Conformational Rigidity of Specific Pyrimidine Residues in tRNA Arises from Posttranscriptional Modifications That Enhance Steric Interaction between the Base and the 2′-Hydroxyl Group†

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ABSTRACT: In order to elucidate roles of the 2′-O-methylation of pyrimidine nucleotide residues of tRNAs, conformations of 2′-*O*-methyluridylyl(3′→5′)uridine (UmpU), 2′-*O*-methyluridine 3′-monophosphate (Ump), and 2′-*O*-methyluridine (Um) in ^2H_2O solution were analyzed by one- and two-dimensional proton NMR spectroscopy and compared with those of related nucleotides and nucleoside. As for UpU and UmpU, the 2′-O-methylation was found to stabilize the C3′-endo form of the 3′-nucleotidyl unit (Up-/Ump-moiety). This stabilization of the C3′-endo form is primarily due to an intraresidue effect, since the conformation of the 5′-nucleotidyl unit (-pU moiety) was only slightly affected by the 2′-O-methylation of the 3′-nucleotide unit. In fact even for Up and Ump, the 2′-O-methylation significantly stabilizes the C3′-endo form by 0.8 kcal·mol^{-1}. By contrast, for nucleosides (U and Um), the C3′-endo form is slightly stabilized by 0.1 kcal·mol^{-1}. Accordingly, the stabilization of the C3′-endo form by the 2′-O-methylation is primarily due to the steric repulsion among the 2-carbonyl group, the 2′-*O*-methyl group and the 3′-phosphate group in the C2′-endo form. For some tRNA species, 2-thiolation of pyrimidine residues is found in positions where the 2′-O-methylation is found for other tRNA species. A common feature is now found between the 2′-O-methylated and 2-thiolated pyrimidine nucleotides; the predominance of the C3′-endo form is due to an enhancement of the intraresidue steric repulsion between the groups in positions 2 and 2′ (and 3′) in the C2′-endo form. Such conformational rigidity of the modified pyrimidine nucleotide residues contributes to the correct codon recognition and thermostability of tRNA molecules.

A transfer RNA (tRNA) is modified at several sites after being transcribed from DNA. Those modifications are probably important for tRNA functions but have been confirmed to be essential in a few cases (Nishimura, 1979). As for 2-thiolation of uridine and cytidine, however, it has been found later that the C3′-endo form is remarkably stabilized (Yokoyama et al., 1979; Watanabe et al., 1979; Sierzputowska-Gracz et al., 1987). The predominance of the C3′-endo form is primarily due to an intraresidue steric interaction between the 2-thiocarbonyl group and the 2′-hydroxy group (Yamamoto et al., 1983). Further, the conformational rigidity of 2-thiolated uridine residues in the first position of the anticodon (position 34) has been found to contribute to the correct codon recognition, and the conformational rigidity of 2-thiolated ribothymidine residues in position 54 in the T loop has been found to contribute to the thermostability of *Thermus thermophilus* tRNA molecules (Yokoyama et al., 1979, 1985; Watanabe et al., 1979; Yamamoto et al., 1983; Horie et al., 1985).

The unmodified yeast tRNA^{Phe} transcript has been found to be folded normally at a higher concentration of Mg^{2+} and aminoacylated by the yeast phenylalanyl-tRNA synthetase in vitro (Sampson & Uhlenbeck, 1988; Hall et al., 1989). However, in the absence of Mg^{2+}, the conformation of the unmodified tRNA transcript is abnormal (Hall et al., 1989). Furthermore, the importance of the modifications for the tRNA identity has been clearly indicated for the case of a hypermodification, cytidine to lysidine, which switches both amino acid and codon specificities (from methionine/AUG to isoleucine/AUA) (Muramatsu et al., 1988a,b).

In the course of elucidating the roles of the 2-thiolation, we have noticed that 2′-O-methylation is also found in any positions of tRNAs where the 2-thiolation has been found (positions 32 and 34 in the anticodon loop and position 54 in the T loop). Therefore, in the present study, the effects of 2′-O-methylation on the conformational properties of UpU,[1] Up, and U were analyzed by one- and two-dimensional NMR spectroscopy. The 2′-O-methylation was also found to stabilize the C3′-endo form through an intraresidue steric repulsion between the 2-carbonyl group and the 2′-methoxy group. Thus, the 2′-O-methylation of the ribose moiety exhibits a conformational effect similar to that of the 2-thiolation of the base moiety. In both cases, the conformational rigidity of the modified pyrimidine residues contributes to the correct codon

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[1] Abbreviations: C, cytidine; Cm, 2′-*O*-methylcytidine; Gm, 2′-*O*-methylguanosine; HOHAHA, two-dimensional homonuclear Hartmann–Hahn spectroscopy; NMR, nuclear magnetic resonance; Tm, 2′-*O*-methylribothymidine; U, uridine; Um, 2′-*O*-methyluridine; Ump, 2′-*O*-methyluridine 3′-monophosphate; UmpU, 2′-*O*-methyluridylyl(3′→5′)uridine; Ump-, 3′-nucleotidyl unit of UmpU; Up, uridine 3′-monophosphate; UpU, uridylyl(3′→5′)uridine; Up-, 3′-nucleotidyl unit of UpU; Ψ, pseudouridine; -pU, 5′-nucleotidyl unit of UmpU or UpU.

