Recognition of a cytosine base lesion by a human damage-specific DNA binding protein

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ABSTRACT

Sodium bisulfite reacts with cytosine and 5-methylcytosine, forming the 5,6-dihydrosulfonate adducts which deaminate to the uracil and thymine adducts, respectively. At alkaline pH, the sulfonate groups are then released, generating uracil and thymine. In DNA, the resulting G:U and G:T base mismatches generated are potential sites of mutagenesis. Using a human damage-specific DNA binding protein as a probe, we have found protein-recognizable lesions in bisulfite-treated DNA and poly d(I-C), but not in treated poly d(A-T) or poly d(A-U). Although this suggests that the lesion recognized is cytosine-derived, there was no correlation between the number of uracils induced and the number of binding sites, suggesting that the protein-bound damage is not a uracil-containing mismatch. Modification of the treatment protocol to reduce elimination of the bisulfite from the base adducts increased the level of binding, suggesting that the protein recognizes a base-sulfonate adduct.

INTRODUCTION

Sodium bisulfite reacts reversibly at the 5,6 carbon-carbon double bond of the pyrimidine bases of nucleic acids, to form the corresponding 5,6-dihydro-6-sulfonate adducts, and subsequently promotes deamination of the cytosine and 5-methyl-cytosine adducts to the uracil and thymine adducts, respectively (1). While pyrimidine-sulfonate adducts are stable under acidic conditions, they decompose at high pH, regenerating the pyrimidine bases (2). Thus, an experimental protocol in which DNA or a synthetic polynucleotide is reacted with bisulfite under acidic conditions, then exposed to base and dialyzed to remove the free bisulfite, should cause only two permanent changes in the substrate, the conversion of cytosine and 5-methylcytosine to uracil and thymine. This reaction sequence is indicated in Fig. 1. These reactions are generally restricted to single-stranded regions of polynucleotides (3) and are favored by low pH and high (>1M) bisulfite concentrations (2). Bisulfite is often employed in targeted mutagenesis studies (4), for the base substitutions it induces will be mutagenic if unrepaired, directing the replacement of

Figure 1. Reaction of bisulfite with cytosine. At low pH, bisulfite adds across the 5,6-double bond of cytosine (A) to form the cytosine-sulfonate adduct (B). This adduct will spontaneously deaminate and at elevated pH the uracil-sulfonate adduct (C) decomposes to generate uracil (D).

a G:C base pair with an A:U base pair in one daughter molecule at the next round of DNA replication. In this regard, the 5-methylcytosine to thymine conversion, although it occurs at a much lower rate than the analogous conversion of cytosine residues to uracil residues (5), could be the more injurious to the cell, because it would escape recognition by the uracil DNA-glycosylase repair system (6).

The biological effects ensuing from treatment of cells or viruses with bisulfite are considered to be due primarily to bisulfite-catalyzed deamination reactions (7). G:C to A:T transition mutations are seen in microorganisms when the treatment conditions are chosen to favor the deamination reaction; that is, high bisulfite concentration and low pH (4,8). At lower bisulfite concentrations (1-10 mM) and neutral pH, bisulfite may not be directly mutagenic, although it appears to enhance the mutagenicity of other agents (9).

We are studying a binding protein isolated from human placenta which has the ability to recognize lesions introduced into DNA by a variety of agents, including UV light (10), γ -rays, a superoxide-generating system (11), a methylene-blue photooxidation system (12), hydrogen peroxide (13), nitrous acid and sodium bisulfite (14). The lesions recognized presumably reside in single base moieties of the DNA, since neither damages involving the backbone, such as strand breaks, single-stranded regions and apurinic sites, nor damages involving two bases, such as thymine dimers and psoralen diadducts, are substrates for the protein (10,14). In this paper, we present a further examination of the binding protein's ability to identify a lesion in nucleic

acids treated with bisulfite. The data indicate that the protein does not recognize the predominant lesion produced by bisulfite, the uracil base mispaired with guanine or inosine, but rather is specific for another form of damage, most probably sulfonate adducts.

