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Mechanism of DNA Cleavage and Substrate Recognition by a Bovine Apurinic Endonuclease†

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Received November 3, 1988; Revised Manuscript Received January 23, 1989

ABSTRACT: The location of the phosphodiester bond cleaved by homogeneous Mg^{2+}-dependent apurinic endodeoxyribonuclease (EC 3.1.25.2; APE) of bovine calf thymus has been determined by using a 21-mer oligonucleotide containing a single central apurinic site as a substrate. A single product of cleavage consistent with cleavage of the oligonucleotide 5′ to the apurinic site, and leaving a 3′ hydroxyl group, was identified. This enzyme is, therefore, a class II apurinic endonuclease. The substrate specificities of this enzyme have been determined by using a variety of natural and synthetic DNAs or oligonucleotides containing base-free sites. Calf thymus APE has an absolute requirement for a double-stranded DNA and requires an abasic site as a substrate. The presence of a base fragment such as a urea residue, an alkoxyamine group attached to the C′-1 position of the abasic site, or reduction of the C′-1 aldehyde abolishes the APE activity of this enzyme. Synthetic abasic sites containing either ethylene glycol, propanediol, or tetrahydrofuran interphosphate linkages are excellent substrates for bovine APE. These results indicate that APE has no absolute requirement for either ring-opened or ring-closed deoxyribose moieties in its recognition of DNA-cleavage substrates. The enzyme may interact with the pocket in duplex DNA that results from the base loss or with the altered conformations of the phosphodiester backbone that result from the abasic site.

Apurinic and apyrimidinic sites, base-free sites in DNA, are mutagenic lesions for bacterial and mammalian cells (Schaaper & Loeb, 1981; Gentil et al., 1984; Kunkel, 1984; Loeb, 1985). Such sites are produced spontaneously (Lindahl, 1979), by the action of alkylating agents (Brooks & Lawley, 1963) and by the action of the DNA glycosylase repair enzymes (Friedberg, 1985). At AP[1] sites, adjacent phosphate residues are linked by a deoxyribose moiety. The action of X-rays or the antitumor antibiotic bleomycin also produces abasic sites in DNA. However, these base-free sites are linked by altered deoxyribose moieties (Hutterman, 1978; Sugiyama et al., 1988).

Both bacterial and mammalian cells contain enzymes that recognize and cleave DNA containing AP sites, the first step in the repair of such lesions. There are four potential sites for phosphodiesterase action adjacent to an AP site, and apurinic endonucleases (APEs) have been classified according to the phosphodiester bond cleaved (Linn et al., 1981). Class I and III APEs cleave 3′ to the base-free site but leave 3′ hydroxyl and phosphate end groups, respectively. Class II and IV APEs both cleave 5′ to the base-free site but leave 3′ hydroxyl and phosphate end groups, respectively. Thus, class II APEs cleave the phosphodiester bond most 5′ to the base-free site.

Recent studies of the *Escherichia coli* apurinic endonucleases reveal these enzymes to have distinctively different substrate specificities and mechanisms of action. For example, the apurinic endonuclease activity of exonuclease III (a class II apurinic endonuclease) recognizes and cleaves DNA at additional sites, such as urea residues (Kow & Wallace, 1985), and endonuclease III (a class I endonuclease) has been shown to cleave DNA by a novel β elimination reaction (Bailly & Verly, 1987; Kim & Linn, 1988).

A similar understanding of the mechanisms of action and the range of substrates recognized by the multiple forms of mammalian apurinic endonucleases should aid in the elucidation of the repair pathways in mammalian cells for base-free sites. This paper describes studies of the mechanism of action

† Research was supported by USPHS Grant CA 35767 and by a Leukemia Society of America Scholar Award to W.D.H. and by USPHS Grant CA 17395 and NIEHS Grant ES 04068 to A.P.G.
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[1] Abbreviations: AP, apurinic and/or apyrimidinic; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; APE, apurinic endonuclease; SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TEA, triethylamine.

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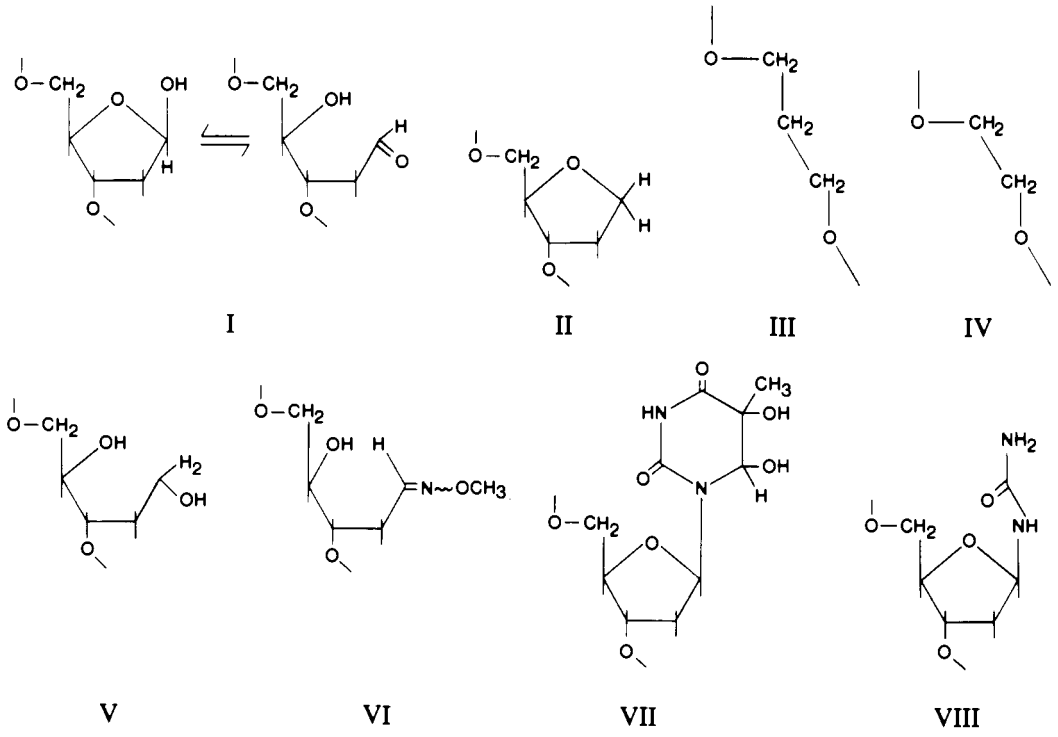

