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^1H NMR studies on the conformational characteristics of 2-thiopyrimidine nucleotides found in transfer RNAs

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ABSTRACT

The molecular conformations of naturally occurring 2-thiopyrimidine nucleosides (5-methylaminomethyl-2-thiouridine, 5-methoxycarbonylmethyl-2-thiouridine and 2-thiocytidine) and 5'-mononucleotides (5-methylaminomethyl-2-thiouridine 5'-monophosphate and 2-thiocytidine 5'-monophosphate) in ^2H_2O solution were elucidated by analyses of the proton NMR spin-coupling constant, nuclear Overhauser effect, and lanthanide-induced shifts and relaxation enhancements. As monomers, these nucleotides are almost exclusively in the ^{3}*E-gg-anti* form, even in the absence of ordinary stabilizing factors of this form; *i. e.*, base-stacking and base-pairing interactions with other nucleotide units. This inherent conformational rigidity of the 2-thiopyrimidine units probably contributes to stability of the conformation of tRNA.

INTRODUCTION

In the primary sequences of $tRNA^{Gln}$, $tRNA^{Lys}$ and $tRNA^{Glu}$ from both prokaryotes and eukaryotes, uridine in the first position of the anticodon, or the "wobble" position, is always modified to 5-substituted 2-thiouridine, whereas cytidine in this position is never modified.[1-3] For example, 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) is found in *E. coli* $tRNA_1^{Gln}$, $tRNA^{Lys}$ and $tRNA_2^{Glu}$, and 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) in yeast $tRNA_2^{Lys}$ and $tRNA_3^{Glu}$ (Figure 1). Recently, 5-carboxymethylaminomethyl-2-thiouridine was found in *B. subtilis* $tRNA_1^{Lys}$.[2] However, 2-thiouridine derivatives have never been found in the wobble position of tRNAs specific to other amino acids. The tRNAs containing 2-thiouridine derivatives recognize codons NAR[4] (more selectively NAA than NAG[3]). The pairing of U in the wobble position of these tRNAs with U or C in mRNA may cause miscoding, which would be lethal to cells. Modification of U to 2-thiouridine de-

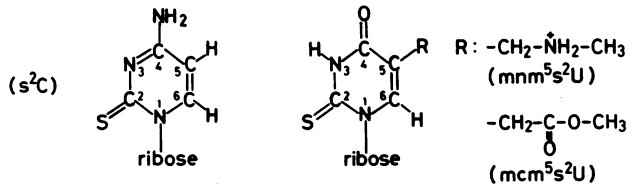

Figure 1. 2-Thiopyrimidine nucleosides

rivatives may be useful in preventing such mispairing.[3]

Furthermore, in *T. thermophilus* HB8 tRNAs, ribothymidine (T) in the TΨC loop is modified to 2-thioribothymidine (s^2T) or 5-methyl-2-thiouridine, probably contributing to thermostability.[5,6] 2-Thiocytidine (s^2C) is located in the anticodon loop of *E. coli* $tRNA_1^{Arg}$ and $tRNA_3^{Ser}$,[1] but its function is not yet clear.[7]

For understanding the significance of these modifications, it is important to take into account various properties of these modified components in comparison with those of unmodified ones. In this context, it is interesting that the secondary structures of polynucleotides containing 2-thiopyrimidine nucleosides (s^2U, s^2C) are extraordinarily stable,[8-11] compared with those of polynucleotides containing unmodified pyrimidine nucleosides (U,C) or 4-thiouridine (s^4U).[12,13] This extraordinary stability has been discussed with respect to the function of 2-thiouridine derivatives in tRNAs.[14] To obtain further information on the function of 2-thiouridine derivatives, it is necessary to investigate the reason for the high stability of modified polynucleotides, and to determine whether similar stability could be introduced into the tRNA structure. s^2U and s^2C have been reported to have about four-fold higher stacking ability than U and C.[15,16] However, this is not necessarily the main reason for the high stability of polynucleotides containing 2-thiopyrimidine derivatives, because s^4U has similar stacking ability to that of s^2U or s^2C, but polynucleotides containing s^4U are not highly stable.[12,13]

The conformational properties of the nucleotide unit backbone have a significant effect on the secondary structure of the polynucleotide chain. The molecular structures of mnm^5s^2U and mcm^5s^2U in crystal have been analysed,[17-19] but it is also impor-

tant to investigate the conformational characteristics of 2-thiopyrimidine nucleotides in aqueous solution. In the present study ^{1}H NMR spectra of naturally occurring modified components (s^2C, mnm^5s^2U, mcm^5s^2U and s^4U) and their 5'-mononucleotides were analysed by measuring spin-coupling constants and nuclear Overhauser effects[20] and by the lanthanide probe method.[21] Results showed that 2-thiopyrimidine nucleotides have remarkable conformational characteristics; 2-thiopyrimidine nucleotides are almost exclusively in the ^{3}*E-gg-anti* form. On the other hand, the fractional population of the ^{3}*E-gg-anti* form in unmodified pyrimidine and 4-thiouracil nucleotides is less than 50%.