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recognition and the thermostability of the tRNA molecules. This will probably serve as a leading concept in elucidating the roles of posttranscriptional modifications in other positions of tRNAs.

Materials and Methods

2′-*O*-Methyluridylyl(3′→5′)uridine (UmpU) and 2′-*O*-methyluridine 3′-monophosphate (Ump) were synthesized as follows. A fully protected uridylyl unit, *S*,*S*-diphenyl-2′-*O*-methyl-5′-*O*-(4-methoxytrityl)uridine 3′-phosphorodithioate was prepared from Um (Kamimura et al., 1982). Removal of the protecting groups from the uridylyl unit gave Ump (Kamimura et al., 1984, and references cited therein). On the other hand, the coupling reaction of the uridylyl unit with 2′, 3′-*O*-dibenzoyluridine followed by deprotection in the usual manner gave UmpU (Kamimura et al., 1984, and references cited therein). Uridylyl(3′→5′)uridine (UpU), uridine 3′-monophosphate (Up), Um, and U were purchased from Sigma Chemical Co.

The sample for NMR measurements was dissolved in ^2H_2O to the concentration of 2 mM for UmpU, 5 mM for Ump, and 10 mM for UpU, Up, Um, and U. The pH (direct pH meter reading) was adjusted to 7.0 for UmpU, UpU, Um, and U. For Ump and Up, the pH was adjusted to 4.0, where the 3′-phosphate group is monoanionic, as is the case for UmpU and UpU at pH 7.0.

The 400-MHz ^1H NMR spectra were recorded on a Bruker AM-400 spectrometer. In two-dimensional homonuclear Hartmann–Hahn spectroscopy (HOHAHA; Davis & Bax, 1985) experiments, 512 free induction decays of 2K data points were accumulated in the phase-sensitive mode using time-proportional phase increment (Bodenhausen et al., 1980) with a mixing time of 100 ms, and the spectra of 1K × 2K data points were obtained with zero-filling prior to two-dimensional Fourier transformation. For UpU and UmpU, the coupling constants $J_{1'2'}$ were obtained from the splitting of the C1′ proton resonance whereas the coupling constants $J_{3'4'}$ were not clearly determined. 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 form and the C3′-endo form were obtained with the formulas [C2′-endo] = $J_{1'2'}/(J_{1'2'} + J_{3'4'})$ and [C3′-endo] = 1 − [C2′-endo] (Altona & Sundaralingam, 1973). For nucleosides and nucleoside 3′-monophosphates, chemical shifts and spin coupling constants at various temperatures were precisely determined by computer simulations of proton NMR spectra with the program NMRSIM (Yamamoto et al., 1983), and the fractional populations were obtained. The temperature dependence of equilibrium constants [C3′-endo]/[C2′-endo] was subjected to a least-square treatment, and the enthalpy difference (ΔH) and the entropy difference (ΔS) between the C2′-endo form and the C3′-endo form were obtained together with their standard deviations.

Results

Proton Resonance Assignments. The 400-MHz ^1H NMR spectra of UmpU, UpU, Ump, Up, Um, and U are shown in Figure 1. For Ump and Um, resonance assignments were performed mainly on the basis of their chemical shifts and confirmed by double resonance experiments. The proton resonances of UmpU and UpU were assigned on the basis of the coupling connectivity found by HOHAHA experiments (Figure 2). The proton resonances of the 3′-nucleotidyl unit (Ump- or Up-) and the 5′-nucleotidyl unit (-pU) were readily identified from the coupling connectivity to the ^{31}P nucleus.

