Isolation and Characterization of Endonuclease VIII from *Escherichia coli*†

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ABSTRACT: Endonuclease VIII, a novel presumptive DNA repair enzyme, was isolated from *Escherichia coli* by FPLC[1] purification. The enzyme was found in strains that contained or lacked endonuclease III and was purified by radial flow S-Sepharose, Mono S, phenyl-Superose, and Superose 12 FPLC. Examination of the properties of endonuclease VIII showed it to have many similarities to endonuclease III. DNA containing thymine glycol, dihydrothymine, β-ureidoisobutyric acid, urea residues, or AP sites was incised by the enzyme; however, DNA containing reduced AP sites was not. HPLC analysis of the products formed by exhaustive enzymatic digestion of damage-containing DNA showed that endonuclease VIII released thymine glycol and dihydrothymine as free bases. Taken together, these data suggest that endonuclease VIII contains both *N*-glycosylase and AP lyase activities. Consistent with this idea, DNA containing AP sites or thymine glycols, that was enzymatically nicked by endonuclease VIII was not a good substrate for *E. coli* DNA polymerase I, suggesting that endonuclease VIII nicks damage-containing DNA on the 3′ side of the lesion. Also, since monophosphates were not released after treating thymine glycol-containing DNA with endonuclease VIII, the enzyme does not appear to have exonuclease activity. The enzyme activity was maximal in 75 mM NaCl or 5 mM $MgCl_2$. Analysis of endonuclease VIII by both Superose FPLC and Sephadex yielded native molecular masses of 28 000 and 30 000 Da, respectively. SDS–PAGE, in conjunction with activity gel analysis, gave a molecular mass of about 29 000 Da. Furthermore, renaturation of the putative active band from SDS–PAGE gave rise to an active enzyme.

Many chemical and physical agents damage DNA by free radical mechanisms. For example, both X-rays (Teoule *et al.*, 1977) and normal oxidative metabolism (Fridovich, 1978; Ames, 1983) produce DNA damages via free radical intermediates. As a result, cells have evolved enzymatic DNA repair systems that can minimize the effects of genetic alterations induced by free radicals (Cohen & Greenwald, 1983) by excising and repairing the lesions. Putative repair endonucleases that specifically recognize oxidized bases have been isolated throughout the phylogenetic kingdom [for a review, see Wallace (1988)].

The electron-rich 5,6 double bond of pyrimidines is particularly sensitive to free radical-induced oxidation (Cohen & Greenwald, 1983). The prototypic enzyme that recognizes damages of this type is endonuclease III from *Escherichia coli*. This enzyme was originally isolated by its ability to nick X-irradiated (Strniste & Wallace, 1975) and heavily UV-irradiated (Radman, 1976) DNA was subsequently shown to recognize a wide variety of DNA substrates (Gates & Linn, 1977a; Katcher & Wallace, 1983; Breimer & Lindahl, 1984; Kow and Wallace, 1985). Thymine glycol, urea, and a number of other radiolysis products of thymine and cytosine are released by the *N*-glycosylase activity of the enzyme (Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984; Doetsch *et al.*, 1987). Interestingly, endonuclease III contains an iron-sulfur cluster (Cunningham, *et al.*, 1989) and its structure has recently been elucidated by X-ray crystallography (Kuo *et al.*, 1992).

E. coli mutants defective in endonuclease III (*nth*) have been isolated (Cunningham & Weiss, 1985); however, these mutants are not hypersensitive to DNA damaging agents such as X-rays that produce substrates for endonuclease III. Since some of the substrates for endonuclease III, including thymine glycols and urea glycosides, have been shown to be replicative blocks to DNA synthesis *in vitro* (Ide *et al.*, 1985; Rouet & Essigmann 1985; Clark & Beardsley, 1986; Hayes & LeClerc, 1986) and lethal lesions *in vivo* (Hariharan *et al.*, 1977; Moran & Wallace, 1985; Laspia & Wallace, 1988), the possibility existed that alternate pathways for the repair of these damages are present in *E. coli*.

In this paper, we report the purification and characterization of a new enzyme activity, endonuclease VIII, that incises damaged PM2 DNA (Melamede *et al.*, 1987). Endonuclease VIII appears to be a novel thymine glycol DNA glycosylase capable of nicking thymine glycol-containing DNA as confirmed by its purification from *E. coli nth* mutants lacking endonuclease III.

MATERIALS AND METHODS

Bacteria and Bacteriophages. The following *E. coli* strains were kindly provided by Dr. B. Weiss: AB1157=*leu*B6 (amber *Sul*II) *thr*-1 Δ (*gpt-pro*A)62 *his*G4 (ochre) *arg*E3 (ochre) *lac*Y1 *gal*K2 *ara*-14 *mtl*-1 *xyl*-5 *thi*-1 *tsx*-33 *rps*L31 *sup*E44 (amber *Sul*I) *rac*, BW9109=Δ(*xth-pncA*)90 + markers of AB1157, BW434=BW9109*nth*-1::*kan* + markers of AB1157, BW435=Δ(*man-nth*)84 + markers of BW9109, BW534=*nfo*-1::kan *nth*-1::*kan* + markers of AB1157, and BW271=KL16-ung1 (KL16=Hfr(PO45)relA1). The *nth* phenotype was introduced, by Dr. M. Maccabee, into a strain lacking uracil–

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[1] Abbreviations: ADA, *N*-(2-acetamido)-2-iminodiacetic acid; AMPSO, 3-[[dimethyl(hydroxymethyl)methyl]amino]-2-hydroxypropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HE, HEPES–EDTA; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; POPSO, piperazine-*N*,*N*′-bis(2-hydroxypropanesulfonic acid); PEG, polyethylene glycol 8000; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TE, Tris–EDTA.