MATERIALS AND METHODS

Materials. A nuclease P_1 digest of unfractionated tRNAs of *E. coli* was fractionated by Dowex 1 column chromatography (3 × 80 cm, formate form) using a linear gradient of 0 - 3 M formic acid. $pmnm^5s^2U$ and ps^2C were collected and purified further by paper chromatography with isobutyric acid : 0.5 N NH_4OH (5:3, by vol.) as the solvent system. s^2C was obtained by treatment of ps^2C with phosphomonoesterase. Synthetic samples of ps^4U and mnm^5s^2U were generous gifts from Prof. T. Ueda, and mcm^5s^2U was a gift from Dr. R. H. Hall. Cytidine was purchased from Yamasa Shoyu Co. Ltd. (Choshi). Nitrates of Pr(III), Eu(III) and Gd(III) of 99.9% purity were obtained from Nakarai Chemical Co. (Kyoto). Lanthanum oxide of more than 99.99% purity was also purchased from Nakarai Chemical Co. and was dissolved in nitric acid to prepare lanthanum nitrate.

Sample Solution. Nucleotides were dissolved in ^2H_2O at a concentration of 5 mM or less; in this concentration range, only slight concentration-dependence of the chemical shifts was observed and thus molecular association was negligible. The pH of sample solutions (the meter reading by a Radiometer PHM26 pH meter) was adjusted below 5.5, so that the phosphate group was monoanionic. For measurement of the nuclear Overhauser effect, the sample solution in an NMR tube was treated with chelating agents to remove paramagnetic impurities. Then, the tube was degassed, filled with nitrogen gas and sealed.

NMR Measurements. 270 MHz ^{1}H NMR spectra of sample solutions at 23°C were recorded on a Bruker WH270 spectrometer. Chemical shifts were measured from sodium 2,2-dimethyl-2-silapentane-5-sulphonate. Spin-lattice relaxation rates were measured by the inversion recovery method. Nuclear Overhauser effects were measured by the gated decoupling method.

Data Analyses. The HITAC 8800/8700 system at the Computer Center of University of Tokyo was used for data analyses. Computer programs, NMRTRY/PLOT and PCS2RX,[22] prepared in our laboratory, were used for spectral simulations and conformation analysis by the lanthanide probe method, respectively.

RESULTS AND DISCUSSION

Spin-coupling Constant Analysis

Spectral Analysis. Assignments of the base and ribose-ring proton signals were made by homonuclear decoupling experiments and by comparing the chemical shifts with those of related compounds and were finally confirmed by spectral simulation using the NMR-TRY/PLOT program. Some of the observed and simulated spectra are shown in Figures 2 and 3. Assignments of the 5' and 5" protons are made so that the H(5') signal appears at a lower field than

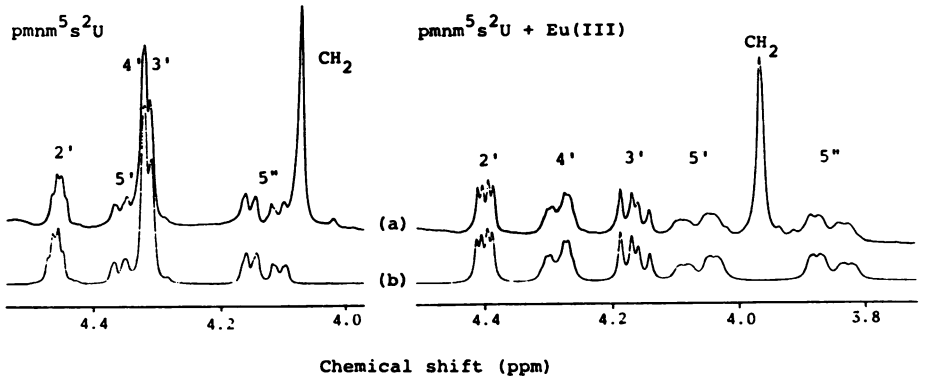

Figure 2. 270 MHz ^{1}H NMR spectra of pmnm^{5}s^{2}U (5 mM) and pmnm^{5}s^{2}U (5 mM) + $Eu(NO_3)_3$ (1 mM) in $^{2}H_2O$ solution at pH 3.5 and 23°C; (a) observed spectra and (b) simulated spectra.