FIGURE 1: Structure of base-free sites in DNA. (I) Apurinic/apyrimidinic (AP) site; (II) tetrahydrofuran; (III) propanediol; (IV) ethylene glycol; (V) reduced AP site; (VI) methoxyamine reacted AP site; (VII) thymine glycol; (VIII) urea residue.

of a bovine apurinic endonuclease recently purified to homogeneity from calf thymus (Henner et al., 1987). Studies are described that define the phosphodiester bond cleaved by this enzyme and structure–activity relationships among its substrates. The use of modified plasmid DNA to study the characteristics of substrate recognition by this enzyme was complemented by the use of synthetic oligonucleotides with several types of abasic sites (Takeshita et al., 1987). Substrates studied (Figure 1) include DNA containing deoxyribose analogues that are incapable of either ring opening (compound II) or ring closure (compounds V and VI) and substrates that lack the ring structures of deoxyribose but retain the phosphodiester backbone (compounds III and IV).

Materials and Methods

Chemicals

Osmium tetraoxide, hydroxylamine, methoxyamine, and dATP were purchased from Sigma. Ethylhydroxylamine was purchased from Aldrich, and sodium borohydride was purchased from Fisher.

Enzymes

Mg^{2+}-dependent bovine apurinic endonuclease (APE) was purified from calf thymus as previously described (Henner et al., 1987), and on SDS–PAGE is a homogeneous protein with a molecular weight of 37 000. *Micrococcus luteus* γ-endonuclease fraction IVb was prepared as previously described (Jorgensen et al., 1987). *E. coli* uracil glycosylase was kindly supplied by Dr. B. Demple, Harvard University. *E. coli* exonuclease III was purchased from New England Biolabs, T4 polynucleotide kinase from Collaborative Research, and terminal deoxynucleotidyl transferase from Bethesda Research Laboratories. DNase I (type IV) was obtained from Sigma and dissolved at 5 mg/mL in 150 mM NaCl.

Preparation of Supercoiled pUC18 DNA Substrates

Supercoiled pUC18 DNA Containing Apurinic Sites or Thymine Glycol Residues. Covalently closed circular (form I) DNA of plasmid pUC18 (Yanisch-Perron et al., 1985) was prepared and quantitated as previously described (Henner et al., 1987). Form I pUC18 DNA, containing 2–3 AP sites/molecule, was produced by heat/acid treatment as previously described (Henner et al., 1987). Thymine glycol residues were produced in pUC18 DNA by osmium tetraoxide treatment as previously described (Jorgensen et al., 1987).

DNA Containing Urea Residues, Reduced AP Sites, and Alkoxyamine-Modified AP Sites. DNA containing urea residues was prepared by a minor modification of the alkaline hydrolysis method described in Jorgensen et al. (1987). pUC18 DNA containing thymine glycols was diluted in buffer to a concentration of 16 mM K_3PO_4–KPO_4 (pH 11.7) and incubated at room temperature for the time indicated in the text. The reaction was stopped by the addition of 3.5% v/v 0.25 M HCl to bring the pH to 7.5. The DNA was then ethanol precipitated, washed twice in 95% ethanol, and resuspended in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. DNA containing reduced AP sites was prepared by incubation of pUC18 DNA containing AP sites at room temperature in 1 M $NaBH_4$ for 90 min. $NaBH_4$ was removed by extensive (>16 h) dialysis at 4 °C against 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. DNA containing alkoxyamine-modified AP sites was produced by a modification of the methods of Weinfeld et al. (1986) and Talpaert-Borle and Liuzzi (1983). pUC18 DNA containing apurinic sites in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA was brought to the indicated concentration of either hydroxylamine, ethylhydroxylamine, or methoxyamine and incubated at 37 °C for the time indicated in the text. The reaction was stopped by chilling on ice, and the DNA was then ethanol precipitated and resuspended in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

Preparation of Oligonucleotide Substrates

Oligonucleotide Substrates. Oligonucleotides A and B were prepared by using an Applied Biosystems automated DNA synthesizer by the oligonucleotide synthesizing facility at the Dana-Farber Cancer Institute, Boston, MA. Oligonucleotides

Table I: Sequence of Oligonucleotides

oligonucleotide	sequence
A	5′ $(dTdC)_5dA(dTdC)_5$ 3′
B	5′ $G(dGdA)_5dT(dGdA)_5$ 3′[a]
C	5′ CAT TCG CGA GGT ACX CAT GGT CTA GAC TC 3′[b]
D	5′ GA GTC TAG ACC ATG CGT ACC TCG CGA ATG 3′[c]

[a] Complementary to oligonucleotide A. [b] X is either uracil (I), tetrahydrofuran (II), propanediol (III), or ethylene glycol (IV). [c] Complementary to oligonucleotide C.

