Cross-Linking of 2-Deoxyribonolactone and Its β-Elimination Product by Base Excision Repair Enzymes†

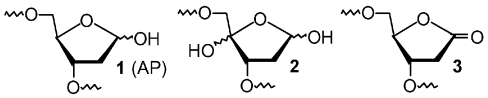
Kelly M. Kroeger,‡,§ Mitsumasa Hashimoto,||,⊥ Yoke Wah Kow,*,|| and Marc M. Greenberg*,‡

Department of Chemistry, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, and Division of Cancer Biology, Department of Radiation Oncology, Emory University School of Medicine, Atlanta, Georgia 30335

Received November 13, 2002; Revised Manuscript Received January 8, 2003

ABSTRACT: 2-Deoxyribonolactone (**3**) is produced in DNA as a result of reaction with a variety of DNA damaging agents. The lesion undergoes β-elimination to form a second metastable electrophilic product (**4**). In this study, DNA containing 2-deoxyribonolactone (**3**) and its β-elimination product (**4**) are generated at specific sites using a photolabile nucleotide precursor. 2-Deoxyribonolactone is not incised by any of the 8 AP lyases tested. One enzyme, *Escherichia coli* endonuclease III, cross-links to **3**, and the lesion strongly inhibits excision of typical abasic sites by this enzyme. Two of the enzymes, FPG and NEIL1 known to cleave normal abasic sites (**1**) by effecting β,δ-elimination form cross-links to the butenolide lesion (**4**). The observed results are ascribable to characteristics of the enzymes and the lesions. These enzymes are also important for the removal of oxidative base lesions. These results suggest that high concentrations of **3** and **4** may exert significant effects on the repair of normal AP site and oxidative base lesions in cells by reducing the cellular activity of these BER enzymes either via cross-linking or competing with binding to the BER enzymes.

Cellular DNA is constantly subjected to endogenous and exogenous agents that alter its chemical structure, which can be mutagenic or lethal if not repaired in a timely and appropriate manner. A variety of enzymatic pathways have evolved to protect the cell from the effects of DNA damage. One pathway, the base excision repair pathway (BER) involves individual removal of modified nucleobases by DNA glycosylases (*1*−*4*). These enzymes are roughly divided (but not exclusively) into two families, which repair pyrimidine and purine derived lesions, respectively (*3*−*6*). Functional homologues of many of the enzymes exist among prokaryotic and eukaryotic species. Some of these enzymes are simple DNA glycosylases, resulting in the release of the nucleobase and concomitant abasic site (**1**, AP)[1] formation. Large amounts of AP sites are also formed independent of BER via spontaneous depurination (*7*). In mammalian cells, Ape1 incises AP sites via 5′-phosphodiester bond hydrolysis (*8*). However, many DNA glycosylases are complex glycosylases that are associated with an AP lyase activity, which can process the AP site after the glycosylase action by inducing β- or β,δ-elimination (*3*). β-Elimination gives rise to fragmented DNA containing an α,β-unsaturated aldehyde, 4-hydroxypentenal, at its 3′-terminus (*9*, *10*). In contrast, β,δ-elimination leads to cleaved DNA containing a 3′-phosphate (*8*). A variety of DNA damaging agents, including the antitumor agents bleomycin and the neocarzinostatin chromophore (NCS), produce structurally distinct abasic sites that are oxidized at the C4 (**2**) or C1 (**3**) positions (*11*−*14*). The 2-deoxyribonolactone (**3**) lesion represents a conservative structural change from an AP site. However, beneath the structural similarity between **1** and **3** lies the very different chemical reactivity of an aldehyde and ester, respectively. We recently showed that 2-deoxyribonolactone (**3**) exploits these differences and similarities to cross-link DNA repair enzymes (*15*, *16*). Herein, we explore the generality of this process with respect to 2-deoxyribonolactone (**3**) and the metastable lesion **(4)** derived from it.



2-Deoxyribonolactone is produced as part of bistranded lesions by NCS and other enediyne type antitumor antibiotics (*12*, *14*, *17*). This lesion is also produced by ionizing radiation, organometallic oxidants, and is a metastable intermediate in DNA damage mediated by copper phenanthroline nucleases (*11*, *18*−*20*). Oxidized abasic site **3** is also formed from the C1′-radical under anaerobic conditions in the presence of the radiosensitizing agent tirapazamine (SR4233) (*21*). 2-Deoxyribonolactone (**3**) undergoes β- and δ-elimina-

† Supported by the NIGMS (GM-63028).

* Corresponding authors. (M.M.G.) E-mail: mgreenberg@jhu.edu. (Y.W.K.) E-mail: ykow@emory.edu.

‡ Johns Hopkins University.

§ Department of Chemistry and Department of Pharmacology and Molecular Sciences, Johns Hopkins University.

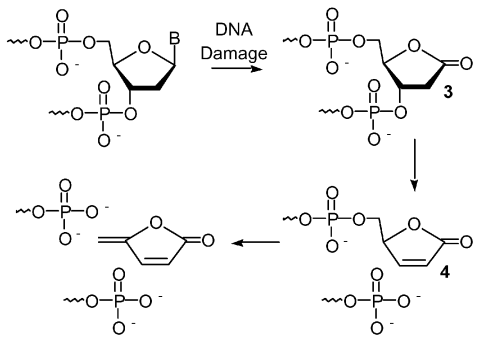
|| Emory University School of Medicine.

⊥ Present address: Frontier Collaborative Research Center, Tokyo Institute of Technology, Yokohama 226-8503, Japan.