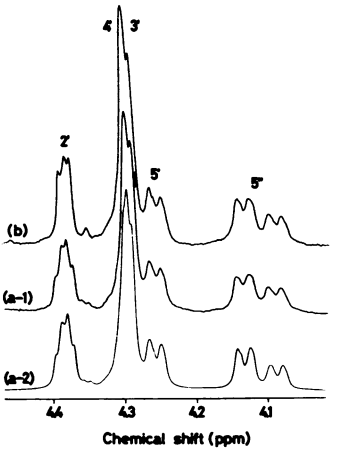

Figure 3.
270 MHz ^{1}H NMR spectra of $ps^{2}C$ (3 mM) in $^{2}H_{2}O$ solution at pH 5.0 and 23°C;
(a-1) observed spectrum,
(a-2) simulated spectrum,
(b) H(1') irradiated spectrum.

the H(5") signal;[23,24] the nomenclature of H(5') and H(5") is shown in Figure 4. The spectra of 2-thiopyrimidine nucleotides are all complicated by signal overlapping and the resultant virtual coupling (Figures 2 and 3). The shift reagent $Eu(NO_3)_3$ was useful for separating the overlapping proton signals of $pmnm^{5}s^{2}U$ (Figure 2) so that more reliable measurements of spin-coupling constants were possible. The simulated spectra with common spin-coupling constants (Table 1) for $pmnm^{5}s^{2}U$ and $pmnm^{5}s^{2}U + Eu(NO_3)_3$ closely agree with the observed spectra as shown in Figures 2(a) and (b). This result indicates that the conformation of $pmnm^{5}s^{2}U$ is not affected by the addition of $Eu(NO_3)_3$. The chemical shifts and spin-coupling constants of $mnm^{5}s^{2}U$, $pmnm^{5}s^{2}U$, $mcm^{5}s^{2}U$, $s^{2}C$,

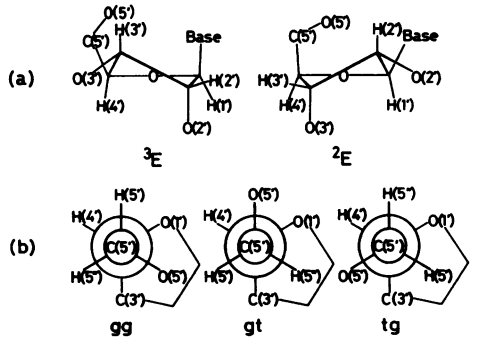

Figure 4.
Local conformations;
(a) ribose ring puckering,
(b) rotation around the C(4')-C(5') bond.

Table 1. Chemical shifts (ppm) and spin-coupling constants (Hz)

	mnm^5s^2U	mcm^5s^2U	s^2C	s^4U	U	C
H(1')	6.607	6.576	6.675	5.86	5.901	5.882
	6.502		6.687	5.931	5.990	5.999
H(2')	4.374	4.384	4.343	4.35	4.341	4.290
	4.457		4.382	4.355	4.363	4.321
H(3')	4.186	4.199	4.154	4.22	4.222	4.190
	4.311		4.292	4.318	4.376	4.337
H(4')	4.178	4.174	4.178	4.14	4.128	4.116
	4.319		4.298	4.279	4.281	4.283
H(5')	4.037	4.021	4.005	3.94	3.907	3.917
	4.332		4.276	4.172	4.160	4.198
H(5")	3.877	3.858	3.855	3.81	3.803	3.801
	4.129		4.114	4.071	4.090	4.093
H(5)	*	*	6.328	6.56	5.887	6.021
	*		6.391	6.622	5.950	6.124
H(6)	8.443	8.211	8.145	7.75	7.862	7.826
	8.485		8.259	7.877	7.991	8.042
CH_2	3.947	3.468	*	*	*	*
	4.069		*	*	*	*
CH_3	2.697	3.719	*	*	*	*
	2.738		*	*	*	*
$\Delta\delta$	0.160	0.163	0.150	0.13	0.104	0.116
	0.203		0.162	0.101	0.070	0.105
$J_{1'2'}$	2.3	2.3	2.1	3.9	4.4	4.0
	2.2		2.4	4.4	4.9	4.1
$J_{2'3'}$	4.8	4.8	4.8	5.4	5.3	5.3
	4.8		4.7	5.0	5.1	5.0
$J_{3'4'}$	7.9	8.0	7.9	5.6	5.5	6.1
	7.6		7.9	5.0	4.3	5.1
$J_{4'5'}$	2.5	2.3	2.3	3.0	3.0	2.9
	2.0		2.0	2.4	2.3	2.4
$J_{4'5"}$	3.2	3.0	3.7	4.3	4.4	4.4
	2.0		2.0	2.6	2.8	3.8
$J_{4'P}$	2.0		2.0	2.1	2.1	2.0
$J_{5'5"}$	-13.2	-13.2	-12.9	-12.7	-12.7	-12.8
	-12.4		-12.1	-11.8	-11.8	-11.8
$J_{5'P}$	4.5		4.4	4.4	4.3	4.2
$J_{5"P}$	4.6		4.7	5.1	5.0	5.1
$J_{5\,6}$	*	*	7.6	7.7	8.0	7.5
	*		7.7	7.6	8.2	7.6

The upper value in each pair of rows is for nucleoside, and lower for the 5'-mononucleotide. Data on pU, pC, s^4U and U are from reference 25, 25, 26 and 27, respectively. *No corresponding item.

