Functional Categorization of the Conserved Basic Amino Acid Residues in TrmH (tRNA (Gm18) Methyltransferase) Enzymes*

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Transfer RNA (Gm18) methyltransferase (TrmH) catalyzes the methyl transfer from S-adenosyl-L-methionine (AdoMet) to the 2′-OH group of the G18 ribose in tRNA. To identify amino acid residues responsible for the tRNA recognition, we have carried out the alanine substitution mutagenesis of the basic amino acid residues that are conserved only in TrmH enzymes and not in the other SpoU proteins. We analyzed the mutant proteins by S-adenosyl-L-homocysteine affinity column chromatography, gel mobility shift assay, and kinetic assay of the methyl transfer reaction. Based on these biochemical studies and the crystal structure of TrmH, we found that the conserved residues can be categorized according to their role (i) in the catalytic center structure of TrmH, we found that the conserved residues can be categorized according to their role (i) in the catalytic center structure of TrmH, (ii) in the initial site of tRNA binding (Lys-90, Arg-166, classified according to their role (i) in the catalytic center structure of TrmH, (ii) in the initial site of tRNA binding (Lys-90, Arg-166, categorized according to their role (i) in the catalytic center structure of TrmH, we found that the conserved residues can be categorized according to their role (i) in the catalytic center structure of TrmH, (ii) in the initial site of tRNA binding (Lys-90, Arg-166, Arg-109—His-71—Met-147 interaction), (v) in the assisted phosphate binding site (His-34), or (vi) in an unknown function (Arg-109). Furthermore, the difference between the $K_a$ and $K_m$ values for tRNA suggests that the affinity for tRNA is enhanced in the presence of AdoMet. To confirm this idea, we carried out the kinetic studies, a gel mobility shift assay with a mutant protein disrupted in the catalytic center, and the analytical gel-filtration chromatography. Our experimental results clearly show that the enzyme has a semi-ordered sequential mechanism in which AdoMet both enhances the affinity for tRNA and induces formation of the tetramer structure.

Transfer RNA (Gm18) methyltransferase (TrmH (classical name, SpoU); tRNA (guanosine-2′-) methyltransferase, EC 2.1.1.34) catalyzes the methyl transfer from S-adenosyl-L-methionine (AdoMet) to the 2′-OH group of the ribose of guanosine at position 18 (G18) in tRNA (1–11). In the reaction, AdoMet is converted to S-adenosyl-L-homocysteine (AdoHcy). G18 is a highly conserved residue in the D-loop of tRNA and is responsible for the formation of the L-shaped threedimensional structure of the tRNA by the D-loop/T-loop interaction meditated through the G18–Ψ55 tertiary base pair (14, 15). Because the 2′-O-methylation of ribose at position 34 stabilizes the C3′-endo form (16), the 2′-O-methyl-G18 (Gm18) modification probably imposes conformational rigidity on the local structure of tRNA. Although the absence of the Gm18 modification in Escherichia coli tRNA has been shown to have no effect on either the activity of the supF amber suppressor tRNA or on the growth rate of cells (9), it has been reported that the growth rate is decreased and that the frequency of the frameshift errors is increased in an E. coli mutant lacking both Gm18 and Ψ55 (17). Thus, the Gm18 modification probably works in conjunction with the other modified nucleosides in the three-dimensional core such as Ψ55. The genes responsible for the Gm18 modification have been experimentally identified as trmH (spoU) in E. coli (9, 10), Thermus thermophilus (5), and Aquifex aeolicus (6) and as trm3 in Saccharomyces cerevisiae (11). The Gm18 modification in tRNA is found in eubacteria and eukaryotes, consistent with the distribution of the trmH and trm3 genes (12, 13). the Gm18 modification is also found in plant organelle tRNA, although the responsible gene has not been identified (12, 13). Furthermore, it has been recently proposed that Sulfolobus solfataricus tRNA Gm18 undergoes Gm18 modification by the C/D box small RNA (sRNA) guide methylation system (18).

Analyses based on the amino acid sequence alignments have shown that several RNA ribose 2′-O-methyltransferases share the common sequence motifs (termed motifs 1, 2, and 3) and, thus, have been classified as SpoU family methyltransferases (10). All of the enzymes characterized in the SpoU family, including T. thermophilus TrmH, are involved in the ribose methylation of tRNA (1–11) or rRNA (19–23) (see Fig. 1). Recently, an archaeal SpoU family enzyme, tRNA (Cm56) methyltransferase (aTrm56), has been identified (24, 25). Thus, SpoU family members are distributed among three domains of life. One of the prominent characters of the SpoU family proteins is the protein fold, which encompasses a deep trefoil knot.
structure, which is produced by threading the polypeptide chain through an untwisted loop (7, 26–32). A similar fold is also found in TrmD (tRNA (m1G37) methyltransferase (EC 2.1.1.31) enzymes (33, 34)). Therefore, the SpoU and TrmD family proteins constitute the SpoU-TrmD (termed SPOUT) superfamily (35), and the protein fold is classified as the class IV fold of the AdoMet-dependent methyltransferases (36).