MATERIALS AND METHODS

Materials Bacteriophage P22 DNA, [^3H]-labelled, was prepared according to the method of Botstein (15). Plasmid pBR322 was prepared as described by Davis *et al* (16). The tritium-labelled alternating copolymers, poly d(A-T) and poly d(A-U) were synthesized in *E. coli* DNA polymerase I-catalyzed reaction mixtures by the procedure of Schachman *et al* (17). Poly d(I-C) was synthesized by the method of Burd and Wells (18). All polymers were purified by chromatography through 1x15 cm columns of Sephacryl S-200. Poly d(A-T) and poly d(I-C) template primers for these reactions were purchased from Collaborative Research. Unlabelled deoxyribonucleoside tri-phosphates and calf thymus DNA were purchased from Sigma, while [^3H]-labelled deoxyribo-nucleoside triphosphates were obtained from New England Nuclear.

The binding protein used was the Fraction VI material purified from human placenta according to the procedure of Feldberg *et al* (13), and contained 0.35 mg protein per ml. Uracil-DNA glycosylase (UNG) was also purified from human placenta. *E. coli* polymerase I was obtained from P.L. Biochemicals and bovine serum albumin (BSA) from Sigma.

Assays The standard binding assay involved a 30 min incubation, at 0°C, of labelled P22 DNA or alternating copolymer with an aliquot of the binding protein, in a reaction buffer containing 10 mM Tris-HCl buffer pH 8 and 75 mM NaCl, in a total volume of 300 μl . After incubation, each assay mixture was diluted with 2 ml of cold 2xSSC and passed through a Millipore HAWP filter. Protein-substrate interaction was determined by counting the dried filters in 2 ml of scintillation fluid.

UNG activity was monitored by the release of acid-soluble radioactivity from a [^3H]-uracil-labelled poly d(A-U) substrate. Following incubation of enzyme and substrate at 37°C for 30 min in 50 mM potassium phosphate buffer pH 7.5 containing 15% glycerol, 2 mg/ml BSA and 0.5 M EDTA, in 60 μl total volume, the reaction tubes were chilled. Calf thymus DNA (0.02 mg) and 1N HCL (80 μl) were added to each tube. The tubes were then centrifuged for 15 min in an Eppendorf centrifuge, and aliquots of the resulting supernatants were counted in 2 ml of Baker RIA scintillation fluid. One unit was defined as the amount of enzyme necessary to release 1 nmole of uracil from poly d(A-U) under

Figure 2. Time Course of Bisulfite Treatment. (A) 5 nmoles of P22 DNA were treated as described in "Methods" for 0, 30, 60 or 120 minutes. Binding assays were performed on 0.18 nmoles of DNA with an excess of binding protein. Points shown are averages of triplicate assays, from which the filter binding in the absence of protein (<5%) has been subtracted.

●—● 2M bisulfite, Δ—Δ 2 M NaCl.

(B) 8.24 nmoles of poly d(I-C) were exposed to 2M bisulfite, 2 M sodium chloride, or no additions in acetate buffer as described in "Methods" for 0, 30, 60 or 120 minutes. Binding assays were performed on 0.1 nmole of polymer with a saturating amount for the 120 minute bisulfite sample. No-protein and 0 minute binding levels were subtracted. Values shown are averages of triplicate assays. ●—● 2 M bisulfite, Δ—Δ 2 M NaCl, □—□ no addition.

these conditions.

