Kinetics of Bisulfite-Induced Cytosine Deamination in Single-Stranded DNA†

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Received June 10, 1992; Revised Manuscript Received January 20, 1993

ABSTRACT: The rate of bisulfite-induced deamination of cytosine to uracil in single-stranded (ss) DNA at physiological temperature and pH was monitored by a sensitive genetic assay. The assay is based on reversion of a mutation in the *lacZα* gene of bacteriophage M13mp2 and employs *ung*⁻ (NR9404) and *ung*⁺ (MC1061) bacterial strains which are isogenic except for uracil glycosylase activity. For ss DNA incubated with 1–50 mM bisulfite and transfected into an *ung*⁻ cell strain, the reversion frequency increased linearly with time of incubation and with concentration of bisulfite. Of 54 revertants sequenced, all were C → T transitions. Reduction in reversion frequency upon transfecting ss DNA into *ung*⁺ cells indicated that the majority of mutations were occurring via a uracil intermediate. Assuming that all revertants arose via uracil, the pseudo-first-order rate constant for deamination in 10 mM sodium bisulfite and 10 mM Hepes-NaOH, pH 7.4, at 37 °C as measured by transfecting into an *ung*⁻ cell strain was 3.5×10^{-10} s^{-1}, as compared to a spontaneous background rate constant of 0.6×10^{-10} s^{-1} in buffer alone.

Bisulfites are used as preservatives in foods and pharmaceuticals and have long been known to cause deamination of cytosine to uracil (Hayatsu et al. 1970; Garrett & Tsau, 1972; Shapiro, 1983), especially at acidic pH. Yet, even though sodium bisulfite is ingested by humans and is considered to be mutagenic at acidic pH, the rate of bisulfite-induced deamination of cytosine in DNA has never been measured under physiologically relevant conditions (37 °C, pH 7.4). Standard chemical methods of analysis have not had the sensitivity to detect the low level of deamination expected. Even though the rates would be slow, the reaction could have considerable mutagenic consequences since the products of deamination (arising from the natural bases cytosine or 5-methylcytosine) would be uracil or thymine, either of which would cause a mutation if not repaired.

The assay described by Frederico et al. (1990) for accurately assessing the rate of spontaneous hydrolytic deamination of cytosine can be used to examine the kinetics of bisulfite-induced deamination at physiologically relevant conditions. The detection system is based on reversion of a mutant of bacteriophage M13mp2 from a colorless to a blue plaque phenotype, resulting from a C → T transition within the *lacZα* gene coding sequence. This approach is highly sensitive; deamination of a single cytosine residue in the M13mp2 genome can be detected at a sensitivity of one deamination event in 10^5–10^6 molecules. In this report, we demonstrate that bisulfite-induced deamination of cytosine can be detected at 37 °C, pH 7.4, and rate constants for the C → U reaction can be calculated.

EXPERIMENTAL PROCEDURES

Materials

Isogenic *Escherichia coli* strains MC1061 [*hsdR mcrB araD139* Δ(*araABC–leu*)*7679*Δ*lacX74 galU galK rpsL thi*] and NR9404 [*hsdR mcrB araD139* Δ(*araABC–leu*)-*7679*Δ*lacX74 galU galK rpsL thi ung1*] to be used as the host cells for transfection of single-stranded (ss) M13mp2C141 DNA were obtained from Tom Kunkel (NIEHS). For visualization of plaque phenotype, cells were plated onto minimal media plates containing *E. coli* strain NR 9099 [Δ-(*pro–lac*) *recA56 ara thi*/F′ (*proAB*, *lacI*^{q}*Z*ΔM15)] obtained from Roeland Schaaper (NIEHS). The mutant M13mp2C141 was the same as that used in Frederico et al. (1990). Sodium metabisulfite anhydride ($Na_2S_2O_5$) was from Sigma Chemical Co. Sephadex G-50 was from Pharmacia, Inc. All other reagents were from previously described sources (Frederico et al., 1990).

Methods

(*A*) *Preparation of DNA.* Single-stranded M13mp2C141 DNA substrate was prepared as described (Frederico et al., 1990) and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) to a final concentration of 2000 ng/μL.

