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Reaction of Bisulfite with the 5-Hydroxymethyl Group in Pyrimidines and in Phage DNAs†

Hikoya Hayatsu* and Makoto Shiragami

ABSTRACT: 5-Hydroxymethylcytosine reacted with bisulfite and, instead of undergoing usual deamination process, gave cytosine 5-methylenesulfonate as the product. The conversion was rapid and quantitative, and the optimum pH was 4.5. The product was isolated as crystals and characterized. Cytosine 5-methylenesulfonate was only very slowly deaminated by treatment with bisulfite. 5-Hydroxymethyl-2′-deoxycytidine 5′-phosphate reacted with bisulfite in the same way as 5-hydroxymethylcytosine. Residues of 5-hydroxymethylcytosine in native as well as denatured T2 DNA were convertible to those of cytosine 5-methylenesulfonate by treatment of the DNA with bisulfite. While it is known that the 5-hydroxymethyl groups of T-even bacteriophage DNA can be enzymatically glucosylated, this observation offers chemical evidence that the 5-hydroxymethyl groups in DNA are situated in such a way that they can readily react with external agents. 5-Hydroxymethyluracil gave uracil 5-methylenesulfonate on treatment with bisulfite. This reaction was much slower than that of 5-hydroxymethylcytosine, and the optimum pH was between 6 and 7.

Bisulfite adds reversibly to the 5,6 double bond of cytosine and uracil, and the cytosine–bisulfite adduct undergoes easy deamination to give the uracil–bisulfite adduct. The bisulfite modification of these pyrimidine bases has been widely used in nucleic acid research (Hayatsu, 1976; Shapiro, 1977).

There are many naturally occurring, as well as synthetic, 5-substituted pyrimidine nucleoside derivatives, and they are subjects of current, chemical and biochemical studies (Bradshaw & Hutchinson, 1977). We have been investigating the reactivities of 5-substituted pyrimidine nucleosides to bisulfite (Shiragami et al., 1975; Hayatsu et al., 1975) and have now encountered an abnormal case for 5-hydroxymethylcytosine (hm^5Cyt)[1] and 5-hydroxymethyluracil

† From the Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan. *Received September 11, 1978.* This work was supported by a grant from Ministry of Education and a grant from Nissan Science Foundation. A preliminary account of part of this paper has been published [(1976) *Nucleic Acid Res., Spec. Publ.*, s31–s32].

* Present address: Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan.

0006-2960/79/0418-0632$01.00/0

(hm^{5}Ura). We wish to report the case in this paper. hm^{5}Cyt and its glucosylated derivatives replace cytosine in the DNA of T-even bacteriophages, and hm^{5}Ura replaces thymine in the DNA of a group of *Bacillus subtilis* bacteriophages (Mandel, 1975). Antibiotic mildiomycin contains an hm^{5}Cyt group in its structure (Harada et al., 1978) and several of the antibiotic polyoxins contain hm^{5}Ura groups (Townsend, 1975).

Experimental Procedures

Materials. hm^{5}Cyt was a product of Sigma, St. Louis, MO. hm^{5}dCMP was synthesized from 2′-deoxycytidine 5′-phosphate by the method of Alegria (1967). hm^{5}Ura was prepared according to Cline et al. (1959). Bacteriophages T2H and T4B were kindly supplied by Dr. M. Honda of the Institute of Medical Sciences, University of Tokyo, and the DNAs of these phages were prepared by the method of Thomas & Abelson (1966). Nuclease P1 was obtained from Yamasa Shoyu, Tokyo.

General Methods. Solvents used for paper chromatography were (1) isobutyric acid–0.5 M ammonium hydroxide (10:6); (2) 1-butanol–acetic acid–water (2:1:1); (3) 1-propanol–concentrated ammonium hydroxide–water (6:3:1); and (4) ethanol–1 M ammonium acetate, pH 7.5 (7:3). Buffer used for paper electrophoresis was 0.03 M sodium phosphate, pH 7.0. Ultraviolet absorption spectra were recorded on a Beckman Acta CIII spectrophotometer. Chemical shifts in NMR spectra were determined using 3-(trimethylsilyl)-propanesulfonic acid as an internal standard.

