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Reactions of 2-Thioribothymidine and 4-Thiouridine with Hydrogen Peroxide in Transfer Ribonucleic Acids from *Thermus thermophilus* and *Escherichia coli* As Studied by Circular Dichroism†

Kimitsuna Watanabe

ABSTRACT: The reaction of thiouridine derivatives with hydrogen peroxide (H_2O_2) at the monomer level as well as in polynucleotides was investigated to check the possibility that the difference or variation in the environment surrounding the thiouridine residues may be simply a reflection of the difference in the reactivity of these residues with H_2O_2. At the monomer level, the reactivity is of the order of 4-thiouridine (s^4U) > 5-[(methylamino)methyl]-2-thiouridine (mnm^5s^2U) > 2-thiouridine (s^2U) ≃ 2-thioribothymidine (s^2T). When these residues are in polynucleotides, but without their C=S groups being hydrogen bonded, such as s^4U in $tRNA^{Phe}$ and $tRNA_f^{Met}$, mnm^5s^2U in $tRNA_2^{Glu}$ from *E. coli*, and s^2T in $s^2T\Psi CGp$, their reactivity is reduced to no more than one-half of that of monomers. All these reactions proceeded by first-order kinetics. Poly(2-thiouridylic acid)/[poly(s^2U)], in which both free and hydrogen-bonded C=S groups exist, reacted with H_2O_2, at first slowly ($k_{initial} = 0.11 \times 10^{-3}$ min^{-1}), corresponding to the reaction of the free C=S group in the double helix, and then rapidly ($k_{final} = 1.5 \times 10^{-3}$ min^{-1}) due to denaturation of the strands. s^2T in *T. thermophilus* $tRNA^{Phe}$ reacted easily when it was denatured by *trans*-1,2-diaminocyclohexanetetraacetic acid treatment, while it reacted very slowly in the native $tRNA^{Phe}$ [$k = (0.06–0.02) \times 10^{-3}$ min^{-1}]. Even the larger rate constant is only one-half of the initial rate constant of poly(s^2U). The charging activity of the H_2O_2-treated $tRNA^{Phe}$ has good correlation with the residual amount of s^2T in the $tRNA^{Phe}$. These results suggest that the s^2T sulfur atom in the native $tRNA^{Phe}$ is probably involved in hydrogen bonding, as reported in the case of the tertiary structure of yeast $tRNA^{Phe}$.

Extreme thermophilic bacteria, *Thermus thermophilus* HB 8 and HB 27, *Thermus aquaticus* YT 1, and *Thermus flavus* AT 62, all have 2-thioribothymidine (s^2T)[1] in place of the usual ribothymidine (T) in the TΨC loop of their tRNAs (Watanabe et al., 1974; Oshima et al., 1977), and this modified nucleoside is thought to be the most responsible for the thermal stability of the tRNAs (Oshima et al., 1976; Watanabe et al., 1976a,b, 1979a, 1980; Davanloo et al., 1979).

On the basis of the tertiary structure of yeast $tRNA^{Phe}$ (Ladner et al., 1975a; Quigley et al., 1975; Rich & Kim, 1978), it is speculated that the s^2T54 residue of the thermophile tRNA is base paired with the m^1A58 residue and that this base pair stacks on the neighboring common base pair G53–C61 and on the interloop base pair Ψ55–Gm18 [the numbering of residues conforms to the proposed rule (Sprinzl et al., 1980)]. s^2T nucleoside is known to take on the 3′-endo–gauche–gauche–anti form preferentially (Watanabe et al., 1979a; Yokoyama et al., 1979). This rigid conformation in the po-

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[1] Abbreviations used: H_2O_2, hydrogen peroxide; C=S, thiocarbonyl group; k, rate constant; $k_{initial}$ and k_{final}, initial and final rate constants, respectively; $t_{1/2}$, half-life; LC, high-pressure liquid chromatography; CDTA, *trans*-1,2-diaminocyclohexanetetraacetic acid; s^2U, 2-thiouridine; s^2T, 2-thioribothymidine; mnm^5s^2U, 5-[(methylamino)methyl]-2-thiouridine; poly(s^2U), poly(2-thiouridylic acid); Cm, $O^{2'}$-methylcytidine; Gm, $O^{2'}$-methylguanosine; m^1A, 1-methyladenosine.

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lynucleotide chain of tRNA is considered to stabilize the stacking, mentioned above, thus contributing further to the stability of the hydrogen bonding of the interloop base pair Ψ55–Gm18. If we assume that tRNA takes on a common tertiary structure (Kim et al., 1974), the sulfur atom attached to C2 of s^2T is probably involved in hydrogen bonding with the amino group of m^1A (see Figure 4b) in the base pair of s^2T54 and m^1A58 of *T. thermophilus* tRNA. Confirmation of this hydrogen bonding will conversely lead to partial support for the possibility that tRNA tertiary structures possess certain features in common, as proposed by Kim et al. (1974).

Hydrogen peroxide (H_2O_2) was used for this purpose, since this reagent is known to preferentially desulfurize the nucleoside having thiocarbonyl (C=S) groups in s^4U (Scheit, 1968) and s^2U derivatives (Chi & Chen, 1957), and under certain conditions, the reactivity of the usual nucleosides should be suppressed to a negligible extent, compared with those of thiopyrimidine nucleoside, as Melzer & Tomlinson reported (1966). Moreover, the H_2O_2 molecule is small enough and electrically neutral so that it is expected to penetrate even into sterically crowded regions without electrostatic interaction (see Discussion). As reported previously (Watanabe et al., 1976a), s^4U and s^2T have characteristic CD bands above 300 nm, derived from the C=S groups, and these bands could be used as reporters for the reaction process of the C=S groups of s^2T and s^4U with H_2O_2.

In this paper, it has been anticipated that the different reactivities of various thiouridine residues with H_2O_2 may reflect the difference in the microenvironment surrounding these residues, and from this it may be deduced that the s^2T sulfur atom is involved in hydrogen bonding in *T. thermophilus* tRNA. The results presented here support this anticipation.