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DNA glycosylase (BW271) using P1 transduction from BW434. Transductants were selected by kanamycin resistance. The phenotype of clone MM001 was confirmed by Mono S chromatography, which showed that endonuclease VIII was present but that uracil–DNA glycosylase and endonuclease III were missing. The *E. coli* strain that harbors the plasmid pLC99 and overproduces endonuclease III, was kindly provided by Dr. B. Weiss. PM2 bacteriophage and host *Alteromonas espejiana* were grown as previously described (Katcher & Wallace, 1983). f1 phage was grown in *E. coli* SMH77 (Hayes & LeClerc, 1986). The genotype of SMH77 is Δ (*pro-lac*), *thr*-1, *his*-4, *thi*-1, *argE3*, *galK2*, *ara*-14, *xyl*-5, *ntl*-1, *tsx*-33, *rps*-L31, *supE44*, *F'pro*$^+$*lacZ M15*. Uracil-containing f1 was produced by growing f1 phage in CJ236 (*dut1*, *ung1*, *thi1*, *relA1*/*pCJ105*(*CM*)).

Enzymes. *E. coli* DNA polymerase I and exonuclease III were purchased from Pharmacia. *E. coli* endonucleases III and IV and uracil–DNA glycosylase were purified by FPLC using procedures similar to those described in this manuscript (unpublished observations).

Preparation of Substrates. Supercoiled PM2 DNA was prepared as previously described (Katcher & Wallace, 1983). Radiolabeled PM2 DNA containing [*methyl*-^{3}H]thymine was prepared by a standard nick translation reaction (Maniatis *et al.*, 1982) and was further purified with a Sephadex G-100 column. The specific activity of the ^{3}H-labeled DNA was 4 × 10^6 cpm/μg DNA.

AP sites were introduced into the DNA by incubation at pH 4.5 and 70 °C for 10 min as described by Lindahl and Andersson (1972). The number of AP sites produced by this treatment was directly quantitated in the absence of any enzyme since AP sites are alkali labile and are cleaved by the fluorometric nicking assay (Kow, 1989).

Thymine glycols were produced in PM2 DNA by treatment with 0.04% osmium tetroxide at 70 °C for 5 min (Katcher & Wallace, 1983). The reaction was stopped by transferring it to an ice bath. The DNA was dialyzed against 1 L of cold TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) for 4 h. The buffer was replaced once, and dialysis continued for another 4 h. About one thymine glycol was introduced per PM2 DNA molecule using these conditions (Kow & Wallace, 1985). The precise number of thymine glycols/DNA molecule was determined by incubating the modified DNA with endonuclease III under conditions of enzyme saturation followed by the fluorometric nicking assay. Tritiated PM2 DNA containing thymine glycol was prepared by treating PM2 ^{3}H-labeled DNA with OsO_4 (0.04%) at 80 °C for 30 min. Under these conditions, about 11% of thymine was converted into thymine glycol as determined by the acetol fragment assay (Katcher & Wallace, 1983).

Urea glycosides were selectively introduced into DNA by treating DNA conrtaining thymine glycols with alkali (Kow & Wallace, 1985). Thymine glycol-containing PM2 DNA was dialyzed against 20 mM KH_2PO_4–KOH (pH 12) and 1 mM EDTA for 24 h at 25 °C followed by overnight dialysis in TE buffer at 4 °C. Thymine glycols in DNA are quantitatively converted to urea by this treatment (Ide *et al.*, 1985).

Dihydrothymidine was incorporated into PM2 DNA *in vivo* by adding 0.25 μg/mL of dihydrothymidine to the culture medium of PM2-infected *A. espejiana* cells every 3 min for 1 h as described (Ide *et al.*, 1987).

β-Ureidoisobutyric acid was produced by incubating dihydrothymine-containing DNA overnight at pH 12 in 20 mM potassium phosphate and 1 mM EDTA as described (Ide *et al.*, 1991). This treatment quantitatively converts the dihydrothymine residues to β-ureidoisobutyric acid (Ide *et al.*, 1987).

f1 phage DNA was isolated from infected wild-type *E. coli* (SMH77) as described by Messing (1983). f1 DNA containing 5% uracil (as determined by HPLC analysis after extensive digestion with uracil–DNA glycosylase) in place of thymine was prepared by growing f1 in *E. coli* strain CJ236 (Ide *et al.*, 1991). Thymine glycols were introduced into f1 DNA by treatment with osmium tetroxide (0.04%). A 10-min treatment of the single-stranded DNA at 37 °C produced approximately two thymine glycols/DNA molecule as determined by HPLC analysis.