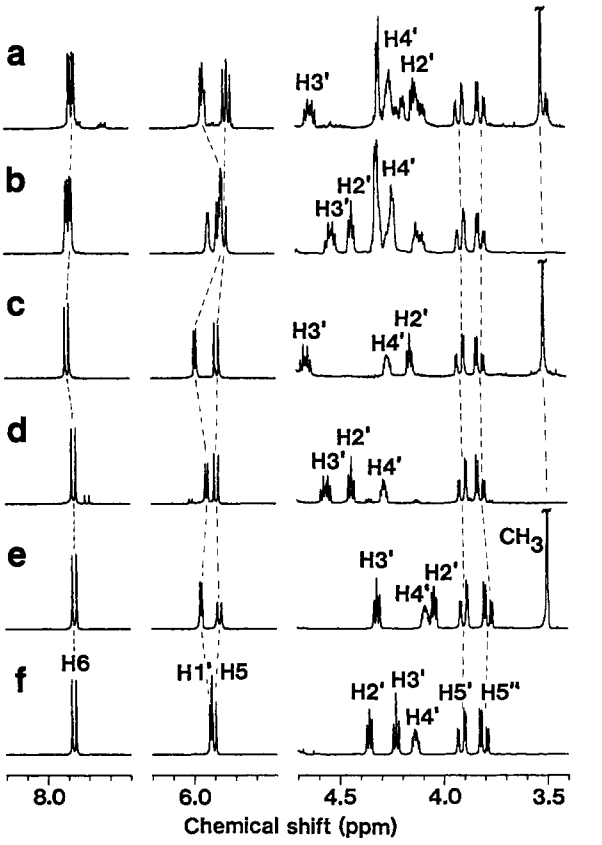

FIGURE 1: 400-MHz ^1H NMR spectra (and resonance assignments) of UmpU (a), UpU (b), Ump (c), Up (d), Um (e), and U (f) in ^2H_2O solution at 28 °C. For UmpU and UpU, the assignments of the 3′-nucleotidyl unit (Ump- and Up-) are shown.

Table I: Fractional Populations (in Percentages)[a] and the Equilibrium Constants [C3′-endo]/[C2′-endo] at 28 °C

	C3′-endo	C3′-endo	[C3′-endo]/[C2′-endo]
UmpU	62	38	1.6
UpU	53	47	1.1
UmpU	64	36	1.8
UpU	63	37	1.7
Ump	56	44	1.3
Up	50	50	1.0
Um	60	40	1.5
U	55	45	1.2

[a] Populations for the underlined nucleoside unit.

NMR Analysis of Dinucleoside Monophosphates. The fractional populations of the C2′-endo and C3′-endo forms at 28 °C are shown in Table I. On 2′-O-methylation of the 3′-nucleotidyl unit (Up-) of UpU, the fractional population of the C3′-endo form of the 3′-nucleotidyl unit is increased from 53% (Up-) to 62% (Ump-); the equilibrium constant [C3′-endo]/[C2′-endo] is increased from 1.1 (Up-) to 1.6 (Ump-). Thus, the 2′-O-methylation was found to stabilize the C3′-endo form. On the other hand, in the case of the 5′-nucleotidyl unit (-pU) in UpU and UmpU, the fractional populations of the C2′-endo form and the C3′-endo form are affected little by 2′-O-methylation of the 3′-nucleotidyl unit (from 63 to 64%). This suggested that the stabilization of the C3′-endo form of the Up- unit by the 2′-O-methylation still occurs in the absence of the 5′-nucleotidyl unit (-pU).

NMR Analysis of Mononucleotides and Nucleosides. In order to elucidate the effect of the 2′-O-methylation on the conformation in the absence of the 5′-nucleotidyl unit, the ribose ring conformations of related nucleotides and nucleosides

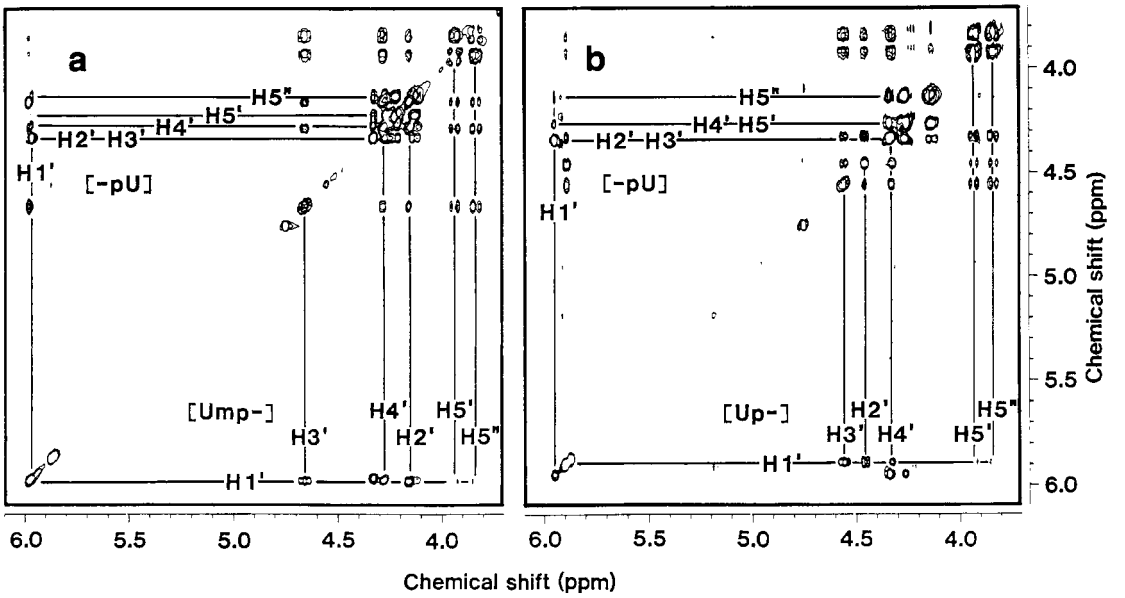

FIGURE 2: HOHAHA spectra of (a) UmpU and (b) UpU in ^2H_2O solution at 28 °C. Coupling connectivities are shown for each nucleotidyl unit (Ump-, Up-, and -pU).

