Differential DNA Recognition and Cleavage by *Eco*RI Dependent on the Dynamic Equilibrium between the Two Forms of the Malondialdehyde−Deoxyguanosine Adduct†

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ABSTRACT: DNA damage may alter the outcome of protein−nucleic acid interactions. The malondialdehyde−deoxyguanosine adduct, 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-α]purin-10-(*3H*)-one (M_1dG), miscodes in vivo and in vitro. M_1dG is an exocyclic adduct that undergoes ring-opening in duplex DNA to form the acyclic adduct, N^2-(3-oxo-1-propenyl)-deoxyguanosine (N^2-OPdG). These two adducts have different effects on DNA polymerase bypass and may affect other DNA processing enzymes. We employed the *Eco*RI restriction endonuclease as a model for the interaction of DNA binding proteins with adducted DNA substrates. The presence of M_1dG in the *Eco*RI recognition sequence impaired the ability of the enzyme to cleave DNA, resulting in only 60% cleavage of the adducted strand and 75% cleavage of the complementary strand. Three adducts of similar structure to M_1dG that are unable to ring-open were cleaved poorly, or not at all, by *Eco*RI. None of the adducts appeared to inactivate or sequester *Eco*RI. Additional studies with *Bss*HII and *Pau*I confirmed these results and demonstrated a positional effect of M_1dG on cleavage efficiency. These data suggest dissimilar modes of protein−nucleic acid interactions based on differences in adduct structure. Comparison of the solution structures of DNA adducts and the crystal structure of *Eco*RI complexed to substrate suggest a model to explain the functional differences.

The recognition of specific DNA sequences by DNA-binding proteins is a crucial regulatory step in cellular processes such as DNA replication, control of gene expression, and the response to and repair of DNA damage. Sites of DNA damage may interfere with these processes by preventing binding or inducing an abnormal interaction between the protein and its cognate recognition sequence. The deleterious effects of DNA adducts are likely mediated in part by their ability to affect binding of DNA by DNA processing proteins, due to either distortion of the DNA helix structure or interruption of the direct readout of the DNA sequence (*1*−*3*).

MDA[1] is a byproduct of lipid peroxidation with mutagenic properties that are likely mediated through its reaction with DNA and subsequent formation of DNA adducts. The major product of the reaction of MDA with DNA is the deoxyguanosine adduct, M_1dG. M_1dG is an abundant adduct in human DNA (*4*−*8*), and site-specific mutagenesis experiments verify that it is a mutagenic lesion (*9*, *10*). The exocyclic ring of M_1dG undergoes ring-opening to form N^2-OPdG when opposite deoxycytidine in duplex DNA (Figure 1) (*11*, *12*). The oxopropenyl moiety of N^2-OPdG extends into the minor groove and causes minimal distortion of the DNA helix (*11*). In contrast to N^2-OPdG, the exocyclic ring of M_1dG is expected to reside in the major groove and distort the DNA helix at the site of the adduct. These contrasting DNA conformations suggest that the two adducts may have significantly different effects on the ability of proteins to interact with adducted DNA. In fact, the effects of M_1dG and N^2-OPdG on DNA replication by purified DNA polymerases are quite dissimilar (*13*). Therefore, it is reasonable to assume that the same may be true for other DNA metabolic processes that are dependent upon the precise recognition and binding of specific DNA elements by regulatory and structural proteins.

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[1] Abbreviations: MDA, malondialdehyde; M_1dG, 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-α]purin-10-(*3H*)-one; N^2-OPdG, 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)1,N^2-(3-oxo-1-propenyl)-deoxyguanosine; PdG, 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)1,N^2-(1,3-propano)-2′-deoxyguanosine; 5,6-dihydro-M_1dG, 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-5,6-dihydropyrimido[1,2-α]purin-10-(*3H*)-one; 1,N^2-ϵdG, 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-9-oxoimidazo-[1,2-α]purine; HO-propyl-dG, 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)1,N^2-(3-hydroxypropyl)-2′-deoxyguanosine; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; MOPS, 3-[*N*-morpholino]-propanesulfonic acid.

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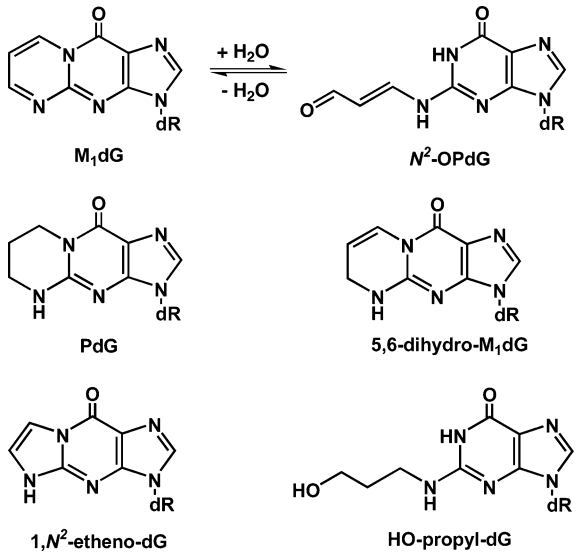

FIGURE 1: Structures of exocyclic adducts.

Type II restriction endonucleases are important models for studying sequence discrimination in DNA−protein interactions. These enzymes are highly selective for their recognition sequence over other DNA sequences, even those differing from the cognate site by only a single base (*14*). The use of short oligonucleotide duplexes as substrates for restriction endonucleases provides a means to probe the effects of functional group modification on enzyme binding and catalysis and to assess the effect of DNA structure and conformation (*15*−*19*). DNA adducts can disrupt the normal action of restriction endonucleases. In fact, the inability of a restriction endonuclease to cleave DNA is often used to confirm the presence of a DNA adduct in the enzyme's recognition site (*20*−*22*).

In the present study, we employed *Eco*RI as a model to test M_1dG's potential to disrupt DNA−protein interactions. *Eco*RI is convenient for the study of specific DNA−protein interactions because it is very well-characterized enzymatically and structurally (*23*−*26*). It recognizes the DNA palindrome, GAATTC, and sequentially cleaves both strands of the DNA duplex at the phosphodiester bond between guanine and adenine residues. Incorporation of a single M_1dG adduct into the *Eco*RI recognition site on one strand of the DNA duplex partially inhibited cleavage of both strands. Further studies were performed with analogues of M_1dG (Figure 1). The results indicated that effects on cleavage are influenced by adduct structure and conformation. We propose a structural basis for these effects based on the cocrystal structure of *Eco*RI with its cognate sequence as well as solution structures of M_1dG analogues.