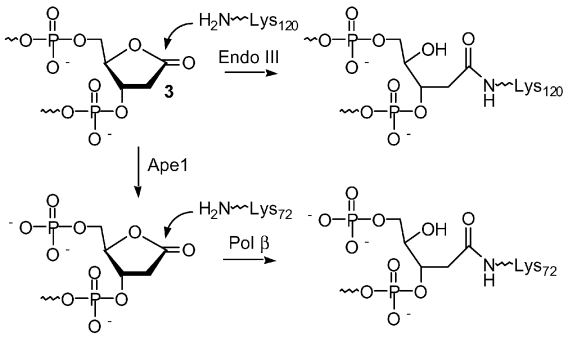
[1] Abbreviations: abasic site, AP; human apurinic endonuclease 1, Ape1; *E. coli* endonuclease III, endoIII; human endonuclease III, NTH1; *E. coli* endonuclease VIII, endoVIII; human endonuclease VIII, NEIL1; formamidopyrimidine DNA glycosylase, FPG; yeast oxoguanine glycosylase 1, yOGG1; human oxoguanine glycosylase 1, hOGG1; yeast endonuclease III, NTG2.

10.1021/bi027168c CCC: $25.00
Published on Web 02/04/2003

Scheme 1: Formation of 2-deoxyribonolactone (**3**) and Its β-Elimination Product (**4**) in DNA



Scheme 2: Cross-linking of 2-deoxyribonolactone (**3**) to Endo III and Pol β



tion in aqueous solution, which is accelerated by copper phenanthroline complexes (Scheme 1) (*20*, *22*). Previous studies in which **3** was produced by NCS indicated that the lesion inhibits DNA repair and is a mutational hotspot (*23*−*26*). In vitro replication of a template containing **3** by Klenow exo$^-$ resulted in preferential incorporation of deoxyadenosine opposite the lesion (*27*). Using a DNA substrate in which 2-deoxyribonolactone (**3**) was produced at a defined site from a photolabile nucleoside precursor, we reported that *Escherichia coli* endonuclease III (endoIII) was irreversibly inhibited in a process involving Lys120 (Scheme 2) (*15*). Cross-linking **3** to endoIII is the first example of irreversible inhibition of DNA repair by a lesion. The lactone (**3**) also cross-links to DNA polymerase β (Pol β) following incision by Ape1 (*16*). Although direct structural evidence was not obtainable in either system, experiments with wild type and mutant enzymes supported the hypothesis that the respective lysine side chains previously implicated in nucleophilic attack on **1** were attacking the carbonyl carbon in **3**, resulting in stable amide bond formation.

Cross-linking to the nucleophilic side chains of two enzymes that are involved in the excision of AP sites provides chemical support for why cells treated with the antitumor antibiotic NCS are resistant to DNA repair and may be biologically significant (*15*, *16*, *23*). The potential significance of inhibition by **3** is underscored further by the fact that the lesion is formed under anaerobic conditions in the presence of the radiosensitizing agent tirapazamine, indicating that the lesion could play a role in the destruction of hypoxic tumor cells (*21*). Furthermore, kinetic studies on the stability of **3** reveal that this lesion is ~20 times more likely to undergo spontaneous elimination in solution than an AP (**1**) site (*22*). The metastable β-elimination product (**4**) is also electrophilic and therefore a potential irreversible inhibitor of BER enzymes containing nucleophiles in their active sites. To our knowledge, no information concerning the reactivity of DNA containing **4** with repair enzymes has been reported. These possibilities and the above observations prompted us to investigate the generality of the cross-linking reaction between repair enzymes and **3** and **4** with a variety of BER enzymes.

EXPERIMENTAL PROCEDURES

Materials and General Methods. All H_2O used was obtained from a Nanopure Barnstead still. Commercially available DNA synthesis reagents were obtained from Glen Research Inc. *E. coli* endonuclease III, endonuclease VIII, and formamidopyrimidine DNA *N*-glycosylase are reagent enzymes routinely prepared in our laboratories. Yeast oxoguanine glycosylase I, yeast endonuclease III, and human oxoguanine glycosylase I are gifts from Dr. Serge Boiteux. Human endonuclease VIII is a gift from Dr. Tapas Hazra. Radionuclides were obtained from Amersham. T4 polynucleotide kinase was obtained from New England Biolabs. Oligonucleotides were synthesized on an Applied Biosystems Inc. 394 DNA synthesizer. DNA manipulations were carried out using standard procedures (*28*). Radioactive samples were counted via Cerenkov counting, using a Packard Tri-Carb 1500 scintillation counter. Phosphorimaging analysis was carried out using a Storm 820 Molecular Dynamics Phosphorimager equipped with Imagequant software (Version 5.1). Oligonucleotide containing **5** (**6**) was prepared as previously described and photolyzed as described below (*29*).

Photolysis and Hybridization. Photolysis was carried out on 50 pmol of 5′-^{32}P-**6** (10 mM MOPS, pH = 6.9, and 100 mM NaCl) in 100 μL. The reaction was photolyzed for 1.5 h at λ_{max} = 350 nm in a Rayonet Photoreactor and then transferred to a 0.6 mL Eppendorf tube. Complement (75 pmol) was added and hybridized by heating the reaction at 55 °C for 5 min, followed by slow cooling to room temperature.

*Generation of Butenolide (**4**) in Duplex DNA (**9**).* To generate butenolide, 2.3 pmol of 5′-^{32}P-**8** was incubated in 100 mM Tris-HCl (pH = 9.9) at 29 °C (*30*). Total reaction volume was 50 μL. To assess percent cleavage based on duration of treatment, an aliquot was taken every 10 min for 90 min then analyzed by 20% denaturing gel.