(*B*) *Incubation of DNA with Various Concentrations of Bisulfite.* Buffer solutions (1 mL) containing 10 mM Hepes-NaOH, pH 7.4, and various concentrations of $NaHSO_3$ were prepared by mixing appropriate amounts of MilliQ deionized water, 1.5 M bisulfite, and 250 mM Hepes and adjusting each mixture to a final pH of 7.4 with NaOH. The bisulfite solution was prepared by adding anhydrous sodium metabisulfite, $Na_2S_2O_5$, to water immediately before mixing with the Hepes. Each buffer was sterilized by filtration through a Millex-GV (0.22 μm, Millipore) membrane into a 1.5-mL polypropylene Eppendorf tube. Then, ss C141 DNA was added to give a final DNA concentration of 20 ng/μL. Each sample (100 μL) was incubated in a sterile glass capillary tube that was placed in a 20- × 150-mm glass culture tube which was capped and then wrapped with aluminum foil. Samples were incubated up to 27 days in the dark in a 37 ± 1 °C water bath. Prior to transfection, the incubated DNA was desalted by centrifuging at 1600 rpm for 4 min through a 1-mL Sephadex G-50 column (made by presterilizing a mixture of 6 g of Sephadex G-50 equilibrated with 100 mL of 10 mM Hepes-NaOH, pH 7.4) and collecting the filtrate; this step separated bisulfite and other small molecules from the DNA, thereby eliminating any possibility of their interfering with the subsequent transfection. The DNA was either used immediately or stored at −70 °C until transfection.

(*C*) *Transfection and Plating.* Transfection was carried out by electroporation, rather than with $CaCl_2$-treated cells

† Supported by American Cancer Society Grant CN-9.

0006-2960/93/0432-3535$04.00/0

as previously used by Frederico et al. (1990), with a System BTX-Electro Cell Manipulator 600 and Bio-Rad Gene Pulser cuvettes (having a 2-mL volume and 2-mm gap between electrodes). One microliter of DNA sample (20 ng/μL) was added to a 50-μL aliquot of NR9404 (*ung*$^-$) or MC1061 (*ung*$^+$) cells (that had been frozen at −70 °C in 10% glycerol, thawed for 10 min at room temperature, and placed on ice before using). The cell/DNA mixture was placed into a chilled cuvette, which was then placed in the BTX safety chamber, and 2.00×10^3 V was applied with a pulse time of 0.01×10^{-3} s. Following the pulse, the cells were immediately mixed with 2 mL of outgrowth medium SOC (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) in a 10- × 100-mm glass test tube. Plating with NR9099 cells and quantitation followed Frederico et al. (1990). In our experience, camouflage of the wild-type phenotype can occur if the number of plaques is greater than 10 000 plaques per plate. Plating conditions were carefully controlled (by diluting samples prior to electroporation to 10–20 ng/μL in order to obtain approximately 5000–7000 plaques per plate), thus ensuring that loss of visualization of wild-type revertants would not occur.

The efficiency of transfection averaged 2000 plaques/ng for unheated control ss C141DNA and varied 2–5-fold from one experiment to another. Efficiency dropped to about 10% of these levels for DNA incubated for longer times at the higher bisulfite concentrations, indicating loss of viability. If deamination to uracil (followed by replication where U codes like T) is the major cause of C → T mutations, then transfection into *ung*$^+$ cells (MC1061) should produce only colorless plaque phenotypes while *ung*$^-$ cells (NR9404) should produce both wild-type blue revertant and colorless plaque phenotypes. In *ung*$^+$ cells, uracil residues (including those resulting from deamination of cytosine) are removed from DNA by uracil glycosylase to produce an abasic site, which gives rise to biologically inactive M13 DNA in the transfection assay. Hence, *ung*$^+$ gives only colorless plaques. Conversely, the *ung*$^-$ cell strain will retain the uracil residues (that can code like thymine) and revertants having the wild-type blue phenotype will result.

(D) Scoring Revertants and Determining DNA Sequences. C141 revertant plaques appear as dark blue plaques against a colorless background. For each DNA sample, at least 10 revertant plaques were picked from the plates, diluted in 50 mM sodium borate, and replated to eliminate false positives. Out of every 100 plaques chosen, 99 were confirmed to be wild-type blue. To determine the specific base change responsible for reversion, DNA sequencing of randomly selected plaques from each experiment was performed using the chain terminator method (Sanger et al., 1977).