C and D were produced as described by Takeshita et al. (1987). The sequences of all oligonucleotides used are shown in Table I. Deblocked oligonucleotides were purified by PAGE, eluted from gel slices, and repurified by absorption to and elution from Nensorb 20 purification cartridge (Du Pont-NEN) according the manufacturer's suggested procedure.

Production of a Single Central AP Site in Oligonucleotides. A central AP site was produced in oligonucleotide A by heat/acid treatment. 5′-^{32}P-End-labeled oligonucleotide A (≤1 ng) was dissolved in 0.3 M HCl and 1 mM EDTA and heated at 70 °C for 60 min. A central AP site was produced in oligonucleotide C (I) by enzymatic removal of the single uracil residue. 5′-^{32}P-End-labeled oligonucleotide C (I) (0–800 ng) was dissolved in 70 mM HEPES buffer (pH 8.0), 1 mM EDTA, and 0.1 unit of uracil glycosylase (final volume 30 μL) and incubated for 15 min at 37 °C. The apyrimidinic oligonucleotide was purified from the reaction mixture on a Nensorb 20 purification cartridge (Du Pont-NEN). The production of AP sites at the single adenine or uracil residue was determined by piperidine hydrolysis (Maxam & Gilbert, 1980). An aliquot was brought to 1 M piperidine (final volume 100 μL) and incubated at 90 °C for 30 min. Piperidine was removed by repeated lyophilization and resuspension in water, and the resulting DNA fragments were analyzed by electrophoresis on 7 M urea–20% PAGE gels. These treatments resulted in a single piperidine-sensitive site in ≥60% of treated molecules for oligonucleotide A (at nucleotide position 11) and in ≥80% for oligonucleotide C I (at nucleotide position 15).

Plasmid Assays

Determination of Endonuclease Activity by Conversion of Form I to Form II Plasmid DNA. Endonuclease activity was measured by the rate of conversion of form I supercoiled substrate DNA to form II nicked/relaxed circles. Substrate DNA (1 μg, 0.5 pmol) was incubated in reaction buffer appropriate for each enzyme at 37 °C for 20 min with the enzyme. Reaction buffers used were 100 mM HEPES–NaOH (pH 8.25) and 3 mM $MgCl_2$ for the bovine APE (Henner et al., 1987), 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA for γ endonuclease (Jorgensen et al., 1987), and 30 mM Tris-HCl (pH 7.5), 16 mM NaCl, and 16 mM $MgCl_2$ for exonuclease III. Agarose gel electrophoresis (0.9% agarose, medium EEO) and scanning densitometry were used to quantitate form I and form II DNA, as previously described (Jorgensen et al., 1987). The number of nicks per molecules was calculated from the relation $n = -\ln$ (form I fraction). One unit of bovine APE produces 1 pmol of nicks/min under these conditions (Henner et al., 1987).

Alkali Lability Assay. One microgram of pUC18 DNA, either normal or containing various base-free sites produced as described above, dissolved in 40 μL of 63 mM sodium phosphate buffer (pH 10.5) was incubated at 37 °C for 2.5 h. The mixture was neutralized by the addition of 4.8 μL of 0.5 M $NaPO_4$ (pH 4.4), and samples were analyzed by agarose gel electrophoresis as for the plasmid assay.

Oligonucleotide Assays

Determination of Apurinic Endonuclease Activity by Cleavage of Base-Free Oligonucleotides. Base-free oligonucleotides were labeled with ^{32}P at the 5′ terminus by using T4 polynucleotide kinase (Maniatis et al., 1982) and purified on a Nensorb 20 purification cartridge (Du Pont-NEN). Reaction conditions were the same as described for the plasmid assay, except that they also contained 100 mM NaCl, 40 ng of the appropriate oligonucleotide of complementary sequence, and 0–200 nM of the 5′-end-labeled base-free oligonucleotide. Incubation was for 5 min at 37 °C. The reaction was terminated by the addition of 6 volumes of cold ethanol. The oligonucleotide products were ethanol precipitated, resuspended, and resolved on denaturing polyacrylamide gels (0.3 or 0.7 mm thick, 7 M urea/20% PAGE). Autoradiographs were obtained as previously described (Henner et al., 1983), and radioactivity was quantitated by scanning densitometry of the resulting autoradiographs.

Terminal Deoxynucleotidyl Transferase. 5′-^{32}P-End-labeled oligonucleotide fragments were eluted from gel slices, purified by using a Nensorb 20 purification cartridge (Du Pont-NEN). Fragments were incubated with 150 units of terminal deoxynucleotidyl transferase in buffer for 30 min at 37 °C. The final assay volume was 30 μL and contained 0.1 M potassium cacodylate (pH 7.2), 2 mM $CoCl_2$, 0.2 mM DTT, and 1.2 mM dATP. The assay mixture was ethanol precipitated and subjected to electrophoresis on acrylamide gels, and autoradiographs prepared as described above.

Preparation of DNase I Cleavage Products. 5′-^{32}P-End-labeled oligonucleotide A (0.1–1 ng, ≤10^6 cpm) was digested with a range of serial 10-fold DNase I dilutions (<90 units) for 10 min at 37 °C. The final assay volume was 20 μL and contained 50 mM Tris-HCl (pH 7.5)/7 mM $MgCl_2$. Digestion was monitored by electrophoresis, and a ladder composed of fragments of 2–21 bases was generated.