T. thermophilus TrmH has many merits as a model protein for structural and functional studies of the SpoU family, because it is a small, stable, and well characterized member of the family (1–5). In recent studies we have determined the crystal structure of the TrmH (7), clarified the residues involved in the AdoMet-binding (7, 8), proposed a catalytic mechanism (7, 8), and identified the roles of the conserved residues in the motifs (8). However, a very important question remains outstanding. The amino acid residues responsible for the contact with the substrate tRNA have not been identified. In the current study, therefore, we have focused on the basic amino acid residues that are conserved only in the TrmH enzymes and not in the other SpoU family proteins to clarify the tRNA binding site. Beyond our expectations, the initial site of tRNA recognition and the subsequent tRNA binding site required for the catalytic cycle to proceed could be distinguished by biochemical experiments. Furthermore, the basic amino acid residues that are involved not only in tRNA binding but also in AdoMet binding (AdoHcy releasing) have been identified. Together with the information from the crystal structures of the TrmH alone and the TrmH-AdoMet complex, the recognition sites of tRNA in TrmH are discussed in comparison to other SpoU family members.

**EXPERIMENTAL PROCEDURES**

**Materials**—[Methyl-14C]AdoMet (1.95 GBq/mmol) and [methyl-3H]AdoMet (2.47 TBq/mmol) were purchased from ICN, cold AdoMet, and AdoHcy from Sigma, and DE52 was from Whatman. The AdoHcy affinity column was synthesized from Whatman. The AdoHcy affinity column was synthesized by CM-Toyopearl 650M column chromatography (liner gradient, 0–250 mM KCl in the buffer A). The mutant protein fractions were assessed by 15% SDS-PAGE. If the purity of the protein was not satisfactory, we repeated the CM-Toyopearl 650M column chromatography after dialysis against the buffer A. The fractions were combined, dialyzed against buffer B (50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2 mM mercaptoethanol, and 50 mM KCl), and concentrated with Centricon YM-10 centrifugal filters (Millipore). The quantity of protein was measured with a Bio-Rad protein assay kit using bovine serum albumin as the standard. The purified proteins were mixed with glycerol (final concentration 50%) and stored at −30 °C.

**Measurements of the Enzymatic Activity**—A standard assay for enzyme purification was performed according to a previously reported method (4, 5). To determine the kinetic parameters for AdoMet, the concentrations of each mutant protein and the yeast tRNA<sub>Phe</sub> transcript were fixed at 0.1 and 8.5 μM, respectively. However, the range of the incubation time was varied (2–30 min) according to the methyl transfer activity of the protein. In a typical assay, [methyl-3H]AdoMet concentrations of 0, 0.5, 1, 2.5, 5, 10, 15, 20, 40, 60, and 80 μM were used. The kinetic parameters for tRNA were determined as previously reported (4). In experiments to assess the enzyme mechanism (see Fig. 4), the tRNA and AdoMet concentrations were varied as follows: tRNA (0, 25, 50, 75, 100, 200, 400, 600 nM) and [methyl-3H]AdoMet (0.5, 1.0, 5.0 μM) (see Fig. 4A) or tRNA (25, 50, and 100 nM) and [methyl-3H]-AdoMet (0, 1.0, 2.5, 5.0, 10, 20, 40, 60 μM) (see Fig. 4B). In each reaction 2.3 nM enzyme, [methyl-3H]-AdoMet, and tRNA were incubated for 2 min at 50 °C in the buffer B.

**Analytical Ado-Hcy Affinity Column Chromatography**—The affinity of the purified proteins for AdoHcy was qualitatively analyzed by the Ado-Hcy column chromatography as described in our previous report (7, 8).

**Gel Mobility Shift Assay**—Standard gel mobility shift assays were carried out as previously reported (8). In brief, the purified protein and 0.05 A₂₆₀₆ unit of yeast tRNA<sub>Phe</sub> transcript were incubated in 20 μl of buffer B at 37 °C for 20 min. 6% polyacrylamide gel (width, 90 mm; length, 90 mm; thickness, 1 mm) was prepared with buffer C (50 mm Tris base, 50 mm acetic acid, and 5 mm magnesium acetate). 4 μl of loading solution (0.25% bromphenol blue and 30% glycerol) was added to each sample and loaded onto the gel immediately. The electrophoresis was carried out at room temperature by constant voltage (100 V) for 1 h. The gel was stained with Coomassie brilliant blue for detection of protein and then with methylene blue for detection of RNA. The quantities of RNA and protein were measured with a Fuji Photo Film BAS2000 imaging analyzer.

