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Degradation of DNA by bisulfite treatment

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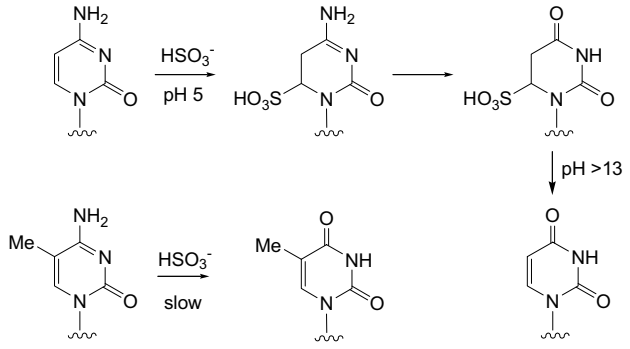
Abstract—A significant level of target degradation was caused by bisulfite treatment for methylcytosine-selective hydrolysis. The depyrimidination proceeded via addition of bisulfite to pyrimidines in DNA. The quantification with real-time PCR after conventional bisulfite treatment showed a large decrease in the amount of full-length DNA.

Gene expression is regulated by the epigenetic modification of biopolymers independent of their primary sequences. In particular, cytosine methylation is a significant modification of chromosomes because it closely relates with gene deactivation,[1] differentiation,[2] and carcinogenesis.[3] Therefore, the detection and quantification for cytosine methylation is mandatory for improving understanding of the functions of cytosine methylation in organisms and for developing early diagnosis.

The *bisulfite method* has been widely applied for epigenotyping at methylation hot spots and for the mapping of cytosine methylation in the promoter region because sodium bisulfite is inexpensive and harmless. In addition, PCR amplification and sequencing after bisulfite treatment ensure high sensitivity without complicated operations.[4] On the other hand, previous research has reported that the bisulfite reaction results in template degradation, although the mechanism and the extent of decomposition are currently unclear.[5]

Herein, we report on DNA degradation caused by treatment with sodium bisulfite. Rate constants for the decomposition processes were determined. A small quantity, but a significant level, of pyrimidine bisulfite adducts was converted into abasic sites via an *N*-glycoside bond cleavage.

The reactions of DNA bases with sodium bisulfite are summarized in Scheme 1. Sodium bisulfite causes the deamination of a cytosine residue in a single-stranded DNA through formation of a 5,6-dihydrocytosine-6-sulfonate intermediate at acidic pH. The deaminated bisulfite adduct is converted into a uracil residue through elimination of bisulfite at alkaline pH. 5-Methylcytosine also yields thymine with sodium bisulfite, but the reaction rate for the bisulfite adduct formation is much slower.[6] The difference in the rate of the adduct formation was exploited in the discrimination between cytosine and methylcytosine. To estimate the rate constants for a series of reactions, we used a common set of reaction conditions that involved treatment of the DNA with 3.1 M sodium bisulfite (pH 5.0), 125 mM hydroquinone, and 400 mM sodium hydroxide at 50 °C for 16 h.[7,8] Alkaline treatment to eliminate bisulfite was performed by adjusting the reaction mixture to pH > 13 with sodium hydroxide and incubating at 25 °C for 5 min. The



Scheme 1.

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rate constants were evaluated as pseudo-first-order reactions by monitoring absorption at 260 nm.

The reactivity of cytosine with sodium bisulfite was examined using **ODN(C8)**, 5′-d(CCCCCCCC)-3′. The absorption due to **ODN(C8)** rapidly decreased during incubation in the reaction mixture containing sodium bisulfite. This absorption loss results from bisulfite adduct formation (Fig. 1a).[9] The rate constant for cytosine was $1.6 \times 10^{-2}\ s^{-1}$ (Scheme 2 and Fig. 1a). The resulting bisulfite adduct is known to be subsequently transformed to uracil with a rate constant of $2.5 \times 10^{-4}\ s^{-1}$.[7,10] The absorption due to **ODN(C8)** completely disappeared within 10 min. The absorption due to **ODN(U8)**, 5′-d(UUUUUUUU)-3′, also disappeared after 15 min incubation in sodium bisulfite solution (Fig. 1b). On the other hand, the adduct formation of thymines was slower. Forty percentage of the absorption of **ODN(T8)**, 5′-d(TTTTTTTT)-3′, still remained even after incubation in sodium bisulfite solution (Fig. 1c). In the case of purine bases in DNA, no decrease in the absorption of **ODN(A8)**, 5′-d(AAAAAAAA)-3′ and **ODN(G4)**, 5′-d(GAGAG GAA)-3′ was observed after incubation for 16 h. The stability of these oligopurine sequences indicates that purine bases and phosphodiester backbones of DNA are insensitive to bisulfite reaction conditions.