RESULTS

Classification of Bovine APE DNA Cleavage

Previous experiments, using DNA sequencing techniques, had demonstrated that this bovine APE preparation cleaves DNA adjacent to AP sites in DNA (Henner et al., 1987). To determine which of the four possible phosphodiester bonds adjacent to AP sites are cleaved by this enzyme, we utilized oligonucleotide A, containing a single purine at the central position in a polypyrimidinic tract. Oligonucleotide A was depurinated at position 11 (60% depurination) without significant production of AP sites at other positions. Following either enzymatic or chemical cleavage, the resulting DNA fragments were analyzed by high-resolution electrophoresis. When 5′-end-labeled and depurinated oligonucleotide A was cleaved by bovine APE in the presence of the complementary oligonucleotide B, a single radiolabeled product was formed (Figure 2) that comigrated with the 10-mer product of DNase I cleavage of 5′-end-labeled oligonucleotide A. DNase I produces DNA fragments with 3′-hydroxyl termini, and a 10-mer is the expected product of cleavage of depurinated oligonucleotide A at a position 5′ to the AP site. This result suggests that the bovine APE cleaves the most 5′ of the four phosphodiester bonds adjacent to the AP site.

Two further experiments were performed to confirm the identity of the product of APE cleavage of depurinated oligonucleotide A as a 10-mer with a 3′-hydroxyl terminus. First,

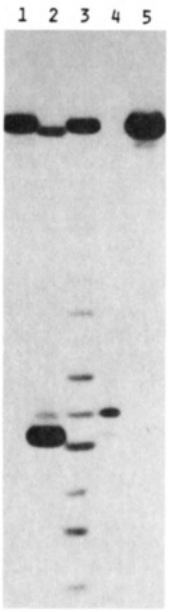

FIGURE 2: Cleavage of depurinated oligonucleotide A by bovine APE. 5′-^{32}P-End-labeled oligonucleotide A was depurinated and incubated either with (lane 4) or without (lane 5) 50 units of bovine APE in the presence of the complementary oligonucleotide B (unlabeled). A sample of 5′-^{32}P-end-labeled oligonucleotide A was left untreated (lane 1), digested with DNase I (lane 3), or depurinated and treated with piperidine (lane 2) as detailed under Materials and Methods. Resolution of the reaction mixtures by 7 M urea/20% PAGE and autoradiography of the gels were performed as described under Materials and Methods.

FIGURE 3: Products of bovine APE cleavage are primers for terminal transferase. 5′-^{32}P-Labeled oligonucleotide A was depurinated, hybridized to oligonucleotide B, and enzyme digested, and specific cleavage products were isolated (described in Figure 4). The fragments from digestion by bovine APE (lanes 1 and 2) or piperidine (lanes 3 and 4) were then incubated in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of terminal deoxynucleotidyl transferase for 10 min at 37 °C. The reaction mixtures were ethanol precipitated, resolved by 7 M urea/20% PAGE, and autoradiographed as described under Materials and Methods.

the APE-induced cleavage product, the product of piperidine treatment of depurinated oligonucleotide A (a 10-mer with a 3′-phosphate group) and the DNase-I-produced 10-mer of the same sequence were isolated from gel slices and again subjected to electrophoresis, either separately or as mixture. As expected, the product of APE cleavage could not be resolved from the DNase I product but migrated distinctly more slowly than the piperidine-induced cleavage product. Second, the APE-generated fragment can serve as a substrate for extension by terminal deoxynucleotidyl transferase, an enzyme known to require a 3′-hydroxyl primer for DNA synthesis (Bollum, 1974). A spectrum of different size fragments, longer than the APE cleavage product, was generated when the APE-generated fragment was incubated with terminal deoxynucleotidyl transferase for 5–30 min. When the APE-generated fragment was extended by terminal deoxynucleotidyl transferase for 30 min, >95% of the fragments were elongated and migrated only 2.0–2.5 cm (150–290 nucleotides) compared to 31 cm migrated by the unextended fragment (Figure 3). These results indicate that the bovine APE is a class II apurinic endonuclease.

Substrate Recognition in Supercoiled Plasmid DNA

Base-free or base-damaged sites can be produced in plasmid DNA without loss of DNA supercoiling. The production of such damaged sites in supercoiled DNA can then be monitored by enzymatic or chemical treatment that converts form I supercoiled DNA to nicked form II DNA. The two forms are separated by agarose gel electrophoresis. We have used plasmid DNA containing AP sites, AP sites modified by reduction, and AP sites modified by alkoxyamine treatment or the damaged thymine products, thymine glycols or urea residues, to explore the substrate specificity of bovine APE. The bacterial apurinic endonucleases *M. luteus* γ endonuclease (a class I AP endonuclease) and *E. coli* exonuclease III (a class II AP endonuclease), whose specificities for some of these base-free substrates have been previously determined, were used as controls in these experiments.

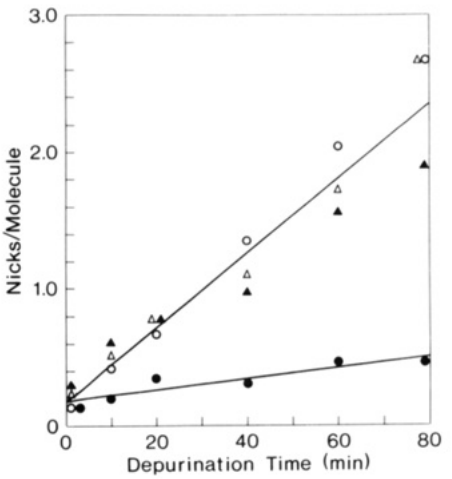

FIGURE 4: Production of sites for apurinic endonucleases in supercoiled plasmid DNA by heat and acid treatment. Form I pUC18 DNA was treated with heat/acid as described under Materials and Methods for the indicated time. The treated DNA was then incubated with either no enzyme (●), bovine AP endonuclease (○), γ endonuclease (△), or exonuclease III (▲). Resolution of form I and form II DNAs by gel electrophoresis, quantitation of DNA, and calculation of the number of enzyme sites per DNA molecule were performed as described under Materials and Methods.