To analyze the effect of AdoMet (see Fig. 5, B and C), the experimental procedure was changed as follows. In the assay shown in Fig. 5B, the R41M protein (0–11 μM) and tRNA (5 μM) were preincubated at 37 °C for 10 min in the buffer B. AdoMet was then added to a final concentration of 50 μM, and the mixture was incubated at 37 °C for 10 min. In the assay shown in Fig. 5C, the R41M protein (0–11 μM) and AdoMet (50 μM) were preincubated at 37 °C for 10 min in the buffer B, tRNA was then added to a final concentration of 5 μM, and the mixture was incubated at 37 °C for 10 min. Loading solution (0.25%
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bromphenol blue and 30% glycerol) was added to each sample, which was then loaded onto the gel immediately.

Analytical Gel Filtration—Analytical gel filtration was performed with an ÄKTAprime chromatography system (Amersham Biosciences) equipped with a Superdex 75 column (10/30; column volume, 23.6 ml) at room temperature. The column was first equilibrated with the buffer D (50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 6 mM 2-mercaptoethanol, 200 mM KCl), and the sample was then injected. The flow rate was 0.5 ml/min. Elution profiles were monitored by the absorption of UV at 280 nm. Formation of an enzyme-AdoMet complex was analyzed as follows. The enzyme (14 μM) and AdoMet (50 μM) were incubated at 37 °C for 10 min in 100 μl of the buffer D and then injected onto the column.

RESULTS

Selection of the Target Sites for the Site-directed Mutagenesis—Fig. 1A shows a multiple amino acid sequence alignment of the typical SpoU family methyltransferases reported thus far (5, 6, 9, 22, 24, 26, 27, 30, 31). We used the ClustalW program to generate the alignment and then modified it according to the publications of Gustafsson (10), Anantharaman (35), and Rinaldi (24). In Fig. 1A, TrmH and aTrm56 are tRNA (Gm18) methyltransferase from eubacteria (5, 6, 9) and tRNA (Cm56) methyltransferase from Archaea (24, 25), respectively; AvrRb (22, 28) and RlmB (21, 31) are rRNA methyltransferases, which have a ribosomal protein-like domain in their N termini; RrmA (26) and YibK (30) are function-unknown proteins, although the amino acid sequence of RrmA strongly suggests that this protein is T. thermophilus ortholog of the E. coli RlmB. The three conserved motifs (motifs 1, 2, 3) in the SpoU family are boxed in red. The conserved arginine residue in motif 1 (Arg-41 in T. thermophilus TrmH) is the catalytic center proposed in our previous studies (7, 8). The 11 blue boxes highlight the basic amino acid residues that are conserved only in the TrmH enzymes. TrmH catalyzes the methyl transfer from AdoMet to the 2′-OH group of the G18 ribose in tRNA (Fig. 1B). In the tRNA docking model of the TrmH dimer (Fig. 1C), these 11 residues are located on the enzyme surface and are accessible to bound tRNA with slight structural changes in the protein or tRNA. We, therefore, expected that some of these residues might be involved in the recognition of tRNA. In this study we substituted these basic amino acid residues with alanine. In addition, the Leu-179 residue was exchanged by stop codon (L179Stop), because the limited proteolysis by trypsin in our previous study showed that the Arg-178 residue was one of the main cleavage sites in TrmH (5). Thus, the L179Stop mutant corresponds to the N-terminal region and the catalytic core of the TrmH. The other 14 mutant proteins used in the tables have been previously reported (7, 8); they each contain the substitution of an amino acid residue in one of the three conserved motifs, and we confirmed the decreased affinity of these mutants for tRNA (see Table 2). All of the proteins were purified to homogeneity as judged by 15% SDS-PAGE (data not shown, see Fig. 2). In this study the yeast tRNA⁹⁰₈₅ transcript was used as tRNA because this is known to be a good substrate RNA for this enzyme (4).

Affinity of the Mutant Proteins for AdoMet—First, we assayed the affinity of the mutant proteins for AdoMet. A convenient method to detect the affinity for AdoMet is the AdoHcy affinity column chromatography (Fig. 2). Because AdoHcy is produced from AdoMet by the methyl transfer reaction, this experiment shows the affinity of the enzyme not only for an AdoMet analogue but also for the direct product of the reaction. As shown in Fig. 2, the wild-type enzyme (Control) bound tightly to the column and was not eluted in the flow-through fraction (Flow-through) and was not eluted by the buffer containing 2 M KCl (Wash). The bound enzyme was eluted from the column by the buffer containing 2 M KCl and 6 M urea (Elution). Ten mutant proteins (R8A, R19A, K32A, H34A, K90A, R109A, R166A, R168A, R176A, and L179Stop) showed the similar elution profiles, demonstrating that the wild-type enzyme and these proteins have substantial affinity for AdoMet. On the basis of these results, we analyzed the kinetic parameters of these proteins for AdoMet (Table 1). The 10 mutant proteins had Kₘ values for AdoMet comparable with that of the wild-type enzyme, consistent with the results of the AdoHcy affinity column chromatography. Remarkably, however, the Vₘₐₓ values of these mutant proteins were considerably lower than that of the wild-type enzyme (Table 2). These decreases in the Vₘₐₓ values can be explained by the lower affinity of the mutants for tRNA, even though they bind to AdoMet sufficiently.