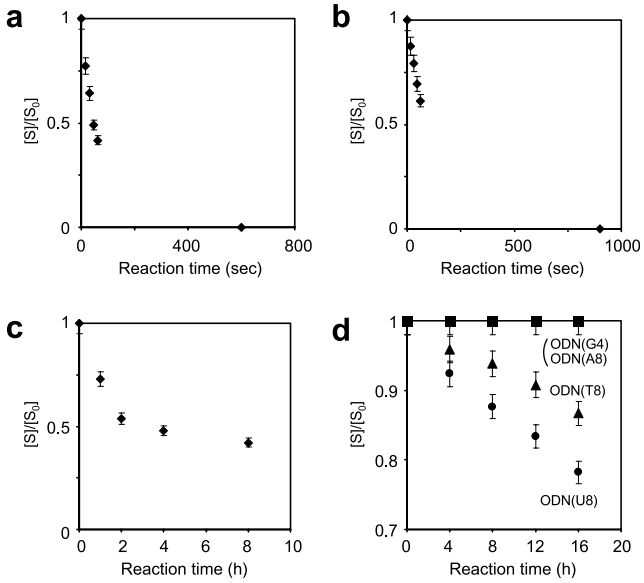
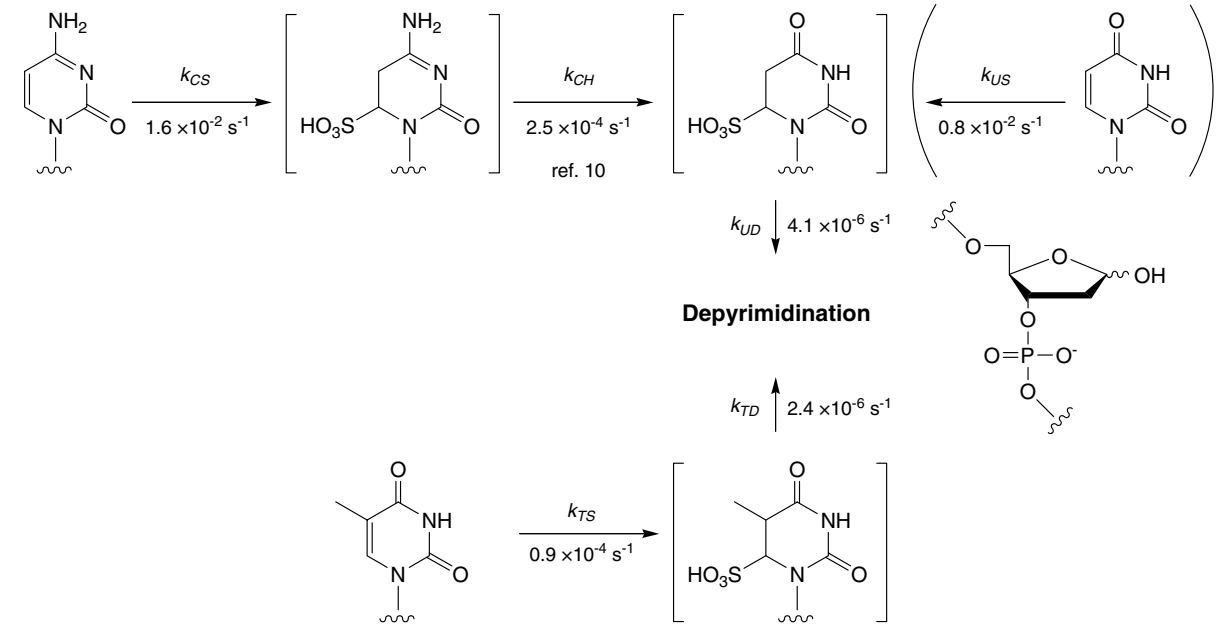