EXPERIMENTAL PROCEDURES

Reagents and Enzymes. T4 polynucleotide kinase and *Bss*HII were from New England Biolabs (Beverly, MA). *Eco*RI and *Pau*I for restriction digestion experiments were from MBI Fermentas (Hanover, MD). *Eco*RI for filter binding experiments was a generous gift from Dr. Linda Jen-Jacobson (University of Pittsburgh, PA). [γ-^{32}P]-ATP was from ICN Biomedicals, Inc. (Costa Mesa, CA). HPLC grade solvents for column chromatography were obtained from Fisher (Pittsburgh, PA) and used as received. Reagent grade chemicals were obtained from Aldrich (Milwaukee, WI). CH_2Cl_2, pyridine, and diisopropylethylamine were distilled from calcium hydride under an argon atmosphere. Nucleoside 2-deoxyribosyl-transferase (EC 2.4.2.6, transferase) was a generous gift from S. Short (Glaxo-Wellcome Inc., Research Triangle Park, NC). Bio-spin 6 columns were from Bio-rad (Bio-rad, Hercules, CA). Micropure-EZ columns were obtained from Millipore (Bedford, MA). Thin-layer chromatography was performed with silica gel F254 (Merck) as adsorbent on glass plates. The chromatograms were visualized by UV (254 nm), fluorescence (excitation, 365 nm), or by staining with sulfuric acid solution, followed by heating. Column chromatography was performed using silica gel 60−100 mesh (Fischer, Pittsburgh, PA).

Oligonucleotides. The 21-mer oligonucleotides contained a unique *Eco*RI restriction site (5′-GAATTC-3′) in the sequence 5′-TATCATGTCT**X**AATTCCCGGT-3′, where X = dG, M_1dG, 5,6-dihydro-M_1dG, PdG, 1,N^2-ϵdG, or HO-propyl-dG. The sequence of the complementary 27-mer oligonucleotide was 5′-AGACCGGGAATTCAGACAT-GATACGGA-3′. The 19-mer oligonucleotides contained a single M_1dG adduct at one of four guanines in a $(CpG)_4$ repeat sequence (5′-GGTGTCC**G$_1$**C**G$_2$**C**G$_3$**C**G$_4$**GCATG-3′, where G_n = M_1dG or dG), comprising a *Bss*HII restriction site. The complementary 19-mer was 5′-catgccgCgcgcgga-cacc-3′. Unmodified oligonucleotides for restriction digest experiments were synthesized by Integrated DNA Technologies (Coralville, IA) and purified by polyacrylamide gel electrophoresis. M_1dG-modified oligonucleotides were synthesized using the method previously described (*27*) and purified by PAGE purification. The PdG-modified oligonucleotides were synthesized following the method described by Marinelli et al. (*28*). 1,N^2-ϵdG-modified oligonucleotides were synthesized and analyzed as described (*29*). HO-propyl-dG-modified oligonucleotides were prepared by reduction of 3-(2′-deoxy-β-D-*erythro*-pentofuranosyl)γ-hydroxy-1,N^2-propano-2′-deoxyguanosine-modified oligonucleotides following the method described by Minko et al. (*30*). 3-(2′-Deoxy-β-D-*erythro*-pentofuranosyl)γ-hydroxy-1,N^2-propano-2′-deoxyguanosine-modified 21-mer oligonucleotides were kindly provided by Thomas M. Harris (Vanderbilt University, Nashville, TN). 5,6-Dihydro-M_1dG-modified oligonucleotides were synthesized as described below.

The unmodified 21-mer:27-mer oligonucleotide duplex in 100 mM NaCl has a calculated T_m = 62 °C, and the unmodified 19-mer:19-mer oligonucleotide duplex has a calculated T_m = 73 °C. M_1dG modification of duplex oligonucleotides lowers the T_m by ∼14 °C (*11*), and PdG-modification lowers the T_m by ∼20 °C (*31*), so the modified DNA molecules should exist as duplexes at the temperatures used for cleavage assays (37 °C for the 21-mer:27-mer duplex containing all the adducts and 55 °C for the 19-mer:19-mer duplex containing M_1dG.

Instrumental Analysis. Samples were injected onto an Ultrasphere ODS reversed phase column (10 mm × 250 mm) from Beckman (Fullerton, CA) and eluted at 1.5 mL/min. A gradient consisting of a starting composition of 95% water/5% acetonitrile and ending with 50% acetonitrile was used as follows: from 0 to 5 min, linear gradient to 10% acetonitrile; from 5 to 20 min, linear gradient to 25% acetonitrile; from 25 to 30 min, linear gradient to 5%

acetonitrile. The eluant was monitored at 260 nm. UV spectra were recorded using a Hewlett-Packard UV/vis model 89500 spectrometer. Mass spectra were obtained on a Finnigan TSQ 7000 triple-quadrupole mass spectrometer in positive ion ESI mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC 300 NMR spectrometer (Billerica, MA) at 300 and 100 MHz, respectively, with D_2O, DMSO-d_6, and $CDCl_3$ as solvent. Chemical shifts are reported in ppm downfield from tetramethylsilane ($\delta = 0$).

Synthesis of 5,6-Dihydro-M$_1$dG-Modified Oligonucleotides. 3-(2′-Deoxy-β-D-erythro-pentofuranosyl)-5,6-dihydropyrimido-[1,2-α]purin-10-(3H)-one (2). M$_1$dG **(1)** was synthesized as previously described (*27*). To a solution of **1** (50 mg, 0.17 mmol) in water (8 mL) was added $NaBH_4$ (31.2 mg, 0.82 mmol). The reaction mixture was allowed to react at room temperature with occasional stirring for 30 min until complete decolorization of the solution was observed. The reaction mixture was neutralized with 1 N HCl, and the product was isolated by extraction on Sep-Pak HLB cartridges (Waters, Milford, MA). The product was eluted with methanol, and the solvent was evaporated under vacuum. The compound was used without further purification (white solid, 41 mg, 81%). ^{1}H NMR (300 MHz; DMSO-d_6): δ 2.19 and 2.46 (both m, 1H, H2′); 3.49 (m, 2H, H5′); 3.79 (m, 1H, H4′); 4.03 (m, 2H, H6); 4.32 (s, 1H, H3′); 4.89 (m, 1H, 5′ OH); 5.26 (d, $J = 3.9$ Hz, 1H, 3′ OH); 5.46 (m, 1H, H7); 6.07 (dd, $J = 7.5$ Hz, 6.3 Hz, 1H, H1′); 7.28 (dt, $J = 8.4$ Hz, 1.9 Hz, 1H, H8); 7.79 (s, 1H, H5); 7.92 (s, 1H, H13). ^{13}C NMR (75.5 MHz; DMSO-d_6): δ 157.8, 153.2, 135.8, 119.3, 107.8, 87.6, 82.3, 70.6, 61.6, 40.0 (2C, CH_2; 2′, 7). $^+$ESI-MS *m*/*z* (rel int): 328.2 $[M + Na]^+$ (7), 306.2 $[M + 1]^+$ (30), 190.1 (13), 142.2 (10), 101.1 (85), 83.2 (100). $C_{13}H_{15}N_5O_4$ M_r: 305.2.

