REPLACEMENT OF RIBOTHYMIDINE BY 5-METHYL-2-THIOURIDINE IN SEQUENCE GTψC IN tRNA OF AN EXTREME THERMOPHILE

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1. Introduction

Transfer RNA from extreme thermophilic bacteria is useful material for studies on the mechanism of protein biosynthesis at high temperature and the structure–function relation of tRNA. There have been some reports on these problems [1–6], but the exact nature of the tRNA of thermophiles is not fully understood. In this work we used tRNA from an extreme thermophilic bacterium *Thermus thermophilus* HB 8, isolated from a hot spring. This bacterium was formerly named *Flavobacterium thermophilum* [7–9], and it can even grow at 85°C. The tRNA was found to contain 5-methyl-2-thiouridine (m^5s^2U) in the sequence G–m^5s^2U–ψ–C–G. These findings strongly suggest that most of the ribothymidine normally present in the GTψC region is replaced by m^5s^2U in tRNA of this thermophile. The m^5s^2U is probably important for the capacity of the tRNA to synthesize protein at high temperature.

2. Materials and methods

2.1. *Materials*

Thermophile tRNA was prepared from *Thermus thermophilus* HB 8 by Zubay's method [10] and further purified as described in the literature [11]. RNase T_2 was purchased from Sankyo Co. (Tokyo). *E. coli* alkaline phosphomonoesterase and snake venom phosphodiesterase were obtained from Worthington Biochemical Co. 2-Thiouridine was synthesized as described by Brown et al. [12]. Authentic 5-methyl-2-thiouridine was prepared from 2′, 3′, 5′-tri-*O*-benzoyl-5-methyl-2-thiouridine which was kindly provided from Dr. M. Sano, Daiichi Pharm. Ltd. by alkaline debenzoylation and purified further by paper chromatography. Synthetic 2-thiouridine-5-acetic acid methyl ester was a gift from Dr. R. H. Hall of McMaster University. Thin-layer glass plates (10 cm × 10 cm) coated with Avicel SF cellulose were products of Funakoshi Pharmaceutical Co. (Tokyo).

2.2. *Paper and thin-layer chromatography and electrophoresis*

The following solvent systems were used for paper and thin-layer chromatography: Solvent system I, isobutyric acid–0.5 M NH_4OH, pH 4.3 (5:3, v/v); II, isopropanol–conc. HCl–H_2O (70:15:15, v/v/v); III, 95% ethanol–1 M NH_4OAC, pH 7.5 (1:1, v/v); IV, butanol–acetic acid–H_2O (5:3:2, v/v/v); V, isopropanol–conc. HCl–H_2O (7:1:2, v/v/v); VI, 95% ethanol–H_2O (4:1, v/v).

Thin-layer electrophoresis was carried out using a glass plate coated with Avicel SF cellulose in 0.05 M triethylammonium bicarbonate buffer, pH 8.0 at 25 V/cm for 15 min.

2.3. *Isolation and characterization of m^5s^2U*

To see whether the thermophile tRNA contained any new modified components, 15 A_{260} units of dialyzed tRNA were extensively hydrolyzed with RNase T_2, and subjected to two-dimensional paper chromatography (Whatman No. 1, 40 cm × 40 cm) using solvent systems I and II, as described previously [13]. For relatively large scale isolation of the new modified nucleoside, hereafter designated as S, RNase T_2 digests of the tRNA (80 A_{260} units) were subjected to two-dimensional paper chromatography (Whatman No. 3 MM, 45 cm × 45 cm) using two sheets of paper. The superimposed spot containing Sp, Tp and 4-thiouridine-3′-phosphate was cut out and eluted with water. The sample was desalted by thin-layer chromatography using solvent system III and then treated with *E. coli* alkaline phosphomonoesterase and separated by thin-layer chromatography using solvent system I. The spot containing S was eluted with water. Desulfurization of S and related compounds and identification of S were carried out essentially as reported previously [14].

