60 ml of buffer B containing 300 mM KCl. The enzyme sample was dialyzed against buffer A containing 200 mM KCl. The relevant fractions were pooled and then washed with 60 ml of buffer B and then 60 ml of buffer B containing 150 mM KCl. The enzyme sample was dialyzed against buffer A containing 300 mM KCl. The fractions were combined, dialyzed against the buffer A, and concentrated with Amicon ultra centrifugal filter device (10,000 M, cut-off) (Millipore). Glycerol was added to the sample to give a final concentration of 50% v/v. Aliquots were then frozen using liquid nitrogen and stored at \(-80^\circ\)C until required. Protein was quantified using a Bio-Rad protein assay kit with bovine serum albumin as the standard.

**Measurements of the Enzymatic Activities**—The standard assay for the purification was carried out by measuring the incorporation of a [methyl-\(^{14}\)C]AdoMet into yeast tRNA\(^{\text{Phe}}\) transcript. The assay mixture (300 ng the protein, 15.3 \(\mu\)M transcript, and 38 \(\mu\)M [methyl-\(^{14}\)C]AdoMet) in 30 \(\mu\)l of buffer C (50 mM Tris-HCl (pH 7.6), 5 mM MgCl\(_2\), 6 mM 2-mercaptoethanol, and 50 mM KCl) was incubated for 5 min at 60 °C. An aliquot (20 \(\mu\)l) was then used for the conventional filter assay. The tRNA transcripts were prepared as reported previously (23) and purified by 10% polyacrylamide gel electrophoresis (7 M urea). Each transcript was annealed (cooling down from 80 to 40 °C for 40 min) in the buffer C before use. Apparent kinetic parameters, \(K_m\) and \(V_{\text{max}}\), were determined from a Lineweaver-Burk plot of the methyl transfer reaction with [methyl-\(^{3}\)H]AdoMet by the filter assay. Briefly, the wild-type transcript and tRNA precursor were assayed at 60 °C, and the other variants were assayed at 50 °C. Methyl transfer was visualized after gel electrophoresis using a Fuji Photo Film BAS2000 imaging analyzer system. A mixture of 100 ng of purified enzyme, 38 \(\mu\)M [methyl-\(^{14}\)C]AdoMet, and 13.5 \(\mu\)M transcript in 30 \(\mu\)l of the buffer C was incubated at 30, 50, or 60 °C for 10 min. The relevant assay temperature of each experiment is stated in “Results.” The reaction mixture (5 \(\mu\)l) was then analyzed by 10% polyacrylamide gel electrophoresis (7 M urea). The gel was stained with methylene blue and dried. The incorporation of \(^{14}\)C methyl group was monitored with a Fuji Photo Film BAS2000 imaging analyzer system. Methyl transfer using poor substrates required an extended incubation period and a large amount of the enzyme. Specifically, the reaction mixture comprised 300 ng of enzyme, 38 \(\mu\)M [methyl-\(^{14}\)C]AdoMet, and 13.5 \(\mu\)M transcript in 30 \(\mu\)l of the buffer C. The mixture was incubated at 50 °C for 30 min, and then the aliquot (5 \(\mu\)l) was loaded onto the gel for analysis.

**Mass Spectrometry**—Yeast tRNA\(^{\text{Phe}}\) (50 \(\mu\)g) was methylated with an excess amount of enzyme and cold AdoMet for 4 h at 60 °C in 100 \(\mu\)l of the buffer C. The RNA was extracted with phenol, recovered by ethanol
precipitation, and loaded onto a 10% polyacrylamide gel (7 M urea). After electrophoresis, the RNA was visualized by UV (254 nm) irradiation on a thin layer plate (Funacell P-254, Japan), excised, and extracted with 400 μL of gel elution buffer (0.5 M ammonium acetate, 10 mM MgCl₂, 1 mM EDTA, and 0.1% SDS). The extracted sample was passed through a Steradisc 13 filter unit (0.2 μm, Kurabo, Japan), and the RNA was recovered by ethanol precipitation. For nucleoside analysis, the sample was completely digested with nuclease P1 and then treated with bacterial alkaline phosphatase. For fragment analysis, methylated or unmethylated control tRNA (25 μg of each) was digested with RNase T1 (2.5 unit) in 25 mM ammonium acetate (pH 5.3) at 37 °C for 1 h and subjected to mass spectrometric analysis. Oligonucleotides produced by RNase T1 digestion were separated and analyzed by LC/MS in negative ion mode as described previously (49, 55).