3-(2′-Deoxy-β-D-erythro-pentofuranosyl)-5′-O-(4,4′-dimethoxytrityl)- 5,6-dihydropyrimido[1,2-α]purin-10-(3H)-one (3). The product, **2,** was placed in a flame-dried 25-mL round-bottomed flask and dried under vacuum in the presence of P_2O_5. Pyridine (5 mL) and diisopropylethylamine (0.08 mL, 0.66 mmol) were added to the flask containing **2,** and the mixture was cooled to 0 °C in an ice bath with stirring under argon. 4,4′-Dimethoxytrityl chloride (137 mg, 0.403 mmol) was added, and the reaction mixture was allowed to warm to room temperature. After 2 h the reaction was quenched with methanol (0.5 mL) and concentrated under vacuum. The mixture was purified by column chromatography on silica gel (0 to 5% methanol in CH_2Cl_2, 0.1% triethylamine) to yield the desired product (69 mg, 85%). $^+$ESI-MS *m*/*z* (rel int): 608.2 $[M + 1]^+$ (10), 303.1 (8), 295.2 (100), 277.1 (94). $C_{34}H_{33}N_5O_6$: 607.2.

3-(2′-Deoxy-β-D-erythro-pentofuranosyl)-3′-O-[(N,N-diisopropylamino)(2-cyanoethyl)phosphinyl]-5′-O(dimethoxytrityl)-5,6-dihydropyrimido[1,2-α]purin-10-(3H)-one (4). The product, **3** (69 mg, 0.114 mmol), and anhydrous 1*H*-tetrazole (10 mg, 0.167 mmol) were placed in a flame-dried 25 mL round-bottomed flask and dried under vacuum in the presence of fresh P_2O_5 overnight. Then anhydrous CH_2Cl_2 (5 mL) was added, followed by 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl phosphoramidite (49 μL, 0.171 mmol). The reaction mixture was stirred under argon at room temperature for 3 h and monitored by thin-layer chromatography on silica gel F254 (Merck), eluted with 1% MeOH in CH_2Cl_2, 0.1% triethylamine. The mixture was then treated with saturated $NaHCO_3$ solution (15 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layer was dried over Na_2SO_4, then filtered and concentrated under vacuum. The residue was purified using HPLC with a Varian model 9012 solvent delivery system and Hewlett-Packard model 9000 diode array detector (column Phenomenex, Luna 5μ Silica, 250 × 10 mm; 50:50 ethanol/hexane, 2 mL/min). The eluant was monitored at 260 nm. The desired diastereomeric products were isolated at 10. 4 and 11.5 min. ^{31}P NMR (300 MHz; DMSO-d_6): δ 147.1, 147.2. $^+$ESI-MS *m*/*z* (rel int): 808.6 $[M + 1]^+$ (18), 459.3 (15), 282.1 (45), 240.9 (22), 143,2 (18), 101.1 (100). $C_{43}H_{50}N_7O_7P$ M_r: 807.9.

Oligonucleotides. Oligonucleotides were synthesized on an Expedite model 8909 nucleic acid synthesis system (PerSeptive Biosystems, Framingham, MA) on a 1 μmol scale using the manufacturer's standard protocol, as described (*27*). The identity of adducted oligonucleotides was confirmed by matrix-assisted laser desorption/ionization mass spectrometry using 3-hydroxypicolinic acid as matrix. Unmodified 21-mer was used as the calibration standard $[M + 1]^+ = 6349.1$. The mass of the 21-mer containing 5,6-dihydro-M$_1$dG $[M + 1]^+$ was 6386.2 (calculated mass = 6385.2).

Restriction Digestion of Adducted Oligonucleotide Substrates. 19-mer, 21-mer and 27-mer oligonucleotides were 5′-radiolabeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP. For *Eco*RI studies, radiolabeled substrates were generated by annealing ^{32}P-labeled 21-mer modified or unmodified oligonucleotides with an equimolar amount of ^{32}P-labeled 27-mer complement. For *Bss*HII and *Pau*I studies, substrates were generated by annealing 19-mer modified or unmodified oligonucleotides with ^{32}P-labeled 19-mer complementary strand in a 1:1 molar ratio. Annealing was performed in buffer (50 mM MOPS, pH 7.4, 10 mM $MgCl_2$), and reactions were incubated at 90 °C for 3 min, followed by slow cooling. Substrates were purified from free [γ-^{32}P]-ATP by passage through Bio-spin 6 columns. Nonlabeled substrates were generated by annealing modified or unmodified oligonucleotides with complement oligonucleotide under the conditions described above. Restriction digests (10 μL) contained 5 nM ^{32}P-labeled substrate, 495 nM unlabeled substrate, 50 mM MOPS, pH 7.9, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, and 5 nM *Eco*RI, *Pau*I, or *Bss*HII restriction endonuclease. The reactions were carried out for specified times at 37 °C for *Eco*RI and *Pau*I experiments and 50 °C for *Bss*HII experiments, then quenched by the addition of 10 mM EDTA in 90% formamide. The products of the reaction were resolved on a 20% denaturing gel using the Ultrapure Sequagel sequencing system (National Diagnostics, Atlanta, GA). The positions of the bands were visualized by autoradiography and imaged using a FujiFilm FLA-5000 phosphorimaging analyzer (Fujifilm, Tokyo, Japan). Cleavage of the *Eco*RI substrate generates two 5′-^{32}P-labeled products of different size. The 21-mer oligonucleotide 5′-cleavage product is an 11-mer, whereas the 27-mer 5′-cleavage product is an 8-mer. Thus, cleavage of both strands of the oligonucleotide substrate may be monitored simultaneously. The experiments with *Bss*HII and *Pau*I were designed to monitor only cleavage of the unmodified strand of the substrate. The extent of cleavage was determined by quantifying the radioactivity of each band, then dividing the radioactivity in the band corresponding to cleavage product

by the sum of the radioactivity of cleaved and uncleaved oligonucleotide to give percent cleavage.