ps^2C, C and ps^4U, obtained by spectral simulation, are listed in Table 1, together with those of pU and pC,[25] s^4U,[26] and U.[27]

Conformation of the Ribose Ring. It was assumed that the ribose ring exists as an equilibrium mixture of two puckered forms, ^2E and ^3E [Figure 4(a)],[28] and the fractional population of the ^3E form was calculated from $J_{3'4'}/(J_{1'2'} + J_{3'4'})$ and is listed in Table 2. The fractional populations of the ^3E form of 2-thiopyrimidine nucleotides (≈80%) are much higher than those of other pyrimidine nucleotides (≲60%). The ^3E populations of s^2C and ps^2C, which do not have any 5-substituent, are also as much higher than those of C and pC as the ^3E populations of the 5-substituted 2-thiouridine derivatives. This shows that the positively charged methylaminomethyl group, or the more bulky methoxycarbonylmethyl group, at the 5-position of the pyrimidine base does not contribute to the high ^3E population. Similarly, the differences between the ^3E population of nucleosides (mnm^5s^2U and s^2C) and that of the corresponding 5'-mononucleotides ($pmnm^5s^2U$ and ps^2C) are negligible, indicating that the phosphate group at the 5'-position does not appreciably affect the $^3E \rightleftharpoons {}^2E$ equilibrium of 2-thiopyrimidine nucleotides. On the other hand, the ^3E population of ps^4U and s^4U are not much different from those of pU and U. All these observations indicate that the greater stability of the ^3E form than the ^2E form is due to the inherent nature of nucleosides with 2-thiolated pyrimidine bases.

Conformation about the C(4')-C(5') Bond. The local conformation about the C(4')-C(5') bond was examined by standard analysis

Table 2. Populations (%) of ^3E and *gg* forms.

	mnm^5s^2U	mcm^5s^2U	s^2C	s^4U	U	C
^3E	78	78	79	59	56	60
gg	82	87	79	66	65	66
	$pmnm^5s^2U$		ps^2C	ps^4U	pU	pC
^3E	78		77	53	47	55
gg	100		100	90	89	77

with three staggered forms [Figure 4(b)],[29] using $J_{4'5'}$ and $J_{4'5''}$. The calculated fractional populations are listed in Table 2. The *gg* populations of 2-thiopyrimidine nucleosides are much higher than those of other pyrimidine nucleosides. Similarly, 2-thiopyrimidine 5'-mononucleotides ($pmnm^5s^2U$ and ps^2C) are exclusively in the *gg* form, while less than 90% of the population of other nucleotides is in the *gg* form. (The comparisons of *gg* populations must be made either among nucleosides or among 5'-mononucleotides, because in general the *gg* form is more stable in 5'-mononucleotides than in the corresponding nucleosides.[30])

The *gg* population may also be estimated from the chemical shift difference between H(5') and H(5") signals (listed in Table 1 as $\Delta\delta$).[24,31] Values for $\Delta\delta$'s of 2-thiopyrimidine nucleotides are much the largest, which suggests that the *gg* populations of 2-thiopyrimidine nucleotides are much the highest. $\Delta\delta$'s of nearly 0.2 ppm are almost equal to the extrapolated value of 0.17 ppm estimated for the *gg* form of pyrimidine nucleosides.[24] Thus, it is concluded that 2-thiolation of the pyrimidine bases exclusively stabilizes the *gg* form of the nucleotide unit.

Conformation about the C(5')-O(5') Bond. $J_{5'P}$ and $J_{5''P}$ of 2-thiopyrimidine 5'-mononucleotides are nearly equal to those of other nucleotides in Table 1. Accordingly, the local conformation about the C(5')-O(5') bond is not affected by 2-thiolation of the bases, in contrast to the significant effect of this modification on the local conformation about the C(4')-C(5') bond.

Analysis of Nuclear Overhauser Effect on $pmnm^5s^2U$

As described in the previous section on the analysis of spin-coupling constants, 2-thiopyrimidine nucleotides were found to have the common conformational characteristic that the ^3E-*gg* form is predominant. However, the conformation about the glycosidic bond can not be deduced from vicinal spin-coupling constants. Therefore, experiments on the nuclear Overhauser effect (NOE)[20] were performed on $pmnm^5s^2U$, as an example of a 2-thiopyrimidine nucleotide. Table 3 lists the relative intensities (I_z/I_0) of the base and ribose protons upon irradiation of various protons of $pmnm^5s^2U$.

