NMR Studies of the Effects of the 5′-Phosphate Group on Conformational Properties of 5-Methylaminomethyluridine Found in the First Position of the Anticodon of *Escherichia coli* $tRNA_4^{Arg}$†

Kensaku Sakamoto,‡ Gota Kawai,§ Satoru Watanabe,‡ Tatsuya Niimi,‡ Nobuhiro Hayashi,§,|| Yutaka Muto,‡ Kimitsuna Watanabe,§ Takahiko Satoh,⊥ Mitsuo Sekine,⊥ and Shigeyuki Yokoyama*,‡

Department of Biophysics and Biochemistry, School of Science, and Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, and Department of Life Science, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 227, Japan

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ABSTRACT: 5-Methylaminomethyluridine (mnm^5U) exists in the first position of the anticodon (position 34) of *Escherichia coli* $tRNA_4^{Arg}$ for codons AGA/AGG. In the present study, the temperature dependence of the ribose-puckering equilibrium of $pmnm^5U$ was analyzed by proton NMR spectroscopy. Thus, the enthalpy difference (ΔH) between the C2′-*endo* and C3′-*endo* forms was obtained as 0.65 kcal·mol^{-1}. By comparison of the ΔH values of pU and $pmnm^5U$, the 5-substitution was found to increase the relative stability of the C3′-*endo* form over the C2′-*endo* form significantly (by 0.56 kcal·mol^{-1}). Furthermore, this conformational "rigidity" was concluded to depend on the 5′-phosphate group, because nucleoside U exhibits only a negligible change in the ribose-puckering equilibrium upon the 5-methylaminomethyl substitution. Further NMR analyses and molecular dynamics calculations revealed that interactions between the 5-methylaminomethyl and 5′-phosphate groups of $pmnm^5U$ restrict the conformation about the glycosidic bond to a low *anti* form, enhancing steric repulsion between the 2-carbonyl and 2′-hydroxyl groups in the C2′-*endo* form. This intrinsic conformational rigidity of the mnm^5U residue in position 34 may contribute to the correct codon recognition.

Since the "wobble" theory was presented (Crick, 1966; Söll et al., 1966), a number of modifications of uridine have been found in the first position of the anticodon (position 34 of tRNA) (Nishimura, 1979; Björk et al., 1987; Sprinzl et al., 1991). It was originally suggested that U(34) can form a base pair only with A or G in the third position of the codon (Crick, 1966; Söll et al., 1966). However, efficient recognition of U in addition to A and G has been found for 5-hydroxyuridine derivatives (xo^5U)[1] in position 34 of $tRNA^{Val}$, $tRNA^{Ser}$, and $tRNA^{Ala}$ (Ishikura et al., 1971; Mitra et al., 1979; Samuelsson et al., 1980; Murao et al., 1982). Thus, the wobble base pairing of $xo^5U(34)$ with the third base of the codon is "extended". In contrast, 5-methyl-2-thiouridine derivatives (xm^5s^2U) in position 34 of $tRNA^{Gln}$, $tRNA^{Lys}$, and $tRNA^{Glu}$ cannot form a base pair with U or C (Sekiya et al., 1969; Agris et al., 1973; Lustig et al., 1981); the wobbling of $xm^5s^2U(34)$ is "restricted". Note here that Gln, Lys, and Glu are each encoded by a set of two degenerate codons terminating in A and G. Therefore, the "restriction of wobbling" for $xm^5s^2U(34)$ probably prevents misdecoding in translation.

From the conformational viewpoint, the ribose ring of U(34) is required to take the C3′-*endo* and C2′-*endo* forms for base pairing with A and U, respectively, in the third position of the codon (Yokoyama et al., 1985; Yokoyama & Nishimura, 1995). Proton NMR analyses have revealed that the modifications of uridine 5′-monophosphate (pU) to pxo^5U and pxm^5s^2U change the ribose-puckering property in contrasting manners: pxo^5U and pxm^5s^2U strongly prefer the C2′-*endo* and C3′-*endo* forms, respectively (Yokoyama et al., 1985). Therefore, it has been suggested that the wobbling properties of the xo^5U and xm^5s^2U residues in position 34 are based on the intrinsic conformational characteristics of these modified uridines (Yokoyama et al., 1979, 1985; Sierzputowska-Gracz et al., 1987). In this context, a nucleotide unit in the C3′-*endo* form is "rigid", whereas that in the C2′-*endo* form is "flexible", in terms of the rotations about the exocyclic bonds (Yokoyama et al., 1981). Thus, the conformational "rigidity" and "flexibility" of these two different types of modified uridines correspond to the "restriction" and "extension", respectively, of wobbling (Yokoyama et al., 1985; Sakamoto et al., 1993).