In some experiments, cleavage reactions were carried out as described above, except that an additional 80 units of *Eco*RI was added to the reaction mixture 2 h after initiation of the assay. The 2 h time-point was chosen since cleavage has reached a plateau for M_1dG-modified substrates by this time. Following the second addition of *Eco*RI, the reaction was monitored for 30 min.

Alternatively, in some experiments, incubation mixtures were prepared in which 500 nM unlabeled adducted substrate or unmodified substrate was incubated for 2 h with *Eco*RI or *Bss*HII. Then 500 nM unmodified substrate, spiked with ^{32}P-labeled unmodified substrate, was added to the reaction mixture, and cleavage was monitored for 2 h.

In other experiments, reactions were prepared under noncleavage conditions, i.e., absence of Mg^{2+}. Radiolabeled and nonradiolabeled oligonucleotides were annealed as described above, but in Mg^{2+}-free buffer (100 mM NaCl, 50 mM MOPS, pH 7.9, 1 mM dithiothreitol). Reaction samples were prepared exactly as for the cleavage assay, except that the buffer was Mg^{2+}-free. *Eco*RI and substrate were allowed to incubate at 37 °C for at least 2 h prior to the addition of 10 mM $MgCl_2$. Cleavage was monitored for 1 h.

DNA Binding Assays. Equilibrium binding assays were performed essentially as described by Robinson and Sligar (*24*). Radiolabeled substrates were generated by annealing 27-mer complementary oligonucleotides with ^{32}P-labeled 21-mer M_1dG-, 5,6-dihydro-M_1dG, or PdG-modified or unmodified oligonucleotides in a 5:1 unlabeled:labeled molar ratio as described above. *Eco*RI (5 nM) was incubated with 0.5 nM ^{32}P-labeled substrate at room temperature for at least 2 h in a buffer containing 20 mM MOPS (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 100 μg/mL bovine serum albumin, and 0.02% Nonidet P-40 nonionic detergent. Glycerol was added to samples to a final concentration of 5% just before loading onto 10% polyacrylamide non-denaturing gels (Proto-gel acrylamide solution, National Diagnostics, Atlanta, GA) in 0.5X TBE buffer. Bands were detected by phosphorimager analysis.

Binding constants were determined using the filter method previously described, with slight modifications (*32*, *33*). ^{32}P-labeled DNA (13.0 pM) was incubated in buffer (20 mM MOPS (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 100 μg/mL bovine serum albumin, and 0.02% Nonidet P-40 nonionic detergent) with *Eco*RI, as indicated. Incubation was at ambient temperature for 1−2 h. Reaction samples were filtered through Micropure-EZ membranes with high affinity for protein but not for double-stranded DNA. The DNA retained on the filter was quantified by liquid scintillation counting. Dissociation constants were calculated by one-site binding (hyperbola) analysis using GraphPad Prism 4.0a software.

RESULTS

DNA Adducts Inhibit Cleavage by EcoRI. To assess the ability of M_1dG to affect recognition and cleavage of DNA by the *Eco*RI restriction endonuclease, we determined the efficiency of cleavage of M_1dG-modified substrates using an in vitro cleavage assay. Radiolabeled oligonucleotide duplexes containing a single M_1dG adduct located within a unique *Eco*RI recognition site were generated (Figure 2A). The adducted strand of the duplex was a different length than its complement, and the oligonucleotides were designed to release ^{32}P-end-labeled cleavage products of different lengths. Thus, simultaneous monitoring of cleavage of both strands of the duplex was achieved (Figure 2D). Figure 2, panels B and C, shows cleavage of unmodified, M_1dG-, PdG-, 1,N^2-ϵdG-, HO-propyl-dG, and 5,6-dihydro-M_1dG-modified substrates by *Eco*RI following incubation with the enzyme for various times. Both strands of the unmodified substrate were cleaved equivalently to a level of 90%. The lack of complete cleavage may indicate the presence of a small amount of impurity introduced during oligonucleotide synthesis although no evidence for impurities was evident in the mass spectra of the individual oligonucleotides. Approximately 60% of the adducted strand and 75% of the complementary strand of the M_1dG-modified substrate were cleaved by *Eco*RI. PdG, 5,6-dihydro-M_1dG, and 1,N^2-ϵdG are analogues of M_1dG that are chemically stable and are unable to ring-open. PdG-modified substrates were poor substrates for *Eco*RI. Cleavage of the adducted strand of the PdG-modified duplex was undetectable, and only 10% of its complementary strand was cleaved. 1,N^2-ϵdG-modifed substrates were cleaved to a similar extent as PdG. The effect of 5,6-dihydro-M_1dG was similar but not quite as severe as that of PdG or 1,N^2-ϵdG. The adducted strand of the 5,6-dihydro-M_1dG-modified substrate was cleaved to a level of about 5%, whereas about 20% of the unadducted strand was cleaved. In all cases, the cleavage reaction reached a plateau within about 2 h. In order to mimic N^2-OPdG, we prepared oligonucleotide substrates containing the ring-opened HO-propyl-dG adduct. Cleavage of the adducted strand of the HO-propyl-dG-modified substrate did not plateau during the time frame of the assay, but the adducted strand was ultimately cleaved to levels comparable to M_1dG-modified strands. The unadducted strand of the HO-propyl-dG-modified substrate was cleaved nearly as well as unmodified substrate.

DNA Adducts Do Not Inactivate EcoRI. To examine whether the reduced cleavage efficiency of modified substrates was due to a sequestration or inactivation of *Eco*RI by the DNA adducts, non-radiolabeled M_1dG-, PdG-, 1,N^2-ϵdG-, or 5,6-dihydro-M_1dG-adducted substrates were incubated with *Eco*RI until the cleavage plateau was reached (2 h). Then ^{32}P-labeled unmodified substrate was added to the reaction mixture, and cleavage was monitored. In all cases, the radiolabeled substrate was cleaved to a similar extent, regardless of whether unmodified substrate or M_1dG-, PdG-, 1,N^2-ϵdG-, or 5,6-dihydro-M_1dG-modified substrates were initially incubated with the enzyme. Figure 3 shows the profiles of cleavage with M_1dG- or PdG-modified substrates and unmodified substrates. The profiles with 1,N^2-ϵdG- or 5,6-dihydro-M_1dG-modified substrates were similar (data not shown). Thus, *Eco*RI retained complete activity following exposure to the DNA adducts.