The relative intensity of the H(6) signal increases by 8%

Table 3. NOE for $pmnm^5s^2U$ (relative intensity, I_z/I_0)

Irradiated	Observed				
	H(6)	H(1')	H(2')	CH_2	CH_3
H(6)		1.05	1.07	1.03	1.00
H(1')	1.02		1.05	1.00	1.00
H(2')	1.08	1.22		1.00	1.00
CH_2	1.17	1.00	0.99		1.05
CH_3	1.02	1.00	1.00	1.03	
H(3'), H(4'), H(5')	1.09	1.08	1.20	1.00	1.00

when the H(2') proton is irradiated, but is less affected by irradiation of the H(1') proton (2%). Similarly, NOE on the H(1') signal is much larger (22%) on irradiation of the H(2') proton than on irradiation of the H(6) proton (5%). These findings indicate that H(6) is much closer to H(2') than to H(1') and that H(1') is much closer to H(2') than to H(6). It is concluded, therefore, that the base is exclusively oriented in the *anti* form relative to the ribose moiety.

The NOE's of the H(6) signal are nearly equal on irradiation of the H(2') proton (8%) and of the H(3') proton [9%: simultaneous irradiation of the H(4') and H(5') protons may have little effect on the H(6) signal]. This indicates that the distances from H(6) to H(2') and to H(3') are nearly equal, which is interpreted in the following manner. When the ribose ring takes the ^3E form rather than the ^2E form, H(3') takes the axial position. Further, it has been pointed out that the χ angle [the dihedral angle for O(1')-C(1')-N(1)-C(6)] is smaller for the ^3E form than for the ^2E form.[30] In such an arrangement of H(6), H(2') and H(3'), the distances from H(6) to H(2') and to H(3') are in fact nearly equal. Thus, the NOE data on $pmnm^5s^2U$ are in accord with the idea of the predominance of the ^3E form.

Lanthanide Probe Analysis of $pmnm^5s^2U$

The above results of local conformation analyses, spin-coupling constants and NOE, have shown that $pmnm^5s^2U$ is almost exclusively in the ^3E-*gg*-*anti* form in aqueous solution. Further analy-

sis of the overall conformation of the pmnm^{5}s^{2}U molecule were performed by the lanthanide probe method.[21] In this method, the spatial arrangements of protons all over the molecule are directly analyzed relative to the lanthanide ion which is bound to the phosphate group. Thus, the results of local conformation analyses can be examined from a different point of view.

Gd(III)-induced Enhancement of Relaxation Rate. $Gd(NO_3)_3$ (≤2 μM) was added to a ^2H_2O solution of pmnm^{5}s^{2}U + $Eu(NO_3)_3$, which has clearly separated signals in the NMR spectrum (cf. Spin-coupling Constant Analysis and Figure 2). The enhancements of proton relaxation rates induced by Gd(III) ion were measured for all non-exchangeable protons, and the relaxation ratios relative to that of H(3') are listed in Table 4.

Gd(III)-induced relaxation-rate enhancement of the ith proton is proportional to $\langle 1/r_i^6 \rangle_{av}$, where r_i is the distance between the ith proton and Gd(III) ion bound to monoanionic phosphate group. For pmnm^{5}s^{2}U in aqueous solution, the relaxation ratio of H(2') signal (0.2) is much smaller than that of the H(3') signal. Accordingly, H(3') must be much closer to the 5'-phosphate group than H(2') is. This is in accord with the high ^3E population (78%) of pmnm^{5}s^{2}U; in the ^3E form, H(3') is in the axial position, while H(2') in the equatorial position is far from 5'-phosphate group. As for pU, however, the relaxation ratio of the H(2') signal (0.6) is larger than for pmnm^{5}s^{2}U, corresponding to the lower ^3E population for pU (56%). For pmnm^{5}s^{2}U, the relaxation ratio of the H(4') signal (0.5) is much smaller than that of the H(3') signal. Since

Table 4. Shift and relaxation ratios of pmnm^{5}s^{2}U

		1'	2'	3'	4'	5'	5"	6	CH_2	CH_3
Shift	obs.	0.3	*	1.0	*	2.0†	2.0†	1.3	0.6	0.4
	calc.	0.5	**	1.0	**	2.7	2.7	2.1	**	**
Relaxation	obs.	0.1	0.2	1.0	0.5	2.0	3.0	0.6	0.4	0.3
	calc.	0.1	0.4	1.0	0.3	2.5	3.4	1.3	**	**

† Not distinguishable from each other because of broadening.
* Not observed. ** Not included in the calculation.

the 5'-phosphate group is further from H(4') in the *gg* form than in the *gt* or *tg* form [Figure 4(b)], the small relaxation ratio of the H(4') signal is in accord with the extremely high *gg* population of $pmnm^5s^2U$.

Pr(III)-induced Shifts. Proton chemical shifts were measured on a ^2H_2O solution of $pmnm^5s^2U$ (1 - 2.5 mM) with $Pr(NO_3)_3$ at twice the concentration of $pmnm^5s^2U$. For correction of the complex formation shifts,[21] $La(NO_3)_3$ was used at the same concentration as $Pr(NO_3)_3$. The shift ratios relative to the H(3') signal are listed in Table 4. The Pr(III)-induced shift of the ith proton is proportional to $\langle(3\cos^2\theta_i - 1)/r_i^3\rangle_{av}$, where θ_i is the angle made by the lines from the Pr(III) ion to the phosphorus and from the Pr(III) to the ith proton.

