[Chem. Pharm. Bull.]
30(11)4126—4133(1982)

Chemical Modification of Cytosine Residues of tRNA^{Val} with Hydrogen Sulfide (Nucleosides and Nucleotides. XL¹⁾)

KAZUNOBU MIURA,^{*,a} TATSUYOSHI IWANO^a, SHIGEKI TSUDA,^a TOHRU UEDA,^a
FUMIO HARADA,^b and NOBUYUKI KATO^b

Faculty of Pharmaceutical Sciences, Hokkaido University,^a Kita-ku, Sapporo, 060, Japan
and Virology Division, National Cancer Center Research
Institute,^b Tsukiji, Chuo-ku, Tokyo, 104, Japan

(Received February 10, 1982)

The conversion of cytosine residue of transfer ribonucleic acid (tRNA) to 4-thiouracil residues was carried out with a hydrogen sulfide-pyridine-water system. In the case of tRNA^{Val} from mouse cells, the cytosine moieties located in unpaired and looped-out regions of the molecule, such as the anticodon and CCA-end regions, were converted. This result shows that the secondary and tertiary structures of tRNA are maintained even in medium containing liquid hydrogen sulfide and pyridine, and hence, this system should be suitable for studies of the higher-order structure of nucleic acids.

Keywords—Sulphydrolysis; 4-thiouracil residue; modification of tRNA^{Val}; mouse cells-tRNA^{Val}; hydrogen sulfide; chemical modification

Since 4-thiouridine was identified as a minor component of transfer ribonucleic acid (tRNA) from *Escherichia coli*,²⁾ many studies have been carried out to clarify the biological role of this nucleoside moiety. For example, intramolecular linking between the 4-thiouracil residue and a cytosine residue of tRNA^{Val} of *Escherichia coli* by photoirradiation gave information about the relation between the tertiary structure and the function of tRNA.^{3–5)} Specific modifications of the 4-thiouracil residue with various reagents have also been carried out.^{6,7)}

In relation to chemical and biophysical studies of 4-thiouracil residue in tRNAs, we have reported an excellent method for the conversion of cytosine nucleosides and nucleotides to their 4-thiouracil counterparts by means of sulphydrolysis.⁸⁾ This method has been successfully applied to the preparation of oligonucleotides containing 4-thiouracil residues from oligonucleotides containing cytosine residues.⁹⁾ Introduction of 4-thiouracil residues into polynucleotides, such as yeast RNA, yeast tRNA and heat-denatured calf thymus deoxyribonucleic acid (DNA) has also been achieved.¹⁰⁾ Moreover, the sulfur-isotope labeling of nucleic acids containing 4-thiouracil residues has been performed by treatment of the nucleic acids with cyanogen bromide followed by substitution with ³⁵S-Na₂S.¹⁾ This reaction should be useful not only for the introduction of 4-thiouracil residues but also for elucidation of the secondary and tertiary structures of nucleic acids, provided that the original ordered structure is not significantly altered in the reaction medium, which contains large amounts of liquid hydrogen sulfide and pyridine.

We report here the highly selective sulphydrolysis of tRNA^{Val} from mouse kidney cells. The results indicate that this reaction system is suitable for studies of higher-order structures of various nucleic acids.

Materials and Methods

Enzymes and Materials for Electrophoresis—RNases T₁, T₂ and U₂ were obtained from Sankyo Co., Ltd. Nuclease P₁ was obtained from Yamasa Shoyu Co., Ltd. RNase A was purchased from Sigma Chemical Co., Ltd. Cellulose acetate (Separax) was obtained from Fuji Film Co., Ltd. Procedures for electrophoresis and fingerprinting were as described in the previous reports.^{11–13)}