Table II: Enthalpy Difference (ΔH, in kcal·mol^{-1}) and Entropy Difference (ΔS, in cal·deg^{-1} mol^{-1}) between the C2′-endo Form and the C3′-endo Form

	ΔH	ΔS
Ump	0.67 (0.08)[a]	1.81 (0.25)
Up	−0.10 (0.08)	−0.32 (0.26)
Um	0.45 (0.01)	0.67 (0.03)
U	0.37 (0.03)	0.86 (0.08)

[a] Standard deviations are in parentheses.

were also analyzed. Figure 3 shows the temperature dependence of equilibrium constants [C3′-endo]/[C2′-endo] of Ump, Up, Um, and U, and the ΔH and ΔS values are listed in Table II. The 2′-O-methylation was found to affect both the ΔH and the ΔS. For mononucleotides and nucleosides, ΔS values depend mainly on the difference in restriction of rotations around the exocyclic bonds. The subject of the present study, however, is to elucidate the role of modified nucleotides in tRNA, where the rotations around the exocyclic bonds are restricted for both the C2′-endo and C3′-endo forms. Thus, the conformational characteristics are discussed here relative to ΔH rather than ΔG or ΔS. In fact, the regulation of codon recognition and the thermostabilization of tRNA by modifications have been explained by considering the ΔH values (Watanebe et al., 1979; Yokoyama et al., 1985).

In Up, the C2′-endo form is slightly more stable (by 0.10 kcal·mol^{-1}) than the C3′-endo form. By contrast, in Ump, the C3′-endo form is more stable than the C2′-endo form by 0.67 kcal·mol^{-1}. This indicates that the 2′-O-methylation of Up stabilizes the C3′-endo form by as much as 0.8 kcal·mol^{-1}. Thus, the stabilization of the C3′-endo form occurs even in mononucleotides, indicating that the stabilization found for UmpU is not primarily due to the base stacking. In fact, the extent of stabilization of the C3′-endo form by 2′-O-methylation for UpU → UmpU ([C3′-endo]/[C2′-endo] = 1.1 → 1.6) is only slightly higher than that for Up → Ump ([C3′-endo]/[C2′-endo] = 1.0 → 1.3), probably because of the *weak* base stacking between uracil bases.

For Um, the C3′-endo form is more stable than the C2′-endo form by 0.45 kcal·mol^{-1}, and this is only slightly higher than the ΔH value (0.37 kcal·mol^{-1}) of uridine. Accordingly, the involvement of the 3′-phosphate group in the stabilization of the C3′-endo form by the 2′-O-methylation is clear from the

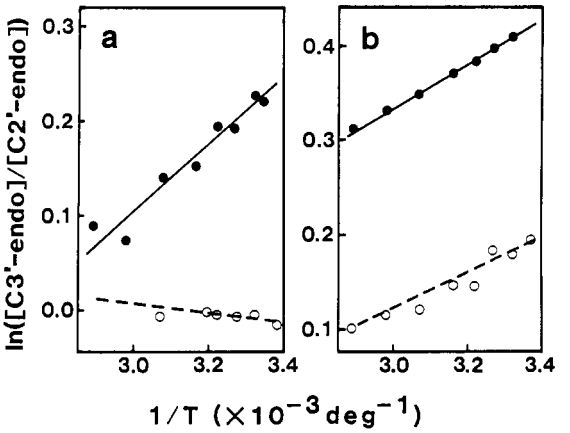

FIGURE 3: Temperature dependence of equilibrium constants of the C2′-endo and C3′-endo forms of (a) Ump (●) and Up (○) and (b) Um (●) and U (○).

comparison of the ΔH values of Ump, Up, Um, and U (Table II); the ΔH value is raised by 0.8 kcal·mol^{-1} for Up → Ump but only by 0.1 kcal·mol^{-1} for U → Um.

Molecular Mechanism of the Stabilization of the C3′-endo Form. Possible conformation models of Ump in the anti form are shown in Figure 4a–d. The dihedral angle (H2′–C2′–O2′–CH_3) was estimated by the molecular mechanics calculation with the program MacroModel (Mohamadi et al., 1990). The 2′-*O*-methyl group was found to take two orientations, g^- with the dihedral angle (H2′–C2′–O2′–CH_3) of about −45° and g^+ with the dihedral angle (H2′–C2′–O2′–CH_3) of about 50°. In the g^- form the 2′-*O*-methyl group is oriented toward the uracil base, while in the g^+ form the 2′-*O*-methyl group in oriented toward the 3′-phosphate group (Figure 5a,b). In the C3′-endo-G^--g^- form (Figure 4a), the 3′-phosphate group is oriented nearly opposite to the 2′-*O*-methyl group, and this 2′-*O*-methyl group does not appear to interfere with the uracil base. On the other hand, the fractional population of the C3′-endo-G^+ form of Ump must be small because of the steric repulsion between the phosphate group and the 5′-hydroxymethyl group (Yokoyama et al., 1981). The C2′-endo-G^+-g^+ form (Figure 4b) is also expected to be stable, since the 2′-*O*-methyl group does not appear to interfere with the uracil base or the 3′-phosphate group. However, in the C2′-endo-G^-