Cleavage of AP Sites. Bovine APE does not cleave normal pUC18 DNA even when present in a 1000-fold excess over that required to completely cleave pUC18 DNA with 3–4 sites/molecule (data not shown). However, depurination of supercoiled pUC18 DNA and subsequent incubation with either bovine APE, *M. luteus* γ endonuclease, or *E. coli* exonuclease III convert form I DNA to form II. The number of sites for cleavage by each of these three enzymes on acid-

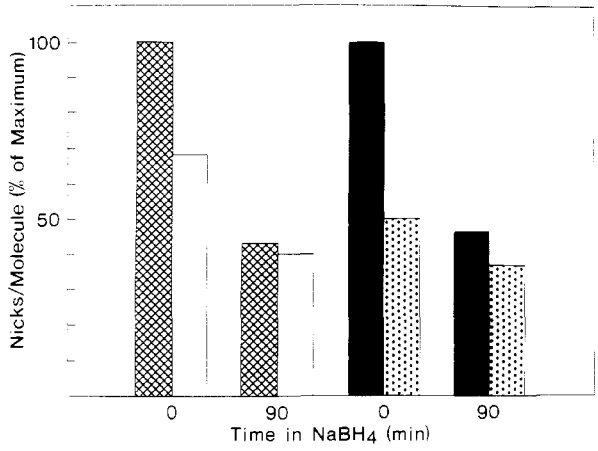

FIGURE 5: Effect of sodium borohydride on the substrate activity of depurinated DNA. A DNA sample containing 2–3 AP sites/molecule produced by heat/acid depurination was treated with sodium borohydride (Materials and Methods). The sodium borohydride treated DNA and control depurinated DNA were then used in the alkali lability assay and the plasmid assay. DNA was incubated in enzyme buffer with (crosshatched bars) or without (open bars) bovine APE or subjected to alkali (solid bars) or neutral conditions (stippled bars). Gel electrophoresis, quantitation of DNA, and calculation of the number of nicks per molecule are described under Materials and Methods. The results are expressed as a percentage of the number of AP sites in the depurinated DNA (standardized to maximum nicks by bovine APE or alkali treatment).

depurinated substrate DNA is approximately equivalent (Figure 4).

Thymine Glycols and Urea Residues as Substrates. Exposure to X-rays or certain oxidative agents produce damaged nucleobases that are substrates for some of the bacterial apurinic endonucleases. For example, *M. luteus* γ endonuclease cleaves DNA containing either thymine glycol or urea residues (Jorgensen et al., 1987), and *E. coli* exonuclease III cleaves DNA containing urea residues (Kow & Wallace, 1985). We wished to determine whether such radiation products are also substrates for cleavage by bovine APE. Bovine APE failed to cleave DNA into which thymine glycols had been introduced by osmium tetraoxide treatment, although such DNA was a substrate for γ endonuclease (Jorgensen et al., 1987). In an experiment to test whether bovine APE can use DNA containing urea residues as substrates, form I DNA containing thymine glycols was treated with mild alkali to convert thymine glycols to urea residues. Aliquots of DNA were removed after 1, 2, 4, 6, and 8 h of mild alkali treatment and incubated either with or without bovine APE. The thymine glycol lesions in DNA were converted to urea residues with increasing time of incubation, as evidenced by the conversion of this DNA to a substrate for exonuclease III. By 8 h all thymine glycols in the plasmid DNA had been converted to urea residues, and there were an equal number of sites for cleavage by either exonuclease III (1.8 sites/molecule) or γ endonuclease (1.9 sites/molecule) but less than 0.2 cleavage site/molecule for bovine APE. Thus, bovine APE cannot utilize thymine glycols or urea residues as substrates for cleavage.

Reduced AP Sites or Alkoxyamine-Modified AP Sites as Substrates. The aldehyde group of the deoxyribose found at an AP site (Figure 1, I) can be reduced by sodium borohydride (Figure 1, V). Reduced deoxyribose residues are incapable of ring closure and are alkali stable. Keubler and Goldthwait (1977) found that a partially purified enzyme from calf liver recognized reduced AP-DNA. However, the AP endonuclease purified from rat liver by Verly et al. (1981) does not recognize such sites that have been reduced. Therefore, DNA containing

Table II: Effect of Alkoxyamine Treatment on Substrate Activity of Depurinated DNA

chemical	dose (mM)	time (min)	% max cleavage[b] APE[c]	% max cleavage[b] alkali[c]
hydroxylamine[a]	0.00	30	100	100
	0.05	30	100	37
	0.10	30	69	13
	0.25	30	16	2
	0.50	30	0	5
ethylhydroxylamine[a]	0.00	30	100	ND[d]
	0.05	30	63	ND
	0.10	30	31	ND
	0.25	30	28	ND
	0.50	30	23	ND
	1.00	30	22	ND
methoxyamine[a]	0.18	0	100	100
	0.18	10	74	94
	0.18	20	80	83
	0.18	30	56	21
	0.18	60	41	21
	0.18	90	39	17

[a] Form I pUC18 DNA was depurinated and then treated with various doses of alkoxyamines for the times indicated (detailed under Materials and Methods). [b] The results, initially calculated as nicks per DNA molecule, are expressed as a percentage of the number AP sites cleaved in depurinated DNA (standardized to maximum nicks by bovine APE or alkali treatment). [c] DNA was incubated in enzyme buffer ± bovine APE for 20 min or subjected to alkali or neutral conditions for 2.5 h. Assays, gel electrophoresis, and DNA quantitation are described under Materials and Methods. [d] ND = not determined.