Preparation and Sequence Analysis of ³²P-tRNA^{Val}—The ³²P-labeled RNAs were prepared by phenol

extraction from mouse kidney cells (C3H2K) cultured in the presence of ^{32}P -phosphate for 24 h as described elsewhere¹³⁾ and the ^{32}P -tRNA^{Val} was isolated by two-dimensional gel electrophoresis (Fig. 1, an arrow denotes the spot of tRNA^{Val}). The product was further purified by gel electrophoresis on 16% polyacrylamide gel containing 7 M urea and was identified as tRNA^{Val} by the comparison of the fingerprints of RNases T₁ and A with those reported for tRNA^{Val} from mouse myeloma cells.¹⁴⁾ Further analysis of the sequence in detail was carried out by further digestion of oligonucleotides with appropriate RNases or nuclease followed by separation and identification of the hydrolysis products by two-dimensional cellulose thin layer chromatography (TLC).

Sulfhydryl analysis of tRNA^{Val}— ^{32}P -tRNA^{Val} (about 500000 cpm, containing 50 μg of yeast tRNA as a carrier) was dissolved in 1 ml of 20 mM HEPES-KOH buffer (pH 7.2)–0.15 M NaCl–10 mM MgCl₂ in a stainless steel container. The container was cooled to -70°C and a mixture of liquefied H₂S (4 ml) and pyridine (2 ml) was added at -70°C . The container was sealed and kept at 28 or 35°C for an appropriate time, then opened. The H₂S was evaporated off, and 4 M NaCl was added to the residual mixture to make 0.8 M NaCl solution, then 2.5 vol of ethanol was added and the suspension was kept overnight at -20°C . The resulting precipitate was collected by centrifugation and dissolved in water, and the precipitation–centrifugation cycle was repeated. The recovery of tRNA was 80–90%.

Determination of the Modification Sites—The modification sites in H₂S-treated tRNA^{Val} were determined by comparison of the fingerprints of RNases T₁ and A digests of untreated and treated tRNA^{Val} and by analyses of newly appeared oligonucleotides formed by hydrolysis of H₂S-treated tRNA^{Val} with RNases.

Fig. 1. Two-dimensional Polyacrylamide Gel Electrophoretic Pattern of Cytoplasmic RNAs of Mouse Kidney Cells

The arrow indicates tRNA^{Val}.

Results

The Nucleotide Sequence of tRNA^{Val} from Mouse Kidney Cells

The RNases A and T₁ fingerprints of tRNA^{Val} from mouse kidney cells are shown in Figs. 2a and 3a. The fingerprints were almost identical with those reported by Piper for tRNA^{Val} from mouse myeloma cells.¹⁴⁾ Further analysis of oligonucleotides on the fingerprints showed that the isolated tRNA was a mixture of two species differing at the wobble position of the anticodon, CAC and IAC, in an approximate ratio of 6: 4. Although it was reported¹⁴⁾ that the tRNA^{Val} from myeloma cells contained only IAC as the anticodon, the RNase T₁ fragment containing CAC anticodon (t-20, C–C–U–C–A–C–A–C–Gp) was also detected at the appropriate position on the fingerprint of Piper.¹⁴⁾ The other partial methylation and partial exchange of the bases at positions 4 (Um), 32 (Cm), 38 (m⁵C), and 43 (G) have been observed previously.¹⁴⁾ It should be noted that the methylation at positions 32 and 38 was observed only in the species having IAC anticodon. The total sequence of tRNA^{Val} from kidney cells was deduced from these results and is shown in Fig. 4.

Since the two species migrate very closely and could not be separated completely even by repeated gel electrophoresis, the mixture was used for the next step.

