Mechanism of Action of *Micrococcus luteus* γ-Endonuclease†

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ABSTRACT: *Micrococcus luteus* extracts contain γ-endonuclease, a Mg^{2+}-independent endonuclease that cleaves γ-irradiated DNA. This enzyme has been purified approximately 1000-fold, and the purified enzyme was used to study its substrate specificity and mechanism of action. γ-Endonuclease cleaves DNA containing either thymine glycols, urea residues, or apurinic sites but not undamaged DNA or DNA containing reduced apurinic sites. The enzyme has both *N*-glycosylase activity that releases thymine glycol residues from OsO_4-treated DNA and an associated apurinic endonuclease activity. The location and nature of the cleavage site produced has been determined with DNA sequencing techniques. γ-Endonuclease cleaves DNA containing thymine glycols or apurinic sites immediately 3′ to the damaged or missing base. Cleavage results in a 5′-phosphate terminus and a 3′ baseless sugar residue. Cleavage sites can be converted to primers for DNA polymerase I by subsequent treatment with *Escherichia coli* exonuclease III. The mechanism of action of γ-endonuclease and its substrate specificity are very similar to those identified for *E. coli* endonuclease III.

Ionizing radiation produces several different types of damage in DNA. Though direct strand breakage has been studied most extensively, base damage is potentially more significant, because stable modified bases may have altered coding capacities. Attempts to quantitate base damage in bacterial and mammalian systems have employed base-damage-specific endonucleases to cleave damaged DNA (Paterson et al., 1976; Schon-Bopp et al., 1977; Skov et al., 1979; Tomilin & Barenfeld, 1979; Schafer et al., 1980; Fornace et al., 1986). These enzymes are believed to be repair enzymes that act on specific types of base damage in DNA as a first step in a multienzyme repair process.

γ-Endonuclease is a Mg^{2+}-independent enzyme activity in extracts of *Micrococcus luteus* that cleaves γ-irradiated DNA at sites of thymines and cytosines (Hentosh et al., 1985). This enzyme is distinct from the *M. luteus* pyrimidine dimer endonuclease, which cleaves UV-irradiated DNA at sites of cyclobutane dimers (Haseltine et al., 1980) and can be distinguished by its different substrate specificity and chromatographic properties (Schon-Bopp et al., 1977; T. J. Jorgensen, unpublished data).

Although the presence of γ-endonuclease in *M. luteus* has been well established (Paterson & Setlow, 1972; Setlow & Carrier, 1973; Wilkens, 1973; Schon-Bopp et al., 1977; Hentosh et al., 1985), the mechanism of action had not been determined. In this paper we describe studies of the mechanism of action of this enzyme using preparations purified 1000-fold from crude extracts. Evidence is presented that γ-endonuclease acts both as an *N*-glycosylase, releasing thymine glycols and probably other altered pyrimidines from DNA, and also as an AP[1] endonuclease to cleave DNA at the resulting base-free sites.

Materials and Methods

Enzymes

M. luteus γ-endonuclease was prepared as described below. *Escherichia coli* endonuclease III was prepared as previously described (Katcher & Wallace, 1983). The Mg^{2+}-dependent bovine AP endonuclease was purified from calf thymus (Henner et al., 1987) and, on polyacrylamide gel electrophoresis, is a homogeneous protein of 37 000 daltons. *E. coli* endonuclease III and DNA polymerase I were purchased from P-L Biochemicals and New England Biolabs.

Preparation of DNA

Phage DNA. PM2 phage DNA was prepared by isolation of phage particles from lysates of infected *Pseudomonas* Bal 31-14 with sucrose gradient centrifugation followed by chloroform/butanol extraction of protein (Wallace et al., 1981).

Plasmid DNA. pUC18 and pBR322 plasmid DNAs were prepared from lysates of transfected *E. coli* HB101 by DNA precipitation followed by cesium chloride density gradient centrifugation (Maniatis et al., 1982).

DNA Copolymer. Poly(dA-[^{3}H]dT) was prepared by an in vitro synthesis reaction (Weiss & Milcarek, 1974; Cunningham & Weiss, 1985). Klenow fragment of DNA polymerase I was added to a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM magnesium acetate, 2 mM 2-mercaptoethanol, 0.01 mM poly(dA-dT) primer, 0.55 mM dATP, and 0.49 mM [*methyl*-^{3}H]dTTP (43 Ci/mmol). The reaction mixture was incubated at 25 °C for 2.5 h and ethanol-precipitated. The precipiate was resuspended in 100 mM NaCl–10 mM EDTA, extracted with phenol, and reprecipitated with ethanol. The precipitate was then lyophilized and resuspended in 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, and

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[1] Abbreviations: AP, apurinic and/or apyrimidinic; EDTA, ethylenediaminetetraacetic acid; DEAE-cellulose, (diethylaminoethyl)cellulose; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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1 mM EDTA and purified by gel filtration on Sephadex G-100 (Pharmacia). The specific activity of the ^{3}H-labeled product was approximately 10.0 Ci/mmol of DNA as nucleotide.

Treatment of DNA

γ-Irradiated DNA. γ-Irradiated DNA was prepared by exposing supercoiled plasmid DNA at 0.2 mM or ^{32}P-end-labeled DNA at 1.0 μM as nucleotide in 10 mM phosphate buffer (pH 7.5) containing 100 mM KI to ^{60}Co γ-irradiation while bubbling with N_2. This procedure reduced the oxygen concentration during irradiation to <6 ppm and produced approximately 20 enzyme-sensitive sites per direct strand break.