These experiments imply that *Eco*RI is not permanently inactivated by any of the DNA adducts tested. However, it is possible that M_1dG and the other adducts form a covalent linkage with *Eco*RI that is reversible in the presence of unmodified substrate. In order to examine whether the DNA adducts used in this study could sequester *Eco*RI by a slowly

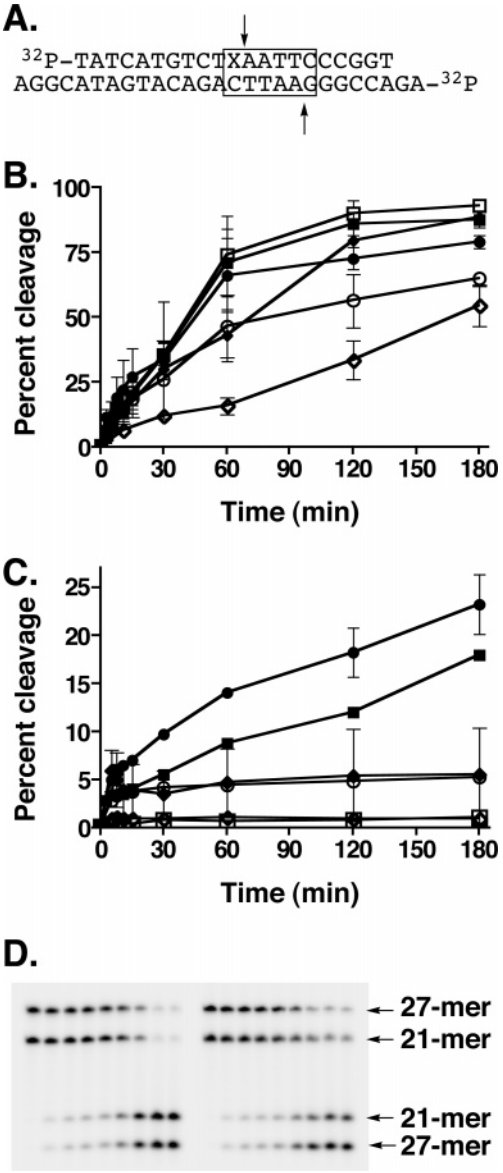

FIGURE 2: Effect of exocyclic DNA adducts on cleavage activity by *Eco*RI endonuclease. Cleavage reactions containing 5 nM ^{32}P-labeled oligonucleotide duplex, 495 nM nonlabeled oligonucleotide substrate, and 5 nM *Eco*RI were incubated at 37 °C for various times, then quenched and subjected to gel electrophoresis and phosphorimager analysis as described in Experimental Procedures. (A) 5′-Radiolabeled duplex containing a single *Eco*RI recognition site. The arrows depict cleavage sites. DNA adducts were contained on the 21-mer strand, as indicated by "X". (B) Cleavage profile of *Eco*RI substrates containing ring-opened adducts: (■) unmodified substrate, 27-mer strand; (□) unmodified substrate, 21-mer strand; (●) M_1dG-modified substrate, 27-mer strand; (○) M_1dG-modified substrate, 21-mer strand; (◆) HO-propyl-dG-modified substrate, 27-mer strand; (◇), HO-propyl-dG-modified substrate, 21-mer strand. (C) Cleavage profile of *Eco*RI substrates containing ring-closed adducts: (■) PdG-modified substrate, 27-mer strand; (□) PdG-modified substrate, 21-mer strand; (●), 5,6-dihydro-M_1-dG-modified substrate, 27-mer strand; (○) 5,6-dihydro-M_1dG-modified substrate, 21-mer strand; (◆) 1,N^2-ϵdG-modified substrate, 27-mer strand; (◇) 1,N^2-ϵdG-modified substrate, 21-mer strand. The values are the mean ± standard deviation from at least four independent determinations. (D) Representative autoradiogram from the *Eco*RI cleavage assay. The bands labeled "21-mer" are the substrate 21-mer oligonucleotide (upper band) and the 11-mer product generated during cleavage by *Eco*RI (lower band), whereas the bands labeled "27-mer" are the substrate oligonucleotide (upper band) and its 8-mer cleavage product (lower band).

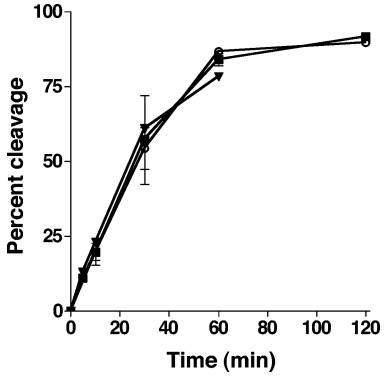

FIGURE 3: Cleavage of unmodified *Eco*RI substrate in competition with modified *Eco*RI substrates. Nonlabeled oligonucleotide duplexes modified with M_1dG, PdG, or unmodified duplexes (500 nM) were incubated with *Eco*RI endonuclease (5 nM) for 2 h at 37 °C, prior to the addition of fresh unmodified *Eco*RI substrate, spiked with ^{32}P-labeled unmodified substrate, to a final concentration of 500 nM. Cleavage of the radiolabeled substrate was monitored for 2 h. The data shown represent cleavage of the 27-mer strand of the radiolabeled duplex in the presence of the indicated competitor substrate. The 21-mer strand of each duplex was cleaved similarly to its complement (data not shown). (■) Unmodified competitor substrate. (▼) M_1dG-modified competitor substrate. (○) PdG-modified competitor substrate. The values are the mean ± standard deviation from three independent experiments.

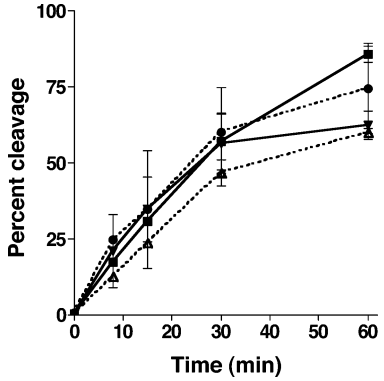

FIGURE 4: Initiation of *Eco*RI cleavage of modified substrates following addition of Mg^{2+}. *Eco*RI endonuclease was incubated for 2 h at 37 °C with ^{32}P-labeled modified substrate in the absence of Mg^{2+}. Cleavage was monitored following addition of $MgCl_2$. The data shown represent cleavage of the 27-mer strand of the substrate duplex. Cleavage of the 21-mer strand of the duplex was similar (data not shown). (■) Unmodified substrate with preincubation in the absence of Mg^{2+}. (▼) M_1dG-modified substrate with preincubation in the absence of Mg^{2+}. (●) Unmodified substrate without preincubation in the absence of Mg^{2+}. (△) M_1dG-modified substrate without preincubation in the absence of Mg^{2+}. The values are the mean ± standard deviation from three independent experiments.

reversible mechanism, ^{32}P-labeled M_1dG-modified duplexes were preincubated with *Eco*RI in Mg^{2+}-free buffer for 2 h to provide sufficient time for covalent binding to occur between enzyme and substrate in the absence of cleavage. $MgCl_2$ was then added to the mixture, and cleavage was monitored (Figure 4). Mg^{2+} is required for cleavage, but not for specific *Eco*RI−DNA binding. If an *Eco*RI−DNA cross-link is responsible for the reduced cleavage observed with adducted substrates, then cleavage should not proceed following addition of Mg^{2+}. However, there was no difference in the cleavage profiles following addition of Mg^{2+} to M_1dG-modified substrates compared to cleavage without preincubation.