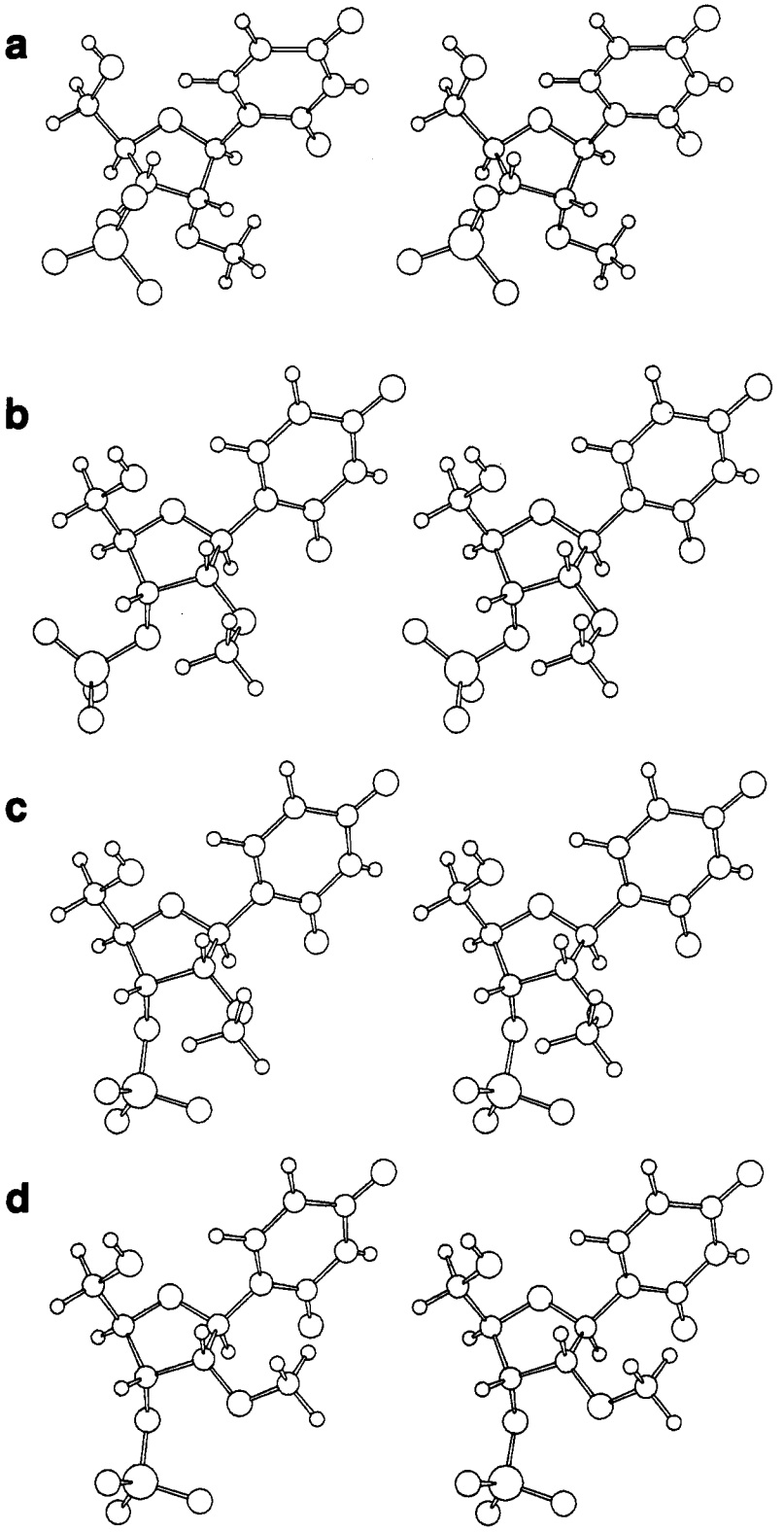

FIGURE 4: Stereoviews of the C3′-endo form (a) and the C2′-endo form (b, c, d) of Ump. The 3′-phosphate group is set in the G^- form with the dihedral angle (H3′–C3′–O3′–P) of −29° (a, c, d) or in the G^+ form with the dihedral angle (H3′-C3′-O3′-P) of 41° (b) as determined for Up (Yokoyama et al., 1981). The uracil base is set in the anti form with the dihedral angle (O4′–C1′–N1–C6) of 37° (a), 45° (b, c), 53° (d) as determined by the molecular mechanics calculation. The 2′-*O*-methyl group is set in the g^- form with the dihedral angle (H2′–C2′–O2′–CH_3) of −47° (a) and −40° (d) or in the g^+ form with the dihedral angle of 57° (b) and 41° (c) determined by the molecular mechanics calculation.

form of Ump, the 2′-*O*-methyl group appears to interfere with the 3′-phosphate group in the g^+ form (Figure 4c) or with the 2-carbonyl group of the uracil base in the g^- form (Figure 4d). Thus, the steric repulsion between the 3′-phosphate group and the 2′-*O*-methyl group will result in the orientation of the 2′-*O*-methyl group toward the uracil base, thus enhancing the steric repulsion between the 2-carbonyl group of the uracil base and the 2′-*O*-methyl group. These indicate that the stabilization of the C3′-endo form by the 2′-*O*-methylation of uridine nucleotide is primarily due to the steric interaction among the pyrimidine base, the 2′-*O*-methyl group, and the 3′-phosphate group.