Thymine Glycol Containing DNA. To prepare thymine glycol containing DNA for assays, PM2 DNA (90–95% form I) was preheated to 65 °C for 2 min, and 4% (w/v) OsO_4 was added to a final concentration of 0.08% followed by further incubation at 65 °C for 5 min. The reaction was stopped by chilling rapidly on ice, and then the mixture was dialyzed extensively against 10 mM Tris-HCl (pH 7.5)–1 mM EDTA at 4 °C. This treatment yielded approximately two thymine glycols per DNA molecule. The average number of thymine glycols per DNA molecule was estimated with *E. coli* endonuclease III as previously described (Katcher & Wallace, 1983). Alternatively, pUC18 DNA was heated at 70 °C in 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, and 0.03% OsO_4 for 90 min. The reaction was stopped by chilling on ice, and OsO_4 was removed by multiple extractions with ethyl ether followed by ethanol precipitation of the DNA. This treatment produced an average of three thymine glycol sites per molecule.

To produce thymine glycol sites in ^{32}P-end-labeled DNA or poly(dA-[^{3}H]dT), the DNA was treated for either 90 min or 4 h, respectively, at 70 °C in 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, and 0.07% OsO_4. The reaction was stopped by chilling in ice, and OsO_4 was removed by multiple extractions with ethyl ether followed by ethanol precipitation of the DNA.

Urea-Containing DNA. Urea-containing DNA was prepared by alkali hydrolysis of thymine glycol containing DNA. Thymine glycol containing PM2 DNA was dialyzed for 24 h at 25 °C against 20 mM KH_2PO_4–KOH (pH 12.0) and 1 mM EDTA and then dialyzed overnight at 4 °C against 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Alternatively, thymine glycol containing DNA was adjusted to pH 12.0 with 1.0 N KOH and incubated for 3 h at room temperature. The pH was then adjusted to 7.5 with 1.0 N HCl. The number of urea residues was estimated with *E. coli* exonuclease III as previously described (Kow & Wallace, 1985) and was equal to the original number of thymine glycols.

Apurinic DNA. Apurinic DNA was prepared according to the method of Lindahl and Andersson (1972) by heat/acid treatment. PM2 DNA was ethanol-precipitated, washed with 70% ethanol, redissolved in 10 mM sodium citrate (pH 4.5) containing 0.1 M NaCl, and incubated at 70 °C for 10 min. The reaction was stopped by chilling rapidly on ice, and the DNA was then precipitated with ethanol, washed with 70% ethanol, dried with N_2, and redissolved in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. This treatment yielded an average of 1.5 apurinic sites per PM2 DNA molecule. The number of apurinic sites was determined by alkaline fluorometry (Kowalski, 1979). In some experiments, apurinic pUC18 DNA containing three sites per molecule was prepared by incubation as above at 70 °C for 80 min.

Another method was used in order to produce a large number of apurinic sites in ^{32}P-end-labeled DNA for sequencing experiments (Nes, 1980; Male et al., 1981). End-labeled DNA was treated with 15 mM methylmethanesulfonate, 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, and 10% ethanol, at 37 °C for 60 min. The DNA was ethanol-precipitated and resuspended in 20 mM NaCl, 20 mM sodium citrate, and 20 mM NaH_2PO_4 (pH 7.4) and then held at 50 °C for 5 h to produce apurinic sites.

Reduced Apurinic DNA. To prepare reduced apurinic DNA, apurinic DNA (40 μg/mL) in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA was brought to 5 M sodium cyanoborohydride and incubated at room temperature for 1 h. Sodium cyanoborohydride was then removed by extensive dialysis at 4 °C. Sodium cyanoborohydride treatment of thymine glycol containing or urea-containing DNA was performed in the same manner as for apurinic DNA. Sodium borohydride was not used since it produced enzyme-sensitive sites.

γ-Endonuclease Assay

Endonuclease activity was measured by conversion of form I supercoiled substrate DNA to form II relaxed circles. Supercoiled DNA was measured either by alkaline fluorometry (Kowalski, 1979) or by agarose gel electrophoresis.

Resolution of intact (form I) from nicked (form II) DNA on agarose gels provided a convenient assay. Substrate DNA was incubated in reaction buffer [10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA] for 20 min with enzyme, and the reaction was stopped by addition of sodium dodecyl sulfate to 1%. The reaction mixture was resolved by agarose gel electrophoresis and stained with ethidium bromide. The gels were photographed, and the amount of form I and form II DNA in each band was determined by densitometer scans of the negative. The number of nicks per molecule was calculated from the relation $n = -\ln$ (form I fraction). Enzyme units were expressed as femtomoles of nicks per minute for nicks produced in OsO_4-treated supercoiled pUC18 DNA contained an average of three enzyme sites per molecule.

Purification of γ-Endonuclease

Crude Extract. γ-Endonuclease was purified from dried *M. luteus* cells (ATCC 4698; Sigma). All purification procedures were performed at 4 °C unless otherwise indicated. A total of 20 g of dried cells was suspended in 1000 mL of wash buffer containing 200 mM sucrose and 10 mM Tris-HCl (pH 8.0). The cells were centrifuged at 5000*g* for 25 min, and the pellet was resuspended in the same volume of wash buffer. After a second centrifugation, the cells were again suspended in the same volume of wash buffer, and 200 mg of lysozyme was added. After 30 min at 30 °C, an equal volume of ice-cold distilled water was added to lyse the cells. The DNA was sheared by sonication and then precipitated by addition of 160 mL of 10% (w/v) streptomycin sulfate over 30 min followed by centrifugation at 10000*g* for 60 min. To the supernatant was added 1030 g of ammonium sulfate over 30 min to produce a 75% saturated solution, followed by 30 min of further stirring and then centrifugation at 10000*g* for 60 min. The precipitate was resuspended in 400 mL of storage buffer [10 mM Tris-HCl (pH 7.6), 10% (v/v) ethylene glycol, 1 mM 2-mercaptoethanol] and frozen at −20 °C (fraction I).