*2-Deoxyribonolactone (**3**) and Butenolide (**4**) Cross-Link Assays.* Cross-linking reactions were carried out at 37 °C in 10 mM MOPS (pH = 7.4), 100 mM NaCl, and 2 mM EDTA. All enzyme and DNA solutions were kept on ice prior to mixing. Enzyme was added (final concentrations of 20, 100, and 500 nM) to 5 nM 5′-^{32}P-**8** (for **3**) or 5 nM 5′-^{32}P-**9** (for **4**). The final reaction volume was 20 μL. Reactions were incubated at 37 °C for 60 min and then transferred immediately to ice, whereupon 20 μL of 2X loading buffer (100 mM Tris-HCl (pH = 6.8), 4% SDS, 150 mM DTT, 20% glycerol, and 0.05% bromophenol blue) was added to each reaction and mixed by pipet. Reactions (10 μL) were loaded onto a 12% SDS PAGE (0.4 mm thick), and the gel was run in TG buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) at 12 mA.

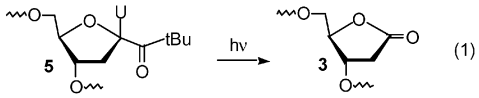
*Inhibition of AP (**1**) Cleavage.* AP (**1**) containing DNA (**12**) was obtained by reacting 5′-^{32}P-**11** (200 nM, 10 pmol)

in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM DTT with UDG (4 units) in a total volume of 50 μL for 1 h at 37 °C. Inhibitor DNA was added (**8**: final concentration 20, 100 nM; **10**: final concentration 100 nM) to 5′-^{32}P-**12** (20 nM), and reactions were treated with 1 nM endoIII at 37 °C. Total reaction volume was 20 μL. Aliquots were taken at 1, 3, 5, 7, and 10 min. Formamide loading dye was added to each aliquot, and samples were run on 20% denaturing gel.

*Lactone (**3**) Cleavage Assays.* Cleavage assays were performed on 5′-^{32}P-**8** (100 nM) in 10 mM Hepes-KOH (pH = 7.4), 100 mM KCl, 10 mM EDTA, and 0.1 mg/mL BSA. Enzyme was added (50 nM final concentration) and incubated at 37 °C for 5, 10, 15, and 20 min. Formamide loading buffer was added to samples, which were placed on ice, then run on 20% denaturing PAGE.

*Butenolide (**4**) Cleavage Assay.* Reactions were carried out on 5′-^{32}P-**9** (40 nM) in 10 mM HEPES-KOH (pH = 7.4), 100 mM KCl, 10 mM EDTA, and 0.1 mg/mL BSA. Enzyme (endoIII, Fpg, endoVIII, NTH1, NEIL1, NEIL2, yOGG1, hOGG1, or NTG2) was added, and reactions were incubated at 37 °C for 30 min. Half of each reaction was treated with 50 mM BME for 1 h at 25 °C. Formamide loading dye was added to all samples, which were then run on a 20% denaturing gel.

***β**-Mercaptoethanol (BME) Treatment.* BME (50 mM) was added to 2.3 pmol of 5′-^{32}P-**8** or 2.3 pmol of 5′-^{32}P-**9** and incubated at 25 °C for 1 h. Total reaction volume was 50 μL. The reaction was then evaporated to dryness and resuspended in 50 μL of H_2O. The presence of a BME adduct was determined by 20% denaturing gel (*31*). Cross-linking reactions with enzymes were then carried out as described above.



(1)

RESULTS

*Reaction between 2-Deoxyribonolactone (**3**) and Base Excision Repair Enzymes.* We recently reported that unlike the unoxidized, normal abasic site (**1**), 2-deoxyribonolactone (**3**) irreversibly inhibits *E. coli* endonuclease III by cross-linking to Lys120 (*15*). Before determining how general this reaction was, we sought to improve conditions for lactone formation from **5** (eq 1) and its cross-linking to endoIII. By photolyzing the radiolabeled oligonucleotide containing **5** (**6**) prior to hybridizing to its complement in MOPS buffer, we were able to achieve 80−90% conversion of the ketone. Ketone conversion was based upon the measured amount of alkali labile lesion formed (**7**) and represents an upper limit for the yield of **3** (*29*). Subsequent incubation of **8** with endoIII produced cross-linked product in yields as high as 50% (based upon the calculated amount of lactone present), an approximately 2.5-fold increase over that originally reported (Figure 1) (*15*). The degree of cross-linking is independent of which terminus of the oligonucleotide containing **5** in **8** is radiolabeled. Further characterization of the constitution of the oligonucleotide in the cross-link was obtained by pretreating the duplex with Tris buffer (100 mM, pH 9.9) for 1 h (25 °C) prior to incubating with enzyme. These conditions cleave the lactone strand (see below) and drastically reduce the extent of cross-linking to endoIII observed (Figure 1) (*30*).

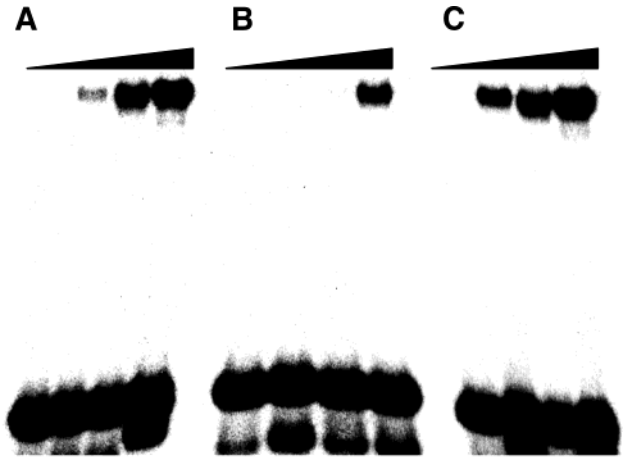


FIGURE 1: Reaction of duplex DNA (**8**) containing 2-deoxyribonolactone (**3**) or its butenolide (**4**) elimination product with endo III. (A) 5′-^{32}P-**8**: 0, 20, 100, and 500 nM endo III. (B) 5′-^{32}P-**9** following treatment of **8** with Tris (pH 9.9): 0, 20, 100, and 500 nM endo III. (C) 3′-^{32}P-**8**: 0, 20, 100, and 500 nM endo III.