Bisulfite Treatments Bisulfite treatment of P22 DNA was carried out at 91°C in 0.10 M sodium citrate containing 2 M NaHSO₃ and titrated to pH 5.4. Following incubation, samples were dialyzed overnight against 0.10 M citrate buffer pH 5.4 at room temperature and then against 10 mM potassium phosphate pH 7.0 at 0°C for 6 hrs. Poly d(I-C) was treated with 2 M NaHSO₃ in 0.50 M sodium acetate, pH 5.4, at 52°C. Poly d(I-C) samples were dialyzed first against 10 mM Tris-HCl, pH 9, 10 mM NaCl, and then against 10 mM Tris-HCl, pH 8, 5 mM NaCl. Control samples were incubated in 2 M NaCl as described above. Solutions were purified through chelex-100 columns (Bio Rad) before use.

Determination of Alkali-Labile Sites/Uracil Residues. The removal of uracil residues by Uracil-DNA glycosylase was used to quantitate this residue since the AP sites generated are labile in base and will form strand breaks in alkaline sucrose gradients. Bisulfite or NaCl-treated polymers were applied to 5 ml linear gradients of 5 to 20% (w/vol) alkaline sucrose (0.3 M NaOH, 0.7 M NaCl, 1 mM EDTA) either directly or following an incubation with UNG (7 units, 2 hrs, 37°C). Samples were centrifuged at 4°C in an SW50.1 rotor along with Hind III-cleaved pBR322 DNA as a molecular weight marker. Alkali-labile sites and uracil residues were determined from number-average molecular weights obtained by the method of Charlesby (19).

Figure 3. Protein Saturation Curves for Bisulfite and UV-Irradiated P22 DNA. P22 DNA was exposed to either NaHSO₃ for 30 min or was irradiated to 60 J/m² and was then assayed with varying levels of binding protein.

● —●— NaHSO₃ △ —△— UV Irradiated

RESULTS

Introduction of Protein-Recognizable Lesions

When native P22 DNA and the alternating copolymer poly d(I-C) were treated with bisulfite at elevated temperatures, protein-recognizable lesions were introduced (Fig. 2) in a time-dependent manner. Control substrates, incubated with sodium chloride in place of sodium bisulfite, sustained comparatively few lesions recognized by the binding protein. The samples taken at each time point were assayed with a saturating amount of binding protein (figs. 3 & 4); thus the values obtained depended only on the level of damage present in the substrate. The basis of the binding assay is the ability of protein, but not of the [³H]-labelled double-stranded polynucleotides, to bind to the nitrocellulose filters. Therefore, only substrate bound by protein will remain on the filter, while the rest will pass through. The level of radioactivity on the filter thus is a sensitive, but indirect, measure of the extent of the binding reaction between the protein and the substrate. Although this assay can detect and quantitate low levels of base damage, it does suffer from two drawbacks. First, it is not possible to determine whether a given substrate molecule has interacted with more than one protein molecule. Second, the assay is sensitive to the size of the substrate molecule. Because the amount of label associated with a smaller substrate fragment will be less than that associated with a larger fragment, the assay is biased in favor of detection of lesions in larger molecules. Both of these factors contributed to the leveling off of the curves in Fig. 2 at less than

Figure 4. Protein Saturation Curves for Bisulfite-Treated Poly d(I-C). Poly d(I-C) was exposed to either NaHSO_3 or NaCl for the times indicated, then assayed with binding protein. The values shown represent specific binding, i.e., the binding levels of the NaCl -treated polymers have been subtracted from those of the NaHSO_3 -treated polymers. □—□ 0 minutes, ■—■ 10 minutes, ●—● 120 minutes.

100% binding. With poly d(I-C), the smaller molecules in the population distribution will be less likely to sustain a protein-binding lesion, while with both polymers the accumulation of strand breaks at long treatment times will tend to generate small fragments. The presence of such undamaged fragments will result in less than total binding.

Figure 3 shows protein saturation curves for P22 DNA treated with either bisulfite or with 254 nm radiation to the same total extent of binding. The similarity in the saturation curves suggests that the protein shows the same affinity for both UV and bisulfite-induced DNA lesions. In contrast to P22 DNA and poly d(I-C), neither poly d(A-U) nor poly d(A-T) showed any increased binding after bisulfite treatment, suggesting that the lesion recognized by the binding protein is derived from cytosine.