AP sites was incubated with sodium borohydride, and samples were taken at various times to assess the extent of cleavage by bovine APE. The degree of reduction was monitored by subjecting a portion of the sample to alkaline hydrolysis. Such an experiment is shown in Figure 5. Reduction of AP sites renders the DNA both alkali stable and no longer a substrate for bovine APE.

AP sites in DNA can also be modified by the formation of an alkoxyamine adduct at the C-1 aldehyde. Such modification prevents furanose ring closure and renders the site alkali stable. The product of methoxyamine reaction at an AP site is compound VI (Figure 1; Vasseur et al., 1986); presumably, hydroxylamine and ethylhydroxylamine form analogous products. Dose-dependent decreases in the number of enzyme nicks per DNA molecule are observed when AP-DNA is incubated with increasing doses of hydroxylamine or ethylhydroxylamine in the presence of an excess of APE (Table II). Such treatment also renders the DNA less alkali sensitive (shown for hydroxylamine). A similar dose-dependent pattern of decrease in enzyme and alkali sensitivity is observed following incubation of AP-DNA with methoxyamine. The time course for such a reaction is shown in Table II. Therefore, alkoxyamines react with AP sites in DNA and render them no longer substrates for bovine APE.

Substrate Recognition in Oligonucleotides Containing Abasic Sites

To further explore the relationship between the structure of base-free sites in DNA and the ability of the bovine enzyme to cleave such substrates, we utilized three oligonucleotides containing synthetic base-free sites. These include three 29-mers whose central position consists of either a tetrahydrofuran moiety (oligonucleotide C II), whose structure differs from the deoxyribose of an AP site by the absence of a hydroxyl group at C′-1, a propanediol moiety (oligonucleotide C III), or an ethylene glycol moiety (olignucleotide C IV). Each of the oligonucleotides was 5′ end labeled and incubated in the presence or absence of APE and a complementary oligo-

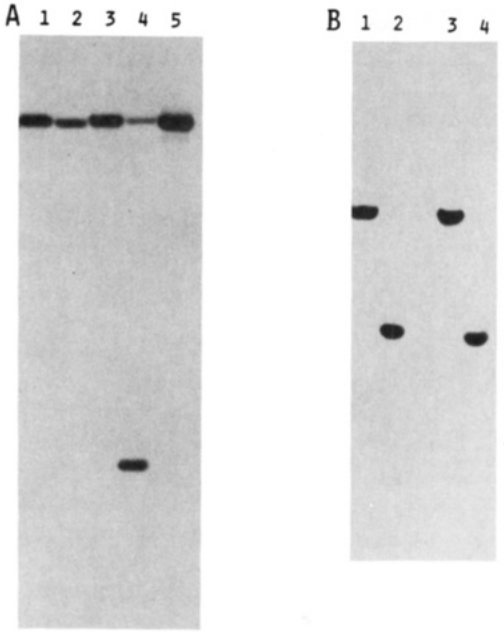

FIGURE 6: (A) Cleavage of oligonucleotide C II is dependent on the presence of the complementary DNA strand. 5′-^{32}P-End-labeled oligonucleotide C (II) was incubated with (lanes 2 and 4) or without (lanes 1 and 3) bovine APE in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of the complementary strand or treated with piperidine (lane 5). Enzyme reactions, piperidine treatment, gel electrophoresis, and autoradiography were performed as described under Materials and Methods. (B) Cleavage of oligonucleotides C III and C IV. 5′-^{32}P-End-labeled oligonucleotides C III (lanes 1 and 2) and C IV (lanes 3 and 4) were incubated with bovine APE (lanes 2 and 4) or without enzyme (lanes 1 and 3) in the presence of the complementary DNA strand. Enzyme reactions, gel electrophoresis, and autoradiography were performed as described under Materials and Methods.

nucleotide, and the products of the reaction were resolved on DNA sequencing gels. The results of such an experiment for oligonucleotide C II, containing a tetrahydrofuran, are shown in Figure 6A. Cleavage is dependent on the presence of both enzyme and complementary oligonucleotide and produces the expected 14-mer oligonucleotide as a product of cleavage. Identical results were obtained for oligonucleotides C III and C IV, containing propanediol and ethylene glycol moieties, respectively (Figure 6B).

Enzyme Kinetics

The results described above allow us to categorize this bovine APE as a class II apurinic endonuclease. That is, it cleaves the most 5′ phosphodiester bond of the base-free site. As C′-1 of deoxyribose does not appear to be the site of phosphodiesterase action, we wished to determine whether the observed inability of bovine APE to cleave the AP sites modified by reduction, methoxyamine treatment, or the presence of a urea residue might be due to either an increase in the apparent K_m for these substrates or a reduction in the V_{max} of the enzyme with these substrates. When plasmid DNA containing these three substrates (concentration of base-free sites 0.1–0.5 pmol/50 μL) was incubated with an amount of APE sufficient to cleave a 100-fold greater number of AP sites, no cleavage was observed (data not shown). Therefore, these C′-1-modified base-free sites are not substrates for this APE under these experimental conditions.

The oligonucleotide assay is more sensitive and better defined than the plasmid assay and therefore was used to study kinetics of the bovine APE. Kinetic parameters were obtained by using the synthetic oligonucleotides C I (following conversion to an AP site by removal of the uracil residue), C II, and CIII. APE was incubated with varying concentrations of each oligonucleotide substrate in the presence of complementary substrate and the amount of cleavage determined following gel electrophoresis of ethanol-precipitated fragments. The apparent K_ms and V_{max}s were determined from Lineweaver–Burk plots. All plots were linear, and the results are summarized in Table III.