In addition to xm^5s^2U, other types of modified uridines, such as 2′-*O*-methyluridine (Um), 5-methyl-2′-*O*-methyluridine derivatives (xm^5Um), and 5-methyluridine derivatives (xm^5U) have been found in position 34 of tRNA species that correspond to two synonymous codons terminating in A and

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‡ School of Science, University of Tokyo.

§ Graduate School of Engineering, University of Tokyo.

|| Present address: Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-11, Japan.

⊥ Tokyo Institute of Technology.

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[1] Abbreviations: mnm^5U, 5-methylaminomethyluridine; mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; mnm^5, 5-methylaminomethyl; NOE, nuclear Overhauser effect; $pmnm^5U$, mnm^5U 5′-monophosphate; pU, uridine 5′-monophosphate; Um, 2′-*O*-methyluridine; Up, uridine 3′-monophosphate; xm^5U, 5-methyluridine derivative; xm^5Um, 5-methyl-2′-*O*-methyluridine derivative; xm^5s^2U, 5-methyl-2-thiouridine derivative; xo^5U, 5-hydroxyuridine derivative.

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G (Sprinzl et al., 1991). The 2′-*O*-methylation of uridine 3′-monophosphate (Up) (Kawai et al., 1992) as well as the 2-thiolation of U and pU (Yokoyama et al., 1979, 1985; Sierzputowska-Gracz et al., 1987) cause the high stability of the C3′-*endo* form (the conformational "rigidity"). Thus, the two totally different types of wobble-restricting modifications cause the same "rigidifying" conformational effect.

On the other hand, it has been suggested that the modification of U to xm^{5}U hardly affects the ribose puckering, on the basis of the conformational properties of xm^{5}U nucleosides (Sierzputowska-Gracz et al., 1987; Agris et al., 1992). However, a preliminary NMR study has indicated that the ribose-puckering equilibrium of 5-methylaminomethyluridine 5′-monophosphate (pmnm^{5}U) is appreciably shifted from that of pU (Sakamoto et al., 1993). This type of xm^{5}U exists in position 34 of *Escherichia coli* $tRNA_4^{Arg}$ (Sakamoto et al., 1993), which recognizes codon AGA primarily and codon AGG much less efficiently (Spanjaard et al., 1990); the wobbling of mnm^{5}U(34) is strongly restricted (Sakamoto et al., 1993). Therefore, in the present study, detailed conformational analyses of mnm^{5}U and pmnm^{5}U were performed by proton NMR spectroscopy and molecular dynamics calculations. Thus, in addition to 2-thiolation and 2′-*O*-methylation, 5-methylaminomethylation was concluded to cause an intrinsic conformational rigidity, which requires the presence of the 5′-phosphate group.

MATERIALS AND METHODS

Materials. The preparation of pmnm^{5}U from a nuclease-P_1 digest of unfractionated *E. coli* tRNAs was previously described (Sakamoto et al., 1993). mnm^{5}U was chemically synthesized (Sekine et al., 1993).