(E) Calculation of Rate Constants. For the cytidine nucleoside *monomer* at high temperatures and neutral pH, the bisulfite-induced deamination is a pseudo-first-order reaction under conditions where the concentration of bisulfite is much greater than that of cytidine (Slae & Shapiro, 1978). Deamination of cytosine *in* DNA here should follow similar kinetics since incubations were carried out with 8×10^{-9} mM DNA and an excess (1–50 mM) of bisulfite. The rate of formation of uracil from deamination of cytosine follows

$$d[U]/dt = k[C][HSO_3^-] \quad (1)$$

where [U] is the concentration of uracil formed at sites 141 and 142, [C] is the concentration of cytosine at sites 141 and 142 in M13mp2C141 DNA, $[HSO_3^-]$ is the concentration of bisulfite, and t is the time of incubation. Since $[HSO_3^-] \gg$ [DNA] and [C] is almost constant (i.e., <0.01% change) during the time of incubation, eq 1 can be integrated to give

$$[U] = k[C][HSO_3^-]t = k'[C]t \quad (2)$$

The reversion frequency F (i.e., mutant fraction) = 2[U]/([C] + [U]) since cytosine can deaminate equally at two sites (see Results). But since [U] ≪ [C],

$$[C] = 2[U]/F \quad (3)$$

Combining eqs 2 and 3 gives the pseudo-first-order rate constant

$$k' = F/2t \quad (4)$$

Thus, by measuring F, the fraction of blue plaques divided by the total number of plaques, and by knowing incubation time, we can obtain the rate constant from eq 4 for a particular buffer. This equation is based on four assumptions which are shown to be valid: (a) there is an equal probability of deaminating to yield a C → T transition at either site 141 or 142 (supported by sequencing data); (b) the bisulfite concentration does not change over the 27 days of incubation (supported by the consistency of rate constants in Table I determined at different times during the incubation); (c) bisulfite converts cytosine to uracil, which then yields C → T revertants (these can be detected by assaying the treated DNA in *ung*$^-$ cells; Frederico et al., 1990); and (d) uracil is the primary intermediate, as shown by removal of the majority of mutants in *ung*$^+$ cells.

RESULTS

Principles of the Assay for Cytosine Deamination. Bisulfite-induced deamination of cytosine to uracil is monitored by using a mutant derivative of the ss DNA bacteriophage M13mp2, which contains a G → C base change at position 141 of the *lacZ*α gene (in the 34th codon of the α peptide) (Frederico et al., 1990). This mutation changed the wild-type ^{141}GCC codon (alanine) to a ^{141}CCC codon (proline), which made the peptide unable to complement β-galactosidase activity in infected *E. coli* host cells. Thus, cells infected with M13mp2C141 mutant phage yield colorless plaques, whereas cells infected with the normal wild-type M13mp2 generate a blue phenotype when plated on X-gal indicator plates.

If a cytosine at positions 141 or 142 deaminates, and if the uracil-containing DNA is used to transfect a strain (NR9404) defective in repair of uracil, the blue phenotype will be restored (because the uracil codes like thymine during the first round of DNA replication in vivo and eventually generates, within the infected cell, progeny that contain either a ^{141}TCC or ^{141}CTC codon. These triplets encode serine and leucine, respectively, both of which result in a functional protein and produce a dark blue plaque phenotype.) Thus, induced deamination events (i.e., revertants) are scored as blue plaques in a field of colorless plaques. Molecules that have deaminated elsewhere but not at either of the two detectable sites will give colorless plaques.

Rate Constants of Bisulfite-Induced Deamination for ss C141 DNA at 37 °C, pH 7.4. M13mp2C141 ss DNA was incubated with increasing concentrations of sodium bisulfite for different time intervals in order to establish the kinetics of bisulfite-induced deamination under physiological conditions of pH and temperature. Following incubation and desalting, the DNA was transfected in the presence of X-Gal into either *ung*$^-$ or *ung*$^+$ host cells and deamination events (revertants) were scored as blue plaques. The reversion