Two mutant proteins (R11A and H71A) showed differences in affinity for AdoHcy as compared with the wild-type enzyme (Fig. 2). The R11A mutant did not absorb to the AdoHcy affinity column and eluted in the flow-through fraction. Thus, the R11A mutant had lost its affinity for AdoMet. Indeed, the methyl transfer activity of the R11A mutant could not be detected (Table 1). By contrast, the H71A mutant was strongly absorbed to the column and was not eluted by 6 M urea under the standard conditions (Fig. 2). The protein was eluted slowly by the repeated treatment with 6 M urea (data not shown). These results indicated that the H71A mutant had increased affinity for AdoHcy as compared with the wild-type enzyme.

The kinetic parameters of the H71A mutant for AdoMet could be measured. The Kₘ value of the H71A protein was not changed significantly as compared with that of the wild-type enzyme; however, the initial velocity of the methyl transfer was decreased to 0.068 that of the wild-type enzyme (Table 1). Thus, the release of the AdoHcy seems to be slower for the H71A mutant than for the wild-type enzyme. To address these changes in the affinity for AdoHcy, we mapped the Arg-11 and His-71 residues on the crystal structure of the TrmH-AdoMet complex (see Fig. 7). The Arg-11 and His-71 residues are located near the dimer interface and contact with the Met-147 residue in the β₆-α₅ connection loop in the other subunit. The β₆-α₅ connection loop corresponds to the conserved motif 3 region (Fig. 1A), and it contains amino acid residues such as Met-144 and Leu-151 that form the AdoMet binding site (7). Substitution of Arg-11 or His-71 seems to affect the affinity for AdoHcy by causing the structural change in the β₆-α₅ connection loop. Thus hydrophobic interaction among the Arg-11, His-71, and Met-147 residues is required for the local structure of the enzyme. Furthermore, it should be mentioned that all three residues, Arg-11, His-71, and Met-147 are conserved only
FIGURE 1. Sequence alignment and structure of tRNA and TrmH. A, multiple amino acid sequence alignment of typical SpoU family methyltransferases. B, cloverleaf structure of the yeast tRNA. The methylation site, G18, is indicated in red. C, tRNA docking model of the TrmH dimer. Two TrmH monomer (blue and green), bound AdoMet molecules (red), and tRNA (orange) are indicated.
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FIGURE 2. AdoHcy affinity chromatography. The affinity of the wild-type and mutant TrmH proteins for AdoHcy was tested by AdoHcy affinity column chromatography. Each protein sample (10 μg each) was loaded onto the column (Control). The column was washed by five column volumes of 50 mM KCl buffer B, and the eluent was combined with the flow-through fraction (Flow-through). The column was then washed by five further column volumes of 2 mM KCl buffer B (Wash). Proteins that absorbed to the column were eluted by five column volumes of buffer B containing 2 mM KCl and 6 M urea (Elution). The protein in each fraction was collected by trichloroacetic acid precipitation and then analyzed by 15% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue.

in TrmH enzymes and not in other proteins in the SpoU family (Fig. 1A). Therefore, the interaction of the Arg-11, His-71, and Met-147 residues may work in conjunction with the local structure of tRNA in release of the product (AdoHcy).

Taking these experimental results together we confirmed that the substitution of each of the nine basic amino acid residues (Arg-11, Arg-19, Lys-32, His-34, Lys-90, Arg-109, Arg-166, Arg-168, and Arg-176) with alanine did not change the affinity for AdoHcy but did decrease the initial velocity of the methyl transfer, and two basic amino acid residues (Arg-8, Arg-19, Lys-32, His-71, Lys-90, Arg-119, Arg-109, Arg-166, Arg-168, and Arg-176) affected only the affinity for tRNA (Fig. 3A). Therefore, the precise $K_a$ value of the wild-type enzyme could not be measured, although it was estimated at around 2.8 μM (Fig. 3A). In the current study we assayed 12 mutant proteins by the same method and expressed the results in terms of five grades (−, ±, +, ++, and ++++) (Fig. 3B). We quantified the intensities of the band with a Fuji Photo Film BAS2000 imaging analyzer and calculated the $K_a$ values. The distinction between the grades − (>8.0 μM) and ± (5.0 − 8.0 μM) was considerably difficult because of the weak intensities of the bands; for example, the difference between R11A and R19A mutant proteins was apparently small. However, it is clear that these mutants classified in the grades − and ± severely decreased the affinity for tRNA. As expected, 10 mutants (R8A, R11A, R19A, K32A, H71A, K90A, R166A, R168A, R176A, and L179STOP) showed decreased affinity for tRNA (Fig. 3B). Among these, the lower affinity of the R11A and H71A mutants can be explained by changes in the local structure of the enzyme as described above. According to the tRNA binding, the Arg-11, His-71, and Met-147 residues may contact with tRNA directly because these residues are located near the elbow region of the bound tRNA in the docking model (Fig. 1C).