DEAE-cellulose Chromatography. Two preparations of crude extract (800 mL) were thawed, dialyzed extensively against 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 1 mM 2-mercaptoethanol, and loaded on a DEAE-cellulose (Whatman DE-52) column (4.5 cm × 37 cm) preequilibrated with the dialysis buffer. The loaded column was washed with 2–3 column volumes of buffer, and the enzyme was then eluted

in the same buffer with a 2000-mL linear gradient of NaCl from 0.05 to 0.30 M. Fractions containing activity (0.05–0.20 M NaCl) were pooled (fraction II).

DNA–Cellulose Chromatography. The pooled fractions from the DEAE-cellulose preparation were dialyzed extensively against 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol and loaded on a column of DNA–cellulose (1 cm × 30 cm; 0.45 mg of DNA/mL of dry cellulose) (Alberts & Herrick, 1971). The column was washed with buffer until the eluent contained <5 μg/mL protein, and bound protein was eluted with a 450-mL NaCl gradient from 0.05 to 1.50 M NaCl in the same buffer. Fractions containing activity (0.15–0.58 M NaCl) were pooled (fraction III).

Phosphocellulose Chromatography. Pooled fractions from the DNA–cellulose column were dialyzed extensively against 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol. The dialyzed preparation was loaded on a column of phosphocellulose (0.9 cm × 1.8 cm; Whatman P-11). The column was washed with 10 mM Tris-HCl (pH 7.6), 125 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol and eluted with a 15-mL salt gradient from 0.125 to 0.60 M NaCl in the same buffer. Active fractions (0.15–0.50 M NaCl) were pooled and stored at −20 °C (fraction IVa).

Mono Q Chromatography. Alternatively, a fast protein liquid chromatography (FPLC) system by Pharmacia was employed. The DNA–cellulose preparation was dialyzed against 50 mM Tris-HCl (pH 8.6) and 1 mM 2-mercaptoethanol. The dialyzed preparation was loaded on a HR 5/5 Mono Q anion-exchange FPLC column. Enzyme was eluted with a 42.9-mL buffer gradient of Tris-HCl (pH 8.6) from 0.05 to 0.80 M. Active fractions (0.60–0.75 M) were stored frozen at −20 °C (fraction IVb). This fraction had a simpler protein profile than fraction IVa, when analyzed by SDS–PAGE (3 bands vs. 15 bands). However, this purification step resulted in low yield and an unstable enzyme preparation.

Gel Filtration Chromatography

The molecular weight of γ-endonuclease was determined by gel filtration chromatography with a Superose 12 gel filtration FPLC column. A 150-μL aliquot of the phosphocellulose preparation (fraction IVa) was injected onto a HR 10/30 Superose 12 column and eluted with 200 mM NaCl, 10 mM Tris-HCl (pH 7.6), and 1 mM EDTA at a rate of 0.4 mL/min. The elution volume of γ-endonuclease was determined by measuring enzyme activity against both thymine glycol containing and apurinic pUC18 DNA in the eluted fractions. The molecular weight of γ-endonuclease was determined on the basis of calibration of elution volumes with protein markers of known molecular weight. Elution volumes of marker proteins were identified by UV absorbance at 280 nm. Bovine serum albumin, carbonic anhydrase, cytochrome *c*, and aprotinin were obtained from Sigma Chemical Co.

Nucleotide Incorporation into γ-Endonuclease-Digested DNA

The ability of γ-endonuclease cleavage sites to act as potential primers for DNA polymerase I was measured by incorporation of radioactive nucleotide precursors into γ-endonuclease-cleaved DNA. Thymine glycol containing PM2 DNA (1 μg) was ethanol-precipitated and redissolved in 0.1 mL of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. γ-Endonuclease (fraction IVa) was added, and the reaction was incubated at 37 °C for 2 h. The enzyme was then inactivated by heating at 70 °C for 10 min. The reaction was then divided into two: To one was added 1 unit of *E. coli* exonuclease III and 10 mM $MgCl_2$, and to the other was added 10 mM $MgCl_2$. Both reactions were incubated at 37 °C for 10 min. As a control, thymine glycol containing DNA (0.5 μg) without pretreatment with γ-endonuclease was incubated with exonuclease III. After 10 min, exonuclease III was inactivated by heating at 70 °C for 10 min. The reaction mixture was then put on ice, and 20 nmol each of dTTP, dCTP, dGTP, and dATP and 1 μCi of [^{3}H]dTTP (67 Ci/mmol) were added. The reaction mixture was brought to 150 μL with 10 mM Tris-HCl (pH 7.5), and the $MgCl_2$ concentration was adjusted to 8 mM. To this was added 1 unit of *E. coli* polymerase I. Immediately after the addition of polymerase, 20 μL was removed from each reaction, placed on glass fiber filters (Whatman GF/A), washed with ice-cold 10% trichloroacetic acid, and washed with 95% ethanol. The reactions were then incubated at 37 °C, and 20-μL samples were taken every 20 min and treated as above. All filters were dried and assayed for radioactivity.

DNA Sequencing Gels

End-labeled DNA restriction fragments were prepared according to Maxam and Gilbert (1980). pBR322 or pUC18 DNA was cleaved with restriction endonuclease *Bam*HI prior to ^{32}P labeling. Labeling at the 3′ ends was performed in a reaction with the Klenow fragment of DNA polymerase I, and all four α-^{32}P-labeled deoxyribonucleoside triphosphates. DNA fragments to be labeled at the 5′ terminus were dephosphorylated with calf alkaline phosphatase. The fragments were then treated with T4 polynucleotide kinase and [γ-^{32}P]ATP. Fragments labeled in only one strand were obtained by cleavage with *Hin*cII or *Hae*II for pBR322 or pUC18, respectively, followed by preparative polyacrylamide gel electrophoresis.