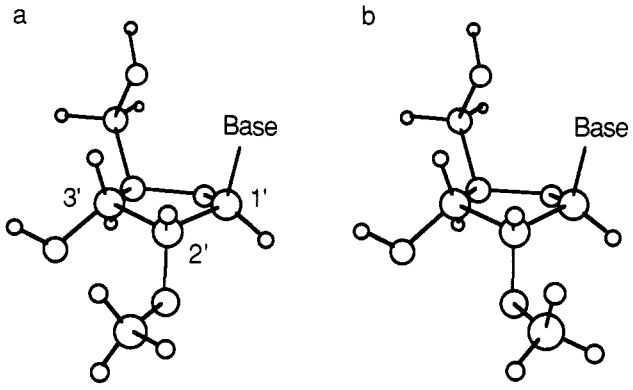

FIGURE 5: The g^+ (a) and g^- (b) forms about the C2′–O2′ bond of the 2′-*O*-methylribose moiety.

DISCUSSION

Effect of 2′-O-Methylation on Nucleotide Conformation. In the present study on the conformations of UmpU, UpU, Ump, and Up in aqueous solution, the C3′-endo form of the Up- moiety has been found to be stabilized by the 2′-O-methylation. In the C3′-endo form of uridine nucleotides, the local conformations around the C4′–C5′ bond and the C3′–O3′ bond have been found exclusively to be the gg form and the G^- form, respectively (Yokoyama et al., 1981). Thus, the 2′-O-methylation of uridine nucleotides causes conformational rigidity through exclusive stabilization of the C3′-endo-gg-G^- form.

The effect of 2′-O-methylation has also been studied previously for model ribonucleotides. The melting temperatures of poly(U) derivatives are remarkably raised by the 2′-O-methylation of uridine residues (Zmudzka & Shugar, 1970). An ordered structure of UmpU is stabilized by the 2′-O-methylation, as shown by a circular dichroism analysis (Drake et al., 1974). These effects of 2′-O-methylation have been explained by the enhancement of base stacking. However, in the present study on Ump and Up, the effect of 2′-O-methylation is also found to be significant even in the absence of base stacking; the 2′-O-methylation of uridine nucleotides is to exclusively stabilize the C3′-endo-gg-G^- form, because of the steric interaction between the groups in position 2 and 2′ (and 3′). This C3′-endo-gg-G^- form of ribonucleotides is the repeating structure of the A-type duplex, and the thermostability of the 2′-O-methylated ribonucleotides may now be explained by the inherent conformational rigidity of the building blocks.

The stabilization of the C3′-endo form by 2′-O-methylation must also occur for cytidine nucleotides since the uridylyl unit and the cytidylyl unit have the 2-carbonyl group of the base in common. In fact, a circular dichroism study on 2′-O-methylated cytidylyl(3′→5′)cytidine has shown that the 2′-O-methylation stabilizes a base-stacked ordered structure (Drake et al., 1974), and furthermore, a ^{1}H NMR analysis has shown that the 2′-O-methylation of the 3′-nucleotidyl unit increases the fractional population of the C3′-endo form of the modified unit in cytidylyl(3′→5′)nucleosides (Cheng & Sarma, 1977).

2′-O-Methylation is also found of guanosine residues but not of adenosine residues in tRNAs (Sprinzl et al., 1989). A circular dichroism study on guanylyl(3′→5′)guanosine has shown that the 2′-O-methylation of the guanylyl unit stabilizes the base-stacked structure (Drake et al., 1974). By contrast, ^{1}H NMR analyses on adenylyl(3′→5′)nucleosides have shown that 2′-O-methylation of the adenylyl unit does not increase the fractional population of the C3′-endo form of the modified unit (Singh et al., 1976). In fact, 2′-O-methylation of poly(A) does not affect the melting temperature of the poly(A)·poly(U) duplex (Bobst et al., 1969).

Comparison between 2′-O-Methylation and 2-Thiolation. As described above, the 2′-O-methylation of pyrimidine nucleotides stabilizes the C3′-endo form and this stabilization is due to the steric interaction among the 2-carbonyl group, 2′-*O*-methyl group, and 3′-phosphate group. On the other hand, the 2-thiolation of pyrimidine nucleotides also stabilizes the C3′-endo form, and this stabilization is due to the steric repulsion between the 2-thiocarbonyl group and the 2′-hydroxy group (Yamamoto et al., 1983). Thus, the effects of the 2′-O-methylation and the 2-thiolation on the nucleotide conformations are similar to each other, in spite of the chemical difference in modifications: one is the ribose modification and the other is the base modification.