Table I: Reversion Frequencies and Rate Constants in *ung*⁻ Cells of Single-Stranded DNA Incubated at 37 °C, pH 7.4

incubation time (days)	no. of sample	$[HSO_3^-]$ (mM)	revertants	total plaques (10^3)	reversion frequency[a] F (10^{-5})	efficiency[b] (plaques/ng of DNA)	rate constant[c] (per site) (10^{-10} s^{-1})
0		0.0 (buffer)	10	112	8.9	5600	
1	1	0.0 (buffer)	20	203	9.8	5100	0.54[d]
	2	1.0	12	107	11	5300	0.66
	3	2.5	12	102	12	2600	1.1
	4	5.0	14	95	15	2300	2.8
	5	10	13	79	16	2000	3.8
	6	50	15	76	20	1900	5.7
3	7	0.0 (buffer)	10	81	12	4100	0.87[d]
	8	1.0	16	88	18	4400	0.92
	9	2.5	16	73	22	3700	1.7
	10	5.0	14	51	27	2600	2.7
	11	10	13	41	32	2100	3.5
	12	50	16	39	41	2000	5.3
9	13	0.0 (buffer)	21	114	18	2900	0.61[d]
	14	1.0	18	58	31	2900	0.81
	15	2.5	16	44	36	2200	1.2
	16	5.0	28	47	60	2400	2.6
	17	10	13	18	72	900	3.5
	18	50	19	19	100	950	5.4
27	19	0.0 (buffer)	9	35	26	2800	0.36[d]
	20	1.0	29	48	60	2400	0.74
	21	2.5	17	17	100	850	1.6
	22	5.0	16	12	133	600	2.3
	23	10	21	11	191	850	3.4
	24	50	26	9.3	280	550	5.4

[a] After incubation of ss M13mp2 C141 DNA (20 ng/μL) at 37 °C in 10 mM Hepes-NaOH buffer, reversion frequencies (F) were determined by transfection into NR9404 *ung*⁻ cells as described under Experimental Procedures. Each F represents the total number of revertants divided by the total number of plaques obtained in three or more separate transfection experiments. The first row in each section is the background F (i.e., no bisulfite) for the corresponding incubation time. [b] The efficiency is defined as the total plaques obtained from a nanogram of DNA for a sample, averaged here for three or more transfection experiments. One microliter of sample ([DNA] = 10–20 ng/μL) was used for each transfection and the transfection mixture (about 2 mL) was equally distributed onto 10 medium plates. Thus, the number of plaques per plate should be double the efficiency. [c] For the buffer plus bisulfite, the pseudo-first-order rate constant per site is obtained from eq 4 after subtracting the reversion frequency of the buffer at the appropriate days of incubation. [d] For the buffer without bisulfite, the pseudo-first-order rate constant per site is obtained from eq 4 after subtracting the reversion frequency of the buffer at zero days of incubation.

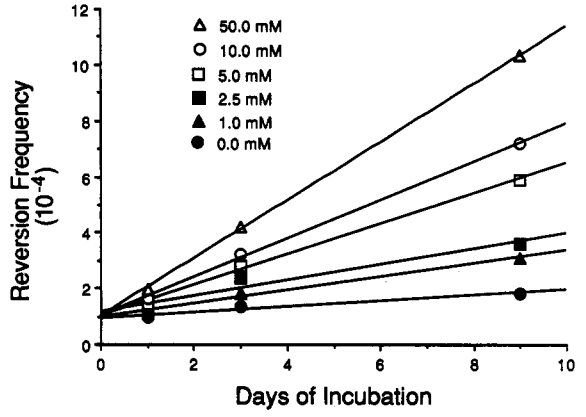

FIGURE 1: Reversion (C → T) frequency of ss M13mp2C141 DNA incubated with varying concentrations of sodium bisulfite in 10 mM Hepes buffer, pH 7.4, at 37 °C and transfected into *ung*⁻ cells.

frequencies and the rate constants of bisulfite-induced deamination are given in Table I.

Time Dependence of Bisulfite-Induced Deamination. For all concentrations of bisulfite (≤50 mM) at pH 7.4, the reversion frequency increased linearly with time (Figure 1). After 9 days of incubation in 10 mM bisulfite, the reversion frequency was 6-fold above the background level. Thus, the presence of bisulfite increases the probability of mutation, linearly over time, as expected for a pseudo-first-order reaction.