Unexpectedly, however, the R109A mutant showed slightly increased affinity for tRNA as judged by the gel shift assay, and the kinetic assay showed that this mutation did not have a significant effect on the $K_m$ value. In contrast, Arg-8, Arg-11, Arg-19, and Lys-32 affected both the tRNA affinity and the methyl transfer activity. Furthermore, the L179Stop mutant markedly decreased both the tRNA affinity and the methyl transfer activity of the enzyme (Fig. 3B and Table 2), demonstrating the importance of the C-terminal region for tRNA binding, although no conserved amino acid residues were present in this region (Fig. 1A).

Taking these results together, we conclude that eight basic amino acid residues (Arg-8, Arg-19, Lys-32, His-71, Lys-90, Arg-166, Arg-168, and Arg-176) are involved in tRNA binding and that alanine substitution of the His-34 or Arg-109 residue does not have a significant effect on tRNA recognition by TrmH (3). Therefore, the substitution of the Arg-109 residue does not have a significant effect on the methyl transfer activity. Furthermore, the L179Stop mutant markedly decreased both the tRNA affinity and the methyl transfer activity as compared with substitution of Lys-32 (Table 2). Thus, these data show that the Lys-32 residue is more important than the His-34 residue.

Unexpectedly, however, the R109A mutant showed unexpectedly increased affinity for tRNA as judged by the gel shift assay, and the kinetic assay showed that this mutation did not have a significant effect on the methyl transfer activity. Furthermore, the L179Stop mutant markedly decreased both the tRNA affinity and the methyl transfer activity of the enzyme (Fig. 3B and Table 2), demonstrating the importance of the C-terminal region for tRNA binding, although no conserved amino acid residues were present in this region (Fig. 1A).

Taking these results together, we conclude that eight basic amino acid residues (Arg-8, Arg-19, Lys-32, His-71, Lys-90, Arg-166, Arg-168, and Arg-176) are involved in tRNA binding and that alanine substitution of the His-34 or Arg-109 residue does not have a significant effect on tRNA recognition. Furthermore, we also confirmed that the C-terminal region (L179Stop) did not reflect on the AdoHcy interaction.

### Table: AdoHcy Affinity Results

| Protein  | Control | Flow through | Wash | Elution |
|----------|---------|--------------|------|---------|
| Wild type|         |              |      |         |
| R8A      |         |              |      |         |
| R11A     |         |              |      |         |
| R19A     |         |              |      |         |
| K32A     |         |              |      |         |
| H34A     |         |              |      |         |
| H71A     |         |              |      |         |
| R109A    |         |              |      |         |
| R166A    |         |              |      |         |
| R168A    |         |              |      |         |
| R176A    |         |              |      |         |
| L179STOP |         |              |      |         |
value incorporates not only binding of substrate tRNA but also releasing of the methylated tRNA, whereas the \( K_d \) value incorporates only the affinity for substrate tRNA. In the TrmH reaction the release of the methylated tRNA is probably slow, which would significantly affect the \( K_m \) value. Indeed, the methylated tRNA transcript inhibits the methyl transfer reaction as a competitive inhibitor (4), suggesting that the TrmH does not recognize the Gm18 modification of the bound tRNA at least in the initial binding process. Thus, our experimental results suggest that Lys-90, Arg-166, Arg-168, and Arg-176 are required for the initial binding event but are not involved in the subsequent stage of the catalytic cycle, including the release of the methylated tRNA. In contrast, Arg-8, Arg-11, Arg-19, and Lys-32 are required for both initial binding and continuation of the catalytic cycle.

The categorized residues are assembled in local regions of the TrmH structure (see Fig. 7A). For example, Arg-8, Arg-11, Arg-19, and Lys-32 residues are assembled around the catalytic center (Arg-41) and the conserved motifs (Asn-35 in motif 1, Glu-124 in motif 2, and Asn-152 in motif 3). These residues are located around the G18 in the elbow region of tRNA (Fig. 1C). As the methyl transfer reaction proceeds, these residues and