Table III: Estimates of K_m and V_{max} for Bovine APE Action on Different Substrates

substrate	oligonucleotide	K_m (nM)	V_{max} (pmol min^{-1} unit^{-1})
AP site	C I[a]	297	0.50
tetrahydrofuran	C II	71	0.03
propanediol	C III	167	0.10
depurinated plasmid DNA		<10	1.00

[a] Oligonucleotide C I that was treated with uracil glycosylase to remove uracil (detailed under Materials and Methods).

Discussion

The results presented in this paper provide a clearer picture of how bovine APE recognizes and cleaves DNA at abasic sites. First, this enzyme is a class II apurinic endonuclease, thus catalyzing hydrolysis of the phosphodiester bond most 5′ to the abasic site. Cleavage at this location excludes β elimination in the mechanism of this enzyme. The action of the enzyme on the synthetic oligonucleotides containing tetrahydrofuran, propanediol, and ethylene glycol interphosphate linkages is also inconsistent with β elimination. These three synthetic linkages are, in fact, alkali and piperidine stable (Chang, unpublished data). Second, duplex DNA is required for bovine APE action. The present experiments cannot distinguish whether a direct interaction between the APE and the complementary DNA strand is required or if the conformation of the strand containing the abasic site is altered when hybridized to the complementary strand in such a way as to facilitate cleavage. Third, while the substrate for this enzyme is by definition an AP site, that is, a base-free deoxyribose residue that provides the interphosphate linkage, there does not appear to be any absolute requirement for a deoxyribose moiety in the recognition or cleavage of base-free sites in DNA by this enzyme. Activity of the APE is only moderately reduced when the interphosphate linkage is changed from deoxyribose to either a slightly modified deoxyribose (tetrahydrofuran), a propanediol, or an ethylene glycol moiety. The ethylene glycol linkage differs from the deoxyribose linkage by the absence of the furanose ring and in the shorter interphosphate bond distance.

Although the results presented above indicate that the bovine APE activity is relatively tolerant of certain modifications to the deoxyribose moiety, other modifications to this group render structures containing the abasic site inactive as substrates. These include reduction of the C′-1 aldehyde moiety to an alcohol and adduct formation at the C′-1 aldehyde by alkoxyamine or the presence of a urea residue on C′-1. Although either reduction or alkoxyamine modification of the C′-1 aldehyde prevents furanose ring closure, the inactivity of these moieties for APE cleavage cannot simply be due to the absence of furanose ring since the ethylene glycol and propanediol linkages are active substrates. One feature, common to all of the inactive substrates studied, is a group substitution adjacent to C′-1 of the AP site. Thus, both the urea residue and the alkoxyamine adducts are at positions normally unoccupied in AP sites per se. Thus, our data are consistent with two requirements for bovine APE recognition of abasic sites. First, the DNA must be in the double-stranded

conformation, and second, the double helix must contain a space or pocket of sufficient size adjacent to the C′-1 position before cleavage at the most 5′ phosphodiester bond can occur. Alternatively, it is possible that the enzyme interacts with some alterations in the conformation of the DNA phosphodiester backbone that are produced by the abasic site.

Highly specific interactions must occur for APE cleavage at an abasic site, as bovine APE and other apurinic endonucleases are highly accurate and efficient in selecting sites for DNA cleavage. Bovine APE cleaves apurinic sites at a rate at least 10^5-fold greater than the rate observed for cleavage of normal DNA. More detailed information concerning the structure of abasic sites in DNA (Kalnick et al., 1988) and the structure of the APE protein itself should further clarify the nature of the interaction between this enzyme and damaged DNA.

This bovine apurinic endonuclease is one of several class II apurinic endonucleases identified in mammalian tissues or cell lines. The enzyme is quite similar to mammalian class II apurinic endonucleases isolated from human tissues and cells (Grafstrom et al., 1984; Linsley et al., 1977; Kane & Linn, 1981; Brent, 1983) and rat tissues (Verly et al., 1981; Ivanov et al., 1988) with respect to apparent molecular weight and Mg^{2+} dependence. The previous report by Verly et al. (1981) of a class II apurinic endonuclease from rat tissue that is also inactive with reduced AP sites as substrates suggests that all mammalian class II apurinic endonucleases so far reported are quite similar in their properties. If so, the observations reported here concerning the substrates for bovine APE may be generalized to other class II enzymes. The most notable difference between the properties of this class II bovine APE and the properties of the other reported mammalian class II apurinic endonucleases is the absence, in bovine APE, of the class I apurinic endonuclease activity, reported to be associated with the class II apurinic endonuclease activity isolated from human placenta (Grafstrom et al., 1982).