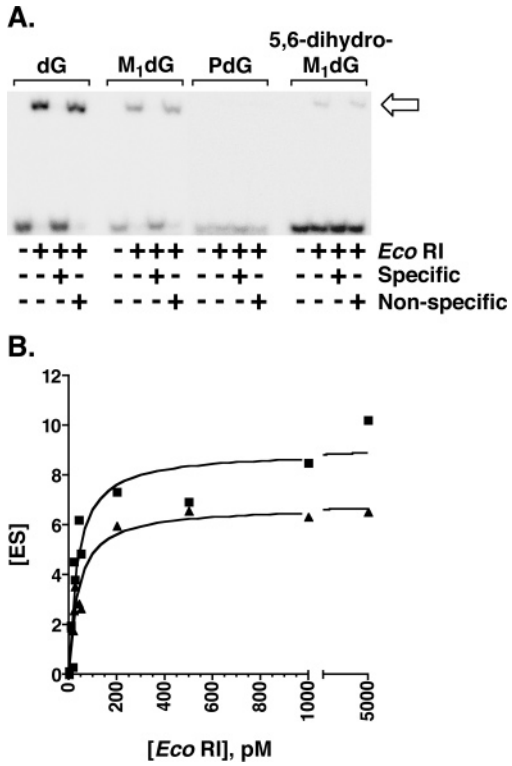

FIGURE 5: Equilibrium binding of *Eco*RI endonuclease to modified *Eco*RI substrates. (A) *Eco*RI (5 nM) was incubated with 0.5 nM ^{32}P-labeled oligonucleotide duplex for 2 h at ambient temperature, and the enzyme−DNA complexes separated on a 10% polyacrylamide non-denaturing gel. The *Eco*RI−substrate complex is indicated by an arrow. 50 nM non-radiolabeled specific competitor (contains *Eco*RI recognition site) or nonspecific competitor substrate was added as indicated. (B) Representative binding isotherm for *Eco*RI recognition of unmodified and M_1dG-modified substrates. Endonuclease:DNA complexes were quantitated by the filter assay (see Experimental Procedures). (■) Unmodified [^{32}P]-labeled DNA (13 pM) or (▲) M_1dG-modified [^{32}P]-labeled DNA was incubated at room temperature with the indicated concentration of *Eco*RI (as dimer).

DNA Adducts Are Recognized Dissimilarly by EcoRI Restriction Endonuclease. The observations described above prompted us to explore *Eco*RI binding interactions with adducted duplexes using an equilibrium electrophoretic mobility shift assay. Mg^{2+} was omitted from the reaction buffer, and a chelating agent (EDTA) was added to the reactions to prevent DNA cleavage. Figure 5A shows a strong shift representing *Eco*RI bound to DNA for both unmodified and M_1dG-modified substrates, but only a very weak or undetectable shift with 5,6-dihydro-M_1dG-modified substrates and PdG-modifed substrates, respectively. These results are consistent with the hypothesis that reduced binding leads to the differences in the cleavage efficiencies described above. The observed gel shifts could be competed away by addition of an excess of non-radiolabeled specific competitor substrate for all substrates tested, but not by the addition of DNA that did not contain the *Eco*RI restriction site. *Eco*RI bound unmodified substrate with a dissociation constant of 52.3 ± 27 pM as measured by a filter-binding assay. This value is consistent with binding constants reported in the literature for *Eco*RI oligonucleotide duplexes (*33*, *34*). Incubation of M_1dG-modified substrates with *Eco*RI yielded a dissociation constant of 53.1 ± 22 pM (Figure 5B). In contrast, *Eco*RI bound very poorly to PdG-modified substrates so that a dissociation constant could not be measured up to 5 μM.

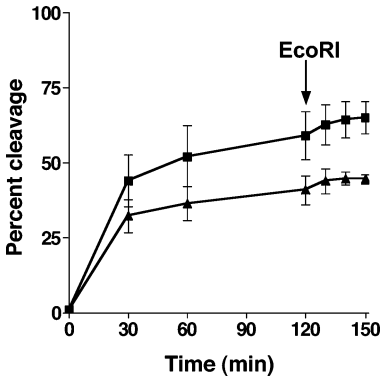

FIGURE 6: Delayed addition of supplemental *Eco*RI to a mixture of M_1dG-modified substrate and *Eco*RI endonuclease. Cleavage reactions containing 5 nM ^{32}P-labeled M_1dG-modified oligonucleotide duplex, 495 nM nonlabeled M_1dG-modified substrate, and 5 nM *Eco*RI were incubated at 37 °C for various times, prior to addition of an extra 80 units of *Eco*RI endonuclease as indicated by the arrow on the graph. The reaction was monitored for an additional 30 min, and analyzed as described in Experimental Procedures. (■) 27-mer strand of duplex. (▲) 21-mer strand of duplex. The values are the mean ± standard deviation from three independent experiments.

A fraction of M_1dG-modified substrate was not cleaved by *Eco*RI during the 2 h incubation. To investigate whether this residual duplex could be cleaved by the addition of fresh *Eco*RI, the cleavage reaction was spiked with additional enzyme after the intial plateau had been reached (2 h). Cleavage was then monitored for an additional 30 min (Figure 6). There was no appreciable increase in cleavage of the M_1dG-modified duplex following the second enzyme addition. Thus, the M_1dG-modified duplex remaining following *Eco*RI digestion appeared to represent a noncleavable species.