Analysis of the Ribose-Puckering Property by ^{1}H NMR Spectroscopy. The sample for NMR measurements was dissolved in ^2H_2O to a concentration of 4 mM for mnm^{5}U and 1 mM for pmnm^{5}U. The p^{2}H (direct pH meter reading) was adjusted to 4.8, where the amino group of the 5-substituent is protonated and the 5′-phosphate group is monoanionic, as in the tRNA. The 400-MHz ^{1}H NMR spectra were recorded on a Brucker AM-400 spectrometer. The assignment of the ribose proton resonances was previously described (Sakamoto et al., 1993). The vicinal coupling constants, $J_{1'2'}$ values, were obtained at various temperatures between 25 and 85 °C, whereas the $J_{3'4'}$ values were not clearly determined because the ^{1}HO^{2}H and H3′ proton signals overlapped each other at high temperatures. Therefore, the value of $J_{1'2'} + J_{3'4'}$ was assumed to be equal to 10 Hz, as found for a number of nucleotides (Altona & Sundaralingam, 1973), and the fractional populations of the C2′-*endo* and C3′-*endo* forms were obtained with the formulas [C2′-*endo*] $= J_{1'2'}/(J_{1'2'} + J_{3'4'})$ and [C3′-*endo*] $= 1 -$ [C2′-*endo*], respectively. The temperature dependence of the equilibrium constant [C2′-*endo*]/[C3′-*endo*] was subjected to a least-squares treatment, and the enthalpy difference (ΔH) and the entropy difference (ΔS) between the two forms were obtained together with their standard deviations.

NOE Analysis of the Conformation about the Glycosidic Bond. Nuclear Overhauser effects (NOEs) on the base and ribose protons upon irradiation of various protons of mnm^{5}U in ^2H_2O were measured with a Brucker AMX-500 spectrometer at a probe temperature of 25 °C. The dihedral χ angle (O4′−C1′−N1−C2) was estimated using the COFLEM program (Yokoyama et al., 1981) on an IRIS indigo XS24 graphic workstation (Silicon Graphics) as follows. On the basis of the fractional populations of the C2′-*endo* and C3′-*endo* forms determined by the spin-coupling constant analysis (39% and 61%, respectively), the NOEs on the base and ribose protons were simulated on the assumption of different *anti*/*syn* populations and χ angles for the two ribose-puckering forms. This calculation was performed for various χ angles and *anti*/*syn* populations, and the root mean squared difference between the simulated and observed NOE values was minimized. Finally, the most probable values of the fractional populations and χ angles of the *anti* and *syn* forms were obtained for the C2′-*endo* and C3′-*endo* forms, together with their standard deviations.

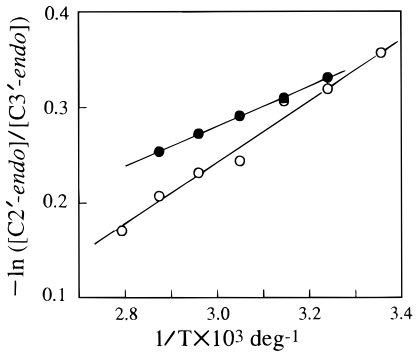

FIGURE 1: Temperature dependence of the equilibrium constants [C2′-*endo*]/[C3′-*endo*] of pmnm^{5}U (○) and mnm^{5}U (●).

Molecular Modeling Calculations. Energy minimization and molecular dynamics calculations were carried out with the program AMBER (Pearlman et al., 1991) on an IRIS indigo XS24 graphic workstation (Silicon Graphics). The force field parameters for the 5-methylaminomethyluracil base were estimated from those for similar functional groups in the AMBER database except for the electrostatic parameters. These parameters were estimated by the *ab initio* molecular orbital calculations for the 1-methyl-5-methylaminomethyluracil with the program GAUSSIAN 86 on a HITAC M-880 system (Frisch et al., 1987). Possible conformations of mnm^{5}U and pmnm^{5}U with the surrounding water molecules were obtained by molecular dynamics calculations of 1 ps at 300 K with a starting conformation in either the C3′-*endo* or C2′-*endo* form. The conformations about the exocyclic bonds, C4′−C5′ and C5′−O5′, were fixed to be the *gg* and *trans* forms, respectively.