2.4. *Isolation of a tetranucleotide containing m^5s^2U and its sequence determination*

Complete RNase T_1 digest of unfractionated thermophile tRNA (4000 A_{260} units) were subjected to DEAE-Sephadex A-25 column chromatography in 7 M urea–0.02 M Tris–HCl, (pH 7.5 with a linear gradient of 0.14 M to 0.7 M NaCl, as described previously [15]. The peak eluted between the peaks of tetranucleotide and pentanucleotide was collected. Digestion of an aliquot with RNase T_2 showed that it contained Sp and ψp. This peak was further purified by DEAE-Sephadex A-25 column chromatography under acidic conditions [16]. The oligonucleotide eluted as the last peak contained approximately equimolar amounts of Sp, ψp, Cp and Gp. This oligonucleotide (60 A_{260} units) was dephosphorylated by treatment with phosphomonoesterase, and purified further by descending paper chromatography using solvent system I. Sequence analysis of the nucleotide was done essentially as described in the literature [15].

3. Results

3.1 *Isolation and identification of S*

The new minor nucleotide, Sp was located in almost the same position as Tp on the two-dimensional paper chromatogram. Based on previous results reported by Kimura-Harada et al. [14], this suggested that Sp may be m^5s^2Up or derivative of it.

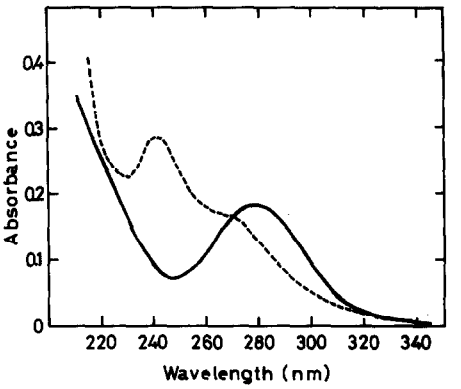

Fig. 1. Ultraviolet absorption spectra of S. pH 2.0 and pH 7.0 (———); pH 12.0 (– – – – –).

Fig. 1 shows the UV absorption spectra of S, isolated as described in Materials and methods. The spectra were identical in all respects with those of synthetic m^5s^2U [14], at three different pH values, indicating that S is 5-methyl-2-thiouridine or a derivative of it. Table 1 shows the R_f values of S on thin-layer chromatography and its relative electrophoretic mobility, in comparison with those of m^5s^2U and related compounds. The mobilities of S were the same as those of synthetic m^5s^2U in all systems tested, but different from those of 2-thiouridine and 2-thiouridine-5-acetic acid methyl ester. When S was desulfurized by the method described in the literature [14], the product behaved identically with ribothymidine on thin-layer chromatography with several solvent systems and on electrophoresis, as shown in table 2. In addition, the UV absorption spectrum of desulfurized S was the same as that of ribothymidine (data not shown). From these results, the new modified nucleoside S was identified unequivocally as 5-methyl-2-thiouridine.

3.2. *Presence of the tetranucleotide, SpψCpGp in the RNase T_1 digest of the thermophile tRNA*

The RNase T_1 digest of the thermophile tRNA was fractionated by DEAE-Sephadex A-25 column chromatography as described in the Materials and methods. Each peak was desalted, digested with RNase T_2, and subjected to two-dimensional thin-layer chromatogra-

Table 1
Relative chromatographic mobilities and electrophoretic mobilities of S, 5-methyl-2-thiouridine and related compounds

	Thin-layer chromatography R_f in solvent system			Electrophoresis R_2', (3') UMP
	I	IV	V	
Nucleoside S	0.60	0.57	0.50	0.18
5-Methyl-2-thiouridine	0.60	0.57	0.50	0.18
2-Thiouridine-5-acetic acid methyl ester	0.64	0.64	0.55	0.32
2-Thiouridine	0.55	0.50	0.43	0.40
Ribothymidine	0.59	0.54	0.57	–0.12
Uridine	0.54	0.43	0.43	0