Comparison of Uracil Residues and Binding Sites Produced by Bisulfite

Since bisulfite treatment results in the conversion of cytosine to uracil, and the lesion recognized by the binding protein is derived from the cytosine base, we examined whether the protein was detecting a uracil-containing base mismatch. Table I gives the results of binding assays performed on bisulfite-treated DNA incubated either with or without a large excess of human uracil-DNA-glycosylase. This enzyme removes uracil residues from DNA, forming apyrimidinic sites, which are not substrates for the binding protein (14). Replacement of recognizable lesions by non-recognizable ones would decrease the level of interaction between the protein and the DNA. However, it is evident that binding was not significantly diminished by uracil-DNA glycosylase pre-treatment, which implies that mismatches containing uracil residues are not the sites recognized by the binding protein.

This conclusion was examined more rigorously by quantitating the numbers

Table I. Effect of Uracil-N-Glycosylase Pre-Treatment on Binding Levels

Treatment	UNG	Binding Protein	%cpm bound	binding sites per molecule
bisulfite	+	+	82	1.7
	-	+	71	1.2
sodium	+	+	32	0.39
chloride	-	+	29	0.34

DNA was treated with either 2 M NaHSO₃ or 2 M NaCl for 60 min as described in "Methods" and then treated with either 7 units of UNG or buffer for one hour at 37°C before assaying with binding protein. All assays were performed in duplicate.

of binding sites and uracils in bisulfite-treated poly d(I-C) and DNA. If uracil-containing mismatches are binding sites for the protein, there should be a direct correlation between the numbers of these lesions. Uracil-DNA glycosylase can be employed to enumerate the uracils present, since the AP sites generated by this enzyme are labile in base and will form strand breaks on alkaline sucrose gradients. The number of binding sites present per molecule of substrate can be calculated from protein-saturation data and the Poisson distribution,

$$m = \ln(1/P_0)$$

where m is the average number of protein-recognized sites per molecule and P₀

Table II. Enumeration of Base Lesions, Uracils, and Apurinic/Apyrimidinic Sites in Treated P22 DNA and Poly d(I-C)

Substrate	Treatment	Time(min)	alkali-labile sites per molecule	binding sites per molecule	uracil sites per molecule
poly d(I-C)	bisulfite	0	0	0	0
		5	0.5	ND*	5
		10	3	0	12
		120	22	2	153**
	sodium chloride	0	0	0	0
		120	2	0.2	0.1
P22 DNA	bisulfite	0	0	0	0
		60	24	0.6	2
	sodium chloride	0	0	0	0
		60	23	0.2	0

* not determined

** estimated value

Figure 5. Effect of Incubation Temperature. P22 DNA, incubated with either 2M sodium bisulfite or 2M sodium chloride in 0.5M acetate buffer at pH 5.4 for 60 minutes at either 0, 43, or 86°C, was employed as a substrate for the binding protein. The values shown are for specific binding. ■—■ 86°C, ○—○ 43°C, ●.....● 0°C.

is the fraction of substrate molecules not bound by the protein.

The outcome of binding assays and alkaline sucrose sedimentation patterns for bisulfite-treated poly d(I-C) are shown in Fig. 4 and summarized in Table II. From the binding assays (Fig. 4), it can be seen that 10 min exposure to bisulfite was ineffective, although a 120 min exposure produced approximately two binding lesions per molecule. The time course for uracil production, however, (Table II) did not coincide with that for binding sites. Short treatment times introduced considerable numbers of uracil residues, yet gave no protein-binding sites in the poly d(I-C). Bisulfite treatment for 120 min produced a modest number of binding sites per molecule, yet this same treatment introduced so many uracil residues that most of the polymer could not be recovered in an acid-precipitable form following UNG treatment and alkaline sucrose sedimentation. There also was no correlation between the numbers of protein-recognized sites and of uracils for a P22 DNA substrate treated for 60 min with bisulfite (Table II). These data indicate that the protein does not recognize inosine-uracil or guanine-uracil base mismatches.