Endonuclease Assays. Endonuclease VIII was assayed using the standard endonuclease III reaction conditions. A 100-μL reaction contained 500 ng of DNA in TE buffer plus 100 mM NaCl. Incubation conditions, unless otherwise indicated, were for 5 min at 37 °C. One unit of endonuclease activity is defined as the amount of enzyme required to nick 0.1 pmol of DNA damage/min at 37 °C.

All endonuclease assays were based on the conversion of cloned circular form I PM2 DNA to open circular form II. Nicking of PM2 DNA was usually monitored using the fluorometric method of Kowalski (1979). This assay does not distinguish between true endonuclease-generated nicks and those caused by *N*-glycosylase activity followed by alkali cleavage, i.e., a glycosylase activity would remove a base and leave an AP site which in turn would be cleaved by the hot alkali step used in the fluorometric assay procedure. Thus, endonucleolytic nicking of alkali labile sites was measured using agarose gel electrophoresis. Horizontal gels containing 0.85% agarose were cast and electrophoresed in TBE (10.8 g of Tris base, 5.6 g of boric acid, and 0.17 g of EDTA/L) for about 3 h and then stained with 0.5 μg/mL ethidium bromide as previously described (Katcher & Wallace, 1983).

The pH optima of endonucleases III and VIII were determined using the assay described above except that TE was replaced by the appropriate Good's buffer (0.01 M, 1 mM EDTA) for the pH being tested (Good *et al.*, 1966). Ada, *N*-(2-acetamido)-2-iminodiacetic acid, was used for pH 6.0, 6.5, and 7.0 buffers; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, was used for pH 7.5; POPSO, piperazine-*N*,*N'*-bis(2-hydroxypropanesulfonic acid), was used for pH 8.0 and 8.5 buffers; and AMPSO, 3-[[dimethyl-(hydroxymethyl)methyl]amino]-2-hydroxypropanesulfonic acid, was used for pH 9.0 and 9.5 buffers.

DNA Polymerase Assay. To determine whether endonuclease VIII was a class I (AP lyase) or class II AP endonuclease, 2 μg of urea- or thymine glycol-containing PM2 DNA was incubated in a 100-μL reaction with endonuclease VIII (0.5 unit, Superose 12 purified), endonuclease III (a class I AP endonuclease), or endonuclease IV (a class II AP endonuclease) for 5 min at 37 °C. Subsequently, a nick translation reaction was performed with the reaction products. DNA polymerase I (0.5 unit) was added and incubated with 5 mM $MgCl_2$, dATP, dCTP, dGTP (100 μM each), and [^{3}H]dTTP (0.1 μM for urea DNA and 0.4 μM for thymine glycol DNA; 67 Ci/mmol) for the indicated times at 37 °C. DNA was precipitated with 10% trichloroacetic acid (final concentration) and filtered through Whatman GFA filters, and the amount of radioactivity incorporated into DNA was determined by scintillation spectrometry.

Bacterial Growth and Cell Lysis. Preparations (5 L) of *E. coli* (either BW435, BW434, or MM001) were grown with aeration in a 12-L flask on a rotary shaker until late log phase.

The cells were centrifuged in a Sorval RC5B refrigerated centrifuge using a GS3 rotor (5000 rpm, 10 min). The bacterial pellet was resuspended in an equal volume of TE buffer containing 2 M KCl and 1 mM β-mercaptoethanol.

Routine preparations consisted of approximately 30 g of *E. coli* paste that was lysed mechanically either with a French press (Aminco) or with glass beads using a Braun homogenizer as described for the purification of endonuclease III (Katcher & Wallace, 1983). If the homogenizer was used, approximately one-third vol of 0.17-mm glass beads was added to the resuspended cells. The cells were then lysed by mechanical shearing (2 min with intermittent CO_2 cooling), and the glass beads and cell debris were removed by centrifugation. The beads were reextracted with an additional volume of TE containing 2 M KCl, and the supernatants were combined.

Large quantities (kilograms) of *E. coli* were grown at the United Technologies Center at the University of Connecticut using a 45-L New Brunswick fermenter. The bacterial pellet was resuspended in an equal volume of TE buffer containing 2 mM NaCl and 1 mM β-mercaptoethanol. The cells were then lysed with a Gaulin mill. An equal volume of 30% PEG in TE buffer containing 1 mM β-mercaptoethanol was added to cell lysates, and the mixtures were kept at 4 °C overnight (final NaCl concentration was 1 M). The precipitate was removed by centrifugation in a Sorval GS3 rotor at 6000 rpm for 20 min.

HPLC/FPLC. All HPLC and FPLC analyses were performed using an LDC/Milton Roy computer command module (CCM) to control Constametric HPLC pumps and acquire data from a variable-wavelength ultraviolet light monitor. The products released from damaged DNA after digestion with endonuclease VIII or uracil–DNA glycosylase were assayed by HPLC on a Whatman ODS-3 column. The column was equilibrated with 0.5% methanol. The samples and authentic markers were injected with a Valco injector and eluted isocratically (0.5 mL/min). Standard deoxyribonucleoside and triphosphate markers were purchased from Pharmacia. The thymine glycol marker was made by the method of Baudish and Davidson (1925).

Chromatography for endonuclease VIII enzyme purification was carried out using the following columns: a 250-mL Sepragen Superflo radial flow column packed with S Sepharose Fast Flow, Mono S cation-exchange column (HR10/10), Mono Q (HR5/5) anion-exchange column, phenyl Superose (HR5/5) hydrophobic column, and Superose 12 gel filtration column; all from Pharmacia.