RESULTS

Conformational Properties of mnm^{5}U and pmnm^{5}U. The equilibrium constants [C2′-*endo*]/[C3′-*endo*] were determined for pmnm^{5}U and mnm^{5}U at various temperatures (Figure 1). Then, the ΔH and ΔS values were obtained, together with their standard deviations (in parentheses), as 0.65 (0.04) kcal·mol^{-1} and 1.46 (0.13) cal·deg^{-1} mol^{-1}, respectively, for pmnm^{5}U, and 0.39 (0.06) kcal·mol^{-1} and 0.61 (0.19) cal·deg^{-1} mol^{-1}, respectively, for mnm^{5}U. The ΔH value for pU has been reported to be 0.09 kcal·mol^{-1}; the relative stabilities of the C2′-*endo* and C3′-*endo* forms of pU are almost equal to each other (Yokoyama et al., 1985). Thus, the 5-substitution of pU to pmnm^{5}U was found to increase the ΔH value significantly (by 0.56 kcal·mol^{-1}). This increase in the ΔH value is comparable to the values of 0.78 and 0.77 kcal·mol^{-1} for the 2-thiolation of pU and the 2′-*O*-methylation of Up, respectively (Yokoyama et al., 1985; Kawai et al., 1992). In contrast, as compared with the ΔH

Table 1: Observed and Simulated NOEs on the Base Proton (H6) and the Ribose Protons (H1′, H2′, H3′, H4′, and H5″) upon Irradiation of Various Protons of mnm^{5}U

irradiated protons	NOEs (%) observed and simulated (in parentheses) for each protons[a]					
	H6	H1′	H2′	H3′	H4′	H5″
H6	−	4.0 (5.1)	8.0 (8.0)	3.0 (3.2)	1.0 (0.0)	0.0 (0.8)
H1′	3.0 (2.3)	−	4.0 (3.2)	0.0 (0.2)	0.0 (1.3)	0.0 (0.0)
H2′	6.0 (6.5)	3.0 (5.6)	−	8.0 (9.0)	0.0 (0.2)	1.0 (0.9)
H3′	3.0 (2.7)	0.0 (0.3)	11 (9.4)	−	−[b]	2.0 (2.0)
H4′	0.0 (0.0)	3.0 (2.4)	3.0 (0.2)	−[b]	−	3.0 (2.0)
H5″	2.0 (0.9)	0.0 (−0.1)	0.0 (1.3)	3.0 (2.7)	5.0 (2.7)	−

[a] The NOE on H5′ could not be determined because the H5′ signal is largely overlapped with the methylene proton signal of the 5-substituent. [b] The NOEs on H3′ and H4′, upon irradiation of H4′ and H3′, respectively, were not included in the calculation, because these proton signals are close to each other.

Table 2: Analysis of the Conformation about the C−N Bond of mnm^{5}U[a]

conformation of mnm^{5}U	χ angle	fractional population
C2′-*endo*−*anti*	−115° ± 2° (−111°)	95% ± 11%
C2′-*endo*−*syn*	nd[b]	5% ± 11%
C3′-*endo*−*anti*	−151° ± 3° (−164°)	60% ± 8%
C3′-*endo*−*syn*	72° ± 2°	40% ± 7%

[a] Fractional populations and χ angles of the *anti* and *syn* forms were estimated for both the C2′-*endo* and C3′-*endo* forms on the basis of the NOEs. The possible values of the χ angle, obtained by molecular dynamics calculations, are also listed in parentheses. [b] The value of the χ angle was not determined because of the small population of this form.

value reported for U (0.37 kcal·mol^{-1}) (Yokoyama et al., 1985), the 5-substitution of U to mnm^{5}U was found to only negligibly stabilize the C3′-*endo* form. This finding is consistent with the previous reports (Sierzputowska-Gracz et al., 1987; Agris et al., 1992). Therefore, the presence of the 5′-phosphate group was concluded to be essential for the intrinsic conformational rigidity of pmnm^{5}U.

Estimation of the χ Angles of mnm^{5}U on the Basis of the NOEs on the Base and Ribose Protons. Two-dimensional NOE spectroscopy has revealed that mnm^{5}U takes the *syn* form as well as the *anti* form about the glycosidic bond (C−N bond), whereas the conformation of pmnm^{5}U is restricted to the *anti* form (Sakamoto et al., 1993). Thus, the conformational characteristics about the C−N bonds of mnm^{5}U and pmnm^{5}U are different from each other. The dihedral χ angle about the C−N bond is strongly correlated with the ribose puckering for pyrimidine nucleotides in the *anti* form (Sundaralingam, 1969). Therefore, the conformations about the C−N bonds of mnm^{5}U and pmnm^{5}U were analyzed in detail to elucidate the molecular basis for their puckering properties.