Restriction fragments of double-stranded pBR322 or pUC18 plasmid DNA, radiolabeled with ^{32}P at the 3′ or 5′ terminus in one strand only, were either irradiated under nitrogen or treated chemically, as described above, to produce fragments with enzyme-sensitive sites. This DNA was then incubated in 50 μL of reaction buffer [10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA] with γ-endonuclease and run on a denaturing 20% polyacrylamide/7 M urea gel. The same end-labeled fragment was treated in parallel with Maxam and Gilbert purine or pyrimidine sequencing reagents and run in the neighboring lane in order to orient the enzyme cleavage pattern along the known sequence of the plasmid DNA. Autoradiography was performed by exposure of the gels to Kodak XAR-5 film for 1–14 days at −70 °C.

High-Performance Liquid Chromatography

Ethanol-soluble radioactivity released from synthetic deoxyribonucleotide copolymer was lyophilized, resuspended in distilled deionized water, and analyzed by high-performance liquid chromatography (HPLC). Samples were eluted from a reverse-phase HPLC column (Waters μBondapak C_{18}; 3.9 mm × 30 cm) with 5% methanol in distilled deionized water. The flow rate was 1.0 mL/min, and 0.3-mL fractions were collected. Five milliliters of scintillation cocktail (Hydrofluor; New England Nuclear) was added to each fraction, and radioactivity was quantitated by liquid scintillation counting.

The elution times of marker compounds were determined by UV absorbance at 230 nm. Thymine and 5,6-dihydrothymine were obtained from Sigma Chemical Co. Thymine glycol (*cis*-5,6-dihydroxy-5,6-dihydrothymine) and thymidine glycol (*cis*-5,6-dihydroxy-5,6-dihydrothymidine) were prepared according to the method of Iida and Hayatsu (1970, 1971)

Table I: Purification of *M. luteus* γ-Endonuclease[a]

fraction	description	[protein] (mg/mL)	sp act. (units/mg)	x-fold purification	yield (%)
I	crude extract	13.2	1 800		100
II	DEAE-cellulose	2.71	11 400	6.3	104
III	DNA–cellulose	0.128	156 000	86	26
IVa	phosphocellulose	0.030	1 700 000	944	5
IVb	Mono Q[b]	0.006	1 825 000	1014	0.88

[a] γ-Endonuclease was purified through a series of chromatographic procedures from crude extract (fraction I) of *M. luteus*. Enzyme isolated through DEAE-cellulose (fraction II) and DNA–cellulose (fraction III) chromatography was subjected to final purification on either phosphocellulose (fraction IVa) or Mono Q (fraction IVb). [b] Mono Q is an anion-exchange column used with the fast protein liquid chromatography system (Pharmacia).

as modified by Frenkel et al. (1981a,b).

RESULTS

γ-Endonuclease activity on thymine glycol containing DNA was purified from dried *M. luteus* cells by a sequence of ion-exchange and affinity chromatographies (Table I). Crude cell extract was bound to DEAE-cellulose and gradient eluted. Active fractions were pooled and further purified by affinity chromatography on DNA–cellulose. Finally, ion-exchange chromatography on either phosphocellulose or Mono Q (FPLC) provided enzyme purified approximately 1000-fold for use in mechanism studies.

Activity with γ-Irradiated DNA. As γ-endonuclease had been purified on the basis of its ability to cleave thymine glycol containing DNA, it was of interest to determine whether the purified preparation retained the activity and sequence specificity of the crude extracts with γ-irradiated DNA. In previous studies of the sequence specificity of cleavage by γ-endonuclease activity of crude extracts, it had been found that cleavage of γ-irradiated DNA occurred at both thymines and cytosines (Hentosh et al., 1985). To determine whether purified γ-endonuclease retained the ability to cleave γ-irradiated DNA at both thymines and cytosines, end-labeled defined-sequence DNA was γ-irradiated and treated with γ-endonuclease, and the resulting fragments were resolved by gel electrophoresis (Figure 1). The location of the fragments by gel autoradiography indicated that cleavage of γ-irradiated DNA occurred at both thymines and cytosines in approximately equal amounts. The identical sequence specificity of cleavage for crude extracts and purified γ-endonuclease suggested that the purified γ-endonuclease was the same enzyme as that in crude extracts responsible for cleavage at γ-radiation-damaged thymines and cytosines.

Substrate Specificity. Since ionizing radiation produces multiple chemically distinct forms of base damage in DNA (Teoule & Cadet, 1978; Hutchinson, 1986), it was preferable to use DNA substrates containing a single chemically defined damage for studies of the mechanism of DNA cleavage by γ-endonuclease. Thymine glycols, urea residues, and apurinic sites are all products of ionizing irradiation of DNA (Teoule & Cadet, 1978), and each can be produced with high specificity by chemical treatment of DNA. Thymine glycols were introduced into DNA by OsO_4 treatment (Burton & Riley, 1966), urea residues were produced by alkali treatment of thymine glycol containing DNA, and apurinic sites were produced by depurination in hot acid. The ability of γ-endonuclease to cleave DNA containing each of these different

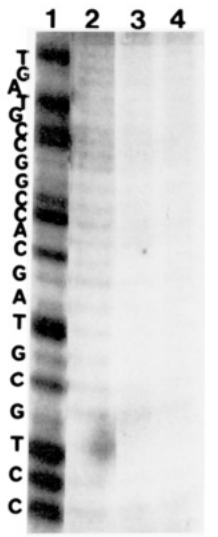

FIGURE 1: Sequence specificity of cleavage on γ-irradiated DNA. 3′-^{32}P-end-labeled DNA was irradiated to 100 Gy, treated with 2 μL of γ-endonuclease (fraction IVa), and subjected to electrophoresis on a DNA sequencing gel (lane 1). Control lanes of irradiated DNA without enzyme treatment (lane 2) and unirradiated DNA with (lane 3) and without (lane 4) enzyme treatment were also run.