Two purification schemes were followed during endonuclease VIII purification. Initially, endonuclease VIII was purified on a Mono S column followed by a Mono Q column. When kilogram-sized batches were processed by the above procedure, recovery of endonuclease VIII activity was variably low from the Mono Q column. Subsequently, the Mono Q column was replaced with a phenyl Superose hydrophobic column followed by a Superose 12 gel filtration column.

The PEG extracts were pumped onto the radial flow column with a peristaltic pump when kilogram quantities were processed. For smaller preparations, samples greater than 5 mL were applied to the FPLC columns by pumping them through the HPLC pump whereas samples smaller than 5 mL were injected in-line onto the columns. As long as the buffers and the columns were kept cool, pumping the samples through the metal tubing and liquid ends of the HPLC pump did not significantly affect enzyme recovery.

Cation-Exchange Chromatography of Endonuclease VIII. For large-scale preparations, the PEG extract was first applied to a radial flow column packed with S Sepharose to take advantage of the extremely high flow rate, prior to chromatography on the Mono S column. The PEG extract (3 L) was diluted 5-fold (<10 mS) and pumped onto the 250-mL column at 60 mL/min. The column was then washed with approximately 500 mL of HE (0.01 M HEPES, pH 7.5, 1 mM EDTA, and 1 mM β-mercaptoethanol) buffer containing 0.15 M NaCl and batch eluted with 250 mL of HE buffer containing 0.30 M NaCl. Sample 50-mL fractions were collected. Alternatively (or subsequently if the radial flow column was first used), the diluted PEG-precipitated extract (approximately 500 mL from 25 g of cells), or the diluted active fractions from the radial flow column, were pumped through the HR10/10 Mono S column (cooled in an ice bath) using an LDC/Milton Roy Constametric III pump equipped with an in-line Pharmacia prefilter at a flow rate of 4 mL/min. After the sample was loaded, the column was washed with HE buffer until the optical density readings returned to baseline. The column was then eluted with a linear gradient of 0–50% buffer B over 160 min. The column was eluted with a flow rate of 2.5 mL/min. Buffer A contained HE plus 1 mM β-mercaptoethanol, and buffer B contained buffer A plus 1 M NaCl. Fractions (8 mL) were collected. The recovery of enzyme activity after the Mono S column was high, often 100%. The activity usually eluted between 0.21 and 0.24 M NaCl.

FPLC Anion-Exchange Chromatography. Prior to loading samples on the Mono Q column, the Mono S fractions with endonuclease VIII activity were pooled and diluted 3-fold with TE buffer or to <10 mS. The Mono Q column was eluted with a 0–50% buffer B linear gradient over 50 min (1 mL/min). Sample 1-mL fractions were collected. Buffer A contained Tris buffer (0.01 M) plus 1 mM β-mercaptoethanol, and buffer B contained buffer A plus 1 M NaCl. The recovery of enzyme activity after Mono Q chromatography was low (~16%), but the specific activity of endonuclease VIII at the Mono Q stage of purification was greater than 1333 unit/mg of protein. The protein concentration in the active Mono Q fraction was at the lower limit of detection both by the Bradford (1976) method and by the silver staining of polyacrylamide gels. Endonuclease VIII typically eluted from the Mono Q column between 0.16 and 0.19 M NaCl, just before endonuclease III, if present (Melamede *et al.*, 1987). At the Mono Q stage of purification, endonuclease VIII only remained active for a few days, perhaps due to the low protein concentration.

When the quantity of endonuclease VIII applied to the Mono Q column was increased significantly during a large-scale preparation, only about 30% of the activity bound to the column even though the column's binding capacity should not have been exceeded. Therefore, Mono Q chromatography has been eliminated from the purification procedure. Endonuclease VIII purified with the Mono Q column showed about five contaminating species when silver stained after SDS–PAGE (data not shown). Many of the reaction parameters of endonuclease VIII described in the Results section used Mono Q-purified endonuclease VIII.

FPLC Phenyl Superose Hydrophobic Column Chromatography. Active fractions from the Mono S column were pooled and diluted 2-fold with HE buffer containing 1 M ammonium sulfate (buffer A). This mixture was then pumped onto an HR5/5 phenyl Superose FPLC column at 0.4 mL/min. The column was developed with a linear gradient that started with 100% buffer A and finished with 99.9% buffer B (HE) in 33.3 min. The flow rate was 0.4 mL/min, and 0.4-mL fractions were collected.

Gel Permeation Chromatography. Gel permeation chromatography was used to determine the native molecular weight of endonuclease VIII. A Superose 12 HR10/30 column was equilibrated with TE buffer containing 1 mM β-mercaptoethanol and 0.5 M NaCl and injected with 0.08 mL of a Mono S fraction. The column was eluted at a flow rate of 0.75 mL/min, and 0.25-mL fractions were collected. Subsequently, for preparative chromatography, 0.5 mL was injected onto the column. The flow rate was 0.5 mL/min with 0.5-min fractions collected.