The NOEs on the base and ribose protons were observed upon irradiation of various protons of mnm^{5}U (Table 1). By NOE simulation, the most probable values of the χ angles and the *anti*/*syn* populations were estimated for the C2′-*endo* and C3′-*endo* forms (Table 2), and the simulated NOEs for these values were in good agreement with the observed data (Table 1). Thus, the χ angle (−115°) obtained for the C2′-*endo*−*anti* form was found to be much "higher" than that for the C3′-*endo*−*anti* form (−151°), as has been pointed out for other pyrimidine nucleosides (Sundaralingam, 1975) (when a value of χ angle is closer to a right angle than others, it is referred to as "higher").

It was difficult to apply this method to pmnm^{5}U, because the H2′ and H3′ proton signals of pmnm^{5}U are largely overlapped and therefore cannot be separately irradiated. Therefore, possible conformations of pmnm^{5}U were computed by a molecular dynamics calculation. A similar calculation was performed also for mnm^{5}U in the *anti* form, and the χ angles thus obtained were found to be consistent with the values estimated by the NOE analysis (Table 2). Thus, the conformational characteristics of mnm^{5}U, and probably of pmnm^{5}U as well, can be discussed on the basis of computer modeling by the molecular dynamics method.

Short-Range Conformational Interrelations between the Ribose Puckering and the χ Angles of mnm^{5}U and pmnm^{5}U. Figure 2 shows the computer-modeled conformations of mnm^{5}U and pmnm^{5}U. In mnm^{5}U (Figure 2A,B), the 6-hydrogen of the uracil ring and the 5′-oxygen possibly interact with each other through a van der Waals contact and may stabilize the conformation about the C−N bond. The χ angle for the C2′-*endo* form is high enough to avoid steric repulsion between the 2-carbonyl and 2′-hydroxyl groups. In this model, the 5-methylaminomethyl group (the mnm^5 group) of mnm^{5}U, which is extended in a plane perpendicular to the uracil ring plane (Hillen et al., 1978), does not appear to be involved in intramolecular interactions that influence the ribose puckering or the χ angle.

On the other hand, the computer modeling of pmnm^{5}U suggested that a hydrogen bond can be formed between the -NH_2^+- moiety of the mnm^5 group and the 5′-phosphate group for both the C2′-*endo* and C3′-*endo* forms (Figure 2C,E). This hydrogen bonding restricts the χ angle to a low *anti* region (−177° and −160° for the C2′-*endo* and C3′-*endo* forms, respectively). For the C3′-*endo* form, a van der Waals contact, in addition to the hydrogen bond, is possibly formed between the groups in positions 5 and 5′ (Figure 2E) and may contribute to the stabilization of the low *anti* form. On the other hand, another possible conformation exists for the C2′-*endo* form (Figure 2D), where a water molecule bridges the -NH_2^+- moiety and the 5′-phosphate group by hydrogen bonding. However, in this water-bridged form, the χ angle is still in a low *anti* region (−155°).

Thus, in contrast to mnm^{5}U, the restriction of the χ angle to a low *anti* region was suggested for both of the two ribose-puckering forms of pmnm^{5}U. The low *anti* forms for the C2′-*endo* form appear to result in steric repulsion between the 2-carbonyl and 2′-hydroxyl groups (Figure 2C,D), while this repulsion may not occur in the C3′-*endo*−*anti* form (Figure 2E). The χ-angle restriction probably causes the preference of the C3′-*endo* form of pmnm^{5}U through the steric hindrance of the groups in positions 2 and 2′.