Table II: Effect of Substrate Reduction on Enzyme Cleavage[a]

DNA substrate		enzyme		
lesion	sodium cyanoborohydride	γ-endonuclease	endonuclease III	exonuclease III
apurinic	–	1.70	1.75	1.50
apurinic	+	0.15	0.20	1.65
thymine glycol	–	1.35	1.25	0.15
thymine glycol	+	1.50	1.55	0.25
urea	–	1.26	1.30	1.45
urea	+	1.36	1.27	1.53

[a] The number of enzyme-sensitive sites per PM2 molecule is shown for DNA containing different lesions that were either treated or not treated with sodium cyanoborohydride.

substrates was measured. Urea, thymine glycol, and apurinic sites were all substrates for the enzyme (Table II). Under identical conditions the rate of nicking of apurinic pUC18 DNA was 2.6 times that for thymine glycol containing pUC18 DNA.

Apurinic Endonuclease Activity. Activity with apurinic DNA substrate and activity with thymine glycol containing DNA substrate comigrated through the purification, including phosphocellulose chromatography and Mono Q chromatography. To determine whether AP endonuclease activity was an inherent component of γ-endonuclease, we attempted to resolve the apurinic and thymine glycol activities by Superose 12 gel chromatography on the high-resolution FPLC system (Figure 2). Both activities eluted at 17.1 mL, which corresponds to a molecular mass of 28 000 daltons. The inability to separate the two activities by this and the other chromatographic procedures suggested that both activities were properties of the same enzyme and also that γ-endonuclease may cleave damaged DNA by a glycosylase/AP endonuclease mechanism.

N-Glycosylase Activity. In order to determine whether γ-endonuclease had *N*-glycosylase activity, we prepared poly(dA-[^{3}H]dT) of high specific activity and treated it with OsO_4 to produce ^{3}H-labeled thymine glycols in the copolymer. Subsequent treatment with γ-endonuclease released ethanol-soluble radioactivity from the copolymer. Analysis by reverse-phase HPLC, using marker compounds for comparison,

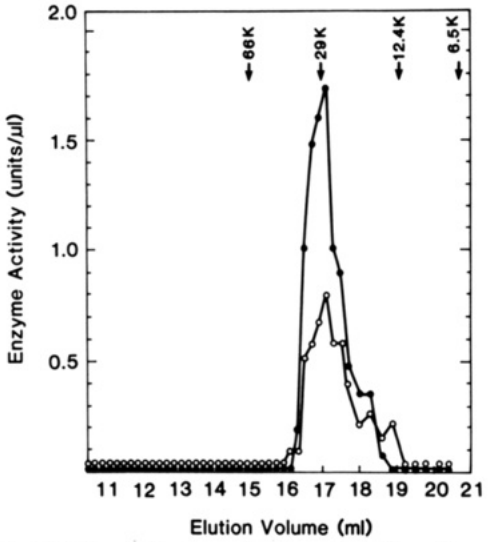

FIGURE 2: FPLC gel filtration of endonucleolytic activity against pUC18 DNA containing thymine glycol or apurinic sites. γ-Endonuclease preparation (fraction IVa) was chromatographed on an FPLC Sepharose 12 gel filtration column as described under Materials and Methods. Fractions were assayed for activity against both thymine glycol containing (open circles) and apurinic (closed circles) pUC18 plasmid DNA. Quantitation of DNA cleavage was by gel electrophoresis. Molecular mass calibration was achieved by determining the elution volumes of proteins of known molecular mass (bovine serum albumin, 66.0 kDa; carbonic anhydrase, 29.0 kDa; cytochrome *c*, 12.4 kDa; aprotinin, 6.5 kDa). Void volume was 9.2 mL.

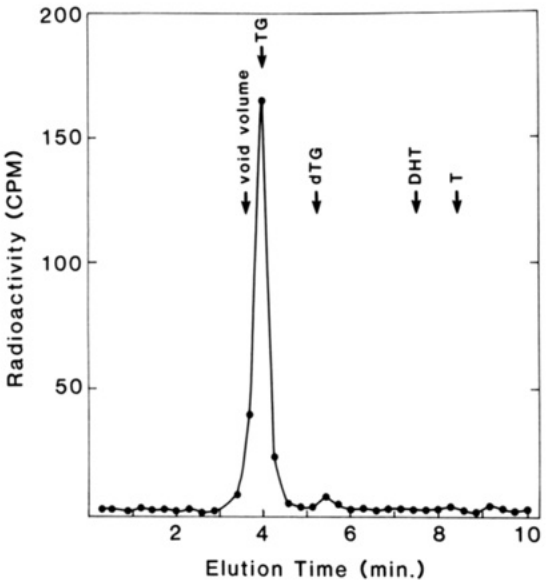

FIGURE 3: HPLC analysis of products released by γ-endonuclease treatment of thymine glycol containing substrate. A synthetic deoxyribonucleotide of poly(dA-dT), labeled with ^3H at the methyl position of thymine, was treated with OsO_4 to produce thymine glycol sites. The copolymer was then treated with γ-endonuclease (fraction IVa). The reaction was stopped by ethanol precipitation, and radioactivity that did not precipitate was analyzed by HPLC. Arrows indicate elution times of marker compounds (TG, thymine glycol; dTG, thymidine glycol; DHT, dihydrothymine; T, thymine). Control samples treated in an identical fashion except for omission of either OsO_4 treatment or γ-endonuclease treatment contained <10 cpm in any fraction.

identified the released radioactivity as thymine glycol (Figure 3). Control experiments indicated that no thymine glycol was released if either OsO_4 or γ-endonuclease were omitted. Therefore, in addition to its endonuclease activity, γ-endonuclease exhibited an *N*-glycosylase activity that released the damaged base.