Materials and Methods

Materials. s^4U was purchased from Sigma Chemical Co., and s^2U and mnm^5s^2U were provided by Dr. H. Kasai, National Cancer Center Research Institute. s^2T and $s^2T\Psi CGp$ were prepared from *T. thermophilus* tRNA as reported previously (Watanabe et al., 1974). *E. coli* $tRNA_f^{Met}$ and $tRNA_2^{Glu}$ were kindly given by Drs. S. Nishimura and Z. Yamaizumi (National Cancer Center Research Institute), respectively. $tRNA^{Phe}$'s from *E. coli* (Seela et al., 1977) and *T. thermophilus* (Watanabe et al., 1980) were purified as reported. Poly(s^2U) was kindly provided by Professor K. H. Scheit of the Max-Planck-Institut für Biophysikalische Chemie (Göttingen, Germany). Hydrogen peroxide (commercial grade, 30% w/v) was used without further purification.

Methods. CD spectra were obtained by using a JASCO J-40 spectropolarimeter. The temperature was set at 20 °C unless otherwise indicated, and was controlled by circulation of a 1:1 mixture of polyethylene glycol and water from a Haake thermostat. UV spectra were obtained with a Shimadzu UV-200 double-beam spectrophotometer at 20 °C.

The sample concentration was determined by measuring the ultraviolet absorption, using the molar extinction coefficients as follows: s^2U, 13.6×10^3 at 275 nm; s^2T, 14.1×10^3 at 272 nm; s^4U, 21.2×10^3 at 340 nm (Dunn & Hall, 1976). The ϵ_{272} value of s^2T was substituted for that of mnm^5s^2U (Watanabe et al., 1979a). The molar extinction coefficients for *E. coli* tRNAs (Adler & Fasman, 1970; Willick & Kay, 1971; Blum et al., 1972) and poly(s^2U) (Bähr et al., 1973) were taken from the literature, and that for *T. thermophilus* $tRNA^{Phe}$ was determined to be 7.5×10^3 at 260 nm (in water) by phosphorus assay (Ames & Dubin, 1960).

H_2O_2 treatment was carried out as follows: 54 μM thiouridine derivatives, 54 μM thiouridine-equivalent $s^2T\Psi CGp$ and poly(s^2U), and 30 A_{260}/mL tRNAs (~54 μM) were mixed with phosphate buffer (0.05 M sodium phosphate, pH 8.0, and 1 mM EDTA) in a volume of 1 mL, into which 12 μL of 30% H_2O_2 was added, and the solution was mixed thoroughly. The solution was kept at 20 °C in the dark except for when it was measured for CD or UV spectra.

The base composition of the nucleosides or tRNA during the reaction with H_2O_2 was analyzed by high-pressure liquid chromatography (Waters Co.), as reported previously (Watanabe et al., 1980). A μBondapack C_{18} column (Waters Co.) was used with a solvent of 0.05 M $NH_4H_2PO_4$, pH 5.1, and 5% methanol (Hartwick & Brown, 1976). A quantitative analysis for each nucleoside was carried out as described previously (Watanabe et al., 1980).

For the amino acid acceptor activity of $tRNA^{Phe}$ during the H_2O_2 reaction process, 1 μL of the solution was taken up at each time into a mixture of 18 μL of water and 1 μL of 1 M β-mercaptoethanol to stop the reaction. The aminoacylation reaction was carried out at 65 °C for 10 min, using a crude aminoacyl-tRNA synthetase fraction prepared from *T. thermophilus* cells (Watanabe et al., 1980).

Results

Reaction of Thionucleosides and $s^2T\Psi CGp$ with H_2O_2. At first, the reactions were studied at the nucleoside level. It is known that s^4U can be converted quantitatively to uridine with H_2O_2 as monitored by ultraviolet absorption spectra centered at 320 nm (Scheit, 1968). For verification of whether s^2U derivatives are also converted to their desulfurized nucleosides with H_2O_2 and whether their reaction process is monitored by CD spectra quantitatively, s^2T was reacted with H_2O_2 while being monitored by CD spectra in the wavelength region from 400 to 290 nm (Figure 1). At the same time, an aliquot of the reaction mixture was taken up at appropriate intervals and loaded onto the chromatograph to check the decrease of s^2T and the increase of its H_2O_2-altered nucleoside quantitatively (Figure 2). EDTA was added to the reaction buffer to minimize the reactivity of H_2O_2 with the usual nucleosides (Melzer & Tomlinson, 1966) except thiopyrimidines.

As shown in Figure 1, the CD band at 319 nm of s^2T decreased with reaction time by first-order kinetics (see Figure 3), and the band disappeared after overnight reaction. Figure 2 shows the LC elution patterns of s^2T reacting with H_2O_2. The peak of s^2T decreased as the reaction proceeded, and a new peak (as indicated by the arrow) appeared concomitantly. The remaining s^2T content was calculated from the peak areas of s^2T, by comparing them with those of guanosine, which was added to the sample solution beforehand as a standard for quantitative analysis. Guanosine was stable against H_2O_2 with the buffer used (Melzer & Tomlinson, 1966) and did not influence the rate constant of s^2T. The results are summarized in the insert in Figure 1, showing a good correlation between the magnitude of the CD band at 319 nm and the s^2T content analyzed by LC. If we assume that the H_2O_2-altered nucleoside is the only one which appeared in the elution profiles in Figure 2, the sum of s^2T and the nucleoside must be constant throughout the reaction. Thus, ϵ_{254} of the H_2O_2-altered nucleoside could be determined as 10.5 ± 0.5, and the increase in the nucleoside with reaction also correlated well with the decrease in s^2T as shown in the insert of Figure 1. Results similar to the above were also obtained in the case of s^2U and mnm^5s^2U (data not shown). Thus, it is evident that the reaction rates of s^2U derivatives with H_2O_2 can be followed by CD signals. In the case of s^4U, it was possible to use both UV and CD signals, giving the same rate constants. However, under the conditions employed, the rate was so fast that it was