Preparation of Cytosine 5-Methylenesulfonate ($CytCH_2SO_3^-$). To a solution of hm^{5}Cyt (312 mg) in water (40 mL) was added sodium bisulfite (1.2 g), and the solution, of which the pH was 5.3, was incubated at 37 °C for 70 min. The solution was cooled in ice and was made to pH 3 by addition of hydrochloric acid. Colorless crystals that precipitated were collected (385 mg; 94%) and were purified by recrystallization from water. Anal. Calcd for $C_5H_7N_3O_4S{\cdot}0.25H_2O$ (zwitterionic form): C, 28.64; H, 3.58; N, 20.05; S, 15.27. Found: C, 28.51; H, 3.67; N, 20.07; S, 15.30. UV: at pH 1 (0.1 N HCl), λ_{max} 285 nm (ϵ 9300), λ_{min} 243 nm (ϵ 900); at pH 7 (0.05 M sodium phosphate), λ_{max} 274 nm (ϵ 5700), λ_{min} 252 nm (ϵ 3400). NMR in NaOD ($pD > 13$): 5-CH_2, 3.92 ppm (s), 6-*H*, 7.66 ppm (s). Ir (KBr): ν_{C-S} 756 cm^{-1}. The pK_a determined spectrophotometrically from the absorbances at 290 nm at various pH values was 4.3.

Rates of the Reaction between Bisulfite and hm^{5}Cyt (and hm^{5}dCMP). Reaction mixtures containing sodium bisulfite and 0.01 M hm^{5}Cyt were incubated at 20 °C in a thermostated water bath. Portions of the mixtures were taken at desired periods and diluted 100-fold with 0.05 M sodium phosphate buffer, pH 7.0. Rise in the absorbance at 288 nm was the measure of the reaction extent: the zero-time absorbance (A_i) was about 0.18 and the final absorbance (A_f) was about 0.33, when the reaction came to a completion. The value, $(A_f - A_t)/(A_f - A_i)$, represents the fraction of hm^{5}Cyt remaining (A_t represents absorbance at time t) and the plots of log $(A_f - A_t)/(A_f - A_i)$ against time t gave straight lines from which the pseudo-first-order rate constants were determined by dividing 0.693 with the time of 50% reaction. The pH values of the reaction mixtures were measured both immediately after the start of the reactions and when the incubations were finished, and they were generally unchanged. In experiments where effect of bisulfite concentration was examined, ionic strength of the reaction mixtures was adjusted to a fixed value by addition of sodium chloride.

Table I: Chromatographic and Electrophoretic Behavior of Compounds

compd	R_f value in solvent[a] 1	2	3	4	mobility in electrophoresis[b] (cm)
hm^{5}Cyt	0.68	0.49	0.56	0.57	0
$CytCH_2SO_3^-$	0.39	0.24	0.47	0.33	4.2
hm^{5}Ura	0.54	0.47	0.56	0.70	
$UraCH_2SO_3^-$	0.16	0.20	0.44	0.53	
hm^{5}dCMP	0.47	0.25			4.2
dCMP-$CH_2SO_3^-$	0.26	0.17			5.2
dAMP	0.60	0.29			
dTMP	0.43	0.36			
dGMP	0.36	0.22			
Glc-hm^{5}dCMP	0.36	0.15			
UMP					4.2

[a] Solvents used were (1) isobutyric acid–0.5 M ammonium hydroxide (10:6); (2) 1-butanol–acetic acid–water (2:1:1); (3) 1-propanol–concentrated ammonia–water (6:3:1); and (4) ethanol–1 M ammonium acetate, pH 7.5 (7:3). [b] Paper electrophoresis was run in 0.03 M sodium phosphate, pH 7, at 200 V/27 cm for 90 min.

The reactions of hm^{5}dCMP were similarly carried out at 37 °C and the reaction extents were determined by use of the absorbance at 288 nm.

Bisulfite Modification of DNA from Bacteriophage T2. An aqueous solution of T2 DNA (40 A_{260nm} units/mL of heat-denatured DNA, or 11 A_{260} units/mL of native DNA) was mixed with an equal volume of 2 M sodium bisulfite, pH 5, and the mixture (final pH 5.2; total volume, 1 mL for denatured DNA, and 1.6 mL for native DNA) was allowed to stand at 37 °C under nitrogen atmosphere. After incubating for a desired period, the pH of the mixture was checked (which was generally the same as the initial value), and the mixture was adjusted to pH 8 by addition of 5 N NaOH. The mixture was dialyzed against 0.1 M sodium phosphate buffer, pH 8, and then against water. The solution was evaporated to dryness under reduced pressure to give the DNA as the residue.