Alternatively, the native molecular weight of endonuclease VIII was determined using Sephadex G100. A column (1 × 50 cm) was packed with Sephadex G100 and equilibrated with 0.1 M NaCl in Tris–EDTA, pH 7.5. Endonuclease VIII, 2 mL (Mono S), was loaded on the column and eluted with the equilibration buffer at a flow rate of 1 mL/min; 1-mL fractions were collected.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed using a BioRad minigel apparatus according to the method of Laemmli (1970). The minigels were silver-stained according to the method of Poehling and Neuhoff (1981).

Activity Gel Electrophoresis. To prepare the oligonucleotide substrate for the activity gel, calf thymus terminal deoxynucleotidyl transferase and chemically synthesized dihydrothymidine triphosphate were used to add one dihydrothymine residue onto the 3′ end of a 20mer (5′-GAGAGATAACCCACAAGAAT) as previously described (Hatahet *et al.*, 1993). The 21mer was then annealed to f1-K12 (Ide & Wallace, 1988) and extended with sequenase in the presence of dT, dG, ddC, and [α-^{32}P]dATP resulting in a 30mer with the sequence 5′-GAGAGATAACCCACAA-GAATdihydrothymineGAGTTAAGC. Treatment of this substrate with endonuclease VIII results in a 9mer as visualized by autoradiography. A second oligonucleotide, 5′-CGGT-GCGGGCCUCTTCGCT, was 5′-end labeled with polynucleotide kinase and [γ-^{32}P]ATP and annealed to f1-K12. This oligonucleotide was used as a control nondamage-containing substrate or treated with uracil–DNA *N*-glycosylase to generate an abasic site at the 12th nucleotide from the 5′-end (5′-CGGTGCGGGCCabasic siteCTTCGCT). When the abasic site-containing substrate was treated with endonuclease VIII, an 11mer with a 3′-P is the visible product.

Detection of endonuclease activity *in situ* was performed following the method of Longley and Mosbaugh (1993) with slight modifications. 15% SDS–polyacrylamide gels were prepared as described before (Laemmli, 1970) and included 2 mM EDTA, 50 μg/mL BSA (not in lanes to be stained), and 0.5–1.0 nM of oligonucleotide substrate. After electrophoresis at 4 °C, SDS was extracted out of the gel at room temperature by two 30-min washes in 10 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol, and 25% (v:v) 2-propanol. For renaturation, the gel was shaken gently overnight at 4 °C in 70 mM HEPES–KOH, pH 7.5, 5 mM β-mercaptoethanol, 1 mM EDTA, 15% glycerol (v:v), and 500 μg/mL BSA. Endonuclease VIII was activated by shaking the gel gently for 2 h at room temperature in renaturation buffer supplemented with either 10 mM $MgCl_2$ or 100 mM NaCl. The lane containing endonuclease VIII was then cut out and placed horizontally in a 20% acrylamide gel containing 8 M urea. Electrophoresis in the second dimension was performed in 90 mM Tris, 90 mM boric acid, pH 8.3, and 2.5 mM EDTA. The gel was then exposed to X-ray film. The position of the renatured endonuclease VIII was detected as a break in the oligonucleotide substrate (30mer) produced by endonuclease VIII cleavage and the appearance of a shorter cleavage product (9mer in the case of dihydrothymine).

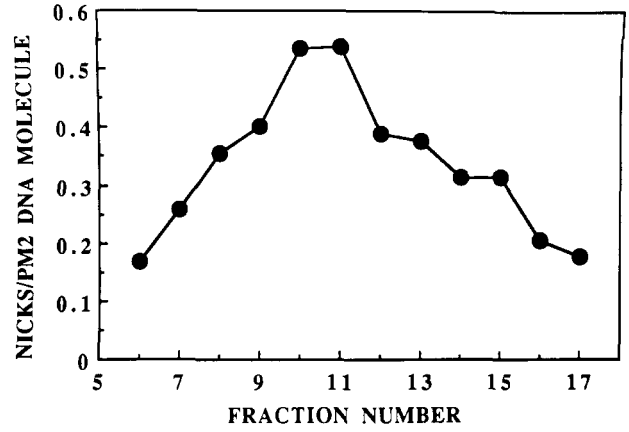

FIGURE 1: S Sepharose fast flow radial flow chromatography of endonuclease VIII. The PEG-precipitated crude extract was chromatographed on a 250-mL Sepragen radial flow column as described under Materials and Methods. Endonuclease activity was determined by fluorometry using OsO_4-treated PM2 DNA (~2 μL, 15-s incubation, 37 °C) as described under Materials and Methods.

Endonuclease VIII was also renatured *in vitro* following SDS–PAGE. Endonuclease VIII, 2 mL (phenyl Superose), was electrophoresed on a preparative (3-mm thick) 15% SDS–polyacrylamide gel. The region of the gel adjacent to the 30-kDa prestained molecular mass standard (Gibco-BRL) was horizontally sliced into several thin segments. Proteins were then eluted from each individual slice and renatured as described before (Hatahet & Fraser, 1989) with slight modifications. The gel slices were mashed finely with a glass rod, resuspended in 4 mL of 10 mM HEPES–KOH, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol, and 0.1% Triton X-100, and incubated on a rocking platform for 2 h at room temperature. The samples were then dialyzed overnight at 4 °C against the same buffer followed by two dialysis steps against the same buffer without Triton and with 15% glycerol (v:v) in the first dialysis and 5% glycerol (v:v) in the second step. Endonuclease activity on both thymine glycol- and urea-containing PM2 DNA was recovered.