Figure 1. Time course for the decrease in the absorption of (a) **ODN(C8)**, (b) **ODN(U8)**, and (c) **ODN(T8)** ($\lambda = 260$ nm) by incubation with sodium bisulfite at 50 °C. The decrease in the absorption of pyrimidine bases was caused by bisulfite adduct formation. The reaction rate for bisulfite adduct formation was calculated from the pseudo-first-order plots. (d) The decrease in the absorption ($\lambda = 260$ nm) of ODNs after bisulfite treatment and subsequent alkaline treatment (5 min, 25 °C).

The adjustment of pH to alkaline values causes elimination of bisulfite from the adduct. We examined the recovery of **ODN(U8)** and **ODN(T8)** from their bisulfite adducts. The addition of 4 M sodium hydroxide to the reaction mixture and subsequent incubation for 5 min at 25 °C were carried out after bisulfite treatment. Twenty-three percentage of absorbance due to **ODN(U8)** and 12% of that due to **ODN(T8)** were unrecovered after 16 h incubation with sodium bisulfite followed by alkaline treatment (Fig. 1d). The amount of unrecoverable DNA increased with longer bisulfite incubation time. Therefore, the failure to return to the original sequences suggests that pyrimidine bases were damaged by incubation with sodium bisulfite. Little change in absorption was observed for **ODN(A8)** and **ODN(G4)**. From the plot of the decrease in absorption versus the reaction time of bisulfite treatment, the decomposition rate constants of pyrimidine bases were determined and are shown in Scheme 2. The rate constants for the bisulfite



Scheme 2.

adducts of uracil and thymine were calculated as $k_{UD} = 4.1 \times 10^{-6}$ and $k_{TD} = 2.4 \times 10^{-6}\ s^{-1}$, respectively. The rate constants k_{UD} and k_{TD} are significant values, which also indicate the decomposition rate constants of the adducts formed by sulfonation and deamination from cytosine and 5-methylcytosine, respectively.

The reaction product formed by incubation with sodium bisulfite was analyzed with MALDI-TOF-MS. The mass spectrum after treatment of **ODN(C1)**, 5′-d(AAAACAAAA)-3′, with bisulfite exhibited not only the production of the bisulfite adduct as a major product ($[M-H]^-$, calcd 2814.93, found 2814.05) but the generation of an abasic site after depyrimidination ($[M-H+2H_2O]^-$, calcd 2675.81, found 2675.85). The mass spectrometric analysis for **ODN(T1)**, 5′-d(AAAATAAAA)-3′, also showed the formation of the bisulfite adduct ($[M-H]^-$, calcd 2827.94, found 2827.68) and an abasic site ($[M-H]^-$, calcd 2638.78, found 2638.54). The decrease in the absorption of DNA observed after alkaline treatment is due to the production of abasic sites through the cleavage of an *N*-glycosyl bond, that is, depyrimidination. Prolonged bisulfite treatment of DNA generates abasic sites due to depyrimidination, rather than long-suspected depurination.[4] Abasic sites are known to cause strand scission readily under heating or alkaline conditions.[11] The DNA fragmentation at abasic sites generated from C and T residues may affect the accuracy of the methylation status quantification using the bisulfite method.

DNA is damaged at pyrimidine sites during reaction with bisulfite, as described above. The amount of target degradation during bisulfite incubation was quantified with real-time PCR (Fig. 2).[12] Bisulfite treatment of a 100-mer DNA, which was designed to prevent primer-binding regions from C → U conversion during reaction, was executed for 16 h under the reaction conditions used above. The threshold cycle (C_T), which was the first cycle in which a signal was detectable, of the PCR samples was fitted to the plots of the standards, and the number of original copies was determined. The value of C_T for the sample without bisulfite treatment corresponded with the standard sample ($C_T \approx 9$, corresponding to 10^{14} copies). On the other hand, after bisulfite treatment for 16 h, this value increased to approximately 20. Only *ca.* 10^{11} copies as PCR-possible DNA remained in the sample solution after 16 h bisulfite incubation. This is 0.1% of the original DNA amount.