To examine the reactivity of DNA inside the phage particles, T2 phage was treated with bisulfite and the reaction was terminated by adjusting the pH to 8. The T2 phages were collected by centrifugation and washed with a phosphate buffer, and then DNA was extracted.

For determination of the base composition, the DNA thus obtained was dissolved in 0.01 M acetate buffer, pH 5, and digested with nuclease P1 (0.1 mg) by incubating at 50 °C for 4 h. The digest was subjected to paper chromatography using solvent 1. In this chromatographic system, five zones corresponding to dAMP, hm^{5}dCMP, dTMP, dGMP + Glc-hm^{5}dCMP, and dCMP-$CH_2SO_3^-$ were obtained (see Table I for R_f values). The 5′-mononucleotides on the chromatogram were eluted with water, the eluate was acidified to pH 1 with hydrochloric acid, and the nucleotides were quantitated spectrophotometrically. In this determination, blank solutions, prepared by soaking appropriate zones of a chromatogram on which no nucleotide had been applied, were used. Molar extinction coefficients employed for this determination were 14 200 at 260 nm for dAMP, 12 900 at 280 nm for hm^{5}dCMP (Lehman & Pratt, 1960), 9650 at 267 nm for dTMP, and 12 300 at 286 nm for dCMP-$CH_2SO_3^-$. The

[1] Abbreviations used: hm^{5}Cyt, 5-hydroxymethylcytosine; hm^{5}dCMP, 5-hydroxymethyl-2′-deoxycytidine 5′-phosphate; hm^{5}Ura, 5-hydroxymethyluracil; $CytCH_2SO_3^-$, cytosine 5-methylenesulfonate; dCMP-$CH_2SO_3^-$, deoxycytidine 5′-phosphate 5-methylenesulfonate; Glc-hm^{5}dCMP, 5(glucosyloxymethyl)deoxycytidine 5′-phosphate; $UraCH_2SO_3^-$, uracil 5-methylenesulfonate.

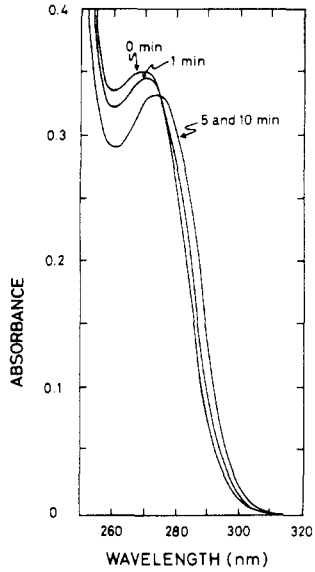

FIGURE 1: Change in ultraviolet absorption spectrum of 5-hydroxymethylcytosine as a function of time of treatment with bisulfite. A solution consisting of 0.005 M 5-hydroxymethylcytosine and 1 M sodium bisulfite, pH 5.0, was incubated at 37 °C. Aliquots were withdrawn and diluted 100-fold with 0.05 M sodium phosphate buffer, pH 7.0, and the spectra of the resulting solutions were determined. No further change in the spectra was observed on standing the diluted solutions for 30 min at room temperature.

amounts of dGMP and Glc-hm^{5}dCMP were determined by calculating from the absorbances at 260 nm and 280 nm of the mixture of these two nucleotides. Molar extinction coefficients used in this calculation were 11 800 (at 260 nm) and 8200 (at 280 nm) for dGMP, and 5000 (at 260 nm) and 12 900 (at 280 nm) for Glc-hm^{5}dCMP (Lehman & Pratt, 1960). Diglucosylated hm^{5}dCMP, which should be present in the T2 DNA at an amount of 1% of total nucleotide (Lehman & Pratt, 1960), was not detectable in this chromatographic system.

For fractionation of nuclease-P1 digest of T4 DNA, solvent 2 was used in paper chromatography (see Table I).

Preparation of Sodium Uracil 5-Methylenesulfonate (*UraCH$_2$SO$_3^-$*). To a solution of hm^{5}Ura (200 mg) in water (10 mL) was added a mixture of sodium sulfite (1.5 g) and sodium bisulfite (0.3 g). The solution, of which the pH was 7.2, was allowed to stand at 37 °C for 45 h and then chilled in a refrigerator for several days. Colorless crystalline material that precipitated was collected (71 mg; 21%) and purified by recrystallization from water–ethanol. Anal. Calcd for $C_5H_5N_2O_5SNa\cdot0.5H_2O$: C, 25.32; H, 2.55; N, 11.81; S, 13.51. Found: C, 25.70; H, 2.56; N, 11.99; S, 13.00. UV: at pH 7 (0.05 M sodium phosphate), λ_{max} 266 nm (ϵ 8200), λ_{min} 235 nm (ϵ 1800); at pH 13 (0.1 N NaOH), λ_{max} 290.5 nm (ϵ 8700), λ_{min} 247.5 nm (ϵ 1800). NMR in D_2O: 5-*CH*$_2$, 4.12 ppm (s), 6-*H*, 7.83 ppm (s).