RESULTS

Identification of Endonuclease VIII Activity. The initial identification of endonuclease VIII was from PEG-precipitated crude extracts prepared from *E. coli* mutants that lacked endonuclease III, either by insertion of the kanamycin gene (BW434) or by deletion (BW435). Endonuclease activity was observed with a PM2 DNA substrate containing thymine glycol but not on undamaged DNA (data not shown). This residual endonuclease activity represented about 10% of the total wild-type activity on a thymine glycol-containing DNA substrate.

Cation-Exchange Chromatography of Endonuclease VIII. Figure 1 shows the elution profile of the nicking activity of endonuclease VIII on PM2 DNA containing thymine glycol after chromatography on a radial flow column that was packed with S Sepharose. The activity was isocratically eluted with HE containing 0.3 M NaCl, and 50-mL fractions were collected. While chromatography on the radial flow column has not been optimized for purification (145-fold), this step has the advantage of speed, simplicity, and high yield, thus serving as an excellent preparatory step for the Mono S FPLC column that gives better resolution.

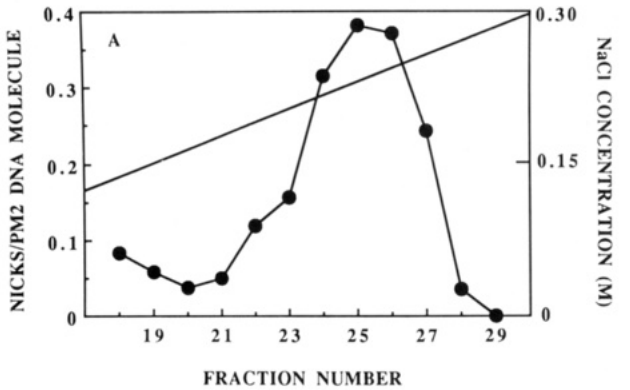

FIGURE 2: Mono S FPLC chromatography of endonuclease VIII. The active fractions from the radial flow were diluted and pumped onto the Mono S column as described under Materials and Methods. Endonuclease activity was determined by fluorometry using OsO_4-treated PM2 DNA (10-μL aliquots, 30-s incubation, 37 °C) as described under Materials and Methods.

Figure 2 shows the profile of endonuclease VIII activity eluted from a Mono S HR10/10 column by a linear NaCl gradient as described in Materials and Methods. Here the activity peaked in fractions 25 and 26. A silver-stained SDS–polyacrylamide gel of the active endonuclease VIII fractions eluted from the Mono S column was still relatively impure (data not shown).

After chromatography on the Mono S column, endonuclease VIII activity remained stable for at least 2 months at 4 °C. Freezing endonuclease VIII, even in the presence of BSA, resulted in complete loss of enzyme activity.

FPLC Phenyl Superose Hydrophobic Column Chromatography. Endonuclease VIII bound tightly to phenyl Superose with the activity eluting at the end of the increasingly aqueous gradient. The most active fraction from the phenyl Superose column was loaded onto both an activity SDS–PAGE and a SDS–PAGE followed by silver staining. There was a very strong correlation between the migration of renatured endonuclease VIII and a polypeptide having an apparent molecular mass around 29 kDa (Figure 3). Activity was also observed in a higher molecular mass fraction, but this did not appear to correlate with endonuclease VIII activity and is probably an exonuclease (data not shown). The substrate used in Figure 3 was DNA containing dihydrothymine, but a strong correlation between this 29-kDa polypeptide and endonuclease VIII activity was also seen when the oligonucleotide contained an abasic site and was also observed with peak fractions of endonuclease VIII from the Mono S column (data not shown). No activity at 29 kDa was observed with control DNA.

The tight binding of endonuclease VIII to the hydrophobic column (Figure 4A) suggests that the protein contains a highly hydrophobic region. A similar hydrophobic domain is not found on endonuclease III, thus the phenyl Superose column is useful for separating endonuclease VIII from endonuclease III when both enzymes are present. Endonuclease III elutes from the phenyl Superose column at about 0.5 M ammonium sulfate.

Figure 4B shows the silver-stained SDS–PAGE of the active fractions eluted from the phenyl Superose column. The intensity of the second of the three bands that runs slightly more slowly than the 30-kDa molecular mass marker appeared to correlate with the activity profile. When this band was cut from the gel and renatured, endonuclease VIII activity was regained (0.3 unit). No renatured activity was found in the adjacent slices, and activity was not recovered from any other