RESULTS

Expression and Purification of A. aeolicus aq065 Protein—To investigate the origin of m7G46 modification in tRNA, we searched for homolog(s) of E. coli yggH in the A. aeolicus genome (NC_000918) by BLAST search. One candidate open reading frame, aq065, was found. The aq065 protein has three amino acid sequence motifs that resemble those of E. coli YggH (Fig. 1A). However, the N-terminal portion of aq065 protein is much shorter than E. coli YggH, and the C-terminal portion does not share homology (Fig. 1B). To characterize this protein, we cloned the target gene by PCR and engineered it for expression in E. coli BL21(DE3)-Codonplus-RIL strain/pET30a vector system as described under “Experimental Procedures.” The purified recombinant protein appeared to be homogeneous by 15% SDS-polyacrylamide gel electrophoresis (Fig. 1C).

The aq065 Protein Has a tRNA (m7G46) Methyltransferase Activity—We tested the methyl transfer activity of the purified recombinant protein using yeast tRNAPhe transcript by the conventional filter assay. The time-dependent experiment at 60 °C clearly showed that 14C methyl group was incorporated into the transcript (data not shown). The kinetic parameters for yeast tRNAPhe transcript were determined at 50 and 60 °C (Table 1). The initial velocity of the enzyme is comparable with other tRNA methyltransferases from A. aeolicus, such as tRNA(Gm18) methyltransferase (23) and tRNA(m1G37) methyltransferase.3 However, the kinetic analysis revealed that this enzyme has relatively small Km and Vmax values for this transcript. The small Km may suggest that many more amino acid residues are involved in the substrate RNA recognition as
compared with the other tRNA methyltransferases. This assumption is in line with the results using the mutant RNA variants described in "Results." Furthermore, the slow $V_{\text{max}}$ may be caused by the relatively poor reactivity of N7 atom of the guanine (6).

To identify the modified nucleoside and the precise position
of the methylated site, we employed LC/MS using electrospray iontrap mass spectrometry (Figs. 2 and 3). Yeast tRNA<sup>Phe</sup> transcript was methylated by aq065 protein using unlabeled AdoMet at 60 °C for 4 h. For nucleoside analysis, the methylated transcript was completely digested with nucleases P₁ and bacterial alkaline phosphatase and then subjected to mass spectrometric analysis as described previously (49, 55). As shown in Fig. 2, m7G nucleoside was eluted at 18.8 min by LC. Mass signals of the protonated molecule of the m7G nucleoside (m/z = 298) and the fragment ion derived from the base moiety (m/z = 166) were clearly detected. The position of the modified site was determined by RNase T₁ fragment mapping (Fig. 3). The methylated RNA was completely digested with RNase T₁ and subjected to mass spectrometric analysis. The fragments were separated on LC (Fig. 3A, top). When the guanine base was modified to m7G, RNase T₁ did not cleave the site. As shown in Fig. 3A, the methylated fragment m⁷GUCCUGp (fragment 11 at 31 min) was clearly identified as a triple-charged negative ion (m/z = 1944.2). Fragment 11 corresponds to the nucleotides at positions 46–51 in the transcript (Fig. 3B). We also detected trace amounts of the unmodified fragment UCCUGp (fragment 9 at 30 min), indicating an incomplete m⁷G46 modification of the substrate tRNA (Fig. 3A). The methylation efficiency was estimated to be around 90% in this experiment. Furthermore, all other fragments derived from yeast tRNA<sup>Phe</sup> were analyzed (Fig. 3B), and no m7G modification was detected in any of them. Based on these results, we concluded that the aq065 protein has a tRNA (m7G46) methyltransferase activity. According to the nomenclature established for the E. coli gene encoding tRNA (m7G46) methyltransferase, we tentatively renamed aq065 as A. aeolicus YggH (accession number AB167817), although there is still some debate concerning the gene name of the E. coli enzyme as described by De Bie et al. (47).