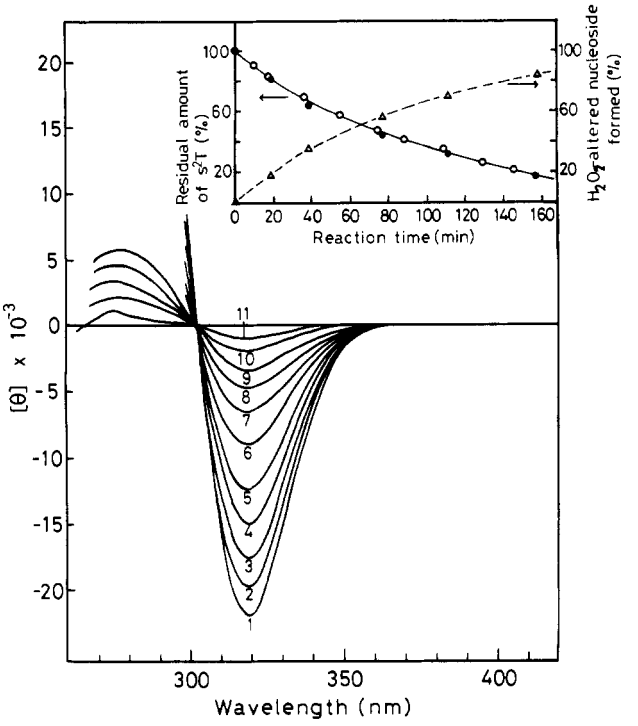

FIGURE 1: CD spectral change of s^2T in the reaction with H_2O_2. s^2T (54 μM) in the presence of 40 μM guanosine was reacted with H_2O_2 at 20 °C. CD spectra were obtained with the scan speed of 10 nm/min, and the intensity of the CD band at 319 nm was measured before reaction (curve 1), and at 10 (2), 21 (3), 36 (4), 55 (5), 88 (6), 104 (7), 151 (8), 183 (9), 236 (10), and 300 min (11) after the onset of reaction. Insert: Correlation curve between the time-dependent residual amount of the s^2T content (percent) monitored by CD spectra at 319 nm (○) and that obtained by LC analysis (●) shown in Figure 2; (△) H_2O_2-altered nucleoside formed by the reaction (see text).

Table I: Kinetic Data for Various Thiouridine Nucleosides and the tRNAs Housing These Nucleosides in the Reaction with H_2O_2

sample	probe	$t_{1/2}$ (min)	$k \times 10^3$ (min^{-1})
s^2U		64.5	10.7
s^2T		68.5	10.1
s^4U		14.7	47
mnm^5s^2U		30.5	22.7
$s^2T\psi CGp$	s^2T	90.0	7.7
E. coli			
$tRNA_f^{Met}$	s^4U	21.7	32
$tRNA^{Phe}$	s^4U	18.3	38
$tRNA_2^{Glu}$	mnm^5s^2U	60.0	11.6
T. thermophilus			
$tRNA^{Phe}$ (intact)	s^4U	18.8	37
	s^2T	~30000.0[a]	0.06 ($k_{initial}$) 0.02 (k_{final})
$tRNA^{Phe}$ (denatured)	s^4U	17.0	41
	s^2T	320.0	2.2
poly(s^2U)	s^2U	3780.0[a]	0.11 ($k_{initial}$) 1.5 (k_{final})

[a] Half-life where the reaction proceeds to 50% completion, although the reaction does not obey first-order kinetics.

more convenient to use the UV absorption method.

Figure 3 shows the time course of s^2T, s^2U, mnm^5s^2U, s^4U, and $s^2T\Psi CGp$ which reacted with H_2O_2. In each case, the reaction process shows first-order kinetics at least up to an 80% decrease in the original material. The half-life ($t_{1/2}$) and the rate constant (k) of each sample are listed in Table I. As indicated in Figure 3 and Table I, s^4U reacts the most with H_2O_2, and the order of reactivity is $s^4U > mnm^5s^2U > s^2U \simeq s^2T$. The rate constant of $s^2T\Psi CGp$ was found to be $^{10}/_{13}$ that of the s^2T monomer, suggesting that some stacking effect

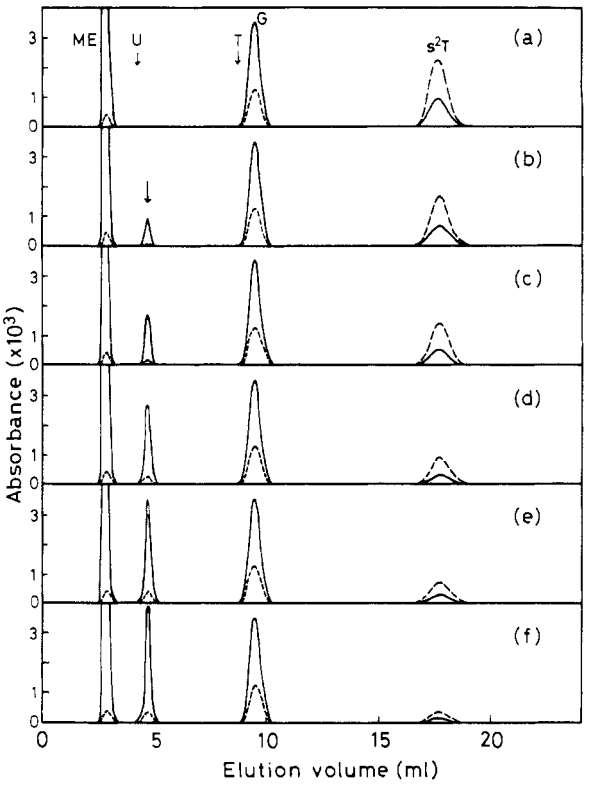

FIGURE 2: Elution patterns of s^2T and the H_2O_2-altered nucleoside by LC: (—) 254 nm, (---) 280 nm. At each time, 1 μL of the reaction mixture was mixed with 2 μL of 1 M β-mercaptoethanol, followed by loading onto the column. The reaction times were 0 (a), 19 (b), 38 (c), 77 (d), 110 (e), and 157 min (f). ME, U, and T are the elution positions of β-mercaptoethanol, uridine, and ribothymidine, respectively. The arrow in the second panel shows the elution position of the H_2O_2-altered nucleoside formed.

influences the reactivity of the s^2T base with H_2O_2.