During treatment at low pH and high temperature, apurinic and apyrimidinic (AP) lesions also are introduced. By comparing the number-average molecular weights of the DNA or polymer substrate at each time point to that of the untreated (0 min) sample, the numbers of AP sites were calculated. The data of Table II indicate that AP sites were introduced at a faster rate than were binding sites, and confirm that the binding protein does not recognize AP sites, as has been shown previously (14). Instead, the protein must be directed towards another, rarer form of damage produced by bisulfite.

Figure 6. Effect of Maintaining Treated P22 DNA at low pH. P22 DNA was treated for 60 minutes at 86°C with either 2M sodium bisulfite or 2M sodium chloride, then dialyzed vs. either a pH 5.4 buffer or a pH 9.0 buffer. The protein saturation curves shown here represent the specific binding levels. Δ — Δ pH 5.4 dialysis, $\bullet$ — $\bullet$ pH 9.0 dialysis.

Effect of Reaction Conditions on Lesion Production

The treatment protocol we employed involved incubation of the bisulfite and substrate at an elevated temperature, to induce partial melting and permit sulfonate adduct formation, followed by cooling and high pH to reanneal the single-stranded regions and reverse any adducts. This heating step was found to be necessary for introduction of the lesions recognized by the binding protein. Fig. 5 contains the protein saturation data for P22 DNA treated with bisulfite for 60 min at 0, 43 and 91°C. It is evident that an increase in the reaction temperature and thus in the extent of melting, resulted in increased numbers of binding sites, with only 0.2 per DNA molecule at 43°C but 0.9 at 91°C.

An alternate candidate for the lesion identified by the binding protein, and one which would have a strong temperature-dependence for its introduction, is a sulfonate adduct. Initially, we considered this improbable, since many reports in the literature have suggested that these adducts are unstable to alkali. To examine this possibility further, we modified the bisulfite treatment conditions to favor preservation of base-sulfonate adducts. Since the adducts are stabilized at low pH, the dialysis buffer pH might be expected to affect the number of binding sites. When bisulfite-treated P22 DNA was dialyzed at pH 5.4, the level of specific binding was indeed found to be increased relative to that seen for the DNA dialyzed at pH 9 (Fig. 6). Finally, the inclusion of an effective free radical scavenger (hydroquinone, 100 mM) during the bisulfite treatment had no effect on damage introduction, suggesting that the binding lesions were not introduced via a free radical mechanism.

Both the temperature dependence of damage introduction and the pH

Figure 7. Lesions recognized by the damage-specific DNA binding protein. Uracil-sulfonate adduct (A), thymine glycol (B) and guanine 4,8-endoperoxide (C).

stability of the protein-binding lesion are consistent with the lesion being the sulfonate adduct. Unfortunately, the high concentrations of bisulfite required for damage introduction preclude a direct radiochemical test of this hypothesis. This work does suggest the possibility, however, that sulfonate adducts may be more stable in double-stranded substrates than has been previously realized, and that the binding protein may provide a sensitive probe for this base lesion.

DISCUSSION

The damage-specific DNA binding protein purified from human placenta can recognize a lesion introduced into DNA or poly d(I-C), but not poly d(A-T) or poly d(A-U), by sodium bisulfite. From these data, which suggest that the lesion is confined to the cytosine base, and from the chemistry of bisulfite-pyrimidine reactivity, it was initially concluded that the protein recognized the guanine-uracil or inosine-uracil mismatched base pairs. Bisulfite treatment did deaminate cytosine bases to uracils; however, these lesions outnumbered the binding sites, and their removal had little effect on the level of binding. Addition of bisulfite to cytosine is indicated by polymer specificity, the requirement for single-strandedness of the polymer and the apparent lack of involvement of hydroxyl radicals in binding lesion production. Thus, the possibility that the lesion actually was a sulfonate adduct was considered. Although we were not able to use radioactively labelled bisulfite to probe directly for the presence of adducts, due to the extremely high levels of bisulfite required for the reaction and the consequent low specific activity of incorporated label, the data given here suggest this may be the case.