Time Course of Bisulfite Reaction with hm^{5}Ura. A solution (2.5 mL) containing 0.01 M hm^{5}Ura and 1.6 M sodium bisulfite was incubated at 37 °C. At desired periods, aliquots (50 μL each) were taken and diluted with 0.3 M Tris-HCl buffer (pH 9.0; 2.5 mL). After the diluted solution was allowed to stand for 1 h at room temperature to regenerate hm^{5}Ura from 5,6-dihydro-5-hydroxymethyluracil 6-sulfonate that was formed, the solution was adjusted to pH 7 with 3 M sodium acetate buffer (pH 3; ca. 0.2 mL) and A_{285nm} value was recorded. The rise in A_{285nm} reflects the formation of UraCH$_2$SO$_3^-$; $(A_f - A_t)/(A_f - A_i)$ represents the fraction of hm^{5}Ura remaining, A_f (0.6) being the absorbance at the completion of the reaction, A_t that at time t, and A_i (0.3) that at the start of the reaction. Plots of log $(A_f - A_t)/(A_f - A_i)$ against time gave straight lines from which the pseudo-first-order rate constants were obtained.

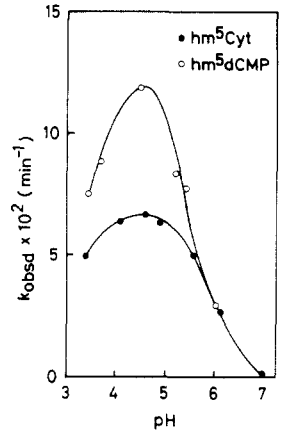

FIGURE 2: The pH–rate profiles for the conversion of 5-hydroxymethylcytosine and its nucleotide into cytosine 5-methylenesulfonate and its nucleotide. (●) hm^{5}Cyt, 0.01 M, 0.5 M sodium bisulfite, 20 °C; (○) 0.01 M hm^{5}dCMP, 1 M sodium bisulfite, 37 °C.

Results

Reaction of hm^{5}Cyt with Bisulfite. A rapid reaction was found when hm^{5}Cyt was treated with bisulfite. Thus, the ultraviolet absorption spectrum obtained after incubating hm^{5}Cyt (0.005 M) in 1 M sodium bisulfite, pH 5, at 37 °C and diluting the mixture 100-fold with 0.05 M sodium phosphate buffer, pH 7, was different from that of hm^{5}Cyt. As Figure 1 shows, the change in spectrum was completed by the 5-min incubation, and the resulting spectrum suggested that the pyrimidine ring was not affected but rather a modification had taken place on the substituent at position 5.

By carrying out a preparative experiment, the product was isolated as a crystalline material in almost a quantitative yield, and it was identified as cytosine 5-methylenesulfonate. Thus, the compound was analyzed as an inner salt of cytosine 5-methylenesulfonate; mobility in paper electrophoresis was consistent with the sulfonate structure (Table I); NMR spectrum gave signals corresponding to 6-H and 5-methylene protons; and IR showed a band assignable to ν_{C-S}. Ultraviolet absorption properties determined for the pure compound indicate that the spectral change shown in Figure 1 was the one that could be expected for a quantitative conversion of hm^{5}Cyt to CytCH$_2$SO$_3^-$. (See Scheme I.)