Reaction of s^4U and mnm^5s^2U in E. coli tRNAs with H_2O_2. Next, the reactivity of s^4U and mnm^5s^2U in the tRNA molecule was investigated under the same conditions as above, by using *E. coli* $tRNA_f^{Met}$, $tRNA^{Phe}$, and $tRNA_2^{Glu}$. On the basis of the tertiary structure of yeast $tRNA^{Phe}$ (Rich & Kim, 1978), both thionucleosides stack on neighboring bases in the tRNA molecule, but their sulfur atoms are not involved in hydrogen bonding; s^4U8 is base paired with A14 and stacks on the C13–G22–m^7G46 base triple in $tRNA_f^{Met}$ and $tRNA^{Phe}$ (Figure 4a), while mnm^5s^2U34 stacks on the neighboring U35, the middle letter of the anticodon triplet in $tRNA_2^{Glu}$. Thus, they could be good models for the free C=S group in the stacked polynucleotide chain.

The positive band at 335 nm derived from s^4U in $tRNA_f^{Met}$ and $tRNA^{Phe}$ and the negative band at 335 nm of mnm^5s^2U in $tRNA_2^{Glu}$ (Willick & Kay, 1971) decreased as the reaction proceeded and vanished when the reaction was completed, showing that all the thiouridine residues were converted to the H_2O_2-altered forms (data not shown). The time course and the rate constants are shown in Figure 3 and Table I, respectively. The rate constants of the s^4U residues in $tRNA_f^{Met}$ and $tRNA^{Phe}$ were from 1.3 to 1.5 times as small as that of the s^4U monomer. The rate constant of mnm^5s^2U in $tRNA_2^{Glu}$ was one-half that of the mnm^5s^2U monomer. These results imply that the stacked nucleosides around the thiouridine residues protect the thiouridines from H_2O_2 attack, with the result that the rate constants become lower.

Reaction of Poly(s^2U) with H_2O_2. In order to understand the reactivity of s^2U derivatives in the polynucleotide chain whose sulfur atoms are involved in hydrogen bonding, poly-

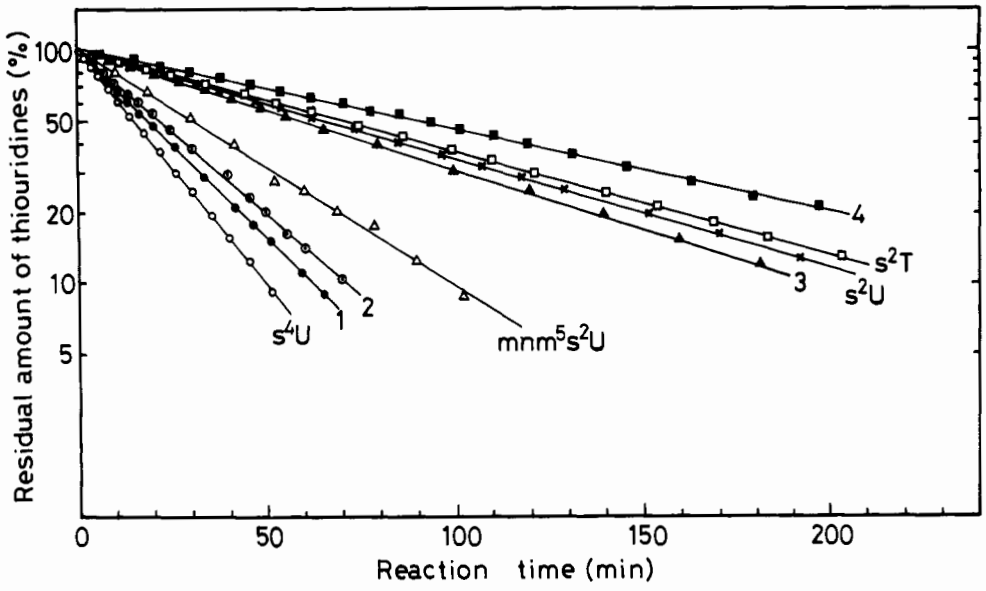

FIGURE 3: Time course of the reactions with H_2O_2 of s^4U, mnm^5s^2U, s^2U, s^2T, and s^4U in *E. coli* $tRNA^{Phe}$ (1) and *E. coli* $tRNA_f^{Met}$ (2), mnm^5s^2U in *E. coli* $tRNA_2^{Glu}$ (3), and s^2T in $s^2T\Psi CGp$ (4). The reactions were followed by UV spectra at 330 nm for the s^4U monomer and at 335 nm for tRNAs and by CD spectra at 332 nm for mnm^5s^2U, 336.5 nm for $tRNA_2^{Glu}$, 325 nm for s^2U, 319 nm for s^2T, and at 322 nm for $s^2T\Psi CGp$.

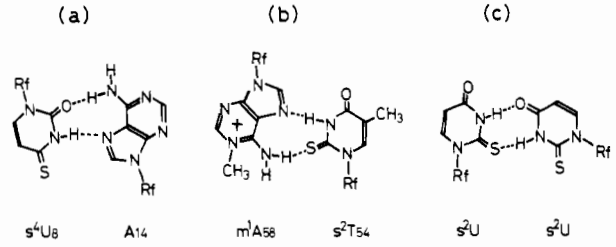

FIGURE 4: Base-pairing schemes of s^4U8 and s^2T54 in *T. thermophilus* tRNA and s^2U in poly(s^2U). The former two base pairs (a and b) were speculated, on the basis of the reported model for yeast $tRNA^{Phe}$ (Rich & Kim, 1978), and the third one (c) is cited from Mazumder et al. (1972).

(s^2U) was taken as a model material (Bähr et al., 1973). The three-dimensional structure of poly(s^2U) has been determined by the analysis of an X-ray fiber diffraction pattern to be a double-stranded helix with unsymmetric base pairing (Mazumder et al., 1974); the sulfur atom of s^2U in one strand (α chain) is concerned with base pairing, while that in the other strand (β chain) is free, as shown in Figure 4c. This will serve as a good model for both the free and hydrogen-bonded C=S groups.