DISCUSSION

5-Methylaminomethylation of pU Causes an Intrinsic Conformational Rigidity. For a variety of mononucleotides and nucleosides, the ΔH and ΔS values between the two ribose-puckering forms have been obtained by NMR analyses (Watanabe et al., 1979; Yokoyama et al., 1985; Kawai et al., 1992; Agris et al., 1992). The difference in the ΔS value mainly depends on the flexibility in the rotations about the exocyclic bonds, whereas these rotations are restricted for both the C2′-*endo* and C3′-*endo* forms in tRNA. Therefore, the conformational characteristics of nucleotide residues in tRNA are reasonably discussed in terms of the ΔH value rather than the ΔS or ΔG values (Watanabe et al., 1979;

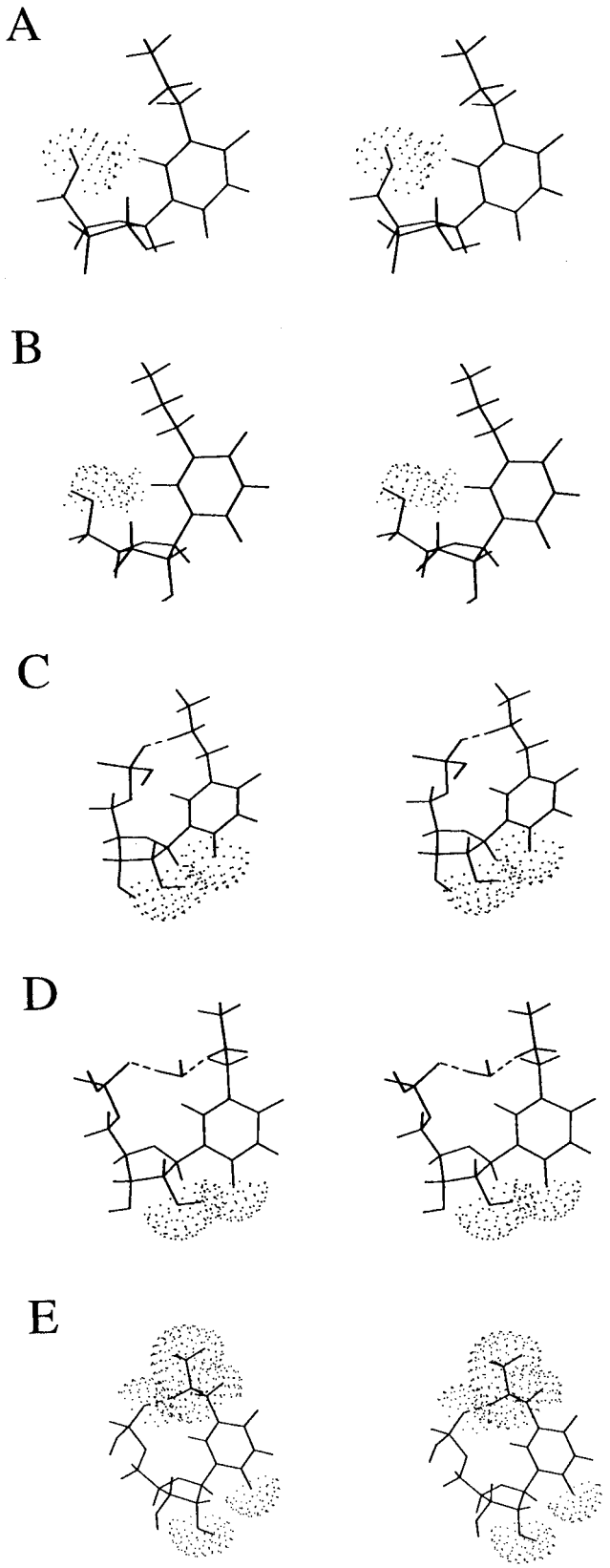

FIGURE 2: Stereoviews of the possible conformations of mnm^{5}U and pmnm^{5}U: the C2′-*endo*−*anti* form (A) and the C3′-*endo*−*anti* form (B) of mnm^{5}U, and the C2′-*endo*−*anti* form (C, D) and the C3′-*endo*−*anti* form (E) of pmnm^{5}U. The dotted spheres are of the van der Waals radii. The broken lines between the mnm^5 and 5′-phosphate groups represent hydrogen bonds. Molecular graphics images were produced using the MidasPlus software system (Ferrin et al., 1988) from the Computer Graphics Laboratory, University of California, San Francisco.