The rate of the conversion of hm^{5}Cyt to CytCH$_2$SO$_3^-$ was measured by use of the difference in ultraviolet absorption between hm^{5}Cyt and CytCH$_2$SO$_3^-$. The reactions of 0.01 M hm^{5}Cyt in 0.5 M bisulfite at various pH values proceeded by the pseudo-first-order rate law, and the optimum pH for the reaction was 4.5 (Figure 2). Effect of the concentration of

Scheme I

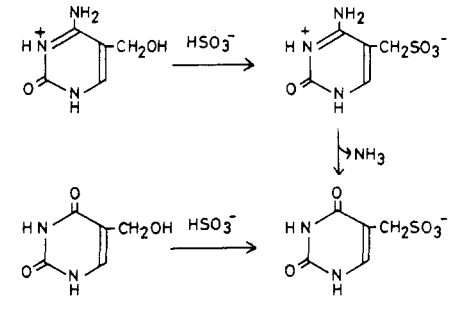

Table II: Pseudo-First-Order Rate Constants for the Reaction of 5-Hydroxymethylpyrimidines with Bisulfite

substrate[a]	pH	temp (°C)	[s][b] (M)	k (min^{-1})	$k/[s]$	$k/[s]^2$
hm^5Cyt	5.5	20	0.50	0.043	0.086	0.172
			0.75	0.100	0.133	0.178
			1.00	0.195	0.195	0.195
			1.50	0.435	0.290	0.193
hm^5Cyt	5.5	0[c]	0.50	0.025	0.050	0.100
			1.00	0.090	0.090	0.090
			2.00	0.380	0.190	0.095
hm^5dCMP	5.5	37	0.50	0.019	0.038	0.076
			1.00	0.080	0.080	0.080
			1.50	0.195	0.130	0.087
hm^5Ura	7.0	37	0.80	0.0033	0.0041	0.0052
			1.00	0.0055	0.0055	0.0055
			1.20	0.0074	0.0062	0.0051
			1.60	0.014	0.0088	0.0055
			2.00	0.019	0.0095	0.0048

[a] Concentration of the substrates was 0.01 M. [b] [s], concentration of total bisulfite buffer. [c] Run in an ice bath.

total bisulfite buffer on the rate was measured, and the results given in Table II indicate that the rate is not linear to the bisulfite concentration but it is linear rather to the square of the bisulfite concentration.

Reaction of hm^5dCMP with Bisulfite. The nucleotide hm^5dCMP reacted with bisulfite in the same manner. The product, isolated by paper chromatography, showed properties consistent with the 5-methylenesulfonate structure. The mobility in paper electrophoresis and the R_f values in paper chromatography are given in Table I. Ultraviolet absorption spectra of the product were similar to those of $CytCH_2SO_3^-$: at pH 1, λ_{max} 291 nm and λ_{min} 248 nm; at pH 7, λ_{max} 279 nm and λ_{min} 254 nm. The pH dependence of the reaction rate was similar to that for hm^5Cyt, the optimum pH being 4.5 (Figure 2). The rate was proportional to the square of the bisulfite concentration as was the case for hm^5Cyt (Table II). In one set of experiments, the rate of the sulfonate formation from hm^5dCMP was compared with that from hm^5Cyt. The rate for the reaction of hm^5Cyt with 0.5 M sodium bisulfite at pH 5.5 and 37 °C was 0.107 min^{-1}, which was about five times greater than that for the reaction of hm^5dCMP under the same conditions (see also Table II).

Reaction at the Polynucleotide Level. In T4 DNA, the hydroxymethyl substituent at positions 5 is completely blocked by glucosyl and the diglucosyl groups (Lehman & Pratt, 1960). In T2 DNA, in contrast, 25% of the hm^5Cyt residues are unglucosylated, the remaining 70% being monoglucosylated and 5% diglucosylated (Lehman & Pratt, 1960).

T2 DNA, either native or heat denatured, was treated with 1 M sodium bisulfite at pH 5.2 and 37 °C for various periods. The modified DNA was analyzed by digesting it with nuclease P1 into mononucleotides and fractionating the nucleotides with paper chromatography. Table III shows the base compositions and the extents of the modification found for the DNA samples. It is seen that approximately 50% of the hm^5Cyt residues in denatured DNA was rapidly modified and the remaining portion of the hm^5Cyt residues was unreactive. It is noteworthy that hm^5Cyt in native T2 DNA was also reactive toward bisulfite. This contrasts sharply to the inactiveness of cytosine toward bisulfite in double-stranded polynucleotides (Shapiro et al., 1973).

In this experiment, it was also noted that Glc-hm^5dCMP residues were unreactive (Table III). The nucleotide Glc-hm^5dCMP was prepared by digesting T4 DNA with nuclease P1 and fractionating the mononucleotide mixture by paper chromatography. When Glc-hm^5dCMP thus obtained was treated with 0.5 M sodium bisulfite at pH 5 and 37 °C for 2 h and the treated solution was submitted to paper chromatography, the Glc-hm^5dCMP was quantitatively recovered. As expected, T4 DNA (denatured) was not affected by treatment with 0.5 M sodium bisulfite at pH 5 and 37 °C for 1 h (data not shown).