Dose Dependence and Rate Constants of Deamination. With bisulfite in large excess, the rate equation would appear pseudo-first-order, where $d[U]/dt = k'[C]$. When the fraction of cytosine converted to uracil (i.e., reversion frequency, F) in *ung*⁻ cells is plotted as a function of time for various concentrations of bisulfite (in Figure 1), the graphs for each concentration of bisulfite yield a straight line, each having a correlation coefficient greater than 0.95. From the slope of each line, the site-specific pseudo-first-order rate constants (k') can be obtained and are as follows: 0.60, 0.78, 1.4, 2.6, 3.6, and 5.5 × 10^{-10} s^{-1} for 0.0, 1.0, 2.5, 5.0, 10, and 50 mM bisulfite, respectively. Although the observed reversion frequencies and corresponding rate constants k_1' of deamination increase with increasing concentration of bisulfite (see Figure 1 and Table I), they are not directly proportional to the bisulfite concentration because deamination with bisulfite is a complex reaction, as shown in the proposed pathway of Shapiro (1983) in Figure 2. As expected, however, the data show a dose-dependent relationship between deamination and bisulfite concentration.

Confirmation of Deamination. Two approaches were used to determine whether or not the observed revertants were caused by deamination. (i) DNA samples incubated with 10 mM bisulfite were used to transfect both *ung*⁻ and *ung*⁺ host cells (see Table II). Reversion frequency was lowered over 2-fold in *ung*⁺ vs *ung*⁻ strains, indicating that the majority of the revertants were removed by cells containing uracil glycosylase. This decrease was observed in buffers with and without bisulfite and for DNA incubated for both 4 days and 27 days. Rate constants were found to be 3.5 × 10^{-10} s^{-1} upon transfection into an *ung*⁻ cell strain and 1.4 × 10^{-10} s^{-1} in an isogenic *ung*⁺ strain (Table II), as compared to a background rate constant of 0.6 × 10^{-10} s^{-1} in buffer alone (Table I). The

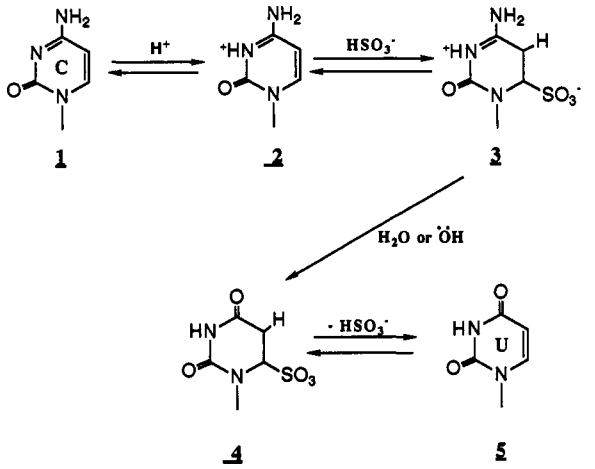

FIGURE 2: Proposed pathway for bisulfite-induced deamination of monomeric cytosine (C) to uracil (U) [from Shapiro (1983)]. Cytosine, **1**, with a pK_a near 4 (i.e., $pK_a = 4.17$ in the nucleoside and 4.56 as the 5′-nucleotide) (Blackburn & Gait, 1990), would be mostly in the unprotonated state at pH 7. Protonated cytosine, **2**, reacts with bisulfite by reversible addition across the 5,6 double bond to afford the dihydrocytosine intermediate, **3**. Deamination of **3** to the dihydrouracil derivative, **4**, is a step that is slower than the initial addition. This step is subject to general base catalysis, with bisulfite, sulfite, and pyrosulfite all acting as active catalysts. Thus, bisulfite plays an effective role in two separate steps in the reaction scheme, and deamination rates rise with increasing bisulfite concentration. The last step in the reaction is the loss of the added bisulfite to give uracil, **5**. The equilibrium constant for formation of the adduct, **4**, is high at neutral pH. Removal of the bisulfite is therefore needed to complete the conversion of **4** to **5**. The decomposition of the uracil–bisulfite adduct may require hours at neutral pH, but it is accelerated by alkali or by increased concentration of bisulfite (Slae & Shapiro, 1978).