Yokoyama et al., 1985; Kawai et al., 1992). Thus, on the basis of the large ΔH values between the C2′-*endo* and C3′-*endo* forms, it is concluded that (p)xm^{5}s^{2}U (Yokoyama et al., 1985; Agris et al., 1992) and Ump (Kawai et al., 1992) are conformationally rigid.

The present study revealed that the 5-substitution of pU to pmnm^{5}U significantly stabilizes the C3′-*endo* form, and that the 5′-phosphate group is essential for this stabilizing effect. The C3′-*endo* form is strongly correlated with the G^- form about the C3′−O3′ bond and the *gg* form about the C4′−C5′ bond (Yokoyama & Miyazawa, 1985). Furthermore, the conformation about the C−N bond of pmnm^{5}U is restricted to the *anti* form (Sakamoto et al., 1993). Thus, pmnm^{5}U by itself predominantly takes the *gg*−C3′-*endo*−G^-−*anti* form, which corresponds to the standard A-RNA helical conformation (Quigley et al., 1975). Thus, 5-methylaminomethylation, as well as 2-thiolation and 2′-*O*-methylation, causes an intrinsic conformational rigidity, in spite of their different chemical natures.

It has been reported that U(34) of *E. coli* tRNA^{Gln}, tRNA^{Lys}, and tRNA^{Glu} is modified to 5-methylaminomethyl-2-thiouridine (mnm^{5}s^{2}U) (Sprinzl et al., 1991). The relative stability of the C3′-*endo* form over the C2′-*endo* form of pmnm^{5}U (0.65 kcal·mol^{-1}) is smaller than that of pmnm^{5}s^{2}U [1.2 kcal·mol^{-1} (Yokoyama et al., 1985)]. However, in *E. coli* tRNA$_4^{Arg}$, the C3′-*endo* form of mnm^{5}U(34) may be further stabilized by base stacking with C(35), whereas the stacking interaction between mnm^{5}s^{2}U(34) and U(35) is probably very weak. Thus, in the tRNA molecule, the base-stacking interaction probably cooperates with the intrinsic conformational rigidity of mnm^{5}U(34).

Molecular Basis for the Conformational Rigidity of pmnm^{5}U. It has been reported that the electronic characteristics of the 5-substituents of xm^{5}U affect the ribose-puckering properties (Egert et al., 1980; Uhl et al., 1983). This effect may cause the slight difference between the ΔH values of U and mnm^{5}U but cannot explain the conformational rigidity of pmnm^{5}U, which depends on the presence of the 5′-phosphate group. The interactions between the mnm^5 and 5′-phosphate groups of pmnm^{5}U probably restrict the χ angle to a low *anti* region. Low χ angles result in steric repulsion between the 2-carbonyl and 2′-hydroxyl groups in the C2′-*endo* forms of pyrimidine nucleosides and nucleotides (Sundaralingam, 1969, 1975). Therefore, the χ-angle restriction in pmnm^{5}U may cause the preference of the C3′-*endo* form. Recently, a derivative of pxm^{5}U, a cyclouridine nucleotide having a covalent bonding liker between the uracil 5-position and the 5′-phosphate group has been found to take exclusively the C3′-*endo* form, with the χ angle possibly restricted to a low *anti* region (Seio et al., 1995). This report supports our conclusion that interaction between the groups in positions 5 and 5′ can cause a conformational rigidity through regulating the dihedral angle around the glycosidic bond.

Because of the structural similarity to mnm^{5}U, the stabilizing mechanism may hold for another type of xm^{5}U, 5-carboxymethylaminomethyluridine, as found in the tRNAs from mitochondria (Martin et al., 1990) and *Mycoplasmas* (Andachi et al., 1989; Tanaka et al., 1991). On the other hand, 5-methoxycarbonylmethylation occurs on U(34) of cytoplasmic tRNAs from eukaryotes (Sprinzl et al., 1991), and a strong restriction of wobbling has been reported for 5-methoxycarbonylmethyluridine (mcm^{5}U) in position 34 of yeast tRNA^{Arg} (Weissenbach & Dirheimer, 1978). However, the