5′-d(GTC ACG TGC TGC A**X**A CGA CGT GCT GAG CCT)
6 X = **5**
7 X = **3**

5′-d(GTC ACG TGC TGC A**X**A CGA CGT GCT GAG CCT)
3′-d(CAG TGC ACG ACG TAT GCT GCA CGA CTC GGA)
8 X = **3**
9 X = **4**
10 X = **5**
11 X = dU
12 X = **1**

5′-d(GTC ACG TGC TGC A A CGA CGT GCT GAG CCT)
3′-d(CAG TGC ACG ACG TAT GCT GCA CGA CTC GGA)
13

Seven additional BER enzymes from a variety of species were then screened for their ability to effect incision of 2-deoxyribonolactone (**3**) in **8**. 2-Deoxyribonolactone was not a substrate for any of these enzymes. No cross-linking of **3** to any of these enzymes was observed at concentrations as high as 100 nM (Table 1). Small amounts (<10%) of cross-linking were observed when the lactone lesion was incubated with very high concentrations (500 nM) of FPG, hOGG1, yOGG1, NTG2, and endoVIII (see Supporting Information).

*Generation of Duplex DNA Containing the Butenolide Incision Product (**4**) of 2-Deoxyribonolactone (**1**) and Its Interaction with Base Excision Repair Enzymes.* Initial reports by Sigman et al. suggested DNA containing 3′-terminal butenolide fragments are very labile and readily undergo δ-elimination to yield 3′-phosphate termini (*32*). More recently, Sugiyama and Oyoshi showed that butenolide (**4**) containing fragments derived from 2-deoxyribonolactone (**3**) were observable by LC/MS upon treatment with Tris buffer (pH 9.9) (*30*). The metastable nature of **4** was confirmed in detailed kinetic studies, which show that β- and δ-elimination occur with comparable rate constants (*22*). Butenolide generation from **3** in **8** was optimized with respect to time and temperature under Sugiyama's buffer conditions

Table 1: Percent Cross-Linking of 2-Deoxyribonolactone (**3**) in Duplex DNA (5′-^{32}P-**8**) as a Function of BER Enzyme Concentration

Percent 2-deoxyribonolactone (**3**) cross-linked			
	[Enzyme] (nM)		
enzyme	20	100	500
endoIII	1.2	9.6	54
NTH1			2
yOGG1			5
hOGG1			7
NTG2			2
FPG			3
endoVIII			6
NEIL1			2

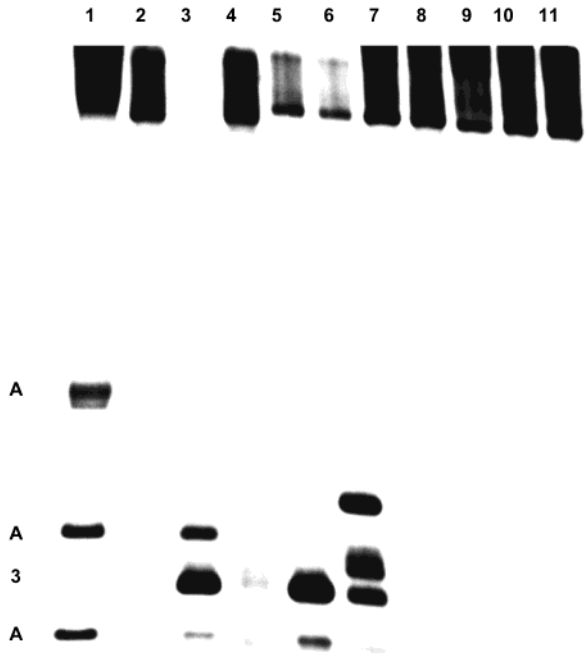


FIGURE 2: Generation and trapping of the β-elimination product (**4**) of 2-deoxyribonolactone (**3**) in **9**. Lane 1: dA sequencing reaction. Lanes 2−6: 5′-^{32}P-**8**. Lanes 7−11: 5′-^{32}P-**10**. Lanes 2 and 7: no further treatment. Lanes 3 and 8: piperidine (1 M, 90 °C, 20 min). Lanes 4 and 9: β-mercaptoethanol (50 mM, 25 °C, 60 min). Lanes 5 and 10: Tris (pH 9.9, 10 mM, 25 °C, 1 h). Lanes 6 and 11: Tris (pH 9.9, 100 mM, 25 °C, 1 h) + β-mercaptoethanol (50 mM, 25 °C, 60 min).

(*30*). Reaction progress was monitored by denaturing PAGE separation and phosphorimaging analysis of 5′-^{32}P-**8**. Although no cleavage of **8** is observed in the absence of Tris (pH 9.9), we were unable to detect **4** directly by denaturing PAGE, as only fragments containing 3′-phosphate termini were observed. DNA containing **5** (**10**) is not cleaved under any of these conditions. Evidence for the intermediacy of the butenolide (**4**) was obtained via trapping with β-mercaptoethanol (Figure 2). Multiple adducts, in addition to 3′-phosphate, are observed. These observations are consistent with our previous report on thiol trapping of fragments derived from **3** via reaction with *N*,*N*′-dimethylethylenediamine (*31*). The 3′-phosphate is believed to result from adventitious cleavage of unreacted **3** upon precipitation, as has been observed previously (*29*, *31*). However, we cannot rule out the possibility that **4** is incompletely trapped by thiol. The recent kinetic studies on **3** mentioned above indicate that it would be fruitless to try to convert **3** completely to the butenolide (*22*). On the basis of the published rate constants for elimination from **3** and **4**, we strove to achieve approximately 50% conversion. This was achieved in 1 h at 25 °C, a period that is consistent with the recently measured half-life for **3** under similar conditions (*22*).