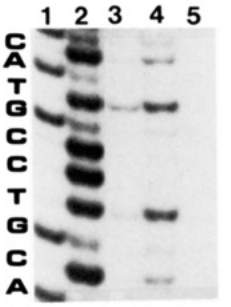

FIGURE 4: Specificity of cleavage for thymine glycol containing DNA. 3′-^{32}P-end-labeled DNA was treated with OsO_4 to produce thymine glycol at sites of thymine. This DNA was then either further treated with 2 μL of γ-endonuclease (fraction IVa) (lane 3; 58 000 cpm loaded) or 1.0 M piperidine at 90 °C (lane 4; 52 000 cpm loaded) or left untreated (lane 5; 40 000 cpm loaded) and run on a 20% polyacrylamide/7 M urea gel. Sites of cleavage were compared with cleavage produced by Maxam and Gilbert purine (lane 1; 29 000 cpm loaded) and pyrimidine (lane 2; 31 000 cpm loaded) reactions.

Sequence Specificity of Cleavage. To determine the location of endonuclease cleavage in relation to the DNA lesion, 3′-^{32}P-end-labeled DNA was incubated with OsO_4 to produce thymine glycol sites. When this DNA was treated with γ-endonuclease, resulting fragments migrated on sequencing gels in a pattern consistent with cleavage at thymines (Figure 4). Hot piperidine treatment, which is known to cleave DNA at sites of thymine glycol (Friedmann & Brown, 1980), produced the same cleavage pattern. These data indicate that γ-endonuclease recognized thymine glycol produced from thymine in DNA and cleaved the phosphodiester backbone adjacent to the nucleoside containing the damaged base. A smaller amount of cleavage was also seen at cytosines, suggesting that γ-endonuclease also recognized a small amount of cytosine damage produced by OsO_4 (Dizdaroglu et al., 1986).

To confirm that γ-endonuclease also cleaves apurinic DNA specifically at the sites of missing bases, 3′-end-labeled plasmid DNA was depurinated, predominantly at guanines, by methyl methanesulfonate and heat treatment (Nes, 1980; Male et al., 1981) and incubated with γ-endonuclease. Analysis of the resulting fragments by DNA sequencing gels (Figure 5) showed that the DNA was cleaved at sites of purines and produced the same cleavage pattern as that produced by a bovine Mg^{2+}-dependent AP endonuclease, demonstrating that γ-endonuclease can recognize and cleave at apurinic sites in DNA and that the presence of a damaged pyrimidine is not required for endonuclease action.

DNA Termini Produced by γ-Endonuclease Cleavage. Purified γ-endonuclease was used, in two types of experiments, to identify the 3′ end group produced by cleavage of thymine glycol containing DNA. DNA polymerase I has a highly specific requirement for 3′-hydroxyl termini to prime polymerization. When thymine glycol containing DNA was treated with either γ-endonuclease or exonuclease III, which removes 3′-phosphates and phosphate-linked sugar residues, the DNA was an inefficient primer for DNA synthesis catalyzed by DNA polymerase I [<0.07 pmol of nucleotide incorporation min^{-1} (μg of DNA)$^{-1}$]. However, sequential digestion using γ-endonuclease followed by exonuclease III did convert the DNA to a good substrate for DNA polymerase I as indicated by the increased rate of precursor incorporation [0.38 pmol of nucleotide incorporated min^{-1} (μg of DNA)$^{-1}$]. These results are consistent with γ-endonuclease producing either a 3′-phosphate or a baseless deoxyribose residue as a 3′ terminus of cleavage.

5′-^{32}P-end-labeled DNA fragments containing 3′-phosphate or 3′-phosphate/deoxyribose groups have distinctly different

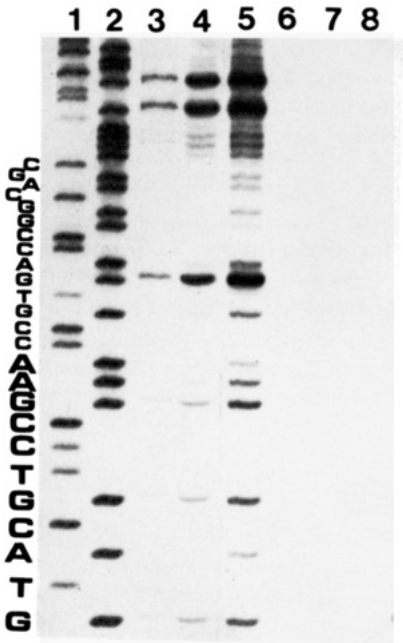

FIGURE 5: Sequence specificity of cleavage for DNA containing AP sites. 3′-^{32}P-end-labeled DNA was depurinated by methyl methanesulfonate and heat treatment. This DNA was then either left untreated (lane 3; 17 000 cpm loaded) or incubated with 1 μL of bovine AP endonuclease (lane 4; 8000 cpm loaded) or 1 μL of γ-endonuclease (fraction IVa) (lane 5; 18 000 cpm loaded) and run on a 20% polyacrylamide/7 M urea gel. Sites of cleavage were compared with DNA cleaved by Maxam and Gilbert pyrimidine (lane 1; 6000 cpm loaded) and purine (lane 2; 7000 cpm loaded) sequencing reactions. γ-Endonuclease cleaved at sites of purines specifically as did the calf thymus AP endonuclease. This enzyme cleavage could be seen above a small background of breakage at purines caused by the depurination treatment. Control DNA lacking apurinic sites showed no cleavage when either left untreated (lane 6; 9000 cpm loaded), treated with bovine AP endonuclease (lane 7; 7000 cpm loaded), or treated with γ-endonuclease (lane 8; 5000 cpm loaded).