Table 2
Relative chromatographic and electrophoretic mobilities of desulfurized nucleosides, ribothymidine and related compounds

	Thin-layer chromatography R_f in solvent system VI	Electrophoresis R_2', (3')UMP
Desulfurized S	0.61	0
Ribothymidine	0.61	0
Desulfurized 5-methyl-2-thiouridine	0.61	0
Desulfurized 2-thiouridine 5-acetic acid methyl ester	0.39	0.54
5-methyl-2-thiouridine	0.65	0.18
2-thiouridine 5-acetic acid methyl ester	0.72	0.31
Uridine	0.53	0.05

phy to detect the modified nucleoside S. It was found that only the peak located between those of tetra- and pentanucleotide contained S. The oligonucleotide containing S was further purified as described in Materials and methods. The nucleotide composition of the RNase T_2 digest of the purified tetranucleotide was found to be Sp, ψp, Cp and G in the ratio, 0.97:0.92:1.04:1.00. Complete hydrolysis of the oligonucleotide by snake venom phosphodiesterase yielded S as the sole nucleoside, indicating that S is located at the 5'-OH end. The oligonucleotide ($2\,A_{260}$ units) was partially hydrolyzed by snake venom phosphodiesterase, and the digest was subjected to two-dimensional thin-layer chromatography as described previously [15]. The RNase T_2 digest of the dinucleotide, thus isolated contained Sp and ψ in the ratio of 1.2:1.0. From these results, the sequence of the tetranucleotide was concluded to be S–ψ–C–Gp.

4. Discussion

The modified nucleoside isolated from the thermophile tRNA was characterized unequivocally as 5-methyl-2-thiouridine (m^5s^2U). It was found that m^5s^2U was present in the sequence, G–m^5s^2U–ψ–C-G, in the tRNA. This strongly suggests that m^5s^2U is located in the GTψC-region of the thermophile tRNA, where it replaces ribothymidine. This possibility is supported by the following evidence. When

purified thermophile $tRNA_{f1}^{Met}$ was cleaved chemically at the site of 7-methylguanosine, by the procedure of Wintermeyer and Zachau [17], the quarter of the molecule derived from the CCA-end contained one mole each of $m^5 s^2 U$ and pseudouridine, but no ribothymidine (K. Watanabe and S. Nishimura, unpublished results).

Analysis of the nucleotide composition of unfractionated thermophile tRNA by two-dimensional paper chromatography indicated the presence of ribothymidine also in approximately two thirds the amount of $m^5 s^2 U$. However, no ribothymidine was detected in the tetranucleotide fraction of the RNase T_1 digestion of the tRNA. This suggested that ribothymidine is not present in G–T–ψ–C–G but in another sequence.

So far, three 2-thiouridine derivatives have been found in tRNAs. Namely 5-methylaminomethyl-2-thiouridine, 2-thiouridine-5-acetic acid methyl ester and $m^5 s^2 U$ have been isolated from the tRNAs of *E. coli*, yeast and rat liver, respectively. However, unlike $m^5 s^2 U$ in the thermophile tRNA, these all seem to be located in the first position of the anticodon [14,18,19]. The presence of a 2-thiouridine derivative has been suggested to a specific interaction with A in the codon sequence [18,20].

Several other cases of replacement of G–T–ψ–C sequence by another sequence have recently been reported. G–T–ψ–C is replaced by G–A–U–C in initiator tRNAs in several eukaryotic cells [21-25]. Wheat germ $tRNA^{Gly}$ and $tRNA^{Thr}$ contain G–U–ψ–C instead of G–T–ψ–C [26]. Rabbit liver $tRNA^{Lys}$ contains 2′–O–methyl ribothymidine in place of ribothymidine [27]. Recently, the specific interaction of the T–ψ–C–G sequence in tRNA with ribosomes, possibly through 5S RNA, has been demonstrated [28,29]. Thus it is very likely that $m^5 s^2 U$ is necessary instead of ribothymidine in the majority of thermophile tRNA for the interaction of the GTψC region with 5S RNA of 50S ribosomes at high temperature. Studies on this possibility are now in progress.