Table 2: Percent Cross-Linking of Butenolide (**4**) in Duplex DNA (5′-^{32}P-**9**) as a Function of BER Enzyme Concentration

Percent butenolide (**4**) cross-linked			
	[Enzyme] (nM)		
enzyme	20	100	500
endoIII			4
NTHI			2
yOGG1			5
hOGG1			6
NTG2			4
FPG	0.4	22	100
endoVIII			23
NEIL1	8	28	50

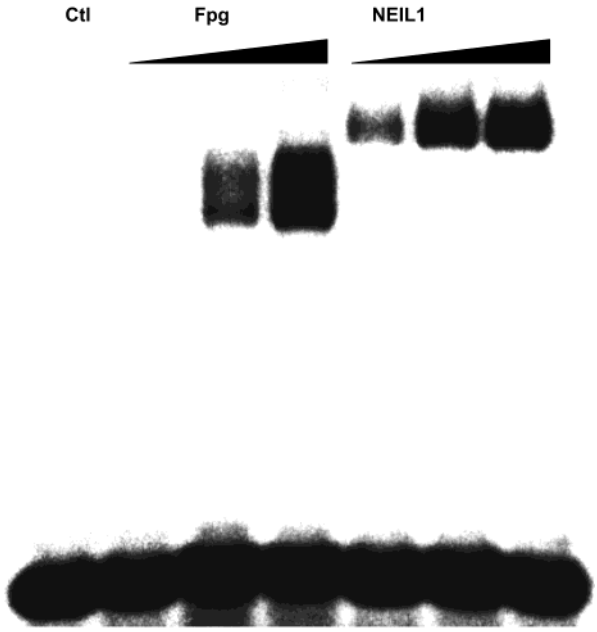


FIGURE 3: Cross-linking of duplex DNA (5′-^{32}P-**9**) containing butenolide **4** to FPG and NEIL1. Ctl: no reaction. [FPG] and [NEIL1]: 20, 100, and 500 nM.

Several enzymes, including endoIII, yielded small amounts of cross-linked products upon reaction with solutions containing butenolide **4** (**9**) at high concentration (500 mM, Table 2). Given that some **3** was still present, one could not rule out that the cross-link observed in the presence of these enzymes, and especially in the case of endoIII, was not due to reaction with this lesion. However, FPG and human endonuclease VIII (NEIL1) showed a significant amount of cross-linking over the 20−500 nM enzyme concentration range (Table 2, Figure 3). One should note that the extent of cross-linking reported in Table 2 takes into account the fact that only 50% of the DNA contains butenolide (**4**). Corroborative evidence suggesting that the cross-linked products were the result of reaction with the oligonucleotide fragment containing the 3′-butenolide was sought because of our inability to obtain mass spectral evidence. As expected, no cross-linking was observed upon reaction of the duplex (**13**) resulting from complete excision of **3** (data not shown).

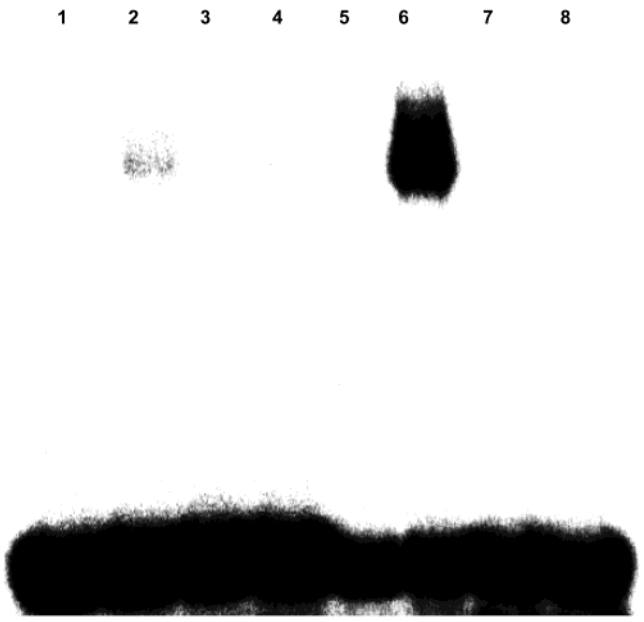


FIGURE 4: Effect of pretreating 5′-^{32}P-**9** containing butenolide **4** with β-mercaptoethanol on cross-linking to FPG. Lane 1: 5′-^{32}P-**8**. Lane 2: 5′-^{32}P-**8** + FPG (500 nM). Lane 3: 5′-^{32}P-**8** + β-mercaptoethanol (50 mM, 25 °C, 60 min). Lane 4: 5′-^{32}P-**8** + β-mercaptoethanol (50 mM, 25 °C, 60 min) + FPG (500 nM). Lane 5: 5′-^{32}P-**8** Tris (pH 9.9, 100 mM, 25 °C, 1 h) (5′-^{32}P-**9**). Lane 6, 5′-^{32}P-**9** + FPG (500 nM). Lane 7: 5′-^{32}P-**9** + β-mercaptoethanol (50 mM, 25 °C, 60 min). Lane 8: 5′-^{32}P-**9** + β-mercaptoethanol (50 mM, 25 °C, 60 min) + FPG (500 nM).

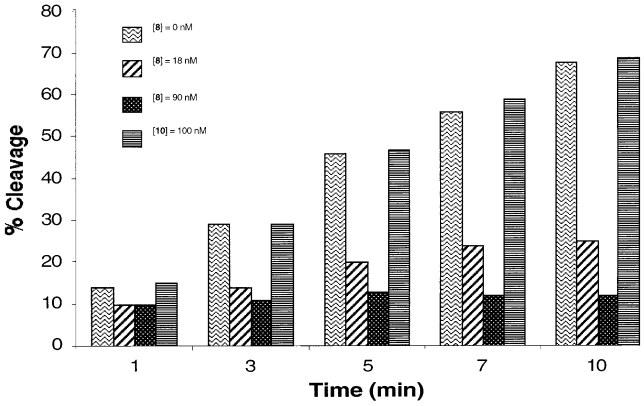


FIGURE 5: Effect of **8** and **10** on endoIII (1 nM) cleavage of 5′-^{32}P-**12** (20 nM).