### Table 1

**Kinetic parameters for AdoMet**

| Variant name | \( K_m \) (\( \mu \text{mol/mg} \)) | \( V_{max} \) (\( \mu \text{mol/mg h} \)) | Relative \( V_{max}/K_m \) | AdoHcy binding assay | Reference/Source |
|--------------|---------------------------------|---------------------------------|--------------------------|----------------------|------------------|
| Wild type    | 10                              | 10                              | 1                        | ++                   | Ref. 5           |
| R8A          | 71                              | 0.19                            | 0.0026                   | ++                   | This work        |
| R11A         | ND                              | ND                              | ND                       | -                    | This work        |
| R19A         | 19                              | 2.1                             | 0.11                     | ++                   | This work        |
| K32A         | 17                              | 0.29                            | 0.017                    | ++                   | This work        |
| H34A         | 67                              | 0.34                            | 0.0051                   | ++                   | This work        |
| N35A         | 83                              | 0.51                            | 0.0061                   | ++                   | Ref. 7           |
| N35Q         | 2,100                           | 0.037                           | 0.000018                 | ++                   | Ref. 8           |
| N35D         | 670                             | 0.016                           | 0.000024                 | -                    | Ref. 8           |
| R41A         | 4,600                           | 0.062                           | 0.000013                 | -                    | Ref. 7           |
| R41K         | 300                             | 0.41                            | 0.0041                   | ++                   | Ref. 7           |
| R41M         | 920                             | 0.076                           | 0.00083                  | ++                   | Ref. 8           |
| H71A         | 44                              | 3.0                             | 0.068                    | ++                   | This work        |
| R90A         | 24                              | 3.4                             | 0.14                     | ++                   | This work        |
| R109A        | 23                              | 6.6                             | 0.29                     | ++                   | This work        |
| E124A        | 28,000                          | 0.46                            | 0.000016                 | -                    | Ref. 7           |
| E124Q        | ND                              | ND                              | ND                       | -                    | Ref. 8           |
| E124D        | 14                              | 0.17                            | 0.0012                   | ++                   | Ref. 8           |
| N152A        | 800                             | 0.040                           | 0.000050                 | -                    | Ref. 7           |
| N152D        | ND                              | ND                              | ND                       | ++                   | Ref. 8           |
| N152E        | ND                              | ND                              | ND                       | ++                   | Ref. 8           |
| R166A        | 21                              | 1.7                             | 0.081                    | ++                   | This work        |
| R168A        | 20                              | 1.7                             | 0.085                    | ++                   | This work        |
| R176A        | 24                              | 2.6                             | 0.11                     | ++                   | This work        |
| L179Stop     | 45                              | 1.1                             | 0.024                    | ++                   | This work        |

### Table 2

**Kinetic parameters for tRNA**

| Variant name | \( K_m \) (\( \mu \text{mol/mg} \)) | \( V_{max} \) (\( \mu \text{mol/mg h} \)) | Relative \( V_{max}/K_m \) | Gel mobility shift assay | Reference/Source |
|--------------|---------------------------------|---------------------------------|--------------------------|----------------------|------------------|
| Wild type    | 62                              | 4.9                             | 1                        | ++                   | Ref. 8           |
| R8A          | 270                             | 2.0                             | 0.094                    | -                    | This work        |
| R11A         | 100                             | 3.2                             | 0.41                     | -                    | This work        |
| R19A         | 120                             | 0.24                            | 0.025                    | +                    | This work        |
| K32A         | 80                              | 0.27                            | 0.043                    | +                    | This work        |
| H34A         | 2000                            | 0.54                            | 0.034                    | +                    | Ref. 8           |
| N35A         | 100                             | 3.2                             | 0.41                     | +                    | Ref. 8           |
| N35Q         | 2000                            | 0.54                            | 0.034                    | +                    | Ref. 8           |
| N35D         | 120                             | 0.24                            | 0.025                    | +                    | This work        |
| R41A         | 80                              | 0.27                            | 0.043                    | +                    | This work        |
| R41K         | 20                              | 1.7                             | 0.085                    | +                    | This work        |
| R41M         | 24                              | 2.6                             | 0.11                     | +                    | This work        |
| H71A         | 45                              | 1.1                             | 0.024                    | +                    | This work        |
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FIGURE 3. Gel mobility shift assay. A, affinity of the wild-type TrmH for the yeast tRNA\textsuperscript{B} transcript was analyzed by the gel mobility shift assay using conditions described under "Experimental Procedures." The gel was double-stained with Coomassie Brilliant Blue and methylene blue. As the protein concentration increased, a tetramer-tRNA complex was formed in addition to the dimer-tRNA complex. Thus, it was difficult to determine the precise $K_d$ value, although it was estimated to be around 2.8 $\mu$M. B, affinity of the mutant TrmH proteins for the yeast tRNA\textsuperscript{B} transcript was analyzed by the gel mobility shift assay. Bands of the dimer-tRNA complex are shown. The estimated $K_d$ values for the mutant proteins binding to the transcript are represented by five grades (++, +++, +, −, −−, −−−, > 8.0 $\mu$M).

tRNA seem to change the structure of the enzyme. These structural changes, including release of the tRNA, are reflected in both the $K_m$ and the $K_d$ values. In contrast, Lys-90, Arg-166, Arg-168, and Arg-176 are located in the region of the enzyme that contacts the three-dimensional core of tRNA (Fig. 1C and see Fig. 7A). Because these residues did not influence the $K_m$ value, they seem to function only in the initial tRNA recognition event.