When poly(s^2U) is reacted with H_2O_2, both a negative CD band at 328 nm and a positive band at 296 nm (Bähr et al., 1973) decreased in parallel (data not shown). The time couse in Figure 5 shows a unique feature in that it appears to be comprised of at least two steps. The reaction proceeded slowly in a linear manner for about 40 h, at the rate constant $k_{initial}$ of 0.11×10^{-3} min^{-1}; the rate constant then became several to ten times faster, finally becoming 1.5×10^{-3} min^{-1}. On the basis of the base-pairing scheme of poly(s^2U) (Figure 4c), it is supposed that the initial step, with the small rate constant, corresponds to the reaction of the free C=S group of s^2U in

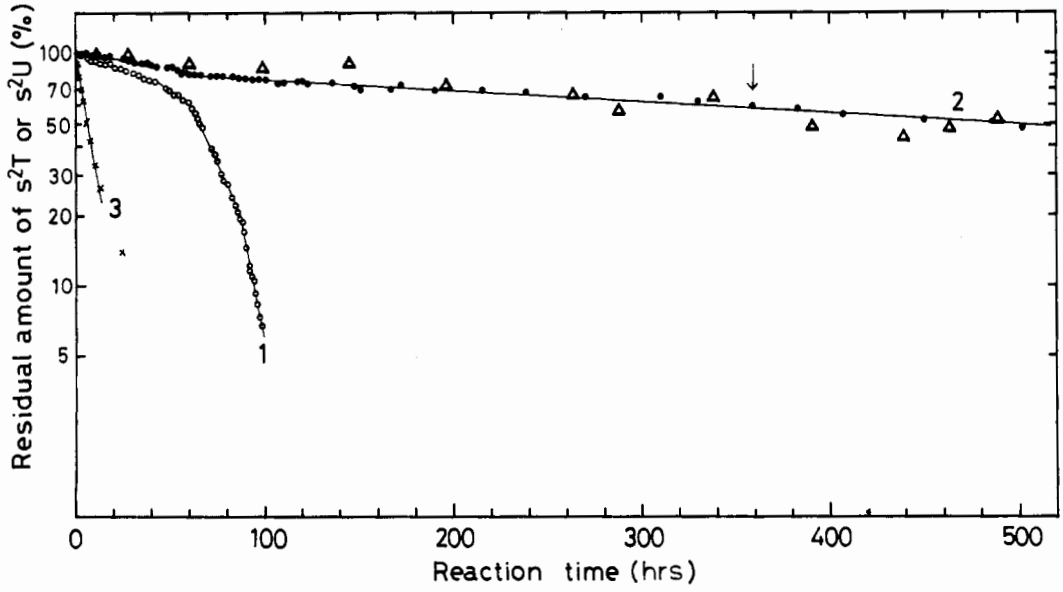

FIGURE 5: Time course of the reactions with H_2O_2 of poly(s^2U) (1, ○), *T. thermophilus* $tRNA^{Phe}$ intact form (2, ●), and its CDTA-treated denatured form (3, ×). The reactions were followed by CD spectra at 327.5 nm for poly(s^2U), at 310 nm for intact $tRNA^{Phe}$, and at 325 nm for denatured $tRNA^{Phe}$, as shown in Figure 6. (△) Charging activity of $tRNA^{Phe}$ intact form; 100% charging corresponded to 1.2 nmol of phenylalanine/A_{260} units of tRNA.

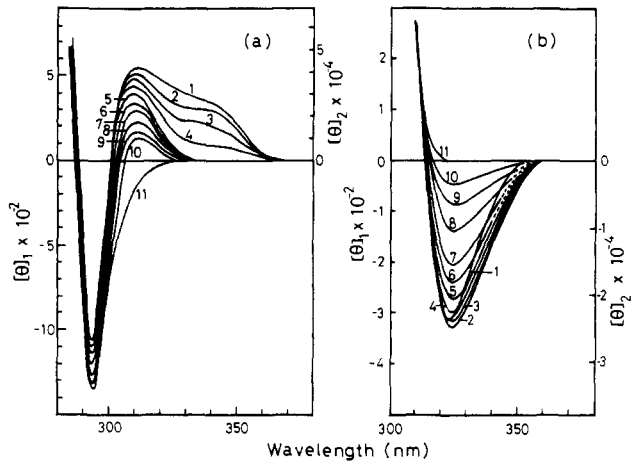

FIGURE 6: CD spectral change of *T. thermophilus* $tRNA^{Phe}$ in the reaction with H_2O_2. The reaction of intact $tRNA^{Phe}$ in the buffer was monitored by the $[\theta]$ value at 310 nm (a), and the reaction times were 0 min (curve 1), 9 min (2), 24 min (3), 51 min (4), 3.4 h (5), 30.5 h (6), 53.1 h (7), 150.5 h (8), 360 h (9), and 500 h (10). Curve 11 shows the CD spectrum (control curve) of $tRNA^{Phe}$ without s^4U or s^2T (see text). The reaction of CDTA-treated $tRNA^{Phe}$ in water was monitored by the $[\theta]$ value at 325 nm (b), and the reaction times were 0 min (curve 1), 30 min (2), 40 min (3), 50 min (4), 1.4 h (5), 2.5 h (6), 3.75 h (7), 7.5 h (8), 13 h (9), 24 h (10), and 72 h (11). $[\theta]_1$ and $[\theta]_2$ were calculated per mol of tRNA and per mol of thiouridine residue, respectively.

the β chain. Scheit & Faerber (1973) studied the interaction of poly(s^2U) with hydroxymercuribenzenesulfonate (HOHgBzS) and concluded that during the binding process of HOHgBzS to s^2U residues the double strand of poly(s^2U) was unfolded in a cooperative manner, all the s^2U residues being finally bound with the reagent. A similar phenomenon may have occurred in the reaction with H_2O_2; the H_2O_2 reaction induces the unfolding of the poly(s^2U) strand. This process may correspond to the final step with the large rate constant, since the final rate constant of poly(s^2U) is similar to the rate constant of s^2T in the denatured $tRNA^{Phe}$ of *T. thermophilus* ($k = 2.2 \times 10^{-3}$ min^{-1}), as described below (Figure 5 and Table I). Thus, we were able to measure only the rate constant of the free C=S group of s^2U in the β chain, but not that of the hydrogen-bonded C=S group in the α chain, which is supposed to be much smaller than the former.