Role of 2′-O-Methylation in Codon Recognition. Um is found in the first position of the anticodon (position 34) of tRNAs specific to glutamine (*Tetrahymena*) and Cm is found in position 34 of tRNAs specific to methionine and tryptophan (Sprinzl et al., 1989). Note that the residue in position 34 of yeast phenylalanine tRNA is in the C3′-endo form (Ladner et al., 1975; Quigley et al., 1975). The modification of U to Um in this position further stabilizes the intrinsic C3′-endo form, as is the case for the modification of U to 2-thiouridine derivatives (Yokoyama et al., 1985).

2-Thiouridine derivatives in the first position of anticodon 34 are found in tRNAs specific to glutamine, lysine, or glutamic acid (Sprinzl et al., 1989), which have two degenerate codons terminating in adenosine or guanosine. If a pyrimidine residue in the first position of the anticodon forms a short "wobble" base pair with a pyrimidine residue in the third position of the codon, the anticodon residue is required to be converted from the ordinary C3′-endo form to the C2′-endo form (Yokoyama et al., 1985). Thus, the 2-thiolated uridine residues there contribute, through their conformational rigidity (inherent stability of the C3′-endo form over the C2′-endo form), to strict recognition of the cognate codons and avoid misrecognition of noncognate codons terminating in uridine or cytidine (Yokoyama et al., 1985). Similarly, the 2′-O-methylation of uridine residue in position 34 of *Tetrahymena* glutamine tRNA is for avoiding misrecognition of the histidine codon CAU or CAC, thus contributing to the correct recognition of glutamine codons CAA and CAG. Further, because of the inherent stability of the C3′-endo form in Cm, the modification of C to Cm in position 34 of methionine tRNA and tryptophan tRNA is also to contribute to the correct recognition of the methionine codon AUG and the tryptophan codon UGG, respectively. Thus, the conformational rigidity of nucleotides in position 34 is essential for the correct recognition of codons terminating in adenosine and guanosine.

Modified pyrimidine nucleosides have also been found in position 32 of the anticodon loop of tRNAs (Sprinzl et al., 1989), including Um in alanine tRNA (*Bombyx mori*) and glutamic acid tRNA (*Escherichia coli*), Cm in aspartic acid tRNA and arginine tRNA (bovine liver), and 2-thiocytidine in serine tRNA and arginine tRNA (*E. coli*). Note that the nucleotide residue in position 32 of yeast phenylalanine tRNA is found to be in the C3′-endo-anti-G^- form (Ladner et al., 1975; Quigley et al., 1975). In this position of tRNA, 2′-O-methylation and 2-thiolation can stabilize the anticodon loop structure and indirectly contribute to correct recognition of codons.

Role of 2′-O-Methylation for Thermostability of tRNA. 2′-O-Methylated uridine nucleotides have also been found in

position 54 (in the T loop) of tRNAs, including Um and 2′-*O*-methylribothymidine (Tm) in initiator methionine tRNA from *Sulfolobus acidocaldarius* and lysine and glutamic acid tRNAs from *Drosophila* and mammals (Sprinzl et al., 1989). Note that the ribothymidine residue in position 54 of yeast phenylalanine tRNA is in the C3′-endo-anti-G⁻ form (Ladner et al., 1975; Quigley et al., 1975). Accordingly, the 2′-O-methylation in this position can further stabilize the intrinsic tertiary structure around the corner of the L-shaped structure and thus contribute to the thermostability of tRNA from *S. acidocaldarius*. In fact, the contents of 2′-O-methylated pyrimidine nucleotide residues in *Bacillus stearothermophilus* (Agris et al., 1973) and 2-thiolated pyrimidine nucleotide residues in *Thermus thermophilus* (Watanabe et al., 1976) have been found to increase as the culture temperatures are raised. This is remarkably similar to the case of 2-thiolation; the modification of U to 2-thioribothymidine in position 54 of tRNAs from *T. thermophilus* (Watanabe et al., 1974) enhances the thermostability of tRNAs through the stabilization of the C3′-endo form (Watanabe et al., 1979; Horie et al., 1985). Furthermore, in extremely thermophilic archaebacteria, 2′-O-methylation and 2-thiolation have also been found in position 54 (Edmonds et al., 1987). Thus, the conformational regidity due to modifications is essential for the thermostability of tRNAs.

In addition to position 54, 2′-O-methylated nucleoside Cm has been found in position 56 (in the T loop) of tRNAs from archaebacteria such as *Halobacteria* (Sprinzl et al., 1987). The cytidine residue in this position of yeast phenylalanine tRNA also takes the C3′-endo-anti-G⁻ form (Ladner et al., 1975; Quigley et al., 1975). Accordingly, the 2′-O-methylation of the cytidine residue there may contribute to the thermostability of tRNAs.

Finally, it should be noted that the structures of the anticodon loop and the T loop of yeast phenylalanine tRNA have been found to be similar to each other (Kim & Sussman, 1976; Quigley & Rich, 1976; Sundaralingam et al., 1976) and that positions 32 and 34 in the anticodon loop closely correspond to positions 54 and 56, respectively. As described above, the 2′-O-methylation and/or 2-thiolation are exclusively located there, indicating that these are the key positions for maintaining the loop conformation.