Semi-ordered Sequential Mechanism—In this study we used two methods, determination of the $K_d$ value by the gel shift assay and measurement of the $K_m$ value by the methyl transfer reaction, to analyze the affinity of the mutants for tRNA. However, there is a considerable difference between two values; the $K_d$ and $K_m$ values of the wild-type enzyme are around 2.8 and 0.1 $\mu$M, respectively. The $K_d$ value was estimated in the absence of AdoMet, whereas the $K_m$ value was measured in the presence of AdoMet. Therefore, this discrepancy can be explained if the enzyme has enhanced affinity for tRNA in the presence of AdoMet.

To test this possibility we carried out a kinetic study in which we changed both the AdoMet and the tRNA concentrations (Fig. 4). In general, a two-substrate reaction assay requires that one substrate is fixed at an excess concentration. In this case, however, we intentionally carried out the kinetic assays at various concentrations around the $K_m$ values for AdoMet and tRNA. As expected, in accordance with an increase in the AdoMet concentration, the $K_m$ value for tRNA was decreased (Fig. 4A), demonstrating that the enzyme showed enhanced affinity for tRNA in the presence of AdoMet. By contrast, when the concentration of tRNA was varied, the $K_m$ value for AdoMet changed but not significantly (Fig. 4B). These experiments clearly show that the catalytic mechanism of the TrmH is a semi-ordered sequential mechanism. Based on this finding, we compared the crystal structures of TrmH alone and TrmH-AdoMet complex (see Fig. 7B). Although we could not conclude the mechanism structurally, the structural differences could be observed as described in the discussion section.
Recently, we used Western blotting analysis to quantify the amount of TrmH in living *T. thermophilus* cells. Our preliminary results showed that the TrmH concentration in late log phase cells cultured at 75 °C is 0.1–1.0 μM, although estimation of the precise volume of the cells was difficult. Thus, in living cells the concentration of the TrmH protein seems to be too low to form the tetramer structure observed *in vitro* study. In living cells, the concentration of AdoMet is expected to be much higher than the *K_m* value for AdoMet. Therefore, almost all TrmH proteins probably exist *in vivo* as the AdoMet-bound form, and the majority of them seem to behave as a dimer. If the TrmH protein is localized to one area in a living cell (for example, as part of the tRNA-processing-modification complex), however, it may form a tetramer structure.

**DISCUSSION**

Here we have demonstrated the roles of the conserved basic amino acid residues in the TrmH enzymes. As a result, these residues can be categorized as having a role (i) in the catalytic center (Arg-41), (ii) in the initial site for tRNA binding (three-dimensional core recognition) (Lys-90, Arg-166, Arg-168, and Arg-176), (iii) in the tRNA binding site required for continuation of the catalytic cycle (elbow region recognition) (Arg-8, Arg-19, and Lys-32), (iv) in the structural element involved in release of AdoHcy (Arg-11–His-71–Met-147 interaction), (v) in the assisted phosphate binding site (His-34), or (vi) in unknown function (Arg-109). The positions of these residues are highlighted on the TrmH structure (Fig. 7A). Of these residues it is not clear whether those in category (i) maintain contact with the tRNA through the whole catalytic cycle; however, it is clear that the substitution of these residues with alanine has no obvious effect on the release of the methylated tRNA. The gel mobility shift assay showed that the individual substitution of these residues had a weak effect on the affinity of the enzyme for tRNA, suggesting that they support the correct binding of
Basic Amino Acid Residues in TrmH Enzymes

![Diagram of Basic Amino Acid Residues in TrmH Enzymes](image)

**FIGURE 7. Summary of the functions of the conserved basic amino acid residues and structural changes caused by AdoMet.** A. A summary of the functions of the conserved basic amino acid residues. The basic amino acid residues categorized under "Discussion" are shown on the TrmH dimer structure. One subunit of the dimer is indicated in **green**, and the other is indicated in **cyan**. The residues in the **cyan** subunit are indicated with a prime (e.g., Arg-8'). The bound AdoMet molecules are indicated in **red**, Arg-41' (dark blue) forms the catalytic center, which functions in the transfer of methyl from AdoMet bound in the green subunit. The residues involved in the initial binding of tRNA (Lys-90', Arg-166', Arg-168', and Arg-176') are indicated in **purple**. The residues required for continuation of the catalytic cycle (Arg-8', Arg-19', and Lys-32) are indicated in **blue**. The structural elements involved in release of AdoHcy (Arg-11', His-71', and Met-147) are indicated in **orange**. The assisted phosphate binding site (His-34) is indicated in **gray**. The residue whose function remains unknown (Arg-109) is indicated in **magenta**. B, comparison of the structure of TrmH alone and TrmH-AdoMet complex. The structure of TrmH alone (orange) is superimposed onto that of TrmH-AdoMet complex (cyan). The amino acid residues analyzed in the current study are indicated by stick models. The residues in the TrmH alone and TrmH-AdoMet complex are indicated in **magenta** and **dark blue**, respectively. The N-terminal amino acid residues (1–8) were disordered in the structure of TrmH alone.