More direct evidence that cross-linking because of reaction with butenolide **4** was obtained by examining the effect of β-mercaptoethanol on product formation. No cross-linking was observed in the presence of high concentrations (500 nM) of either FPG (Figure 4) or NEIL1 (see Supporting Information) when **4** was trapped with β-mercaptoethanol (Figure 2) prior to incubation.

2-Deoxyribonolactone (3) Inhibition of AP (1) Excision by EndoIII. Qualitative evaluation of the binding affinity of endoIII for duplex DNA containing **3** was obtained indirectly. Because excision of AP sites (**1**) by EndoIII is well-studied, we examined the ability of 2-deoxyribonolactone in an otherwise identical duplex to inhibit this reaction (Figure 5). Radiolabeled DNA containing **1** (**12**) was prepared via reaction of **11** with UDG. Treatment with NaOH (1 M, 37 °C, 30 min) showed that **1** was produced in >99% yield, and <2% of the material underwent adventitious cleavage. The actual concentration of lactone containing DNA was based upon conversion of the ketone to NaOH labile material, as previously described. In these experiments, this was accomplished by radiolabeling an aliquot of the photolysate. Adventitious cleavage of **3** was found to be as high as 8%. Since it is uncertain when scission occurred, the lactone concentration was not adjusted, and those noted (Figure 5) represent an upper limit.

Cleavage of **12** steadily increased over 10 min in the presence of endoIII (1 nM) and was unaffected by 100 nM DNA containing the ketone precursor (**10**). At approximately equal amounts of lactone and AP site, slightly greater than 50% inhibition of cleavage of **12** was observed in the first five minutes of the endonuclease III reaction. Cleavage reached a plateau in the presence of **3** that was inversely proportional to the concentration of the inhibitor. Interestingly, the amount of cleavage was approximately independent of lactone concentration in the first minute of the reaction. At the highest lactone concentration (90 nM), the maximum amount of cleavage was reached in the first minute and represents approximately one-sixth of the total amount of cleavage of **1** observed in 10 min in the absence of any additional DNA.

DISCUSSION

2-Deoxyribonolactone (**3**) and its β-elimination product (**4**) are commonly generated DNA lesions whose structures resemble the more widely observed AP site (**1**) and its respective β-elimination product. Given the structural similarities, we expected these oxidized abasic sites to be recognized by BER enzymes that repair **1**. The lactone lesions also possess an electrophilic carbonyl group potentially suitable for reaction with the amine side chains implicated in BER enzyme lyase activity. In contrast to **1**, the lactones (**3** and **4**) do not require any equilibration or prior enzymatic reaction to reveal their carbonyl groups, which are inherently less electrophilic than the aldehyde in **1**. However, nucleophilic attack on the carbonyl of either lactone would result in a permanent covalent bond instead of a transient Schiff base. Three synthetic methods have been developed in the past several years that enable one to prepare DNA containing **3** in a controlled manner at a defined site (*29*, *33*, *34*). These methods facilitate examining the reactivity of **3** and provide means for generating **4** in useful quantities in DNA. In this study, DNA containing 2-deoxyribonolactone (**3**) was generated using the method developed in our laboratory (eq 1) (*29*).

2-Deoxyribonolactone (**3**) is not a substrate for any of the enzymes studied but does efficiently cross-link to endoIII. Excess endoIII is required for obtaining high yields of cross-linked product. Gel-shift experiments were carried out in an attempt at determining whether this was due to weak binding and/or slow covalent bond formation. EndoIII binds DNA containing a reduced AP site with modest affinity ($K_{obs} = 2 \times 10^6$ to 2×10^7 M^{-1}) (*35*). We were unable to observe specific binding of endoIII to lactone. Only nonspecific binding was observed at high concentrations of enzyme. This in itself does not affirm that binding of endonuclease III to lactone is much weaker than a reduced AP site but could reflect a high off-rate. Indeed, the ability of DNA containing **3** to inhibit endoIII cleavage of AP sites suggests that binding

by the two abasic sites is comparable (Figure 5). Addition of approximately equal amounts of deoxyribonolactone containing DNA to endoIII AP site reactions reduces cleavage by more than one-half. Furthermore, the amount of **1** cleaved in the same time period when 5-fold excess of lactone DNA (**8**) is present is approximately one-sixth that produced in the absence of any potential inhibitor. These data suggest that **3** is bound with approximately equal affinity by endoIII as is a typical AP site. We believe that the observed plateau in the cleavage of **1** is reflective of the irreversible cross-linking of endoIII by **3**, which occurs more rapidly at higher concentrations.

EndoIII is the only enzyme that cross-links to **3** at concentrations $\leq$100 nM. Although quantitative information regarding the lyase activity of all the BER enzymes used in this study is not available, we believe that the literature suggests that endo III, FPG, and endo VIII are more effective than yOGG1, hOGG1, and NTG2 at nicking AP sites (*36*−*42*). Others have shown that the AP lyase activity of FPG and endoIII is significantly higher than their glycosylase activities (*38*−*40*). In contrast, the AP lyase activities of the oxoguanine glycosylases (OGG1) are significantly lower than their glycosylase activities (*36*, *41*, *42*). We propose that endoIII is distinguished from FPG and NEIL1 by the chemical nature of the putative nucleophiles in the respective enzyme active sites. EndoIII utilizes a lysine, which is a primary amine; whereas FPG, endoVIII, and NEIL1 employ more hindered N-terminal prolines to excise AP sites (*37*, *38*, *43*, *44*). Studies on other enzymes support the possibility that the lysine residue in endo III is more nucleophilic than the proline groups in FPG and endo VIII (*45*, *46*). The deoxyribonolactone (**3**) substrate also plays a role in selectivity. Although a recent report suggests that the hemiacetal form of an AP site may also react with BER enzymes, it is generally believed that the acyclic carbonyl containing isomer of **1** is the substrate attacked by Lys120 of endoIII (*9*, *10*, *47*). The more rigid cyclic nature of the lactone may make accessibility of **3** poorer than that of the typical AP site. In addition, esters are inherently less electrophilic than aldehydes. Both properties of 2-deoxyribonolactone (**3**) may deter reaction with the proline nucleophiles present in FPG, endoVIII, and NEIL1 as compared to reaction with the lysine residue in endoIII.