Reaction of s^2T in T. thermophilus $tRNA^{Phe}$ with H_2O_2. As reported previously (Watanabe et al., 1976a), a change in the CD signal of s^2T in the tRNA is dependent on the tRNA conformation. When the tRNA exists in the native state, there appears a positive band at 310 nm which is assigned to s^2T, whereas in the denatured state of tRNA s^2T shows a negative band centered at 325 nm; this band is exactly the same as that of the free s^2T nucleoside. These findings strongly suggest that s^2T in the native tRNA somehow interacts with other nucleoside residues. Therefore, two forms (native and denatured) of *T. thermophilus* $tRNA^{Phe}$ were prepared; the native form was obtained by the conventional preparation and the denatured form by treatment with CDTA, followed by an extensive dialysis against water (Watanabe et al., 1980). The latter form was reacted with H_2O_2 without buffer, since, when a phosphate or even 1 mM EDTA was added to the sample solution, the sample again exhibited a positive signal at 310 nm, showing that the tRNA was refolded back, even if partially, to the native conformation.

The H_2O_2-induced CD spectral changes in these two forms of $tRNA^{Phe}$ are shown in Figure 6. In the denatured form of $tRNA^{Phe}$, the CD spectra changed rapidly from curve 1 to curve 2 in Figure 6b, with the rate constant of 41×10^{-3} min^{-1},

Table II: Nucleoside Composition of *T. thermophilus* $tRNA^{Phe}$ before and after Reaction with H_2O_2[a]

	$tRNA^{Phe}$	
nucleosides	before reaction	after reaction (360 h)
A	11.1	11.5
U	9.9	9.3
G	23.0	23.0
C	22.0	20.5
ψ	0.8	1.0
m^7G	2.1	0.3
s^4U	1.0	0.0
s^2T	1.0	0.57
Cm	0.6	0.5
Gm	1.0	1.0

[a] m^1A overlapped with U in the elution profile of LC and could not be analyzed. *T. thermophilus* $tRNA^{Phe}$ consists of multispecies; the species used in the present study is different from that used in the previous report (Watanabe et al., 1980), and, thus, the base compositions of both species are slightly different.

due to the degradation of the s^4U residues. The negative band at 325 nm then gradually decreased with $k = 2.2 \times 10^{-3}$ min^{-1} and disappeared after a reaction time of 72 h. This latter change corresponds to the degradation of the s^2T residues, with the rate constant being about one-fifth of that of the free s^2T nucleoside, showing that s^2T in the polynucleotide chain, even if unfolded, is protected from H_2O attack. In the native form of $tRNA^{Phe}$, the CD spectra rapidly changed from curve 1 to curve 5 in Figure 6a. This change must be due to the degradation of the s^4U residues, judging from the rate constant ($k = 37 \times 10^{-3}$ min^{-1}). Thus, this curve 5 may be taken as the control curve representing the native $tRNA^{Phe}$ having 100% s^2T but no s^4U. The 310-nm band then decreased very slowly, as an indication of the degradation of the s^2T residues, but could still be observed even after 500 h, as shown by curve 10 in Figure 6a. A very long period of time must be necessary for obtaining the CD curve representing the $tRNA^{Phe}$, following the complete degradation of the s^2T residues. Therefore, this curve was substituted by the curve of *E. coli* $tRNA^{Phe}$, the s^4U of which was completely lost in the reaction with H_2O_2. It is shown as curve 11 in Figure 6a. This procedure may be justified by the observation that $tRNA^{Phe}$'s from *T. thermophilus* and *E. coli* are very similar in their secondary structures as well as in their base compositions, except for the presence or absence of s^2T (Watanabe et al., 1980, and unpublished results).

The remaining content of s^2T (χ) may be calculated from the CD spectra in Figure 6a as

$$\chi = \frac{[\theta](\text{each curve at each time}) - [\theta](\text{curve 11})}{[\theta](\text{curve 5}) - [\theta](\text{curve 11})}$$

where $[\theta]$ is taken at 310 nm. The validity of this equation was confirmed by LC analysis. After 360 h of reaction, a portion of the sample solution was taken up and loaded onto the chromatograph after degrading it into nucleosides. The base conposition of the $tRNA^{Phe}$ sample was calculated from the elution profile of LC as reported (Watanabe et al., 1980), together with that of the intact $tRNA^{Phe}$ before reaction. The results are shown in Table II. Almost all nucleosides, except for s^4U, m^7G, s^2T, and cytidine, remained unchanged in their content before and after reaction. m^7G and s^4U were almost or completely lost in the $tRNA^{Phe}$ after reaction, and, in addition, about 1 mol of cytidine residue out of 22 mol of the original residues reacted with H_2O_2. The remaining content of s^2T was 0.57, which is in good agreement with the content (0.6) determined by the CD method as above (see the arrow

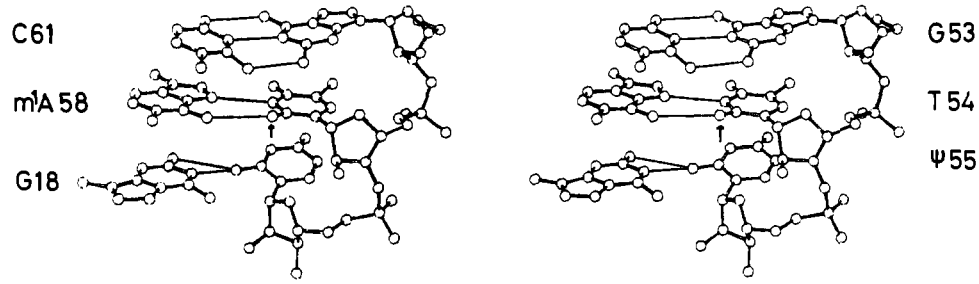

FIGURE 7: Stereoview of base pairing and stacking around T54, m¹A58, Ψ55, G18, G53, and C61 in the crystal structure of yeast $tRNA^{Phe}$ (Ladner et al., 1975b). The arrows show the O-2 of T54, which is replaced by the S-2 of s^2T54 in the case of *T. thermophilus* $tRNA^{Phe}$.