Conformation Analysis. For $pmnm^5s^2U$ in aqueous solution, the shift and relaxation ratios were calculated from the atomic coordinates, using the computer program PCS2RX.[22] For the base moiety, the atomic coordinates of 2',3'-isopropylidene-mnm^5s^2U were taken from x-ray crystallographic data.[18] For the ribose moiety, the typical structures of the ^3E and ^2E forms were adopted.[32] The calculation was made for an equilibrium mixture of the ^3E-*gg-anti* form (78%) and the ^2E-*gg-anti* forms (22%). The conformation about the O(5')-P bond was treated as an equilibrium mixture of three staggered forms,[21,22] and the fractions of these three forms were adjusted in the procedure of refinement. The calculated values of shift and relaxation ratios (Table 4) agree satisfactorily with the observed values, indicating that the conclusions obtained by spin-coupling constant and NOE analyses are in accord with those by the lanthanide probe method. Therefore, these studies show quantitatively that $pmnm^5s^2U$ is almost exclusively in the ^3E-*gg-anti* form.

Possible Mechanism for Stabilization of the ^3E-*gg-anti* Form in 2-Thiopyrimidine Nucleotides

The present study showed a remarkable conformational characteristic of 2-thiolated pyrimidine bases directly due to 2-thiolation: 2-thiopyrimidine nucleotides are almost exclusively in the ^3E-*gg-anti* form in aqueous solution. As this form is stabilized generally by base-stacking or base-pairing interaction with other

nucleotide units, it is surprising that the ^3E-*gg*-*anti* form is significantly stabilized in monomers of 2-thiopyrimidine nucleotides.

The long C=S double bond (≃1.66 Å) and large van der Waals radius of the sulfur atom (1.85 Å) stabilizes the *anti* form but not the *syn* form. With regard to ribose ring puckering, significant stabilization of the ^3E form is observed for nucleosides (s^2C, mnm^5s^2U and mcm^5s^2U) as well as for corresponding mononucleotides, while the phosphate group at the 5'-position does not necessarily affect the $^3E \rightleftharpoons {}^2E$ equilibrium. Accordingly, the predominance of the ^3E form is due to destabilization of the ^2E form by the interaction between the large thiocarbonyl group and either H(1') or 2'-OH group in the ^2E-*anti* form, but is not due to the interaction between the base and the 5'-exocyclic group. The high ^3E population favors the high *gg* population, because the *gg* form is known to be much more stable when the ribose ring takes the ^3E form rather than the ^2E form.[21,33]

Effect of 2-Thiolation on RNA Conformation

In a polynucleotide chain of the RNA-A form, nucleotide units take the ^3E-*gg*-*anti* form and the dihedral angle (ϕ') of C(4')-C(3')-O(3')-P is -151°,[34] designated here as the g^- form. If a ribose ring in the chain takes the ^3E form, the *gg* and g^- forms are exclusively stabilized,[21,22] so that the local conformation of the chain is suitable for the RNA-A form. On the other hand, if the ribose ring takes the ^3E form rather than the ^2E form, the local conformational properties of the polynucleotide chain are very different. Firstly, the conversion from the ^3E form to ^2E form changes the direction of the chain by about 75°, as a result of the rotation about the C(3')-C(4') bond, so that a more extended backbone is produced by the ^2E form than by ^3E form.[29] Secondly the *gt* and *tg* forms are somewhat stabilized with the ^2E form, the chain is more flexible with the ^2E form than with the ^3E form. Thus, the high stability of the ^3E-*gg*-*anti* form inherent to the 2-thiopyrimidine nucleotides greatly contributes to the stability of the secondary structure of the RNA-A form.

Effect of Base Stacking. Polynucleotides containing s^2U or s^2C form extraordinarily stable secondary structures,[8-11] while

the stability of polynucleotides containing s^4U are only a little higher[12] or rather lower[13] than those of polynucleotides containing U instead of s^4U. Previously, this extraordinary stability of 2-thiopyrimidine polynucleotides was ascribed to the enhanced base stacking ability of 2-thiolated bases.[9,14,16] However, 4-thio- as well as 2-thiopyrimidine nucleosides (*e.g.* s^2U, s^2C and also s^4U) have about four-fold higher self-association constants in aqueous solution, namely, higher stacking abilities, than those of non-thiolated pyrimidine nucleosides.[15,16] In the previous interpretations, therefore, the different stabilities of polynucleotides containing 2-thio- and 4-thiopyrimidine nucleosides was attributed to the difference in their stacking patterns; in base stacking of polynucleotide chains, a thiocarbonyl group in the 2-position of pyrimidine base can contribute to the interaction with the adjacent base [so-called S(2)-N(1) stacking[14]], whereas a thiocarbonyl group in the 4-position cannot stack with the adjacent base and so can not contribute to the stacking interaction.[9,14,16] However, this interpretation has not necessarily been confirmed. Generally it is not reasonable to assume that a thiocarbonyl group at the 4-position cannot contribute to the stacking interaction. For example, in the sequence of purine-pyrimidine, the 4-substituent of pyrimidine base can stack to the purine ring. On the other hand, in the present study, we found that only 2-thiolation stabilizes the *^{3}E-gg-anti* form and that 4-thiolation does not. Further, as described above, stabilization of the *^{3}E-gg-anti* form probably contributes to the stability of the secondary structure of RNA-A chain. Consequently, it is more reasonable to attribute the extraordinary stability of polynucleotides containing 2-thiopyrimidine nucleosides to their conformational rigidity than to the base-stacking ability of bases.