migration on DNA sequencing gels (Gordon & Haseltine, 1981). Therefore, to determine which 3′ end group is produced by γ-endonuclease, 5′-end-labeled DNA was treated with OsO_4 and cleaved with γ-endonuclease, and the DNA fragments were resolved by electrophoresis (Figure 6). As noted above, cleavage occurred at sites of thymines. However, the migration of γ-endonuclease-cleaved DNA was slower than that of the DNA chemically cleaved at thymines by a Maxam and Gilbert sequencing reaction. As the chemical cleavage produces a 3′-terminal phosphate group (Maxam & Gilbert, 1980), such a structure is not the product of γ-endonuclease cleavage. The slower migration seen for enzyme-cleaved DNA is consistent with an uncharged baseless sugar residue as the 3′ end group of cleavage.

In contrast, similar experiments with 3′-end-labeled DNA (Figure 4) demonstrated identical migration of γ-endonuclease-cleaved DNA and chemically cleaved DNA. Since chemically cleaved DNA contains 5′-phosphate groups (Maxam & Gilbert, 1980), the data suggest the 5′ end group produced by purified γ-endonuclease is a phosphate group. These results are in agreement with previous work by Hentosh et al. (1985), who demonstrated a 5′-phosphate group as the end product of cleavage when *M. luteus* crude extract was used to cleave γ-irradiated DNA.

γ-Endonuclease Cleavage of Reduced AP Sites. Acid depurination of DNA produces base-free deoxyribose moieties in DNA. There are two resonance structures for the baseless sugar—a "closed" furanose form and an "opened" form containing C-1′ aldehyde group. The C-1′ aldehyde is required

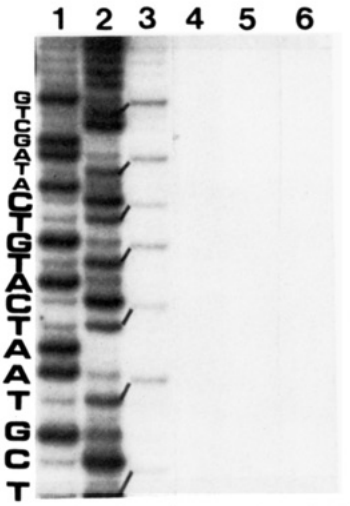

FIGURE 6: Relative migration of DNA cleaved chemically or by γ-endonuclease on DNA sequencing gels. 5′-^{32}P-end-labeled DNA was either chemically cleaved by Maxam and Gilbert sequencing reactions at purines (lane 1) and pyrimidines (lane 2) or treated with OsO_4 and then cleaved with 2 μL of γ-endonuclease (fraction IVb) (lane 3). Control lanes of DNA treated with OsO_4 only (lane 4), treated with γ-endonuclease only (lane 5), and untreated (lane 6) were also run. (Black bars indicate the displaced migration of enzyme-cleaved DNA relative to chemically cleaved DNA.)

for alkali hydrolysis of AP sites (Jones et al., 1968). We wished to determine whether the presence of a C-1′ aldehyde is also required for enzymatic cleavage of AP sites by γ-endonuclease.

We have compared the extent of cleavage of PM2 DNA containing either AP sites, thymine glycols, or urea residues, which had either been exposed to sodium cyanoborohydride or not been treated. This reducing agent converts the C-1′ aldehyde to a C-1′ hydroxyl group. The effect of such reduction on cleavage by either γ-endonuclease, *E. coli* endonuclease III, or *E. coli* exonuclease III was measured (Table II).

Sodium cyanoborohydride reduction of AP sites markedly decreased the extent of cleavage by γ-endonuclease and endonuclease III. Cleavage of AP DNA by exonuclease III was not affected by reduction. Thymine glycol containing or urea-containing DNA does not have the C-1′ aldehyde to be reduced; therefore, exposure of this DNA to sodium cyanoborohydride had no effect on the extent of cleavage by the three endonucleases. These data suggest that the C-1′ aldehyde group is required for cleavage at AP sites by γ-endonuclease.

Discussion

γ-Endonuclease of *M. luteus*, an enzyme that cleaves γ-irradiated DNA, was purified from crude extracts to allow studies of its mechanism of action. The substrate specificity of cleavage indicated that the enzyme recognized different types of pyrimidine damage such as thymine glycols and urea residues, as well as apurinic sites.