5'-Leader and 3'-Trailer Sequences in tRNA Precursor Are Not Required for the Recognition of A. aeolicus YggH—In vivo, m7G46 modification occurs in the tRNA precursor before removal of the 5'-leader and 3'-trailer RNAs (57). First, we tested the effect of these RNAs on A. aeolicus YggH activity. We chose E. coli tRNA<sup>Gly</sup> (GGU) precursor as a model because m7G46 modification of this precursor in living E. coli cells has been reported (57). We prepared E. coli tRNA<sup>Gly</sup> precursor and the mature size transcript; a CCA sequence was added at 3'-end of the mature size transcript instead of the 3'-trailer sequence (Fig. 4A). The methyl transfer activities to them were compared with each other. As shown in Fig. 4B, both RNAs were efficiently methylated, and kinetic parameters for them were determined by the filter assay (Table II). Although the initial velocity for the precursor was slightly slower due to an increase in Kₘ, it was not significantly different. The increase of Kₘ for the precursor may be caused by nonspecific interaction of the enzyme with the 5'-leader and/or 3'-trailer RNAs. Thus, our results demonstrate that the 5'-leader and 3'-trailer RNAs are not required for substrate recognition of A. aeolicus YggH. Nevertheless, this modification to the precursor RNA probably occurs in vivo.

Substitution of the Variable Loop of Yeast tRNA<sup>Phe</sup> Transcript by the E. coli tRNA<sup>Gly</sup> Variable Loop—The three-dimensional structure of yeast tRNA<sup>Phe</sup> is now well established (Refs. 58 and 59 and see Fig. 9). Therefore, we selected yeast tRNA<sup>Phe</sup> as a model substrate instead of E. coli tRNA<sup>Phe</sup> for further studies. For comparison of the A. aeolicus YggH with the E. coli protein, we tested one yeast tRNA<sup>Phe</sup> variant whose variable loop is substituted by the E. coli tRNA<sup>Gly</sup> variable loop (Fig. 5). E. coli tRNA<sup>Gly</sup> has a special variable loop composed of only four nucleotides. In living E. coli cells, unlike other class I tRNAs, tRNA<sup>Gly</sup> does not undergo m7G modification (1, 2). Thus, E. coli YggH does not recognize tRNA<sup>Gly</sup> as a substrate. As shown in Fig. 5B, this variant was not methylated at all by A. aeolicus YggH, demonstrating that the A. aeolicus enzyme strictly recognizes the size of the variable loop. This recognition mechanism of the A. aeolicus YggH is common with that of E. coli YggH.

Truncated Variants of Yeast tRNA<sup>Phe</sup> Transcript—To clarify the essential region in tRNA, we prepared seven truncated yeast tRNA<sup>Phe</sup> variants (Fig. 6). The experiments were carried out at 30 and 50 °C after annealing of the RNAs since temperature-induced structural changes were expected. To our surprise, methyl group incorporations into the truncated RNAs at 30 °C apparently coincided with those at 50 °C (data not shown). In addition to the conventional filter assay, we employed gel electrophoresis and imaging analysis to detect very slow methyl transfer (Fig. 7). Fig. 7 shows the results at 50 °C. Kinetic parameters are given in Table III. The RNA fragment corresponding to nucleotides at positions 34–48 (Fig. 6B) of the full-length tRNA was not methylated, suggesting that A. aeolicus YggH does not simply recognize the sequence of the variable loop. Thus, the three-dimensional structure of the RNA molecule is a critical factor for the methyl transfer reaction to

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**Table II**

|          | Kₘ (nm) | Vₘₐₓ (nmol ng⁻¹ h⁻¹) |
|----------|---------|----------------------|
| Mature size transcript | 110     | 160                   |
| Precursor | 150     | 180                   |