Effect on tRNA Structure. It is important to consider whether conformational rigidity can also be introduced into the tRNA structure by 2-thiolation. In the tertiary structure of yeast $tRNA^{Phe}$,[35] the nucleotide units in the positions corresponding to the 2-thiolated sites [Cm(32) corresponding to s^2C, Gm(34) to mnm^5s^2U and mcm^5s^2U in the wobble position, and T(54) to s^2T in thermophile tRNAs] all take the *^{3}E-gg-anti* form. Assuming that the conforma-

tions of all tRNAs are largely similar to that of yeast $tRNA^{Phe}$, the local conformation required of the 2-thiolated components is also the $^{3}E\text{-}gg\text{-}anti$ form in the native tRNA structure. Because of the exclusive preference of the $^{3}E\text{-}gg\text{-}anti$ form, 2-thiopyrimidine nucleosides contribute to the enhanced stability of native tRNA conformations.

2-Thiouridine derivatives in the wobble position have been found only in tRNAs corresponding to codons NAA and NAG. Without this modification, these tRNAs should have anticodons UUN. The sequence U-U is the least stable component in the helical ordered structure of RNA-A chain ($^{3}E\text{-}gg\text{-}anti$ form), because of the weak base-stacking ability of U and the relative weakness of base pairs involving U. Thus, the conformation of unmodified UUN anticodons will be less stable than those of anticodons with other sequences. Accordingly, it may be necessary that the conformation of wobble position is directly fixed in the $^{3}E\text{-}gg\text{-}anti$ form by the rigidity of the 2-thiouridine derivatives in these tRNAs. It is likely, therefore, that 2-thiouridine derivatives in the wobble position prevent the undesirable U-U or U-C mispairing[3] or enhance the efficiency of the tRNA functions in protein biosyntheses.[36]

A hydrogen bond involving a thiocarbonyl group is weaker than one involving a carbonyl group.[37] However, in some of very stable secondary structures of polynucleotides containing 2-thiopyrimidine nucleosides,[8,11] the 2-thiocarbonyl group is involved in hydrogen-bonding interaction. Thus, the conformational rigidity of 2-thiopyrimidine nucleosides not only offsets the disadvantage of the weak S··H-N hydrogen bond, but also results in enhanced stability of the secondary structure.

CONCLUSION

In the present study, it is shown that a simple modification, such as replacement of the oxygen atom in the 2-position of a pyrimidine base by a sulfur atom, probably causes remarkable stabilization of the essential conformation of tRNAs. This stabilization is not primarily due to enhancement of base-stacking interactions, but to fixation of the ribose-base conformation in the $^{3}E\text{-}gg\text{-}anti$ form. In the present study, monomers were investigated

so that the conformational characteristics of modified nucleotide units were elucidated, without being influenced by interactions between units. For detailed investigations on the correlation between the structures and functions of tRNAs, the conformational properties of nucleotide units need be taken into account, in addition to direct base-base interactions.