These studies have been greatly aided by the use of synthetic oligonucleotides containing a single chemically defined abasic site. The plasmid assay is limited in the range of substrate concentrations that can be studied because of the method used to visualize the DNA (detailed under Materials and Methods). It is not possible to use the plasmid assay, with nonlabeled plasmid, to do kinetics studies because the enzyme rates are essentially the same at the range of substrate concentrations possible. The oligonucleotide assay permits a large range of substrate concentrations to be used to determine enzyme activity and has a smaller standard deviation than the plasmid assay. The oligonucleotide assay has the additional advantage that the lesion in such oligonucleotides is better defined than in plasmids containing several abasic sites. Such modified oligonucleotides are also more versatile, allowing the synthesis of sequences with a high frequency of either natural or structurally modifed abasic sites. However, there are clearly important differences between oligonucleotides and plasmid DNA as substrates for bovine APE. For example, the K_m of the bovine APE for oligonucleotides containing abasic sites (297 nM) is much greater than for the same sites in plasmid DNA (<10 nM). Differences have also been reported in the K_m values estimated for a mammalian glycosylase when oligonucleotides and calf thymus DNA are compared as substrates (Male et al., 1987). In assessing the role of these and other enzymes in cellular DNA repair, it should be realized that kinetics of repair of lesions in chromatin may well differ from those observed in vitro by using plasmid or oligonucleotide substrates. The K_m values estimated for apurinic endonucleases from other sources vary substantially, ranging from 1 to 4 nM for partially purified AP endonucleases from human cells and tissues (Kuhnlein et al., 1976; Linsley et al., 1977) to 8 μM, estimated by Shaper et al. (1982) for enzyme fractions partially purified from human placenta. These values may vary from those obtained for the bovine APE in part because they represent distinct forms of mammalian AP endonucleases. They may also reflect differences in the substrates used to estimate activity, as found when the bovine system was characterized.

Repair of AP and other abasic sites in DNA is clearly a multistep, multienzyme process. Apurinic endonucleases of both class I and class II have been identified in mammalian tissues and cell lines (Brent, 1983). The class I apurinic endonucleases, enzymes that cleave AP sites immediately 3′ to the deoxyribose moiety, differ from class II enzymes not only by the phosphodiester bond cleaved but by differences in size, chromatographic behavior, and cofactor requirements and by the presence of an associated thymine glycol glycosylase activity. AP sites in DNA are substrates for cleavage by either class I or class II apurinic endonucleases. However, the products of cleavage by these two enzymes differ. For each enzyme a phosphate-linked, base-free sugar moiety remains attached to the DNA as a DNA 3′ terminus following class I cleavage or as a 5′ terminus following class II cleavage. In vivo, it is possible that such residual sugar moieties may be removed by the action of a second class of apurinic endonucleases. However, other mechanisms for removal of these lesions can also be envisioned. The relative importance of each of the apurinic endonuclease classes in repair of AP and other base-free sites in vivo and in the usual sequence of cleavage if both of these classes are involved is yet to be determined.

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DNA Sequence Specificity of Mitomycin Cross-Linking†

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Received August 3, 1988; Revised Manuscript Received January 13, 1989

ABSTRACT: Using a gel electrophoresis assay, we show that the target DNA sequence cross-linked by *N*-methylmitomycin A, its aziridinomitosene, and mitomycin C is CpG, in strong preference over GpC. The yield per CpG site increases as the number of successive CpG sequences increases. Molecular modeling reveals no systematic difference between the energies of mitomycin cross-links at CpG in comparison with GpC. However, the distance between guanine amino groups in CpG sequences is nearly the same as the distance in the cross-linked adduct, whereas the amino group separation at GpC sites is substantially larger in the starting DNA than in the adduct. We suggest that the favorable placement of the second reaction center in CpG greatly accelerates the second step in the cross-linking reaction. As shown by a competition assay, mitomycins bind A·T and G·C sequences noncovalently equally well, even though the only sequence that yields appreciable cross-linking is CpG. *N*-Methylmitomycin A and its aziridinomitosene are found to be better cross-linking agents than is mitomycin C.

Since their discovery in 1956 (Hata et al., 1956), mitomycins have received much attention due to their potency as chemotherapeutic agents (Remer, 1956) although most of the work has been focused on one member of the group, mitomycin C (MC).[1] MC has three major functional groups: quinone, carbamate, and aziridine, but the drug is not active in the quinone form (Figure 1). Activation requires either enzymatic or chemical reduction (Iyer & Szybalski, 1964a), or mild acidic treatment (Tomasz & Lipman, 1979), converting MC to alkylating agents capable of monofunctional and/or bifunctional covalent interaction with DNA (Iyer & Szybalski, 1964; Mercado & Tomasz, 1972). MC acts directly on DNA to inhibit DNA synthesis (Goldberg & Friedman, 1971), thus preventing cell division and diminishing cell viability. Although monofunctional alkylation occurs 10–20 times more frequently than bifunctional alkylation (Iyer & Szybalski, 1964; Weissbach & Lisio, 1965), cross-linking has been considered to be the source of the lethal effect on cancer cells (Iyer & Szybalski, 1967). More recently, monofunctional alkylation has been reported to cause significant DNA damage (Mercado & Tomasz, 1972; Small et al., 1976; Kinoshita et al., 1971). Besides damage caused by alkylation, superoxide radicals formed upon reoxidation of the mitomycin hydroquinone induce single-strand breakages (Lown & Beigleiter, 1976; Tomasz, 1976).

Characterization of the DNA–mitomycin complex has been difficult, since early studies showed that only a small fraction of added mitomycin incorporates into DNA, about one per several hundred nucleotides (Weissbach & Lisio, 1965). In addition, the activated intermediate is short-lived and difficult to isolate (Iyer & Szybalski, 1964; Patrick et al., 1964). In spite of these problems, investigators have been able to determine the functional groups involved in mitomycin–DNA binding. Iyer and Szybalski hypothesized that the cross-linking of DNA by MC involved C_1, after loss of the methoxy group and subsequent opening of the aziridine ring, and C_{10}, after displacement of the carbamoyl group (Iyer & Szybalski, 1963, 1964). Support for Iyer and Szybalski's proposal has been provided by many investigators. Both Tomasz and Mercado

†Supported by Grant CA 15583 from the National Cancer Institute.

[1] Abbreviations: MC, mitomycin C; NMA, *N*-methylmitomycin A; MS, the aziridinomitosene of NMA (Danishefsky & Egbertson, 1986).

0006-2960/89/0428-3901$01.50/0