The inherent properties of the BER enzymes also provide an explanation for the cross-linking of **4** to FPG and NEIL1 but not endoIII or any of the other BER enzymes mentioned above that exhibit relatively weaker lyase activity. The butenolide fragment (**4**) is structurally analogous to the β-elimination product formed enzymatically from AP sites. The reluctance of endoIII and the other BER enzymes to cross-link to **4** correlates with these enzymes' AP lyase activities. None of these enzymes induce β,δ-elimination from **1**. Therefore, it is not surprising that they do not recognize and cross-link to the butenolide (**4**), which is structurally analogous to the 4-hydroxypentenal formed from β-elimination of **1** (*48*). In contrast, FPG and NEIL1 catalyze β,δ-elimination from **1** via a covalent iminium ion intermediate formed by the repair enzyme with the initially formed β-elimination product 4-hydroxypentenal. The 3′-terminal butenolide (**4**) is structurally similar to the 4-hydroxypentenal intermediate bound within the active site of FPG or endoVIII (*48*). Studies with mutant forms of these proteins were not carried out. However, we suggest that the N-terminal amino acids in FPG and NEIL1 are responsible for cross-linking. This is the most conservative explanation based upon what is known about these enzymes (*3*, *4*). However, neither attack by other nucleophiles present in the enzyme active site or 1,4-conjugate addition cannot be ruled out. Incised abasic sites are known to react with thiols and amines via 1,4-conjugate reactions (*31*, *49*). Finally, we do not know why *E. coli* endoVIII does not cross-link to **4**. The lack of reaction may be attributable to the subtle differences between the active sites of homologous but distinct enzymes.

In principle, cross-linking can occur via conjugate addition to the carbon−carbon π-bond or carbonyl group of **4**. There is no experimental evidence to eliminate the former pathway. Reaction of **4** with β-mercpatoethanol removes the π-bond but also alters the structure of the lesion as a whole (*31*, *49*). Hence, the elimination of cross-linking by prior incubation of **4** with thiol supports the proposal that the butenolide is the substrate but does not allow one to draw conclusions regarding the site of nucleophilic attack by the enzymes. In the absence of affirmative data, application of Ockham's razor requires that we propose that cross-links of **4** to FPG and NEIL1 occur by nucleophilic attack on the butenolide's carbonyl group.

CONCLUSIONS

2-Deoxyribonolactone (**3**) and its β-elimination product (**4**) are produced by a variety of DNA damaging agents. The structural similarity between these lesions and an AP site (**1**) and its respective β-elimination product results in the irreversible inhibition of some BER enzymes via cross-linking to the appropriate amino acid side chain implicated in nucleophilic attack of AP sites. There is a correlation between cross-linking and individual enzyme lyase activity. Only enzymes with more substantial AP lyase activity cross-link either of the lesions. There is also a correlation with substrate structure. None of the enzymes form cross-links with both lesions. The butenolide (**4**) is excised only by those enzymes that effect δ-elimination of AP sites. It is unknown whether these processes occur in vivo, but the ramifications of irreversible inhibition of DNA repair suggest that the appropriate investigations are warranted.

ACKNOWLEDGMENT

We thank Dr. Serge Boiteux for a gift of yOGG1, NTG2, and hOGG1 and Dr. Tapas Hazra for NEIL1.

SUPPORTING INFORMATION AVAILABLE

Phosphorimaging analysis of enzyme reactions with 5′-^{32}P-**8** and effect of β-mercaptoethanol on cross-linking of **4** with hendo VIII. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Lindahl, T. (2000) *Mutation Res. 462*, 129−135.
2. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA Mutagen. Repair*, ASM Press, Washington, DC.
3. David, S. S., and Williams, S. D. (1998) *Chem. Rev. 98*, 1221−1261.
4. Ide, H. (2001) *Progress in Nucleic Acid Research and Molecular Biology, Vol. 68, Base Excision Repair* (Moldave, K., Ed.) pp 207−222, Academic Press, San Diego.