To examine whether or not T2 DNA inside bacteriophage particles is susceptible to the treatment with bisulfite, T2 phage was treated with 1 M sodium bisulfite at pH 5.3 and 37 °C for 24 h, and the DNA was extracted from the treated phage and analyzed. No change was observed in the hm^5dCMP fraction as well as in the other nucleotides (Table III). It is obvious that the DNA in phage particle is highly protected against the attack of reagent from outside.

Reaction of hm^5Ura with Bisulfite. The hydroxymethyl substituent at position 5 of uracil reacts with bisulfite in a fashion similar to that of cytosine giving uracil 5-methylenesulfonate ($UraCH_2SO_3^-$) as the product. The product was isolated as the sodium salt and characterized by elemental analysis, ultraviolet absorption, and NMR spectra. This compound was identical with the product of bisulfite-mediated deamination of $CytCH_2SO_3^-$ (see below) with respect to its R_f values in four solvents systems (Table I). The sulfonate formation from hm^5Ura was much slower than that from hm^5Cyt. The rate was measured spectroscopically and the pseudo-first-order rate constants found for the reaction of 0.01 M hm^5Ura with 1.6 M sodium bisulfite at 37 °C were 0.0034 min^{-1} at pH 4.8, 0.013 min^{-1} at pH 6.0, 0.012 min^{-1} at pH 7.1, and 0.0023 min^{-1} at pH 8.1. Therefore, the pH optimum for the reaction lies between 6 and 7. In this reaction, addition of bisulfite across the 5,6 double bond of hm^5Ura also took place depending on the conditions employed. This was found

Table III: Base Composition of Bisulfite-Treated T2 DNA

form of T2 DNA to be treated	time of treatment[a] (h)	mol % of nucleotide						% of modification
		dAMP	dTMP	dGMP	Glc-hm^5-dCMP	hm^5dCMP	dCMP-$CH_2SO_3^-$	
denatured	none	33.5	31.7	18.9	11.1	4.9		
	0.5	32.3	31.5	19.2	11.5	3.8	1.6	30
	1	32.4	32.4	19.2	11.4	2.8	1.8	39
	2	32.9	31.1	19.3	11.5	2.6	2.6	50
	5	33.0	31.2	19.6	11.2	2.3	2.8	55
	24	32.6	31.1	18.9	11.0	2.2	4.3	66
native	2	32.2	31.6	19.3	11.7	3.8	1.5	28
	5	32.8	33.3	17.9	11.5	2.7	1.7	40
DNA inside phage	24	34.1	31.4	18.9	11.1	4.9	0	0

[a] Conditions: 1 M sodium bisulfite, pH 5.2, 37 °C.

by directly measuring NMR spectrum of the reaction mixture. Thus, upon incubation of 0.05 M hm^{5}Ura in 2 M sodium bisulfite in D_2O, at pD 5 and 37 °C for 105 min, two singlet signals corresponding to the 5-CH_2 of two isomeric 5,6-dihydro-5-hydroxymethyluracil 6-sulfonates were observed at 4.22 and 4.33 ppm. From the peak heights it was estimated that the ratio, hm^{5}Ura/hm^{5}Ura–bisulfite adduct, was 5/2. For the reactions at pD 7, these signals were not observed, indicating that the adduct can accumulate only in acidic conditions. It should be noted that the bisulfite adduct formation was not observable for either $UraCH_2SO_3^-$ or $CytCH_2SO_3^-$ in NMR studies.

Effect of bisulfite concentration on the rate of the reaction between hm^{5}Ura and bisulfite was investigated, and the results shown in Table II indicated that the rate was proportional to the square of the bisulfite concentration.

Bisulfite-Mediated Deamination of $CytCH_2SO_3^-$. It was found that $CytCH_2SO^-$ was very slowly deaminated to give $UraCH_2SO_3^-$ on treatment with bisulfite. The $UraCH_2SO_3^-$ formed was identified by paper chromatography with the $UraCH_2SO_3^-$ prepared from hm^{5}Ura (see above). The deamination was studied by directly measuring the NMR spectrum of a mixture of $CytCH_2SO_3^-$ and bisulfite in D_2O. Thus, 50% deamination of $CytCH_2SO_3^-$ was observed on treatment with 3 M sodium bisulfite at pD 5.5 and 37 °C for 28 h (77% deamination at 72 h). For comparison, deamination of cytosine (Shapiro et al., 1970; Hayatsu et al., 1970) and 5-methylcytosine (Shiragami et al., 1975) under the same conditions was investigated, and the approximate half-lives found were 6 min for cytosine and 8 h for 5-methylcytosine.