Conformational Characteristics Essential for tRNA Functions. In the present study, the 2′-O-methylation as well as the 2-thiolation of pyrimidine nucleotide residues in the anticodon loop (positions 32 and 34) and in the T loop (positions 54 and 56) is found to bring about conformational "rigidity" through stabilization of the intrinsic C3′-endo-anti-G- form, thus contributing to correct codon recognition and/or thermostability. On the other hand, the conformational "flexibility" of 5-hydroxyuridine derivatives in the first position of the anticodon has been found to be important for the role of tRNAs specific to valine, alanine, threonine, and serine, which have four degenerate codons (Yokoyama et al., 1985). Thus, the specific conformational characteristics introduced by posttranscriptional modifications are important for regulation of tRNA functions. X-ray crystallographic studies have also suggested the importance of steric interactions due to the posttranscriptional modifications for correct codon recognitions; the roles of the hypermodifications of adenosine at position 37 (adjacent to the anticodon) have been proposed to prevent the nucleotide residue at this position from reading the nucleotide adjacent to the codon (Parthasarathy et al., 1974a,b, 1977). Further, the crystal structures of some other modified nucleotides, such as 4-acetylcytidine and 2,2-dimethylguanosine, have also been analyzed (Parthasarathy et al., 1978; Ginell & Parthasarathy, 1978). However, in order to examine the applicability of the concept proposed above, in the present, it was necessary to perform NMR analyses of dynamic properties such as conformational equilibrium for these modified nucleotides in relation with tRNA functions.

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Site-Specific Binding Constants for Actinomycin D on DNA Determined from Footprinting Studies†

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ABSTRACT: We report site-specific binding constants for the intercalating anticancer drug actinomycin D (Act-D), binding to a 139-base-pair restriction fragment from pBR 322 DNA. The binding constants are derived from analysis of footprinting experiments, in which the radiolabeled 139-mer is cleaved using DNase I, the cleavage products undergo gel electrophoresis, and, from the gel autoradiogram, spot intensities, proportional to amounts of cleaved fragments, are measured. A bound drug prevents DNase I from cleaving at ~7 bonds, leading to decreased amounts of corresponding fragments. With the radiolabel on the 3′ end of the noncoding strand (A-label), we measured relative amounts of 54 cleavage products at 25 Act-D concentrations. For cleavage of the 139-mer with the label on the 3′ end of the coding strand (G-label), relative amounts of 43 cleavage products at 11 Act-D concentrations were measured. These measurements give information about ~120 base pairs of the restriction fragment (~12 turns of the DNA helix); in this region, 14 strong and weak Act-D binding sites were identified. The model used to interpret the footprinting plots is derived in detail. Binding constants for 14 sites on the fragment are obtained simultaneously. It is important to take into account the effect of drug binding at its various sites on the local concentration of probe elsewhere. It is also necessary to include in the model weak as well as strong Act-D sites on the carrier DNA which is present, since the carrier DNA controls the free-drug concentration. As expected, the strongest sites are those with the sequence (all sequences are 5′ → 3′) GC, with TGCT having the highest binding constant, 6.4×10^6 M^{-1}. Sites having the sequence GC preceded by G are weak binding sites, having binding constants approximately 1 order of magnitude lower than those of the strong sites. Also, the non-GC-containing sequences CCG and CCC bind Act-D with a binding constant comparable to those of the weak GGC sites. The analysis may reveal drug-induced structural changes on the DNA, which are discussed in terms of the mechanism of Act-D binding.

Actinomycin D, Act-D (Figure 1), is one of the most intensely studied anticancer drugs (Gale et al., 1981). Numerous investigations have shown that the agent exhibits its antitumor effects by binding to double-stranded DNA, thereby blocking transcription. The binding mechanism involves intercalation of the phenoxazone ring system of the drug via the minor groove of DNA at GC-rich sites (Gale et al., 1981).

Footprinting studies on Act-D using DNase I (Lane et al., 1983; Scamrov & Beabealashvilli, 1983; Fox & Waring, 1984) and Fe–MPE (van Dyke et al., 1982) showed that the highest affinity sites have the dinucleotide sequence 5′-GC-3′. Model building and a single-crystal X-ray structural analysis (Sobell 1973) indicated that the specificity of Act-D for this sequence is due to hydrogen bonding between the 2-amino group of guanine of DNA and the threonine moiety located in the cyclic peptide of the drug.

Although 5′-GC-3′ is the preferred binding site, there are other sites. The duplex $d(CGTCGACG)_2$, which does not contain 5′-GC-3′, is able to strongly bind two actinomycin D molecules in a highly cooperative manner (Snyder et al., 1989). Further, in an effort to measure Act-D binding specificity, Rill et al. (1989) examined the DNA cleavage sites of the photoaffinity probe 7-azidoactinomycin D on several DNA re-

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