Several DNA repair endonucleases are now known to combine both *N*-glycosylase and AP endonuclease actions including pyrimidine dimer endonuclease of *M. luteus* (Gordon & Haseltine, 1981), the pyrimidine dimer specific den V protein of bacteriophage T4 (McMillan et al., 1981; Nakabeppu & Sekiguchi, 1981; Warner et al., 1981), *E. coli* endonuclease III (Demple & Linn, 1980; Katcher & Wallace, 1983), and calf thymus redoxyendonuclease (Doetsch et al., 1986). Our evidence indicates that γ-endonuclease is another member of this class of *N*-glycosylase/AP endonuclease (Figure 7). With

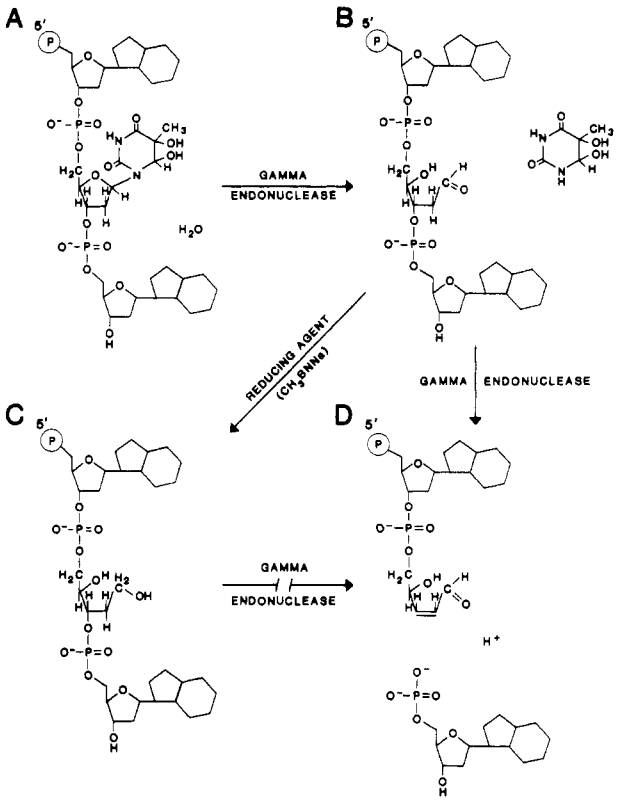

FIGURE 7: Mechanism of γ-endonuclease. γ-Endonuclease is proposed to cleave irradiated DNA at damaged pyrimidines by a two-step mechanism. Damaged pyrimidines, such as thymine glycol shown here (A), may be recognized and removed from the DNA by N-glycosidic cleavage (B). The resulting apyrimidinic site would then be cleaved at the phosphodiester bond immediately 3′ to the site (D), leaving 3′-sugar and 5′-phosphate termini. Reduction of the C-1′ aldehyde (C), however, inhibits cleavage of the apyrimidinic site by γ-endonuclease.

thymine glycol containing DNA, free thymine glycol was released. Furthermore, endonuclease activity with thymine glycol containing or apurinic DNA migrated together throughout the purification.

The results presented here also provide some evidence concerning the chemical mechanism by which the apurinic endonuclease activity of γ-endonuclease cleaves DNA. The apurinic endonuclease of this enzyme is a class I apurinic endonuclease (Linn et al., 1981) producing 5′-phosphate and 3′ baseless sugar termini. Other bacterial enzymes that combine *N*-glycosylase and apurinic endonuclease activities are also of the class I type (Friedberg, 1984). At least two distinct chemical mechanisms for catalysis by class I apurinic endonucleases are possible (Grafstrom et al., 1982). Alternative mechanisms include hydrolysis of the C-1′ phosphoester linkage or β-elimination (elimination β to the C-1′ aldehyde of an apurinic site). Reduction of the C-1′ aldehyde of apurinic sites essentially abolishes the cleavage of AP DNA by either γ-endonuclease or endonuclease III, but not by exonuclease III. Since exonuclease III can cleave DNA without N-glycosidic release of urea (Kow & Wallace, 1985) and can also cleave reduced AP sites, it is unlikely that this enzyme cleaves DNA by a β-elimination reaction. Our results are consistent with, but cannot be considered formal proof of, catalysis of β-elimination as the mechanism of the apurinic endonuclease activities of γ-endonuclease and endonuclease III.

γ-Endonuclease of *M. luteus* and endonuclease III of *E. coli* are quite similar in their substrate specificities. Both enzymes cleave DNA containing urea residues, thymine glycols, or apurinic sites. Neither enzyme cleaves reduced apurinic sites or undamaged DNA (Table II; Katcher & Wallace, 1983). For both enzymes, cleavage of γ-irradiated DNA occurs at both cytosine and thymine sites (Figure 1, Helland et al., 1986). In addition, the mechanism of action of these enzymes is quite similar. Both are *N*-glycosylases (Figure 3; Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984), and both have associated class I apurinic endonuclease activities that appear to catalyze β-elimination reactions. The conservation of such similar enzymatic activities in these two prokaryotic species suggests that they confer significant selective advantage to organisms normally exposed to a variety of DNA-damaging agents under natural conditions. The recent evidence that there is a bovine endonuclease with similar substrate specificity and mechanism of action (Doetsch et al., 1986; Helland et al., 1986; P. W. Doetsch, personal communication) suggests that enzymes of this class may be very widely distributed among evolutionarily diverse species.

γ-Endonuclease and endonuclease III have been utilized in several previous studies of DNA damage and repair as probes for DNA base damage produced by ionizing radiation or other DNA-damaging agents (Paterson & Setlow, 1972; Paterson et al., 1976; Skov et al., 1979; Fornace et al., 1986) or as a reagent to release damaged bases from DNA for identification and quantitation (Breimer & Lindahl, 1985a,b). The better understanding of the substrate specificity and mechanism of action of γ-endonuclease provided by the results reported here should facilitate the use of this enzyme in future studies of DNA damage and repair.

ADDED IN PROOF

Bailly and Verly (1987) have recently shown that the products of *E. coli* endonuclease III treatment of DNA containing abasic sites are the same as those produced by alkali treatment, further suggesting that catalysis of β-elimination is the mechanism of cleavage for that enzyme.

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