M_1dG Affects Activity of BssHII and PauI Restriction Endonucleases. In order to examine whether M_1dG could affect the activity of other restriction endonucleases as well as *Eco*RI, we determined the cleavage efficiency of *Bss*HII restriction endonuclease on M_1dG-modified substrates. *Bss*HII recognizes the 6 base-pair palindrome, 5′-GCGCGC-3′. Four different 19-mer oligonucleotides containing a single M_1dG adduct in a $(CpG)_4$ repeat sequence were synthesized. The oligonucleotide sequence contains a single *Bss*HII recognition site, thus M_1dG could be positioned at any one of the deoxyguanosine bases within the recognition sequence, as well as at the 3′ adjacent guanine. *Bss*HII substrates were generated by annealing the modified oligonucleotide with its ^{32}P-labeled 19-mer complement (Figure 7A). All four M_1dG-modified substrates were cleaved to a lesser extent than the unmodified duplex (Figure 7B). The extent of cleavage (<55%) was somewhat lower than that achieved with *Eco*RI. Interestingly, the effect of M_1dG on *Bss*HII cleavage was dependent on the location of M_1dG, with cleavage of substrates modified at $G_1 > G_4 > G_2 > G_3$. *Bss*HII experiments were performed at 50 °C whereas the studies with *Eco*RI had been conducted at 37 °C. In order to more closely resemble the conditions used during *Eco*RI experiments, we performed a parallel study with *Pau*I, which functions optimally at 37 °C. *Pau*I is an isoschizomer of *Bss*HII, and thus recognizes the same cognate sequence. *Pau*I

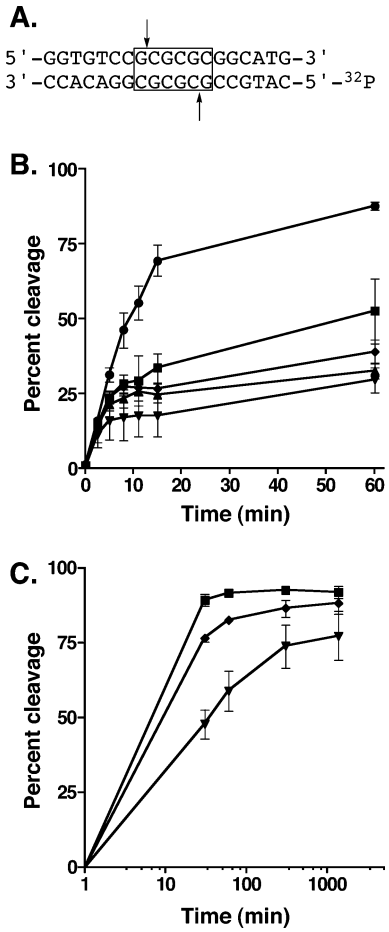

FIGURE 7: Effect of M_1dG on cleavage by *Bss*HII and *Pau*I restriction endonucleases. Cleavage reactions containing 5 nM ^{32}P-labeled oligonucleotide duplex, 495 nM nonlabeled oligonucleotide substrate, and 5 nM restriction endonuclease were incubated for various times, then quenched and subjected to gel electrophoresis and phosphorimager analysis as described in Experimental Procedures. Data represent cleavage of the unmodified strand of the substrate. (●) Unmodified substrate. (■) M_1dG-modification at G_1. (▲) M_1dG-modification at G_2. (▼) M_1dG-modification at G_3. (◆) M_1dG-modification at G_4. (A) Structure of substrate duplex containing a single *Bss*HII or *Pau*I restriction site. Sites of cleavage are indicated by arrows. (B) Profile of *Bss*HII cleavage or (C) *Pau*I cleavage. The values are the mean ± standard deviation from three independent experiments.

cleaved M_1dG-modified substrates less efficiently than unmodified substrates at short time-points (Figure 7C). The dependence on the adduct location within the recognition site for cleavage by *Pau*I differed from that of *Bss*HII, with $G_3 > G_1 \approx G_2 > G_4$. Although the overall rates of cleavage were slower for *Pau*I than for *Bss*HII, an extended time-course revealed that M_1dG-modified substrates were eventually cleaved to extents comparable to unmodified substrates.

DISCUSSION

DNA damage can elicit a range of cellular responses, but the relationship between adduct structure and the resultant cellular response is not fully understood. It is well-documented that damaged bases alter DNA metabolism by DNA-binding proteins (*35*). Transcriptional bypass of uracil by RNA polymerase results in the synthesis of mutant protein (*36*); 8-oxo-deoxyguanosine inhibits binding of AP-1 and Sp1 transcription factors, which could result in faulty regulation of gene expression (*37*); *cis*-platin adducts recruit the high mobility group protein, HMG1, which may play a role in growth regulation (*38*); and M_1dG and PdG block transcription by RNA polymerase (*39*).

*Eco*RI is a useful model for studying the effects of M_1dG on recognition by DNA-binding proteins. The endonuclease has been extremely well-characterized, and the molecular basis for substrate specificity has been studied extensively (*14*, *16*, *40*, *41*). Several crystal structures are available of *Eco*RI in complex with oligonucleotide substrates (*23*, *42*). These structures reveal a torsional kink in the DNA helix that unwinds the DNA and widens the major groove in the recognition site. *Eco*RI interacts with DNA exclusively in the major groove, and makes contacts with DNA bases solely within the recognition sequence. *Eco*RI active site residues hydrogen-bond with deoxyguanosine and the two 3′-deoxyadenosines. Arg-200 participates though a water molecule in two hydrogen bonds with O^6 and N7 of the terminal deoxyguanosine and is thought to be a critical residue for sequence discrimination at the DNA−endonuclease interface. Removal of N7 or the O^6 oxygen atom of deoxyguanosine results in poorer binding of the recognition site by *Eco*RI, consistent with the importance of hydrogen-bonding to Arg-200 (*43*). However, analysis of Arg-200 mutants indicates that cleavage specificity is determined by factors in addition to Arg-200−deoxyguanosine interactions, such as discrimination of the sequence-dependent conformation of the DNA (*40*).

In this study, we assessed the effects of a series of exocyclic DNA adducts positioned at the 5′-deoxyguanosine of the *Eco*RI recognition site on cleavage by the restriction enzyme. The rates of cleavage on the adduct-containing and complementary strands were directly compared by using single-strands of different lengths, which were labeled at the 5′-position with ^{32}P. Cleavage of modified and unmodified duplexes was compared in parallel incubations. The alternate approach of constructing duplex DNAs containing both an adduct-containing and an unmodified *Eco*RI site was not attempted because of the technical difficulties in synthesizing and purifying long, adduct-containing oligonucleotides. The results with separate modified and unmodified oligonucleotides were quite reproducible and provided a quantitative estimate of the impact of the individual adducts on duplex cleavage.