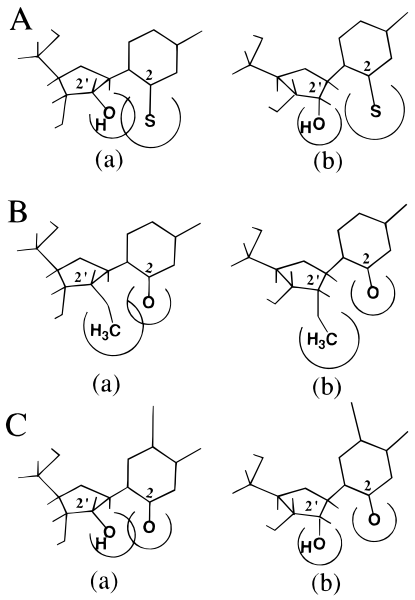

FIGURE 3: Schematic drawings showing that the similar mechanisms function in the stabilization of the C3′-*endo* form for 2-thiouridine, Um, and pmnm^{5}U. 2-Thiolation (A), 2′-*O*-methylation (B), and 5-methylaminomethylation (C) each cause steric repulsion between the groups in positions 2 and 2′ in the C2′-*endo* form (a) rather than in the C3′-*endo* form (b).

conformational properties of pmcm^{5}U have yet to be elucidated.

Stabilizing mechanisms similar to that for 5-methylaminomethylation have been suggested for the "bulky" modifications, 2-thiolation and 2′-*O*-methylation. Each of these modifications increases the conformational rigidity through steric hindrance between the groups in positions 2 and 2′ (Yamamoto et al., 1983; Kawai et al., 1992) (Figure 3). In this context, these "bulky" substitutions may cooperate with the 5-substitution to increase the conformational rigidity. Interestingly, xm^{5}U(34) in a number of prokaryotic and mitochondrial tRNAs is further modified to either xm^{5}s^{2}U or xm^{5}Um (Sprinzl et al., 1991). A study of 2-thiouridine and mnm^{5}s^{2}U has shown that the effect of 2-thiolation is additive to that of 5-methylaminomethylation (Yokoyama et al., 1985). 5-Carboxymethylaminomethyl-2′-*O*-methyluridine (cmnm^{5}Um) has been found in *E. coli* tRNA^{Leu} (Sekine et al., 1987) and *Mycoplasma* tRNA^{Leu} and tRNA^{Trp} (Andachi et al., 1989; Tanaka et al., 1991). A conformational study of pcmnm^{5}Ump is necessary to determine if the 5-substitution and 2′-*O*-methylation cooperatively increase the conformational rigidity.

Intrinsic Conformational Rigidity of mnm^{5}U(34) May Contribute to the Restriction of Wobbling. The 5-methylaminomethylation occurring on U(34), as well as the 2-thiolation and 2′-*O*-methylation, correlates with the strongly restricted wobbling (Sakamoto et al., 1993). It was now established that all of these modifications each confer the conformational rigidity on the uridine nucleotide unit. This supports the molecular mechanism of the wobbling restriction (Yokoyama et al., 1979, 1985; Sierzputowska-Gracz et al., 1987; Kawai et al., 1992), which is based on the modeling study suggesting that the ribose ring of U(34) takes the C3′-*endo* form for the base pairs with A and G, while the ribose puckering of U(34) is required for it to be converted from the C3′-*endo* form to the C2′-*endo* form for base pairing with U (Yokoyama et al., 1985). Thus, the 5-substitution of U(34) to mnm^{5}U(34) may contribute to preventing the base pairing with U, as well as to stabilizing the base pairs with A and G, as has been proposed for xm^{5}s^{2}U(34) and xm^{5}Um(34).

On the other hand, by computer modeling, it has been suggested that an interresidue hydrogen bond can be formed between the -NH_2^+- moiety of the mnm^5 group of mnm^{5}s^{2}U-(34) and the 2′-hydroxyl group of U(33) (Hillen et al., 1978). Similar hydrogen bonding is possibly formed also for mnm^{5}U(34), xm^{5}U(34), and xm^{5}Um(34) each having the 5-aminomethyl moiety. Therefore, in addition to the intrinsic conformational rigidity of these anticodon residues, this interresidue interaction possibly contributes to the wobbling restriction. However, it remains to be determined if this hydrogen bond is actually formed in the anticodon loop of tRNA.

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