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**Fig. 5. Assay of the methyl transfer activity to a variant, which has a variable loop of E. coli tRNA<sup>Gly</sup>.** A, the variable loop of yeast tRNA<sup>Phe</sup> was substituted by that of E. coli tRNA<sup>Gly</sup>. Outlined letters indicate the altered nucleotides. B, results of the assay of the methyl transfer activity to the wild-type (lane 1) and the variant (lane 2). The RNAs treated with the purified YggH protein and [1⁴C]AdoMet at 60 °C for 10 min were analyzed by 10% polyacrylamide gel (7 M urea) electrophoresis. The gel was stained with methylene blue (left, MB staining), and the corresponding autoradiogram is shown (right, autoradiogram).
When the anticodon- and T-arms are formed (Fig. 6C), the methyl transfer reaction into the transcript can be observed. The imaging analyzer system detected very slow methyl transfer under the conditions described under "Experimental Procedures" (Fig. 7, C and D). The kinetic parameter analysis showed that the decrease of the activity was caused by a decrease of $V_{\text{max}}$. The small $K_m$ value means that the enzyme effectively captures and releases the truncated RNA in the turnover of the reaction complex. Furthermore, this result clearly shows that the structure of the variable loop inserted into the two stems is an absolute requirement for the methyl transfer reaction. In contrast, when the anticodon and aminoacyl stems are formed (Fig. 6D), recovery of the methyl acceptance activity was not observed (Fig. 7, C and D). This result suggests that the T-arm structure plays a key role in the recognition of the substrate RNA. To confirm this idea, we individually deleted four domain structures of tRNA: aminoacyl stem, D-arm, anticodon-arm, and T-arm (Fig. 6, E–H). Intriguingly, not all truncated variants lost methyl acceptance activity, although deletion of the T-arm did cause a dramatic decrease in activity (Fig. 7). These results suggest the existence of multiple recognition sites dispersed on the tRNA structure, of which the T-arm is clearly important. It is also clear that the L-shaped structure of tRNA is not required for the methyl transfer reaction. This is in line with RNA recognition mechanisms of several tRNA modification enzymes, which modify the nucleotide(s) in the three-dimensional core of tRNA (15, 22, 23, 29). Furthermore, several truncated variants (Fig. 6, C, E, G, and H) displayed small $K_m$ values in comparison to that of the full-length transcript. This result may indicate that disruption of the L-shaped structure is necessary for methyl transfer in the enzyme-tRNA complex to occur.

**Disruptions of Stem Structures around the Variable Loop—**

To confirm the importance of T-arm, we tested two variants; one has a disrupted T-stem (Fig. 8A), and the other has a disrupted anticodon-stem (Fig. 8B). The methyl acceptance activity of the variant with the disrupted T-stem (Fig. 8A) was not detectable by the imaging analyzer system. Together with the results obtained from the truncated variants (Figs. 6 and 7), the most important site for the methyl transfer reaction appears to be on the T-stem. In contrast, the variant disrupted anticodon-stem (Fig. 8B) was clearly methylated, although the initial velocity was considerably decreased via increase of the $K_m$ value. These results indicate that YggH recognizes the G46 residue from the T-stem side.
Disruptions of Tertiary Base Pairs in the Three-dimensional Core—As described above, we have demonstrated the importance of the T-stem. Nevertheless, the variant with a deleted T-arm was still methylated (Fig. 6H and 7). This apparent discrepancy can be resolved by considering the formation of tertiary base pairs between the D-arm and the extra loop in the variant with the deleted T-arm, as found in mitochondria tRNAs from nematoda (48, 56). Thus, the variable loop structure allows the formation of tertiary base pair(s), which probably compensates for the lack of T-arm. In yeast tRNAPhe, the m7G46 residue is located in the three-dimensional core of tRNA (Fig. 9A) and forms a m7G46-C13-G22 tertiary base pair (Fig. 9B). This tertiary base pair is stacked among A9-U12-A23, G15-C48, and m2G10-C25-G45 tertiary base pairs in the core (Fig. 9C). Although the N7 atom of G46 residue is located on the surface of yeast tRNAPhe, disruption of the core is necessary for the recognition of the entire G46 base. This is in line with the results from the truncated variants where several variants showed small $K_m$ values as compared with the full-length transcript. Therefore, we tested the effect of the disruption of the tertiary base pair(s) on the methyl acceptance activity. We made 10 variants possessing a point mutation; eight were either disrupted or had altered tertiary base pairing, and two had disrupted base pairing in the stem (Fig. 10A). In addition, we made five variants possessing double mutations that had disrupted base pairing (Fig. 10B). The results are summarized in Table IV. These results reveal that no tertiary base pair is essential for the RNA recognition. However, the mutations around G46 have an important effect on the methyl acceptance activity. The variants 9A→U, 9A→C, 15G→C, and 13C→G/22G→C) have relatively poor methyl acceptance activities. Because these mutation sites are nearest to G46 in the core (Fig. 9C), the decrease in methyl acceptance activities seems to be caused not only by disruption of the tertiary base pairs but also by the alteration of the stacking effect between the mutation site and G46. In contrast, disruptions of the C25-G10-G45 tertiary base pair had no significant effect on the methyl acceptance activity. Furthermore, the disruption of the interaction between the D- and T-loop (54U→A/55U→A variant) had only a small effect on the methyl acceptance activity. Furthermore, it was found that the 65G→C variant, which disrupted the C49-G65 base pair in the T-stem, displays low methyl acceptance activity, consistent with the importance of the T-stem structure.