Sulfhydryl analysis of tRNA^{Val}

First, the sulfhydryl analysis of ^{32}P -tRNA^{Val} was performed at 28°C for 72 h. The presence of 4-thiouracil residues in H₂S-treated tRNA was confirmed by nuclease P₁ digestion followed by two-dimensional cellulose thin layer chromatography (Fig. 5, pS=4-thiouridylate). A fingerprint of the RNase A digest of H₂S-treated tRNA^{Val} is shown in Fig. 2b. Three new spots, As-1 to -3, were observed. As-1 was identified as 4-thiouridylate by direct comparison of the mobility with that of authentic 4-thiouridylate on the fingerprint. On the fingerprint

Fig. 2. RNase A Fingerprints of Untreated (a) and H_2S -treated (35°C) (b) tRNA^{Val}

The sequences of oligonucleotides are summarized in Table II.

Fig. 3. RNase T_1 Fingerprints of Untreated (a) and H_2S -treated (35°C) (b) tRNA^{Val}

The sequences of oligonucleotides are summarized in Table III.

of the RNase T_1 digest, four new spots of oligonucleotides (Ts-1, -3, -4, and -5) were observed.

When the temperature of sulfhydryllysis was raised to 35°C an additional spot (Ts-2) was observed on the RNase T_1 fingerprint (Fig. 3b).

Sequence Analysis of Oligonucleotides containing 4-Thiouracil Residues

Oligonucleotides produced by RNase A Digestion—Spot As-1 was identified as 4-thiouri-

formation from Sp. Similar inconsistencies have been encountered in other cases.

Oligonucleotide Ts-3 was assigned as A-S-A-C(m⁵C)-Gp or A-C-A-S-Gp derived from A-C-A-C(m⁵C)-Gp (t-6) from the results of RNases A and T₂ digestions. Although C-38 was partially replaced with m⁵C, its corresponding 4-thio derivative was not detected in the RNase T₂ digest. It is reasonable to expect that the displacement of the 4-amino function of m⁵Cp should be retarded by the presence of the 5-methyl group as compared to that of Cp.

Ts-4 was identified as a mixture of 3 species of monothiated t-20. Limited digestion of Ts-4 with RNase U₂ gave C-C-U-C-A>p and C-C-U-U-A>p, C-A>p and U-A>p, and C-Gp and U-Gp which means that cytosine at three positions was modified. The nucleotide composition of Ts-4 is consistent with this assumption. The sequence of Ts-5 was determined to be as shown in Table I, and one cytosine residue of Ts-4 is further modified. RNase U₂ digestion gave the same oligonucleotide fragments as in the case of Ts-4. Although the nucleotide compositions as determined by RNase T₂ digestion were not satisfactory, the direction of migration of the spots of t-20→Ts-4→Ts-5 on the fingerprint is reasonable if the exchange of C to S has occurred.

It is assumed that the first consecutive sequence of cytidylate in t-20 is unmodified, since the sequence of C-C-(Cm)-U-Ip (t-13, the RNase T₁ digest of other species having IAC anticodon) was found to be unmodified.

All nucleotide fragments produced by RNases A and T₁ digestions of tRNA^{Val} and H₂S-treated tRNA^{Val} are summarized in Table II and III.

The extent of conversion of cytosines at the anticodon region (positions 34, 36 and 38) was estimated as 30—35% and that of cytosines at the 3'-terminal was 50% at 35°C. The

TABLE I. Nucleotide Compositions determined by RNase T₂ Digestion, and Products of Further Digestion of RNase T₁ Fragments from H₂S-treated tRNA^{Val}