When P22 DNA was dialyzed at either a low or high pH after bisulfite treatment, to minimize or maximize the reversal of adducts, respectively, and then assayed with binding protein, it was found that a higher level of

specific binding was obtained under conditions which would preserve adducts (Fig. 6). The uracil-sulfonate adduct, unlike its cytosine counterpart, is stable at neutral pH, both in the free base form (1) and in polynucleotides (2). In both the treated DNA and poly d(I-C), a small number of uracil-sulfonate adducts may be "trapped" in the renatured double-stranded structure where they are relatively stable. These adducts, or the disruption of the base stacking which they cause, are probably the lesions identified by the binding protein.

The biological effects of bisulfite, most notably the G:C to A:T transition mutations, have been studied in several different systems (1,4,8). Several authors have hypothesized the presence of sulfonate adducts in the DNA in order to interpret their results (8,21). For example, Hayakawa and Sekiguchi (21) studied the survival and mutation frequency of bisulfite-treated SP02 phage in strains of *B. subtilis* either having or lacking the uracil glycosylase. The phage plated on the strain lacking this enzyme exhibited poorer survival and increased mutation frequency. It was also observed that a period of exposure to alkali following bisulfite treatment augmented, in a time-dependent manner, phage survival on the wild type hosts, while it decreased survival on the mutant hosts. These data imply first, that base-sulfonate adducts are lethal lesions and second, that adducts can persist in phage kept at pH 9 for at least nine hr after bisulfite treatment.

That sulfonate adducts can persist in double-stranded DNA has been shown in studies with microorganisms (8,22), and not surprisingly, the presence of even small numbers of sulfonate adducts can interfere with the proper functioning of DNA. For instance, the ability of single-stranded calf thymus DNA to serve as a substrate for *E. coli* DNA-polymerase I is compromised by the presence of sulfonate adducts; however, restoration of the bases from the adducts permits DNA synthesis to continue in a normal fashion (20). Minor DNA lesions may exert considerable biological effects as do, for example, the bipyrimidine (6,4) adducts produced by 254 nm ultraviolet light. These lesions form preferentially at mutational "hotspots" in the *lac I* gene of *E. coli*, while pyrimidine dimers, the major photoproducts, may be responsible for only a small portion of the mutations caused by uv irradiation (23,24).

The binding protein used in this study recognizes lesions in more than one type of base in DNA. Ultraviolet light is known to produce a thymine lesion recognized by the binding protein, while a methylene blue photooxidation system produces a guanine lesion (10,12). From the data presented in this paper, it appears that a cytosine lesion also is identified.

The similarity between UV and NaHSO_3 -treated DNA in the protein saturation curve (Fig. 3) as well as the fact that both UV and NaHSO_3 -treated unlabeled DNA compete equally well against a UV-irradiated [^3H] P22 DNA substrate, suggest that there is a single binding site for both types of damage. Earlier work in our laboratory has also revealed that photodynamically-damaged DNA competes as well as UV-damaged DNA for a UV-irradiated labeled substrate. These results indicate that the different forms of damage all bind at a single site, suggesting that there must be some feature(s) common to the multiple lesions which are bound. The structures of three products, which have been tentatively identified as binding lesions (from thymine, guanine and cytosine, respectively) are shown in Fig. 7. One alteration these three products share is saturation of the carbon-carbon double bond at the 5,6 position of the pyrimidines and the analogous 4,5 position of guanine. Saturation of the carbon-carbon double bond results in the destruction of the planarity of the damaged base, which subsequently would disrupt the stacking of the affected base with its neighbors. It is possible that disruption of base stacking, and not the individual base lesions, may be the actual alteration recognized by the binding protein.

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