DISCUSSION

In this paper we have demonstrated that the open reading frame aq065 from \textit{A. aeolicus} encodes a protein that has a tRNA (m7G46) methyltransferase activity. The purified protein catalyzed methyl transfer to the N7 atom of G46 in a tRNA transcript, as confirmed by LC/MS. Based on the experimental results, we renamed aq065 as \textit{A. aeolicus} yggH (accession number AB167817) according to the nomenclature established for the \textit{E. coli} gene. However, comparison of the \textit{A. aeolicus} and

| Transcript | Features | $K_m$ nm | $V_{max}$ nmol mg$^{-1}$ h$^{-1}$ | Relative $V_{max}/K_m$% |
|------------|----------|----------|------------------|----------------------|
| A          | Full length | 110      | 110              | 100                  |
| B          | Corresponding to positions 34–48 | Not detectable$^b$ | Not detectable$^b$ |
| C          | Deletions of aminoacyl-stem and D-arm | 40       | 0.6              | 1.5                  |
| D          | Deletions of T-arm and D-arm | 60       | 90               | 150                  |
| E          | Deletion of D-arm | 120      | 90               | 75                   |
| F          | Deletion of amino acid-stem | 80       | 90               | 113                  |
| G          | Deletion of anticodon-arm | 50       | 10               | 20                   |
| H          | Deletion of T-arm | 90       | 110              | 100                  |

$^a$The relative $V_{max}/K_m$ values are expressed with respect to that of the full length transcript, which was taken as 100%.

$^b$“Not detectable” means that the methyl transfer was not detectable by the gel electrophoresis and imaging analyzer system as shown in Fig. 7.

**FIG. 8.** Two variants of yeast tRNAPhe disrupted the stem structures. A, the T-stem structure was disrupted by the change of the stem sequences. B, the anticodon-stem structure was disrupted by the change of the stem sequences.
E. coli YggH proteins highlighted considerable differences in terms of the overall size of the two enzymes and the locations of the conserved sequences (Fig. 1, A and B). During the course of this study, De Bie et al. (47) reported that E. coli yggH encodes the tRNA \( \text{m}^7 \text{G46} \) methyltransferase activity in E. coli. They compared the amino acid sequence of E. coli YggH with those of the other methyltransferases and found that E. coli YggH has several motifs conserved among the Rossmann-fold methyltransferases (47). These conserved motifs probably constitute parts of the catalytic domain. The key sequences used for our Blast search corresponded to the conserved motifs highlighted by De Bie et al. (47). As described under “Results,” we found only one candidate open reading frame when screening the A. aeolicus genome. A. aeolicus and E. coli YggH proteins probably

**FIG. 9. The location of m\(^7\)G46 in the L-shaped tRNA structure and the tertiary base pairs.** The Protein Data Bank accession number of yeast tRNAPhe is 1EHZ. This figure was generated by RasMac Version 2.6 with slight modifications. A, the m\(^7\)G46 nucleotide in the L-shaped structure of yeast tRNA\(^\text{Phe}\) is indicated in red. B, the C13-G22-m\(^7\)G46 tertiary base pair is shown by the ball and stick model. Carbon, nitrogen, oxygen, and phosphorous atoms are indicated in white, blue, red, and yellow, respectively. C, the location of the C13-G22-m\(^7\)G46 tertiary base in the three-dimensional core is shown by a wire-frame model. The tertiary base pairs, G15-C48, A9-U12-A23, and m\(^7\)G10-C25-G45 are indicated in green, blue, and light blue, respectively. The C13-G22 base pair and m\(^7\)G46 are indicated in brown and red, respectively.