In conclusion, we have described the decomposition of pyrimidines in DNA after bisulfite treatment and the quantification of the decomposed product. Hydrolysis of cytosine to uracil using sodium bisulfite is an effective reaction for discriminating between cytosine and methylcytosine. However, a critical level of degradation was caused via depyrimidination during bisulfite treatment. Lengthy incubation with bisulfite produced abasic sites, which resulted in strand damage. Even if most of the DNA sample were damaged, gene analysis would still be possible because the survival sample is amplified with PCR. However, the DNA sample should be carefully treated for methylation status quantification, and pleiotropic analysis including chemical or molecular biological techniques is essential to obtain more reliable methylation data.

a Forward primer: 5'-TGGAAAATTTGTTAGAGTTA-3'
Reverse primer: 5'-TACTATTAACCAACTAAAAA-3'
Target:
5'-TGGAAAATTTGTTAGAGTTACTTTGCCTCTGTGTCATATT
TGGATACACAGGTCGGCCGCCTCTTGAGTGCTTTGGACGA
TTTTTAGTTGGTTAATAGTA-3'

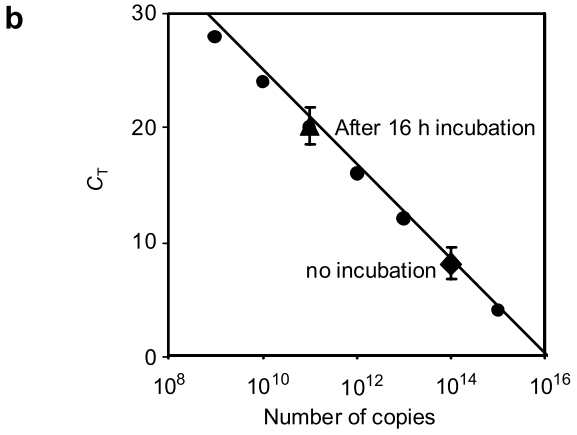


Figure 2. Quantification of the full-length DNA with real-time PCR. (a) Target DNA and PCR primers used in this experiment. (b) Quantification of the full-length target DNA before/after a 16 h bisulfite incubation. The target DNA (10^{14} copies) before/after a 16 h bisulfite incubation was amplified in a buffer solution containing primers, SYBR Green I, dNTPs mix, and *TaKaRa Ex Taq* polymerase. See Ref. 12 for experimental information on the detailed PCR conditions. The threshold cycle (C_T) was determined as the first detectable cycle from the amplification curves. A 10-fold dilution series, from approximately 10^{15} to 10^9 copies of the target DNA, is shown as a standard plot.

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8. *Bisulfite treatment.* The sample DNA (50 μM) was incubated in a reaction mixture (20 μL) containing 3.1 M sodium bisulfite (pH 5.0), 125 mM hydroquinone, and 400 mM sodium hydroxide at 50 °C. After a definite time of incubation, 20 μL of 4 M sodium hydroxide was added. After the incubation at 25 °C for 5 min, 40 μL of glacial acetic acid was added. The rate constants were evaluated as pseudo-first-order reactions by monitoring absorption at 260 nm.
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12. *Real-time PCR.* The PCR cocktail was assembled as follows: 1 μL of target-containing solution (target DNA was 10^{14} copies) before/after a 16 h bisulfite incubation, 2.5 μL of 10 μM each primer, 2.5 μL of 1/5000× SYBR Green I solution in DMSO, 2 μL of 2.5 mM dNTP mix, 10× attached buffer, 1.25 U *TaKaRa Ex Taq* polymerase, and deionized water to a final volume of 25 μL. The PCR was performed by denaturing at 95 °C for 5 s and annealing at 60 °C for 10 s. The extension step was at 72 °C for 15 s. The process of PCR amplification was monitored by the fluorescence of SYBR Green I, and the threshold cycle (C_T) was determined as the first detectable cycle from the amplification curves.