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REFERENCES

1. Gauss, D. H., Grüter, F. and Sprinzl, M. (1979) Nucleic Acids Res. r1-r19
2. Yamada, Y. and Ishikura, H. (1978) private communication
3. McCloskey, J. A. and Nishimura, S. (1977) Acc. Chem. Res. 10, 403-410
4. Abbreviations: N, any unspecified nucleoside; R, adenosine or guanosine; A, adenosine; G, guanosine; U, uridine; C, cytidine; mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; mcm^5s^2U, 5-methoxycarbonylmethyl-2-thiouridine; T, ribothymidine; Ψ, pseudouridine; s^2T, 2-thioribothymidine; s^4U, 4-thiouridine; $pmnm^5s^2U$, 5-methylaminomethyl-2-thiouridine 5'-monophosphate; ps^2C, 2-thiocytidine 5'-monophosphate; ps^4U, 4-thiouridine 5'-monophosphate; pU, uridine 5'-monophosphate; pC, cytidine 5'-monophosphate; Cm, 2'-*O*-methylcytidine; Gm, 2'-*O*-methylguanosine; m^1A, 1-methyladenosine; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.
5. Watanabe, K., Oshima, T. and Nishimura, S. (1976) Nucleic Acids Res. 3, 1703-1713
6. Watanabe, K., Shinma, M., Oshima, T. and Nishimura, S. (1976) Biochem. Biophys. Res. Commun. 72, 1137-1144
7. Kruse, T. A., Clark, B. F. C. and Sprinzl, M. (1978) Nucleic Acids Res. 5, 879-892
8. Bähr, W., Faerber, P., and Scheit, K.-H. (1973) Eur. J. Biochem. 33, 535-544
9. Scheit, K.-H. and Faerber, P. (1975) Eur. J. Biochem. 50, 549-555
10. Faerber, P., Scheit, K.-H. and Sommer, H. (1972) Eur. J. Biochem. 27, 109-115
11. Faerber, P. (1974) FEBS Lett. 44, 111-116
12. Simuth, J., Scheit, K.-H. and Gottschalk, E. M. (1970) Biochim. Biophys. Acta 204, 371-380
13. Cramer, F., Gottschalk, E. M., Matzura, H., Scheit, K.-H. and Sternbach, H. (1971) Eur. J. Biochem. 19, 379-385
14. Mazumdar, S. K., Saenger, W. and Scheit, K.-H. (1974) J. Mol. Biol. 85, 213-229

15. Plesiewicz, E., Stępień, E. and Wierzchowski, K. L. (1975) Stud. Biophys. 48, 93-96
16. Plesiewicz, E., Stępień, E., Bolewska, K. and Wierzchowski, K. L. (1976) Nucleic Acids Res. 3, 1295-1306
17. Hillen, W., Egert, E., Lindner, H. J. and Gassen, H. G. (1978) FEBS Lett. 94, 361-364
18. Kasai, H., Nishimura, S., Vorbrüggen, H. and Iitaka, Y. unpublished results
19. Hillen, W., Egert, E., Lindner, H. J. and Gassen, H. G. (1978) Biochemistry 17, 5314-5320
20. Noggle, J. H. and Schirmer, R. E. (1971) The Nuclear Overhauser Effect, Academic Press, New York
21. Inagaki, F., Tasumi, M. and Miyazawa, T. (1978) Biopolymers 17, 267-279
22. Yokoyama, S., Inagaki, F., Tasumi, M. and Miyazawa, T. (1978) Sixth International Biophysics Congress Abstract, p. 319
23. Remin, M. and Shugar, D. (1972) Biochem. Biophys. Res. Commun. 48, 636-642
24. Davis, D. B. and Rabczenko, A. (1975) J. Chem. Soc. Perkin II, 1703-1711
25. Lee, C.-H., Ezra, F. S., Kondo, N. S., Sarma, R. H. and Danyluk, S. S. (1976) Biochemistry 15, 3627-3639
26. Hruska, F. E., Ogilvie, K. K., Smith, A. A. and Wayborn, H. (1971) Can. J. Chem. 49, 2449-2452
27. Blackburn, B. J., Grey, A. A., Smith, I. C. P. and Hruska, F. E. (1970) Can J. Chem. 48, 2866-2870
28. Altona, C. and Sundaralingam, M. (1973) J. Amer. Chem. Soc. 95, 2333-2344
29. Lee, C.-H. and Sarma, R. H. (1976) Biochemistry 15, 697-704
30. Sundaralingam, M. (1973) in: Conformation of Biological Molecules and Polymers, The Jerusalem Symposia on Quantum Chemistry and Biochemistry, Bergman, E. D. and Pullman, B., eds., Vol. V, pp. 417-456, Academic Press, New York
31. Hruska, F. E., Wood, D. J., McCaig, T. N., Smith, A. A. and Holy, A. (1974) Can. J. Chem. 52, 497-508
32. Yathindra, N. and Sundaralingam, M. (1975) in Structure and Conformation of Nucleic Acids and Protein-Nucleic Acid Interactions, Sundaralingam, M. and Rao, S. T., eds., pp. 649-676, University Park Press, Baltimore
33. Hruska, F. E. (1973) in: Conformation of Biological Molecules and Polymers, The Jerusalem Symposia on Quantum Chemistry and Biochemistry, Bergman, E. D. and Pullman, B., eds., Vol. V, pp. 345-358, Academic Press, New York
34. Arnott, S., Hukins, D. W. L., Dover, S. D., Fuller, W. and Hodgson, A. R. (1973) J. Mol. Biol. 81, 107-122
35. Stout, C. D., Mizuno, H., Rubin, J., Brennan, R., Rao, S. T. and Sundaralingam, M. (1976) Nucleic Acids Res. 3, 1111-1123
36. Sen, G. C. and Ghosh, H. P. (1976) Nucleic Acids Res. 3, 523-535
37. Todoriki, H., Nishimura, Y., Hirakawa, A., Tsuboi, M. and Higuchi, S., private communication