**FIG. 10. Variants with disrupted or altered base pairs.** A, point mutations are individually introduced into yeast tRNAPhe transcript to disrupt or alter the base pairs. The cloverleaf structure of wild-type tRNAPhe is shown. The arrows connect the mutation site and the substituted nucleotides. B, double mutations are individually introduced into yeast tRNAPhe transcript as indicated by arrows. The substituted base pair or nucleotides are shown by arrows.

### TABLE IV

**Kinetic parameters for single- or double-mutated variants**

The relative \( V_{\text{max}}/K_m \) values are expressed with respect to that of the wild-type transcript, which was taken as 100%.

| Transcript | Feature | \( K_m \) (nM) | \( V_{\text{max}} \) (nmol mg\(^{-1}\) h\(^{-1}\)) | Relative \( V_{\text{max}}/K_m \) (%) |
|------------|---------|----------------|--------------------------------|----------------------------------|
| Wild-type  | —       | 40             | 68                            | 100                              |
| 9A→G       | Decrease of hydrogen bonds in A9-A23-U12 tertiary base pair | 110             | 150                           | 91                               |
| 9A→U       | Disruption of A9-A23-U12 tertiary base pair | 210             | 150                           | 48                               |
| 9A→C       | Alteration of stacking effect between A9 and G46 | 180             | 130                           | 48                               |
| 15G→C     | Disruption of G15-C48 tertiary base pair | 160             | 140                           | 58                               |
| 21A→G     | Alteration of stacking effect on three-dimensional core | 70              | 120                           | 114                              |
| 21A→U     | Formation of A14-U21 base pair | 70              | 120                           | 114                              |
| 45G→A     | Disruption of C27-G43 base pair in anticodon-stem | 70              | 70                            | 67                               |
| 45G→C     | Disruption of G10-C25-G45 tertiary base pair | 40              | 60                            | 100                              |
| 65G→C     | Disruption of G10-C25-G45 tertiary base pair | 40              | 60                            | 100                              |
| 12U→C23A→G| Disruption of A9-U12-A23 tertiary base pair | 60              | 110                           | 122                              |
| 13C→U22G→A| Disruption of C13-G22-G46 tertiary base pair | 50              | 120                           | 160                              |
| 13C→U22G→G| Alteration of C13-G22-G46 tertiary base pair | 220             | 240                           | 72                               |
| 13C→G22G→C| Disruption of C13-G22-G46 tertiary base pair | 300             | 240                           | 53                               |
| 54U→A55U→A| Disruption of tertiary base pairs between D- and T-arms | 300             | 270                           | 60                               |
have the same catalytic mechanism because they are homologous within the catalytic core. However, the protein structure involved in the RNA recognition may be different from each other.

In this paper we investigated the RNA recognition mechanism of A. aeolicus YggH. The 5' and 3' RNAs in the precursor tRNA are not required for the methyl transfer reaction. Likewise, the L-shaped structure of tRNA is not essential for the reaction since the enzyme can catalyze the methylation of truncated RNA fragments. We also demonstrated that the recognition sites are dispersed on the tRNA structure. The most important site is located on the T-stem. The D-arm compensates for deletion of the T-stem through the formation of tertiary base pairs with the variable loop. Our results reveal that the tertiary base pairs in the three-dimensional core are not essential for methylation, although substitutions of the nucleotides around G46 cause a marked decrease in methyl acceptance activity. The tertiary base pairs and the stacking of the bases probably permit entry of the G46 base into the catalytic pocket of the enzyme.

Our results seem to explain a general mechanism of bacterial m7G46 modification in tRNA. Although the primary amino acid sequences of YggH proteins from A. aeolicus and E. coli differ considerably, the RNA recognition patterns are similar. Substitution of the yeast tRNA Phet variable loop by the E. coli tRNA A46 variable loop causes a complete loss of the methyl acceptance activity. The tertiary base pairs and the stacking of the bases are not required for the methyl transfer reaction. Like-
Substrate tRNA Recognition Mechanism of tRNA (m7G46) Methyltransferase from Aquifex aeolicus
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