Discussion

Precedents of this type of reaction can be found in the action of bisulfite on benzyl alcohol derivatives such as epinephrine and *p*-aminobenzyl alcohol (Higuchi & Schroeter, 1959). In these cases, nucleophilic substitution occurs on the benzylic carbon which is activated by an electron-withdrawing group located ortho or para to the benzylic substituent, producing the corresponding benzylsulfonates. A similar sulfonate formation has been observed in the reaction between bisulfite and thiamin (Williams et al., 1935). It has been reported that bisulfite reacts with uracil substituted at position 5 with ribose to give a product which has not been identified (Singhal, 1974).

The ease with which hm^{5}Cyt undergoes this type of reaction may be interpreted as deriving from a strong activation of the 5-methylene carbon by the protonated cytosine ring. The pH–rate profile shown in Figure 2 is consistent with a mechanism in which sulfite ion attacks the protonated hm^{5}Cyt (pK_a = 4.3 (Dunn & Hall, 1975)). The slow reaction of hm^{5}Ura with bisulfite indicates that the activation of the methylene group is weaker in hm^{5}Ura than in hm^{5}Cyt. The optimum pH of the hm^{5}Ura–bisulfite interaction (pH 6 to 7) suggests that the reactive species in this reaction are sulfite ion and the undissociated hm^{5}Ura [pK_a = 9.4 (Dunn & Hall, 1975)].

In the reactions of bisulfite with both hm^{5}Cyt and hm^{5}Ura, the rate was proportional to the square of the bisulfite concentration (Table II). Bisulfite adduct across the 5,6 double bond of hm^{5}Cyt appears not to be involved as an intermediate of the reaction since hm^{5}Cyt in double-stranded DNA can undergo the sulfonation as easily as that in single-stranded DNA (Table III). It was reported by Shapiro et al. (1973) that bisulfite addition to the 6 position of pyrimidine is completely hindered in double-stranded polynucleotides. The possibility that two molecules of bisulfite interact to generate some "reactive species" for the sulfonation is also unlikely because the sulfonate formation from benzyl alcohol derivatives was reported to be first order to the concentration of bisulfite (Higuchi & Schroeter, 1959). The reason why the reaction is second order with bisulfite remains to be an open question.

The bisulfite-mediated deamination of cytosine is greatly slowed down by the presence of 5-methyl group: the rate for the deamination of 5-methylcytosine is almost two orders of magnitude smaller than that for cytosine. The introduction of an electron-repulsive sulfonate group in the 5-alkyl substituent would make the addition of sulfite ion to position 6 more difficult, and, as a consequence, the deamination of $CytCH_2SO_3^-$ is considerably slower than even that of 5-methylcytosine.

The reactivity of bisulfite to hm^{5}Cyt in native, double-stranded DNA is remarkable. This is apparently due to the fact that the site of the reaction is the side chain but not pyrimidine ring itself. It is known that enzymatic glucosylation occurs on hm^{5}Cyt of T4 DNA during the phage infection of *E. coli* (Josse & Kornberg, 1962). This requires that the enzyme and the glucose donor, UDP-glucose, can approach the 5-hydroxymethyl group of DNA. Therefore, the reaction of bisulfite with hm^{5}Cyt in native T2 DNA may be taken as a chemical proof that the 5-hydroxymethyl groups are exposed to external agents.

The reactivity of hm^{5}Cyt with bisulfite both in double- and single-stranded DNA also provides a way to prepare polynucleotides bearing extra anionic charges. Such polynucleotides may be useful in biochemical and biophysical studies.

Acknowledgments

We thank Professor H. Uchida of the Institute of Medical Sciences, University of Tokyo, for providing us facilities to manipulate T-even bacteriophages.