Spot No.	Products of further digestion with					Deduced sequence
	RNase T ₂	Composition ^{a)}	Nuclease P ₁	RNase A	RNase U ₂	
Ts-1	Ap	3.9(4)	pA	Cp		A-A-A-C-A-S-C-A _{OH}
	Cp	2.3(2)	pC	Up		
	Up	0.8(1)	pU	A-Cp		A-A-A-C-A-C-S-A _{OH}
				A-Up		
Ts-2	Ap	3.5(4)	pA	Up		A-A-A-C-A-S-S-A _{OH}
	Cp	2.1(1)	pC	A-Up		
	Up	1.3(2)	pU	A-A-A-Cp		
Ts-3	Ap	2.0(2)		Gp		A-S-A-C(m ⁵ C)-Gp
	Cp+m ⁵ Cp	1.3(1)		A-Cp(A-m ⁵ Cp)		
	Up	0.8(1)		A-Up		A-C-A-S-Gp
	Gp	0.9(1)				
Ts-4	Ap	2.1(2)	pA	Cp	C-A>p	C-C-U-S-A-C-A-C-Gp
	Cp	4.4(4)	pC	Up	U-A>p	
	Up	1.4(2)	pU	Gp	C-Gp	C-C-U-C-A-S-A-C-Gp
	Gp	1.0(1)	pG	A-Cp	U-Gp	C-C-U-C-A-C-A-S-Gp
			Pi	A-Up	C-C-U-C-A>p	
					C-C-U-U-A>p	
Ts-5	Ap	1.9(2)	pA	Cp	C-A>p	C-C-U-S-A-S-A-C-Gp
	Cp	3.7(3)	pC	Up	U-A>p	
	Up	2.4(3)	pU	Gp	C-Gp	
	Gp	1.0(1)	pG	A-Cp	U-Gp	C-C-U-S-A-C-A-S-Gp
			Pi	A-Up	C-C-U-C-A>p	C-C-U-C-A-S-A-S-Gp
					C-C-U-U-A>p	

a) The numbers in parenthesis show the molar ratios expected for the deduced sequence.

TABLE II. Oligonucleotides produced by RNase A Digestion of H₂S-treated (35°C) and Untreated tRNA^{Val}

Spot No.	Sequence	Molar ratio			
		H ₂ S-treated		Untreated	
		Experim.	Theor.	Experim.	Theor.
a-1	Up + Dp + Ψ p	4.0		5.2	
a-1'	U>p + D>p + Ψ >p	1.1	4-6	0.7	4-6
a-2	Cm-Up + Um-Cp	0.4		0.3	
a-2'	Cm-U>p + Um-C>p	0.2	0-2	0.2	0-2
a-3	Cp + m ⁵ Cp	9.5		12.7	
As-1	Sp	1.2	10-13	—	10-13
a-4	A-Cp + A-m ⁵ Cp	2.6		4.1	
As-2	A-Sp	0.8	3-4	—	3-4
a-6	G-Cp	1.8	2	2.0	2
a-7	A-Up	1.3	1	0.9	1
a-8	I-A-Cp	0.3		0.4	
As-3	I-A-Sp	0.15	0-1	—	0-1
a-9	G-m ¹ A-A-A-Cp	1.1	1	0.9	1
a-10	m ² G- Ψ p	1.1	1	1.1	1
a-11	G-Up + G- Ψ p	2.4	1+1	2.2	1+1
a-12	A-G-Up + A-G-Dp	2.6	1+1	2.0	1+1
a-13	G-G-A-A-A-Cp	0.8	1	0.9	1
a-14	G-G-G-Cp	0.4	1	0.5	1
a-15	G-G-Up + G-G-Dp	2.3	1+1	2.1	1+1
a-16	pG-Up	1.1	1	1.0	1
a-17	G-A-A-A-G-m ⁷ G-Dp	1.1	1	0.9	1

TABLE III. Oligonucleotides produced by RNase T₁ Digestion of H₂S-treated (35°C) and Untreated tRNA^{Val}