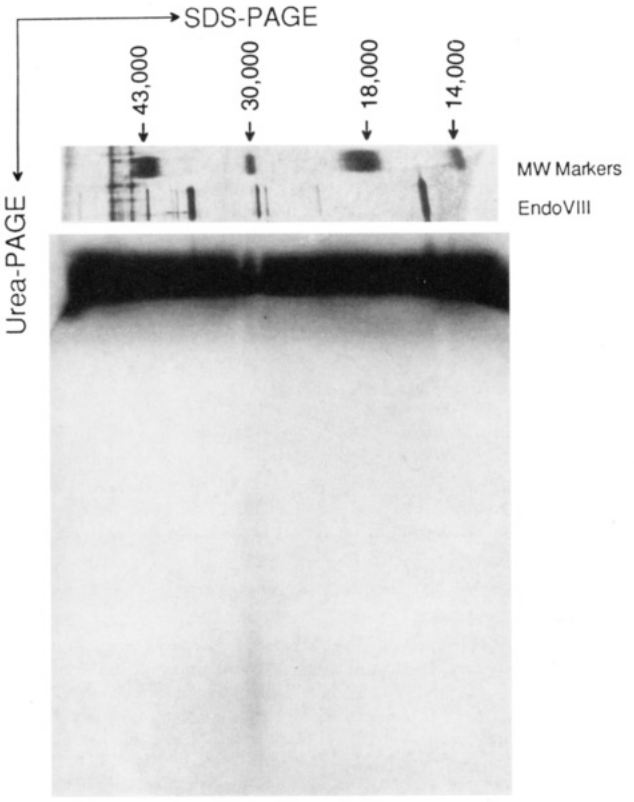

FIGURE 3: *In situ* detection of endonuclease VIII activity. Endonuclease VIII, 20 μL of phenyl Superose fraction 29, was loaded onto both a 15% polyacrylamide gel and a 15% activity polyacrylamide–SDS gel containing a ^{32}P-labeled substrate oligonucleotide with a single dihydrothymine (as described under Materials and Methods). The molecular mass standards used were ovalbumin (43 kDa), carbonic anhydrase (30 kDa), β-lactoglobulin (18 kDa), and lyzozyme (14 kDa). Following electrophoresis, the SDS was extracted, the endonuclease VIII was renatured, and the enzyme-containing lane was placed horizontally in a 20% acrylamide gel containing 8 M urea and then electrophoresed in the second dimension as described under Materials and Methods.

band on the gel. Figure 4C shows the remigration of the renatured enzyme on SDS–PAGE, which again migrates slightly more slowly than the 30-kDa marker.

Superose Gel Permeation Chromatography. Gel permeation chromatography was run to determine the native molecular mass of endonuclease VIII. Both Superose 12 FPLC and Sephadex G100 columns were run as described under Materials and Methods. As shown in Figure 5A, with Superose 12, the endonuclease VIII activity migrated slightly ahead of the 26-kDa chymotrypsinogen marker. Figure 5B shows a native molecular mass of about 30 kDa after conventional analytical sephadex chromatography. SDS–polyacrylamide gel analysis of the fractions containing endonuclease VIII activity from a preparative Superose 12 FPLC column (data not shown) correlated with a molecular mass of approximately 28 kDa. This is in close agreement with the data shown in Figure 4C, showing that renatured endonuclease VIII activity was derived from a protein band that corresponds to 29 kDa. The purification scheme for endonuclease VIII is shown in Table 1.

Reaction Parameters for Endonuclease VIII. Endonuclease VIII and endonuclease III may be compared by a number of their reaction parameters. Figure 6A shows the pH optimum of endonuclease VIII (Mono Q fraction) using thymine glycol-containing DNA as the substrate. Endonuclease VIII, like endonuclease III, was active over a broad pH range. However, endonuclease VIII exhibited an optimum at pH 7.0, whereas