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Photocross-linking Analysis of the Contact Surface of $tRNA^{Met}$ in Complexes with *Escherichia coli* Methionine:tRNA Ligase†

Joseph J. Rosa,‡ Margaret D. Rosa,§ and Paul B. Sigler*

ABSTRACT: Photoinduced covalent cross-linking has been used to identify a common surface of four methionine-accepting tRNAs which interact specifically with the *Escherichia coli* methionine:tRNA ligase (EC 6.1.1.10). tRNA–ligase mixtures were irradiated, and the covalently linked complexes were isolated and digested with T1 RNase (Schimmel & Budzik, 1977). The fragments lost from the elution profile of the T1 RNase digest were considered to have been cross-linked to the protein and therefore in intimate contact with the enzyme. Only specific cognate tRNA–ligase pairs produce covalently linked complexes. The four substrate tRNAs used in this study have substantially different sequences, but all showed a common cross-linking pattern, supporting the view that the sites cross-linked to the enzyme reflect the functionally common contact surface rather than particularly photoreactive regions of tRNA. The cross-linked contact surface is comprised of three regions: (1) the narrow groove of the anticodon stem and its extension into the anticodon loop; (2) the 3′ terminal residues; and (3) the 3′ side of the "T arm". Unlike previous studies with other tRNAs, the D arm is not involved and significant radiation damage is suffered by the tRNA which must be taken into account in the analysis. The results are consistent with and complement chemical modification studies [Schulman, L. H., & Pelka, H. (1977) *Biochemistry 16*, 4256].

A large body of information has been accumulated concerning the specific recognition of tRNA by its cognate amino acid:tRNA ligase. It has become apparent that this interaction is a subtle chemical process which is sensitive to the ionic strength and character of the solvent and no doubt involves regions of tRNA structure that are spatially separated from the site of aminoacylation (see a review, Söll & Schimmel, 1974). A number of strategies have been used in attempts to identify the structural features of tRNA responsible for ligase recognition; they include (a) observing the effects of chemically modifying tRNA [e.g., Schulman & Pelka, 1977 (and references cited therein); Litt & Greenspan, 1972], (b) testing ligase recognition of tRNA fragments (e.g., Thiebe et al., 1972; Oda et al., 1969; Seno et al., 1969), (c) comparing the accessibility of nascent and complexed tRNA to various reagents ranging from the very small tritium nucleus (Schoemaker & Schimmel, 1976) to a very large probe such as a nuclease molecule (e.g., Horz & Zachau, 1973; Dube, 1973; Dickson & Schimmel, 1975), (d) the use of spectral probes such as fluorescence (Lam & Schimmel, 1975), (e) testing ligase interaction with mutant tRNA species (Smith et al., 1970; Celis et al., 1973), (f) comparing sequence homologies among tRNAs recognized by the same enzyme (Roe et al., 1973), and (g) competitive oligonucleotide binding to indicate shielding of complementary sequences by the bound ligase (Schimmel et al., 1972).

While all of these approaches are informative, they have serious limitations. For example, it is difficult to infer with any precision the details of a tRNA–ligase interaction from the inaccessibility of a particular region of tRNA to a chemical or enzymatic probe. Most probes used in analyzing tRNA–protein interactions interact preferentially with single stranded RNA and are therefore likely to point preferentially to contact regions in the tRNA that are devoid of significant secondary (or tertiary) structure. There is no reason to believe the same constraint applies to photocross-linking. Further, interpretations of the disruptive effect of either a chemical or genetic modification are generally compromised by the qualifying consideration of a secondary or indirect conformational effect. The strongest statement of a "protection" or "modification" experiment is therefore usually restricted to the null result; that is to say, if the chemical accessibility of a region in a molecule remains *un*changed in a complex, or if a structural alteration has *no* discernible effect on complex formation, then the reaction sites are considered *not* to be involved in the specific interactions of the complex.

An alternative approach was suggested by the work of Markovitz (1972), who reported that irradiating a complex between DNA and the *Escherichia coli* DNA polymerase I with UV light rendered the complex stable to high salt and denaturants, presumably through photoinduced covalent cross-links between the protein and nucleic acid. This finding was exploited by Schimmel and his co-workers (Schoemaker & Schimmel, 1974; Budzik et al., 1975; Schoemaker et al.,

† From the Department of Biophysics and Theoretical Biology, The University of Chicago, Chicago, Illinois 60637. *Received September 11, 1978.* This work was supported by National Institutes of Health Grant No. GM 15225 and National Science Foundation Grant No. GB 97654. J.J.R. and M.D.R. were supported by U.S. Public Health Service Training Grant GM 780.

‡ Present address: Department of Molecular Biophysics and Biochemistry, Kline Tower, Yale Station, New Haven, CT 06520.
§ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510.

0006-2960/79/0418-0637$01.00/0