in Figure 5). Thus, the CD method using the above equation was applied for calculating the remaining content of s^2T during the reaction. The time course of the reaction is depicted by curve 2 in Figure 5. This reaction appears to proceed in a two-step manner: up to 80 h, the reaction proceeds linearly at a rate constant $k_{initial}$ of 0.06×10^{-3} min^{-1}, and then the reaction rate lowers to $k_{final} = 0.02 \times 10^{-3}$ min^{-1} (Table I). Even the initial rate constant is only about one-half of the $k_{initial}$ of poly(s^2U) (0.11×10^{-3} min^{-1}). Since the reactivity of s^2U and s^2T is almost the same at the nucleoside level, this difference should be ascribable to the different environments of the C=S groups of s^2U and s^2T in the polynucleotide chain.

There is a possibility that during a long reaction period H_2O_2 may lose its potent oxidative ability, so that apparently the reactivity of s^2T in the native $tRNA^{Phe}$ is lowered, resulting in the very small rate constant. Such a situation was avoided in the following experiment. The sample solution, after 500 h of reaction, was divided into two portions; one sample was heated directly in the presence of H_2O_2, while the other sample was dialyzed once against water to remove any H_2O_2, supplied with the buffer again (without H_2O_2), and then heated. In the preceding case, the 310-nm band disappeared at 65 °C and was not recovered even when the solution was cooled to room temperature, showing that H_2O_2 is in fact potent enough to destroy the s^2T residue completely. But in this case, the heating profiles of the sample were similar to those reported for $tRNA_f^{Met}$ (Watanabe et al., 1976a). The 310-nm band disappeared at 83 °C, and, instead, a new negative band appeared at 325 nm which showed the exposure of the s^2T residue out of the tRNA structure due to heat denaturation. When the sample solution was cooled, the 310-nm band reappeared, with the small negative band still being left (data not shown). This clearly indicates that the intact s^2T residue exists in the tRNA sample after 500 h of reaction, and also that H_2O_2 did not damage very much the tRNA molecule as a whole.

Charging Activity of the H_2O_2-Treated $tRNA^{Phe}$. $tRNA^{Phe}$ reacting with H_2O_2 was investigated for its phenylalanine acceptor activity. At an appropriate time, an aliquot of the sample solution was taken up, and an excess of mercaptoethanol was added to neutralize the H_2O_2 activity. The mixture was then checked for its [^{14}C]phenylalanine acceptor activity. The results, indicated by open triangles, are added to curve 2, in Figure 5. $tRNA^{Phe}$ was still almost fully active (more than 95%) after 3 h of reaction and had about 90% of its activity even after 10 h of reaction time. The charging activity showed good correlation with the residual content of s^2T in $tRNA^{Phe}$, suggesting that the $tRNA^{Phe}$ losing the intact s^2T residue is no more chargeable (see Discussion).

Discussion

Desulfurization of thiopyrimidines has been well investigated by using cyanogen bromide at the nucleoside level as well as in polynucleotides (Saneyoshi & Nishimura, 1967, 1970, 1971; Walker & RajBhandary, 1972; Agris et al., 1973; Singhal, 1974). The reaction is highly specific, but it yields thiocyanato derivatives of uridine nucleosides, which still possess the CD band above 300 nm (Saneyoshi et al., 1972). Thus, this reagent is not suitable for a quantitative analysis of the reaction process. In the present study, H_2O_2 was used as the reagent, but in this case side reactions, such as alteration of nucleoside other than thiopyrimidines or polynucleotide chain scission, can occur, especially in the presence of metal ions (Phaese et al., 1968), under alkaline conditions or at high temperatures (Priess & Zillig, 1965; Subbaraman et al., 1971), although these reaction rates are much lower than those with thiopyrimidines.

In order to suppress these side reactions, EDTA was added to prevent any activity of contaminating metal ions (Melzer & Tomlinson, 1966). The H_2O_2 concentration was lowered to 0.1 M, and the reaction was carried out at 20 °C. Under these conditions, tRNA remained almost intact for 3 h of reaction time, and even after 360 h only 1 mol of C and m^7G residues was attacked, in addition to the thiouridines (Table II). Thus, under suitable conditions, H_2O_2 serves as a useful reagent for modifying thiouridine residues selectively in the polynucleotide chain, making it possible to carry out a quantitative analysis of the reaction process.

The H_2O_2-altered form of s^4U was found to be uridine, but that of s^2T could not be identified. From the elution pattern of the H_2O_2-altered form in LC, the nucleoside seems to be a s^2T derivative wherein the sulfur atom has been modified. The observation that the extinction coefficient and the elution position of the nucleoside in LC appeared to be similar to those of uridine (Figure 2) precludes the possibility that the altered form of s^2T is a ring-opened or a disulfide form.

For examination of the reactivity of s^2T in both native and denatured $tRNA^{Phe}$, poly(s^2U) was used as a control, since it has both free and hydrogen-bonded sulfur atoms in the double-stranded helix (Figure 4c). In the tertiary structure of yeast $tRNA^{Phe}$, T54 is base paired with m^1A58, and this base pair is sandwiched between the neighboring base pairs, Ψ55–G18 and G53–C61. These three base pairs form nearly a part of the RNA double helix as shown in Figure 7. If we assume that tRNA takes on a common tertiary structure (Kim et al., 1974), we can superimpose s^2T54 on T54 and Gm18 on G18 on the structure of Figure 7, in the case of *T. thermophilus* $tRNA^{Phe}$. Thus, it is evident that the H_2O_2 molecule can approach the C=S group of s^2T from above or below the base plane; in particular, there is a considerable amount of space between the s^2T–m^1A and Ψ–Gm base pairs, which can assist H_2O_2 in approaching the C=S group. Therefore, it is considered that the availability of the sulfur atom of s^2T in the native $tRNA^{Phe}$ for reaction would be sterically equivalent to that of s^2U residues in poly(s^2U).