Spot No.	Sequence	Molar ratio			
		H ₂ S-treated		Untreated	
		Experim.	Theor.	Experim.	Theor.
t-1	A-A-A-C-A-C-C-A _{OH}	0.4		1.2	
Ts-1	A-A-A-C-A-S-C-A _{OH} + A-A-A-C-A-C-S-A _{OH}	0.6	1	—	1
Ts-2	A-A-A-C-A-S-S-A _{OH}	0.2		—	
t-2	Gp	2.8		5.0	
t-2'	G>p	2.3	5	0.3	5
t-3	C-Gp	2.0	2	2.4	2
t-4	A-Gp	0.1	0-1	0.1	0-1
t-5	m ¹ A-A-A-C-C-Gp	0.8	1	0.8	1
t-6	A-C-A-C(m ⁵ C)-Gp	—		0.4	
Ts-3	A-S-A-C(m ⁵ C)-Gp + A-C-A-S-Gp	0.2	0-1	—	0-1
t-7	A-A-A-Gp	1.0	0-1	0.6	0-1
t-8	pGp	0.6		0.9	
t-8'	pG>p	0.5	1	—	1
t-9, 10	D-Gp + U-Gp	1.8	1+1	2.1	1+1
t-11	U-A-Gp + Ψ -A-Gp	2.1	1+1	1.8	1+1
t-13, 13'	C-C(Cm)-U-Ip + C-C(Cm)-U-I>p	0.3	0-1	0.4	0-1
t-14	m ⁷ G-D-m ⁵ C-m ⁵ C-C-C-Gp	0.9	1	0.9	1
t-15	U- Ψ -C-Gp + Ψ -U-C-Gp	1.8		2.1	
t-15'	U- Ψ -C-G>p + Ψ -U-C-G>p	0.1	1+1	—	1+1
t-16	U-U-U(Um)-C-C-Gp	1.0	1	1.0	1
t-17, 17'	D-D-A-U-C-A-C-m ² Gp + D-D-A-U-C-A-C-m ² G>p	0.8	1	1.0	1
t-20	C-C-U-C-A-C-A-C-Gp	0.2		0.6	
Ts-4	C-C-U-S-A-C-A-C-Gp + C-C-U-C-A-C-A-S-Gp	0.2	0-1	—	0-1
Ts-5	C-C-U-S-A-S-A-C-Gp + C-C-U-C-A-S-A-S-Gp	0.2		—	

sites of sulphydrolysis of tRNA^{val} are shown in Fig. 4, where S→denotes the conversion to 4-thiouridine.

Discussion

We have developed an excellent method for the introduction of 4-thiouracil residues into RNA and DNA by means of conversion of cytosine residues with liquid hydrogen sulfide.¹⁰⁾ In this paper the effect of this system on the higher-order structure of RNA was studied. tRNA^{val} was selected as the substrate for sulphydrolysis, as cytosine residues are present in the anticodon region and the tertiary structure of the tRNA is well elucidated.

The tRNA^{val} was purified from mouse kidney cells and was found to be a mixture of species containing CAC and IAC as the anticodon. A similar result regarding the anticodon of mammalian tRNA^{val} was found in the species from human placenta.¹⁵⁾

The sulphydrolysis was carried out at 28°C and 35°C to see whether any change in the mode of modification occurred. In a previous study¹⁰⁾ the sulphydrolysis of unfractionated tRNA was carried out at 40°C for 12 h. The conversion of about 3 cytosine residues, on average, was observed.

Analysis of the site of modification was performed by preparing fingerprints with RNases and by further sequence determination of the separated oligonucleotides. The sequences of new spots of RNase A digest of H₂S-treated tRNA^{val} (As-1, -2 and -3) were determined as Sp, A-Sp and I-A-Sp, respectively (Fig. 2, Table II). Oligonucleotides from the RNase T₁ digest (Ts-1 to -5) were identified as modified 3'-terminal oligonucleotides (Ts-1 and -2) and three oligonucleotides were derived from the anticodon regions of the species of tRNA^{val} containing CAC and IAC as the anticodon (Ts-3, -4 and -5, Fig. 3, Table III).

From the results of sulphydrolysis of tRNA^{val}, the following conclusions can be reached.

1) The cytosine moieties at the anticodon loop and the CCA end were converted to appreciable extents. No other cytosine moiety, including one in the common GTΨC region, was modified.