Table II: Rate Constants for ss DNA in *ung*⁻ and *ung*⁺ Cell Strains

cell strain	incubation[a] (days)	[HSO_3^-] (mM)	plaques scored: blue	plaques scored: total (10^3)	reversion frequency (10^{-5})	rate constant per site[b] (10^{-10} s^{-1})
NR9404 (*ung*⁻)	0	0	10	112	9.0	*c*
	4	0	10	70	14	*c*
	4	10	12	31	39	3.6
	27	0	9	35	26	*c*
	27	10	26	14	186	3.4
average						3.5
MC1061 (*ung*⁺)	0	0	5	159	3.1	*c*
	0	10	6	54	11	
	4	0	11	146	7.5	*c*
	4	10	19	106	18	1.5
	27	0	9	84	11	*c*
	27	10	14	20	70	1.3
average						1.4

[a] All samples were 8×10^{-9} mM M13mp2C141 ss DNA in 10 mM Hepes buffer, pH 7.4, 37 °C. [b] The calculation of rate constants is the same as in Table I. [c] The background experiments were performed using the same DNA, under the same experimental conditions, but with no bisulfite.

reduction in reversion frequency upon transfection of treated DNA into *ung*⁺ cells (Table II) indicates that the majority of mutations are occurring via a uracil intermediate, consistent with the pathway (in Figure 2) proposed by Shapiro (1983). Bisulfite catalyzes cytosine deamination, generating uracil and regenerating bisulfite. However, there are a residual number of mutants in bisulfite-treated cells (Table II) that are not removed by uracil glycosylase, suggesting either that uracil glycosylase is not completely efficient or that an alternative, minor mechanism of mutation [that must also give C → T transitions; see (ii) below] may be operative. For example, bisulfite-generated free radical damage at our target site may not be repaired by uracil glycosylase and/or may lead to increased miscoding by DNA polymerase, but these are unlikely to contribute much to mutations under these conditions.

(ii) Further confirmation that mutagenesis proceeded via deamination was obtained by sequencing two or three revertants randomly chosen from each of the 24 samples appearing in Table I. Of the total 54 revertants, about half (28) were found to have a T at site 141, indicating deamination at that site. The remaining half (26) had a T at position 142. The equal probability of occurrence of thymine at sites 141 and 142 is expected for a random deamination mechanism. Further, the sequencing results show no correlation of the position of deamination with either the length of incubation or concentration of sodium bisulfite.

DISCUSSION

Bisulfite at very high (2 M) concentrations and acidic pH is known to induce deamination of cytosine to uracil in vitro (Shapiro, 1977); however, it is not known whether bisulfite is mutagenic under physiologically relevant conditions. Our measurements provide the first definitive evidence that millimolar concentrations of bisulfite will induce C → T transitions in ss DNA at pH 7.4 and 37 °C.

The reversion frequency increased linearly with time of incubation and showed a dose-dependent effect (Figure 1). The significant reduction in revertants upon transfecting bisulfite-treated DNA into *ung*⁺ cells indicates that the majority of mutations are occurring via a uracil intermediate (Table II). Further confirmation of deamination was obtained by sequencing DNA from revertant plaques. Every revertant sequenced (out of 54 total) was found to contain a C → T mutation. We conclude that bisulfite induces measurable rates of deamination of cytosine to uracil in DNA.

Deamination of cytosine in DNA by water (called "spontaneous" or hydrolytic deamination) is a common reaction in cells. An estimated 10^2–10^3 spontaneous deamination events occur in a human cell every day,[1] making deamination the second most probable endogenous, mutagenic reaction in cellular DNA (Shapiro, 1981). As shown here, bisulfite catalyzes that process in ss DNA at pH 7.4 and 37 °C. By extrapolation from Table I, it can be seen that 2 mM bisulfite would double the endogenous rate of deamination of ss DNA, and 10 mM bisulfite would result in a 6-fold rate increase. The pseudo-first-order rate constants can be compared with the other reported measurement of the rates of DNA deamination in vitro carried out by Frederico et al. (1990), where spontaneous deamination of cytosine in ssDNA at 37 °C was shown to be 100 times faster than that in dsDNA, with rate constants of 10^{-10} and $<10^{-12}$ s^{-1}, respectively. Since bisulfite increases the rate of deamination of ss DNA, it might be expected to also increase deamination of double-stranded DNA, which may be of greater consequence to the cell since cellular DNA is more often in the double-stranded form. Experiments with dsDNA are in progress.