The presence of most adducts in substrate DNA inhibited cleavage by *Eco*RI to various extents without apparent sequestration or inactivation of the restriction endonuclease. PdG-, 1,N^2-ϵdG-, and 5,6-dihydro-M_1dG-modified substrates were barely cleaved, and PdG-containing oligonucleotides did not appear to bind tightly to *Eco*RI as revealed by electrophoretic mobility shift assays and a filter binding assay. Structural studies on PdG-containing oligonucleotides provide insight into potential PdG−*Eco*RI interactions. PdG appears to exist in two conformations in duplex DNA, only one of which has been fully characterized (*31*, *44*−*49*). PdG rotates about the glycosyl bond to a syn conformation, which allows it to form a Hoogsteen base pair with a protonated cytosine residue opposite it (*31*, *49*). The effective pK_a of the protonated cytosine is 7, which means that somewhat less than 50% of the PdG is in the syn conformation under the conditions of this experiment (*49*). Since the PdG:dC-H$^+$ Hoogsteen base pair projects into the major groove, it is anticipated to disrupt interactions with *Eco*RI,

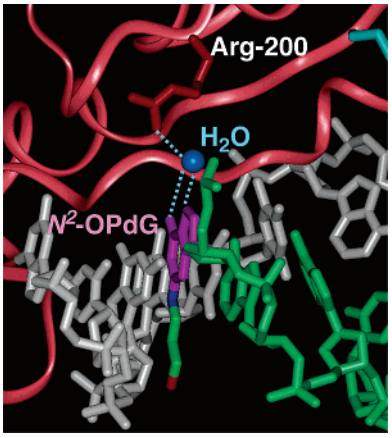

FIGURE 8: N^2-OPdG modeled into the *Eco*RI active site. One monomer of the *Eco*RI dimer is shown interacting with its recognition site in duplex DNA (PDB file 1ERI). The oxopropenyl moiety of N^2-OPdG wraps into the minor groove of the DNA duplex, allowing water mediated hydrogen bond formation between Arg-200 and O^6 and N7 of N^2-OPdG. The model was generated using Insight II 2000 software with an R12000 Silicon Graphics Octane workstation.

particularly with Arg-200. The other conformation of PdG within duplex DNA has not been fully characterized, but it appears to also disrupt *Eco*RI interactions with its substrate as judged by the inability of the enzyme to bind or cleave PdG-containing oligonucleotides. The three-dimensional structures of 5,6-dihydro-M_1dG and ϵdG have not been elucidated, but they may adopt conformations similar to PdG.

In contrast to the other exocyclic adducts, M_1dG did not completely reduce *Eco*RI activity; in fact, approximately half of the modified and unmodified strands of the DNA duplex were cleaved. M_1dG can exist as either the intact exocyclic adduct or its acyclic derivative, N^2-OPdG. When M_1dG is placed opposite dC residues, it rapidly opens to N^2-OPdG (*50*). N^2-OPdG remains in the anti conformation around the glycosyl bond, and the oxopropenyl moiety projects into the minor groove (*11*, *12*). N^2-OPdG hydrogen-bonds to the dC residue on the complementary strand, which helps to stabilize the duplex (*12*). Thus, N^2-OPdG is likely to have little impact on protein−DNA contacts in the major groove and should not be as disruptive to *Eco*RI interactions as PdG (Figure 8). Indeed, the K_d's for binding of *Eco*RI to an unmodified duplex or an M_1dG-modified duplex were identical. Duplexes containing another acyclic adduct, HO-propyl-dG, were cleaved to extents comparable to M_1dG-modified substrates.

Interestingly, *Eco*RI cleavage of M_1dG-modified substrates reached a plateau, suggesting the presence of a noncleavable species that is not in equilibrium with a cleavable species. Addition of fresh enzyme to reaction mixtures that had ceased cleavage did not reinitiate cleavage. No adduct species besides M_1dG and N^2-OPdG are detectable in M_1dG-containing duplexes, but 8-OH-propeno-dG is an intermediate in the conversion of N^2-OPdG to M_1dG as the duplexes denature (Figure 9) (*51*). 8-OH-propeno-dG is ring-closed and so would not be expected to be recognized by *Eco*RI. However, since 8-OH-propeno-dG is in equilibrium with M_1-dG and N^2-OPdG, one anticipates that duplexes containing it would eventually be cleaved. Thus, the formation of 8-OH-propeno-dG may not account for the inability of *Eco*RI to completely cleave M_1dG-containing duplexes.

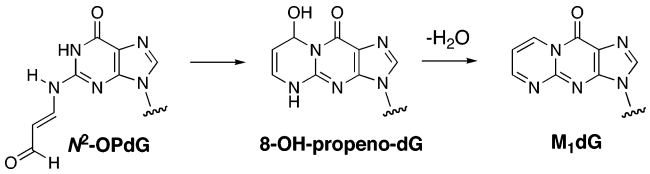

FIGURE 9: 8-OH-propeno-dG is an intermediate in the conversion of N^2-OPdG to M_1dG.

Cleavage assays were also performed with the isoschizomers, *Bss*HII and *Pau*I, to test the generality of the observations with *Eco*RI. Substrates containing M_1dG were modified at one of three deoxyguanosine residues located within the *Bss*HII recognition sequence, as well as at a deoxyguanosine 3′ to the recognition site. *Bss*HII cleavage of all four M_1dG-modified substrates rapidly reached a plateau, and activity was substantially reduced compared to unmodified controls. Interestingly, there appeared to be some sequence-specificity to the effect of M_1dG-modification on cleavage. In contrast, although *Pau*I activity was generally slower than *Bss*HII, it was only modestly affected by the presence of M_1dG in or adjacent to its recognition sequence. In fact, M_1dG-modified substrates were eventually cleaved to comparable levels as unmodified substrates. Thus, a plateau in cleavage similar to that observed with *Eco*RI was observed with *Bss*HII but not with *Pau*I. However, only cleavage of the oligonucleotide complementary to the M_1-dG-modified strand was monitored, so it is possible that *Pau*I cleavage of the M_1dG-modified strand was more greatly affected. Presumably, the contrasting effect of M_1dG on *Bss*HII and *Pau*I activity may be attributed to differences in enzyme active site and substrate recognition; unfortunately, structural data are not available for either endonuclease.

The present study demonstrates that exocyclic DNA adducts alter cleavage of DNA by restriction endonucleases. One exception to this rule is M_1dG, which ring-opens to N^2-OPdG and permits recognition, binding, and cleavage. Thus, DNA−protein interactions appear to be sensitive to the dynamic equilibrium between the two forms of this adduct. Cline et al. recently demonstrated that the exocyclic adduct, PdG, is a strong block to transcription by RNA polymerase whereas M_1dG is a weaker block (*39*). Interestingly, when M_1dG was placed opposite a thymidine residue, it was a stronger block to replication, consistent with the observation that it does not ring-open when it is opposite thymidine. Thus, studies with restriction endonucleases and RNA polymerases reveal a functional sensitivity to the dynamic equilibrium between this exocyclic adduct and its ring-opened derivative. It will be interesting to assess the impact of this equilibrium on the activity of other DNA binding proteins such as transcription factors or repair enzymes.

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