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References

[1] Arcà, M., Frontali, L., Sapora, O. and Tecce, G. (1967) Biochim. Biophys. Acta, 145, 284.
[2] Johnson L. and Söll, D. (1971) Biopolymers 10, 2209.
[3] Zeikus, J. G. and Brock, T. D. (1971) Biochim. Biophys. Acta 228, 736.
[4] Kay, A. C. and Grunberg-Manago, M. (1972) Biochimie 54, 1281.
[5] Agris, P. E., Koh, H. and Söll, D. (1973) Arch. Biochem. Biophys. 154, 277.
[6] Ohno-Iwashita, Y., Oshima, T. and Imahori, K., submitted to Biochem. Biophys. Res. Commun.
[7] Oshima, T. and Imahori, K. (1971) J. Gen. Appl. Microbiol. 17, 513.
[8] Oshima, T. (1972) Molecular Evolution: Prebiological and Biological (Rohlfing, D. L. and Oparin, A. I., eds), pp. 399, Plenum Publishing Co., New York.
[9] Oshima, T. and Imahori, K. (1974) Intern. J. Syst. Bacteriol. in press.
[10] Zubay, G. (1962) J. Mol. Biol. 4, 347.
[11] Von Ehrenstein, G. and Lipmann, F. (1961) Proc. Natl. Acad. Sci. U.S. 47, 941.
[12] Brown, D. M., Parihar, D. B., Todd, S. A. and Varadarajan, S. (1958) J. Chem. Soc. 3028.
[13] Nishimura, S., Harada, F., Narushima, U. and Seno, T. (1967) Biochim. Biophys. Acta 142, 133.
[14] Kimura-Harada, F., Saneyoshi, M. and Nishimura, S. (1971) FEBS Letters 13, 335.
[15] Harada, F., Kimura, F. and Nishimura, S. (1971) Biochemistry 10, 3269.
[16] Kimura, F., Harada, F. and Nishimura, S. (1971) Biochemistry 10, 3277.
[17] Wintermeyer, W. and Zachau, H. G. (1970) FEBS Letters 11, 160.
[18] Yoshida, M., Takeishi, K. and Ukita, T. (1970) Biochem. Biophys. Res. Commun. 39, 852.
[19] Ohashi, Z., Saneyoshi, M., Harada, F., Hara, H. and Nishimura, S. (1970) Biochem. Biophys. Res. Commun 40, 866.
[20] Nishimura, S. (1972) in: Progress in Nucleic Acid Research and Molecular Biology (Davidson, J. N. and Cohn, W. E., eds), Vol. 12, pp. 49, Academic Press, New York and London.
[21] Simsek, M., Petrissant, G. and RajBhandary, U. L. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2600.
[22] Simsek M. and RajBhandary, U. L. (1972) Biochem. Biophys. Res. Commun. 49, 508.
[23] Petrissant, G. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1046.
[24] Simsek, M., Ziegenmeyer, J., Heckman, J. and RajBhandary, U. L. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1041.

[25] Piper, P. W. and Clark, B. F. C. (1973) FEBS Letters 30, 265.
[26] Marcu, K., Mignery, R., Reszelbach, R., Roe, B., Sirover, M. and Dudock, B. (1973) Biochem. Biophys. Res. Commun. 55, 477.
[27] Gross, H. J., Simsek, M., Raba, M., Limburg, K. Heckman, J. and RajBhandary, U. L. (1974) Nucl. Acids Res. 1, 35.
[28] Erdman, V. A., Sprinzl, M. and Pongs, O. (1973) Biochem. Biophys. Res. Commun. 54, 942.
[29] Richter, D., Erdmann, D. and Sprinzl, M. (1973) Nature New. Biol. 246, 132.