5. Asagoshi, K., Yamada, T., Terato, H., Ohyama, Y., Monden, Y., Arai, T., Nishimura, S., Aburatani, H., Lindahl, T., and Ide, H. (2000) *J. Biol. Chem. 275*, 4956−4964.
6. Asagoshi, K., Yamada, T., Okada, Y., Terato, H., Ohyama, Y., Seki, S., and Ide, H. (2000) *J. Biol. Chem. 275*, 24781−24786.
7. Lindahl, T. (1993) *Nature 362*, 709−715.
8. Demple, B., and Harrison, L. (1994) *Annu. Rev. Biochem. 63*, 915−948.
9. Mazumder, A., Gerlt, J. A., Rabow, L., Absalon, M. J., Stubbe, J., and Bolton, P. H. (1989) *J. Am. Chem. Soc. 111*, 8029−8030.
10. Mazumder, A., Gerlt, J. A., Absalon, M. J., Stubbe, J., Cunningham, R. P., Withka, J., and Bolton, P. H. (1991) *Biochemistry 30*, 1119−1126.
11. von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, Taylor & Francis Inc., Philadelphia, PA.
12. Kappen, L. S., and Goldberg, I. H. (1989) *Biochemistry 28*, 1027−1032.
13. Stubbe, J., and Kozarich, J. W. (1987) *Chem. Rev. 87*, 1107−1136.
14. Greenberg, M. M. (1999) *Comprehensive Natural Products Chemistry, Vol. VII, DNA and Aspects of Molecular Biology* (Kool, E. T., Ed.) pp 371−426, Pergammon Press, Oxford.
15. Hashimoto, M., Greenberg, M. M., Kow, Y. W., Hwang, J.-T., and Cunningham, R. P. (2001) *J. Am. Chem. Soc. 123*, 3161−3162.
16. DeMott, M. S., Beyret, E., Bales, B. C., Hwang, J. T., Greenberg, M. M., and Demple, B. (2002) *J. Biol. Chem. 277*, 7637−7640.
17. Xi, Z., and Goldberg, I. H. (1999) *Comprehensive Natural Products Chemistry* (Kool, E. T., Ed.) Vol. 7, pp 553−592, Elsevier, Amsterdam.
18. Neyhart, G. A., Cheng, C. C., and Thorp, H. H. (1995) *J. Am. Chem. Soc. 117*, 1463−1471.
19. Pitié, M., Bernadou, J., and Meunier, B. (1995) *J. Am. Chem. Soc. 117*, 2935−2936.
20. Bales, B. C., Pitie, M., Meunier, B., and Greenberg, M. M. (2002) *J. Am. Chem. Soc. 124*, 9062−9063.
21. Hwang, J.-T., Greenberg, M. M., Fuchs, T., and Gates, K. S. (1999) *Biochemistry 38*, 14248−14255.
22. Roupioz, Y., Lhomme, J., and Kotera, M. (2002) *J. Am. Chem. Soc. 124*, 9129−9135.
23. Povirk, L. F., and Goldberg, I. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3182−3186.
24. Povirk, L. F., and Goldberg, I. H. (1987) *Biochimie 69*, 815−823.
25. Povirk, L. F., and Goldberg, I. H. (1986) *Nucleic Acids Res. 14*, 1417−1427.
26. Povirk, L. F., and Steighner, R. J. (1989) *Mutation Res. 214*, 13−22.
27. Bethet, N., Roupioz, Y., Constant, J. F., Kotera, M., and Lhomme, J. (2001) *Nucleic Acids Res. 29*, 2725−2732.
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, NY.
29. Tronche, C., Goodman, B. K., and Greenberg, M. M. (1998) *Chem. Biol. 5*, 263−271.
30. Oyoshi, T., and Sugiyama, H. (2000) *J. Am. Chem. Soc. 122*, 6313−6314.
31. Hwang, J.-T., Tallman, K. T., and Greenberg, M. M. (1999) *Nucleic Acids Res. 19*, 3805−3810.
32. Kuwabara, M., Yoon, C., Goyne, T., Thederahn, T., and Sigman, D. S. (1986) *Biochemistry 25*, 7401−7408.
33. Kotera, M., Bourdat, A. G., Defrancq, E., and Lhomme, J. (1998) *J. Am. Chem. Soc. 120*, 11810−11811.
34. Lenox, H. J., McCoy, C. P., and Sheppard, T. L. (2001) *Organic Lett. 3*, 2415−2418.
35. O'Handley, S., Scholes, C. P., and Cunningham, R. P. (1995) *Biochemistry 34*, 2528−2536.
36. Hill, J. W., Hazra, T. K., Izumi, T., and Mitra, S. (2001) *Nucleic Acids Res. 29*, 40−48.
37. Burgess, S., Jaruga, P., Dodson, M. L., Dizdaroglu, M., and Lloyd, R. S. (2002) *J. Biol. Chem. 277*, 2938−29344.
38. Rabow, L. E, and Kow, Y. W. (1997) *Biochemistry 36*, 5084−5096.
39. Marenstein, D. R., Ocampo, M. T., Chan, M. K., Altamirano, A., Basu, A. K., Boorstein, R. J., Cunningham, R. P., and Teebor, G. W. (2001) *J. Biol. Chem. 276*, 21242−21249.
40. Kow, Y. W., and Wallace, S. S. (1987) *Biochemistry 26*, 8200−8206.
41. Dherin, C., Dizdaroglu, M., Doerflinger, H., Boiteux, S., and Radicella, J. P. (2000) *Nucleic Acids Res. 28*, 4583−4592.
42. Zharkov, D. O., Rosenquist, T. A., Gerchman, S. E., and Grollman, A. P. (2000) *J. Biol. Chem. 275*, 28607−28617.
43. Hazra, T. K., Izumi, T., Boldogh, I., Imhoff, B., Kow, Y. W., Jaruga, P., Dizdaroglu, M., and Mitra, S. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 3523−3528.
44. Bandaru, V., Sunkara, S., Wallace, S. S., and Bond, J. P. (2002) *DNA Repair 1*, 517−529.
45. Stivers, J. T., Abeygunawardana, C., Mildvan, A. S., Hajipour, G., and Whitman, C. P. (1996) *Biochemistry 35*, 814−823.
46. Highbarger, L. A., Gerlt, J. A., and Kenyon, G. L. (1996) *Biochemistry 35*, 41−46.
47. McCullough, A. K., Sanchez, A., Dodson, M. L., Marapaka, P., Taylor, J. S., and Lloyd, R. S. (2001) *Biochemistry 40*, 561−568.
48. Rabow, L., Venkataraman, R., and Kow, Y. W. (2001) *Progress in Nucleic Acids Research and Molecular Biology, Vol. 68, Base Excision Repair*, (Moldave, K., Ed.) pp 223−234, Academic Press, San Diego.
49. Bailly, V., and Verly, W. G. (1988) *Nucleic Acids Res. 16*, 9489−9496.

BI027168C