Contrary to expectation, poly(s^2U) seems to unfold easily by H_2O_2 attack, as Scheit & Faerber (1973) reported, by using

Table III: Comparison of Chemical Shifts of Methyl and Imino Protons of Modified Nucleosides in the Three Species of tRNA

tRNA	form	chemical shifts (ppm)				
		methyl proton			imino proton	
		T54(s^2T54)	m^1A58	ref	T54(s^2T54)(3NH)	ref
E. coli $tRNA_f^{Met}$	native	1.04		*a*	14.54	*c*
	denatured	1.70				
T. thermophilus $tRNA_f^{Met}$	native	1.04	3.73	*a*	14.58	*c*
	denatured	1.47	3.78			
yeast $tRNA^{Phe}$	native	1.01, 1.49	3.78	*b*	14.30	*d*
	denatured	1.78	3.86			

[a] Davanloo et al. (1979). [b] Kan et al. (1977). [c] Kyogoku et al. (1977). [d] Reid (1979).

HOHgBzS. Thus, we could obtain only the rate constant of the free C=S group in the β chain; this rate constant must be the initial rate constant ($k_{initial} = 0.11 \times 10^{-3}$ min^{-1}) and has a value about twice that of s^2T, in the native $tRNA^{Phe}$.

The rate constant of s^2T in the native $tRNA^{Phe}$ became lower after 60 h of reaction time and remained constant for at least 3 weeks; in addition, 50% of the s^2T residue still remained unreacted following that time. These observations, together with the different initial rate constants of s^2T in $tRNA^{Phe}$ and s^2U in poly(s^2U) (even if the difference is not very much), suggest that these thiouridine residues have different microenvironments, determined either by $tRNA^{Phe}$ or by poly(s^2U). The s^2U in the β chain is not involved in hydrogen bonding, and the s^2T sulfur in the native $tRNA^{Phe}$ is very likely protected by some mechanisms.

Since the steric environment surrounding the s^2T54–m^1A58 base pair is probably not very different from that of the base pairs in double-stranded RNA as discussed above (Figure 7), the protection mechanism may be ascribable to hydrogen bonding. The availability of the hydrogen-bonded sulfur atom for reaction, should this be the case, may be explained by the fluctuation of hydrogen bonds in general, which has been observed even in the native double-stranded DNA (Paleček, 1976) and the following proposal by Bodell & Singer (1979). These authors reported that an electron pair not involved in hydrogen bonding of the hydrogen-bonded oxygens in G, T, and C of double-stranded DNA might be available for alkylation reaction. This situation could be considered to apply to the sulfur of s^2T in the native $tRNA^{Phe}$; consequently, such an electron pair might apparently increase the rate constant of s^2T, resulting in the small difference in the initial rate constants between s^2T in $tRNA^{Phe}$ and s^2U in poly(s^2U).

The residues in the native $tRNA^{Phe}$, which are altered by the reaction with H_2O_2 for 360 h, are s^2T (partially and time dependently altered), s^4U, m^7G, and one C (Table II). Chemical modification of s^4U and m^7G does not influence the charging activity of tRNA (Schimmel & Söll, 1979). If one C which is important for the activity, such as C75, is modified, the tRNA would lose the charging activity to a great extent. The results in Figure 5 show that this is not possible. Therefore, the charging activity of the H_2O_2-treated $tRNA^{Phe}$ can be considered to be well correlated with the residual amount of s^2T in $tRNA^{Phe}$, as shown in Figure 5. This may provide favorable evidence for the involvement of the s^2T sulfur in hydrogen bonding. If the sulfur is not involved in hydrogen bonding for the s^2T–m^1A base pair, the H_2O_2 reaction will not influence the base pair or the conformation of tRNA very much. In fact, the tRNA in which the s^4U residue was modified with cyanogen bromide or further desulfurized by heat or alkaline treatment was still fully active as an amino acid acceptor and in the translation steps as well (Saneyoshi & Nishimura, 1967, 1971; Walker & RajBhandary, 1972). On the other hand, if the s^2T sulfur is hydrogen bonded, the H_2O_2 reaction will break the s^2T–m^1A base pair, thereby inducing a conformational change in tRNA to some extent. This tRNA would no longer remain in its native conformation, with the result that its amino acid acceptor activity would be lost. The results in Figure 5 are explained well on the basis of this assumption.

There is evidence deduced from ^{1}H NMR to indicate that s^2T54 is base paired with m^1A58 in the same manner as T54–m^1A58 base pair in the crystal structure of yeast $tRNA^{Phe}$ (Kyogoku et al., 1977; Davanloo et al., 1979). As shown in Table III, the chemical shift of the methyl proton for s^2T54 in *T. thermophilus* $tRNA_f^{Met}$ (Watanabe et al., 1979b) is the same as that for T54 in *E. coli* $tRNA_f^{Met}$ and also very similar to that for T54 in yeast $tRNA^{Phe}$. The same situation holds for chemical shifts of the methyl protons of m^1A in both *T. thermophilus* $tRNA_f^{Met}$ and yeast $tRNA^{Phe}$. These shifts are dependent to a small extent on the nucleotide sequences around m^1A. The chemical shift of the imino proton for s^2T54 in *T. thermophilus* $tRNA_f^{Met}$ has a value similar to those for T54 in *E. coli* $tRNA_f^{Met}$ and yeast $tRNA^{Phe}$. These results suggest that not only the microenvironments surrounding s^2T54 and m^1A58 but also the manner of base pairing between these residues are the same for *T. thermophilus* $tRNA_f^{Met}$, *E. coli* $tRNA_f^{Met}$, and yeast $tRNA^{Phe}$.

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