The tRNA to the catalytic pocket around the Arg-41 residue. In contrast, the substitution of the residues in category (iii) had a severe effect on both tRNA binding and the methyl transfer activity, demonstrating that these residues are important for the induced fit of the tRNA to the catalytic pocket as well as the release of the product. Until now, we have not been able to visualize the mechanism by which the TrmH-methylated tRNA-AdoHcy complex dissociates after the reaction. However, our current results provided a clue to elucidating this mechanism. In the previous study we showed that the substitution of the Asn-35 residue by Gln enhances the affinity for AdoHcy, and the catalytic cycle was halted (8). In the current study we found that the residues in category (iv) are involved at least in the release of AdoHcy. Taking these results together, the subunit interaction around bound AdoHcy seems to be involved in the release of the AdoHcy. This idea is in line with the fact that all SpoU family members characterized to date (Fig. 1A) have a dimer structure (7, 8, 26–31). In the current study we demonstrated that the C-terminal region of the protein significantly affects tRNA recognition. Recently, the crystal structure of *A. aeolicus* TrmH was solved (27). Our previous study showed that *A. aeolicus* TrmH effectively methylates specific tRNA species (6), whereas *T. thermophilus* TrmH methylates all tRNAs. The obvious difference between these TrmH proteins is the length of the C terminus. Taking these results together with the tRNA modification pattern (12, 13) and the amino acid sequence of *E. coli* TrmH (9), the structure of the C terminus seems to be involved in the specificity of TrmH toward tRNA species despite the low conservation of the amino acid sequence in this region.

Among the SpoU members characterized, *Haemophilus influenzae* YibK protein is the smallest protein (Fig. 1). The crystal structure of this protein was previously solved (30), and more recently, the mechanism underlying the formation of the *H. influenzae* YibK dimers has been studied in detail (38). The striking homology between TrmH and YibK suggests that YibK is an RNA ribose 2'-O-methyltransferases; amino acid residues involved in the catalytic center and AdoMet binding site are conserved in the YibK protein. Furthermore, our computational analysis of the *H. influenzae* genome suggests that the YibK protein is the best candidate for an enzyme of the Gm18 modification system (data not shown), although whether the Gm18 modification occurs in *H. influenzae* tRNA has not been confirmed. However, the N-terminal region is missing in the YibK protein. In the current study, we demonstrated that the basic amino acid residues in the N terminus of the TrmH (Arg-8, Arg-11, and Arg-19) are required both for continuation of the catalytic cycle and for the structural element involved in release of AdoHcy. Lim et al. (30) have predicted that the YibK may require another partner protein (subunit) for its enzyme activity. The experimental results demonstrated in this study support their idea.

The crystal structure of *Bacillus subtilis* TrmB (tRNA (m7G46) methyltransferase (EC 2.1.1.33)) (39–42), which catalyzes the methyl transfer from AdoMet to the N7 atom of the semiconserved G46 in the extra-loop of tRNA, has been reported recently (37). The study revealed that TrmB is a unique variant of the Rossmann-fold methyltransferase (class I fold). In the study Zegers et al. (37) showed that sinuefungin, an analogue of AdoMet, induced the aggregation of the TrmB and tRNA complex. In the current study, we demonstrated that AdoMet enhanced the affinity for tRNA and induced the formation of the tetramer structure of TrmH. Therefore, these phenomena, in which AdoMet and its analogue change the affinity for tRNA and a subunit structure, may be common in tRNA methyltransferases despite the difference of the protein fold. Fig. 7B shows the differences of the crystal structures of TrmH alone and TrmH-AdoMet complex.
marked difference between two structures is the direction of the basic amino acid residues in category (iii), which are required for continuation of the catalytic cycle and located near the elbow region of tRNA in the docking model; these residues in the TrmH-AdoMet complex are opened to the solvent. These structural differences seem to change the affinity for tRNA and affect the subunit structure.

Recently, a new member of the SpoU family, aTrm56 was found in the Archaea domain (24, 25). The amino acid sequence of this archaeal enzyme is considerably different from that of bacterial SpoU members (Fig. 1). Indeed, several amino acid residues in motifs 1–3 are replaced by the other amino acid residues; asparagines in motif 1 (Asn-35 in TrmH) and in motif 3 (Asn-152 in TrmH) are replaced by histidine and glutamic acid, respectively, in aTrm56. In TrmH, these residues form a hydrogen-bond network (Asn-35—Glu-124—Asn-152) and function in tRNA binding (phosphate binding) and protein stability (8). Furthermore, basic amino acid residues investigated in the current study are not found in the aTrm56 sequence. Therefore, the tRNA recognition mechanism of the aTrm56 protein may differ considerably from that of bacterial TrmH. To resolve these issues further studies will be necessary.

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