Deamination rates depend somewhat on the buffer composition, and thus the actual rate constants will vary somewhat depending on the buffer conditions. For example, the

[1] Using the 37 °C experimentally determined rate constant of 1×10^{-12} s^{-1} for deamination of double-stranded DNA in vitro (Frederico et al., 1990) and 3×10^9 base pairs per genome, one can calculate that 10^2–10^3 spontaneous hydrolytic deamination events would occur per day per genome. This assumes, of course, that the cellular environment of DNA is similar to the incubation conditions used in the assay.

background value (0.6×10^{-10} s^{-1} in the absence of bisulfite) reported in Figure 1 for deamination of cytosine at 37 °C is slightly lower than the value (1.0×10^{-10} s^{-1}) reported previously by Frederico et al. (1990) (and also obtained by us, data not shown), which reflects a dependence of deamination on buffer composition.[2] This dependence on ionic strength is in accord with results of Shapiro and Klein (1966) and Shapiro et al. (1974), who observed that the rate of deamination of cytidine and cytosine *monomers* in different buffers varied up to 1.5-fold as the ionic strength increased from 0.01 to 0.2. (Note that their studies had to be carried out at 95 °C in acidic pH in order to obtain measurable quantities of uracil.) Nevertheless, for a given buffer, the deamination rate increases significantly in the presence of bisulfite.

Our assay combines both chemical and biological methods and consequently is highly informative. Damage by the chemical reagent is done in vitro on a defined target, and the subsequent processing and amplification of the DNA is carried out in vivo in cells with different repair capacities. This method has a number of attractive features. (1) By incubating DNA in vitro it is possible to carefully control all environmental variables and to eliminate other molecules and cellular components which could influence deamination. (2) Following incubation, the damaging agent can be removed from the DNA, so that only the damaged target DNA is processed by the cell. (3) By transfecting DNA molecules into a host cell, the damage event can be amplified by the host replicative machinery in vivo. The amplification can be carried out in cells with different repair backgrounds in order to sort out the mechanism and pathway of mutation as well as to obtain information on how the cell copes with a specific lesion. (4) Since deamination can be detected at a single cytosine target, the method allows measurements of site-specific, rather than average, rate constants. (5) The method is very sensitive and allows the calculation of macroscopic rate constants for very slow reactions like deamination. Since the method is amenable to statistical analysis, accurate but small rate constants can be calculated and the effect of exogenous agents, like bisulfite, can be quantitated.

Deamination of cytosine to uracil is mutagenic and, if unrepaired, results in C → T transitions, which are the most frequent type of mutation observed in certain cancer types (Harris, 1991). Since some of these undoubtedly are caused by deamination, any process that accelerates deamination may therefore increase mutagenesis. Hence, it is important to understand and quantitate the effects of various agents on DNA deamination. In cells with effective repair, hydrolytic deamination of cytosine in DNA is probably not mutagenic. However, mutations would be expected in a number of scenarios: (1) if damage exceeded the repair capacity of the cell; (2) if damage occurred in a region of chromatin inaccessible to repair; (3) if deamination of cytosine were catalyzed by a cross-strand effect [e.g., if its guanine pairing partner reacted with a carcinogen, hence labilizing the cytosine to deamination as described by Sowers et al. (1987) and Williams and Shaw (1987)], in which case a doubly mutated (U·X) base pair would result, such that neither base would retain the same genetic code as the original C·G base pair; or (4) if deamination occurred in 5-methylcytosine, which deaminates directly to thymine, in which case a T·G base pair would result. In the absence of a T·G repair mechanism (Lieb, 1985; Weibauer & Jiricny, 1990), the mutation would become fixed, as has been observed in *E. coli*, where 5-methylcytosine residues are hotspots for spontaneous transition mutations (Coulondre et al., 1978; Duncan & Miller, 1980).

In summary, we have used a sensitive assay to determine the rate of bisulfite-induced cytosine deamination in ssDNA under physiologically relevant conditions (37 °C, pH 7.4) and low (millimolar) bisulfite concentrations. The facts that bisulfite catalyzes deamination at rates exceeding those observed in ss DNA and is a prevalent environmental pollutant suggest that further study of its mechanism of action and effect on ds DNA, both in vitro and in vivo, might be important. Such studies are in progress.

ACKNOWLEDGMENT

We thank Ms. S. Barry and Dr. R. Moyer for reading the manuscript.

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[2] Frederico et al. (1990) employed a high ionic strength buffer (100 mM KCl, 50 mM Hepes-KOH, 10 mM $MgCl_2$, and 1 mM EDTA, pH 7.4, ionic strength 0.22), whereas the buffer used in the present study was low ionic strength (10 mM Hepes-NaOH, pH 7.4, ionic strength 0.04).