2) This means that the secondary and tertiary structures of tRNA are maintained in the reaction medium composed of liquid H₂S, pyridine and H₂O in a ratio of 4: 2: 1 by volume. The sulphydrolysis of tRNA^{Pro} from mouse cells,¹⁶⁾ which has no cytosine moiety in the anticodon loop, has also been performed and in this case no change of cytosines other than those of the CCA end was observed (data not shown).

As reagents for the modification of cytosine residues of tRNAs, aqueous methoxyamine¹⁷⁾ and aqueous sodium bisulfite¹⁸⁾ are known. The characteristic feature of the sulphydrolysis may be that the 4-thiouracil residue thus introduced can be further converted to other pyrimidine moieties and can undergo various other transformations, such as photodynamic coupling.³⁻⁵⁾

In conclusion, the present system for the sulphydrolysis of the cytosine moiety can be useful for studies of the higher-order structures of various RNAs and possibly DNAs. Modification studies of ribosomal 5S and 5.8S RNA from mouse cells are currently being undertaken and the results will be reported separately.¹⁹⁾

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

References

- 1) Part XXXIX: K. Miura, S. Sato, K. Takagi, J. Tohyama, and T. Ueda, *Chem. Pharm. Bull.*, **30**, 1069 (1982).
- 2) M.N. Lipsett and B.P. Doctor, *J. Biol. Chem.*, **242**, 4072 (1967).
- 3) A. Favre, A.M. Michelson, and M. Yaniv, *J. Mol. Biol.*, **58**, 367 (1971).

- 4) M. Yaniv, A. Chestier, F. Gros, and A. Favre, *J. Mol. Biol.*, **58**, 381 (1971).
- 5) M. Krauskopf, C.-M. Chen, and J. Ofengand, *J. Biol. Chem.*, **247**, 842 (1972).
- 6) L. Shugart, *Arch. Biochem. Biophys.*, **148**, 488 (1972).
- 7) F. von der Haar, E. Schlimme, and D.H. Gauss, "Procedures in Nucleic Acid Research," Vol. 2, ed. by G.L. Cantoni and D.R. Davies, Harper and Row Publishers, New York, 1971, pp. 643—664.
- 8) a) T. Ueda, M. Imazawa, K. Miura, R. Iwata, and K. Odajima, *Tetrahedron Lett.*, **1971**, 2507; b) T. Ueda, K. Miura, M. Imazawa, and K. Odajima, *Chem. Pharm. Bull.*, **22**, 2377 (1974).
- 9) K. Miura, M. Shiga, and T. Ueda, *J. Biochem. (Tokyo)*, **73**, 1279 (1973).
- 10) K. Miura and T. Ueda, *Chem. Pharm. Bull.*, **28**, 3415 (1980).
- 11) F. Harada and Y. Ikawa, *Nucleic Acids Res.*, **7**, 895 (1979).
- 12) F. Sanger, G.G. Brownlee, and B.G. Barrell, *J. Mol. Biol.*, **13**, 373 (1965).
- 13) F. Harada and N. Kato, *Nucleic Acids Res.*, **8**, 1273 (1980).
- 14) P.W. Piper, *Eur. J. Biochem.*, **51**, 295 (1975).
- 15) E.Y. Chen and B.A. Roe, *Biochem. Biophys. Res. Commun.*, **78**, 631 (1977).
- 16) F. Harada, G.G. Peters, and J.E. Dahlberg, *J. Biol. Chem.*, **254**, 10979 (1979).
- 17) T.I. Jilyaeva and L.L. Kisselev, *FEBS Lett.*, **10**, 229 (1970).
- 18) R.W. Chambers, S. Aoyagi, Y. Furukawa, H. Zawadzka, and S. Bhanot, *J. Biol. Chem.*, **248**, 5549 (1973).
- 19) A Preliminary report has appeared: K. Miura, T. Iwano, T. Ueda, N. Kato, and F. Harada, *Seikagaku*, **52**, 661 (1980).