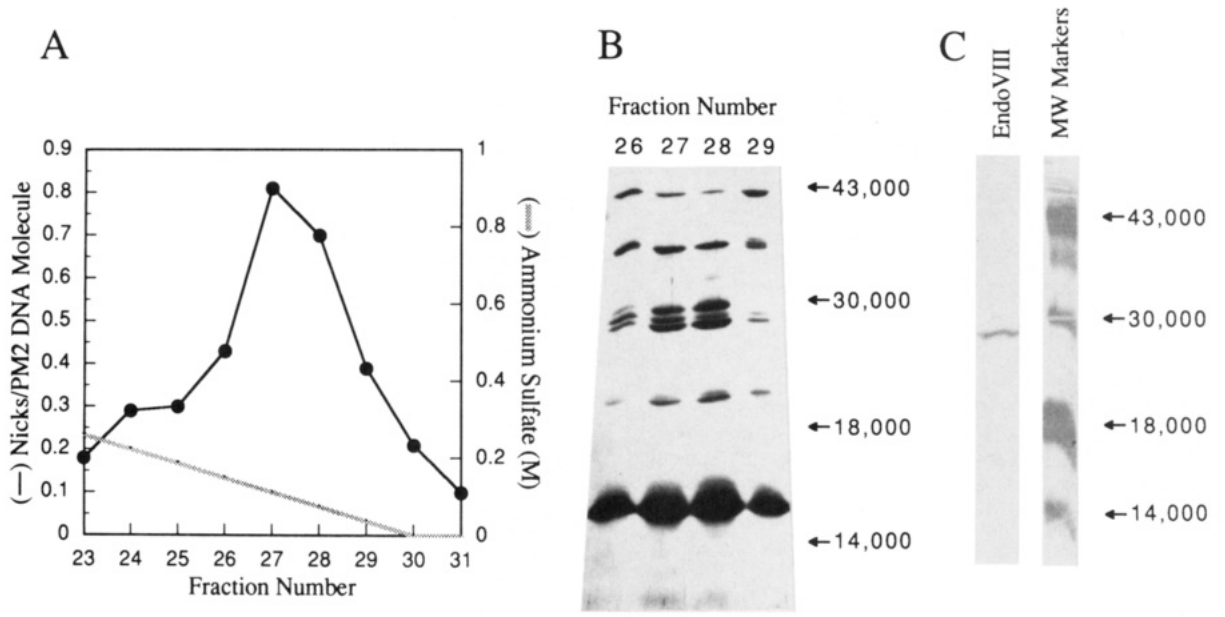

FIGURE 4: (Panel A) Phenyl Superose FPLC chromatography of endonuclease VIII. The active fractions from the Mono S column were made 1 M with respect to ammonium sulfate and pumped onto the HR5/5 phenyl Superose column as described under Materials and Methods. Endonuclease activity (10 μL of a 200-fold dilution, 30-s incubation, 37 °C) was determined by fluorometry using OsO_4-treated PM2 DNA as described under Materials and Methods. (Panel B) SDS–PAGE analysis of phenyl Superose-purified endonuclease VIII. Fractions 26–29, 40-μL aliquots, from the phenyl Superose column were electrophoresed through a 12% polyacrylamide mini-slab gel and silver stained as described under Materials and Methods. (Panel C) SDS–PAGE analysis of pooled peak fractions of endonuclease VIII renatured following preparative SDS–PAGE. 100 μL of protein recovered from a preparative SDS–polyacrylamide gel were electrophoresed on a 15% SDS–polyacrylamide gel followed by silver staining as described under Materials and Methods. The molecular mass standards were as for Figure 3.

the pH optimum for endonuclease III was 8.0. Figure 6B shows that endonuclease VIII had a salt optimum of 75 mM NaCl when thymine glycol-containing DNA was used as the substrate, whereas endonuclease III has a 100 mM NaCl optimum (Katcher & Wallace, 1983). KCl, LiCl, or NH_4Cl could effectively substitute for NaCl in endonuclease VIII reactions (data not shown).

Interestingly, both endonuclease VIII and endonuclease III exhibited full activity when NaCl was replaced with 5 mM $MgCl_2$ (Figure 6C) or $MnCl_2$ (data not shown). This divalent cation effect on endonuclease III activity was not previously observed (Radman, 1976; Gates & Linn, 1977a; Breimer & Lindahl, 1980, 1984; Katcher & Wallace, 1983). Similar results were obtained with endonuclease III that was purified from an overproducing plasmid-bearing strain and yielded a single band after polyacrylamide electrophoresis and silver staining. Furthermore, since these reactions were performed under conditions of limiting enzyme activity, 0.05 unit, the presence of a contaminating activity (in both enzyme preparations) that acts on thymine glycol-containing DNA does not seem likely. The addition of ATP (50 μM) did not affect the activity of endonuclease VIII when thymine glycol-containing PM2 DNA was used as a substrate (data not shown).

Substrate Specificity of Endonuclease VIII. Since the fluorometric assay uses heat and alkali to prevent renaturation of nicked circular DNA, any AP sites present in the DNA are cleaved by the assay procedure. In order to demonstrate whether endonuclease VIII contained endonuclease activity and not simply glycosylase activity, the nicking of a variety of substrates was measured by analyzing enzyme-treated DNA samples on agarose gels. Enzyme saturating conditions, 1 unit of activity, were used to demonstrate any potential minor activities of endonuclease VIII. Saturation was defined with respect to thymine glycol-containing DNA. In Figure 7, it can be seen that endonuclease VIII incised PM2 DNA containing AP sites, thymine glycols, urea glycosides, and dihydrothymine but not UV damages. In contrast, endonuclease VIII was unable to nick PM2 DNA that contained reduced AP sites (Table 2).

In order to examine the possibility that endonuclease VIII and endonuclease V might be the same enzyme, f1 DNA or f1 DNA containing uracil was incubated with endonuclease VIII at pH 9.4 in the presence of 9 mM $MgCl_2$ and 25 mM KCl (Gates & Linn, 1977b). Figure 8 (lanes 1–4) shows that endonuclease VIII did not nick either of these single-stranded substrates under the reaction conditions established for endonuclease V (Gates & Linn, 1977b). In addition, endonuclease VIII did not nick single-stranded f1 DNA containing thymine glycols or uracil (lanes 5–8) under the reaction conditions established for endonuclease VIII. Thus, endonuclease VIII appears to be an activity distinct from endonuclease V.

Figure 9 demonstrates the relative rate of nicking by endonuclease VIII compared to endonuclease III using a variety of potential substrates. Both enzymes preferred to nick PM2 DNA containing urea glycosides over DNA containing thymine glycol. Although endonuclease III has been reported to recognize dihydrothymine-containing DNA (Demple & Linn, 1980), as seen in Figure 9, panels A and B, DNA containing dihydrothymine was a poor substrate for both endonucleases III and VIII (the approximate rate of nicking of dihydrothymine-containing DNA by endonuclease VIII was about 12% that of thymine glycol-containing DNA). DNA that contained β-ureidoisobutyric acid was incised by endonuclease VIII at about 50% the rate at which DNA that contained thymine glycols was nicked. Endonuclease III exhibited a similar level of endonuclease activity against DNA containing the β-ureidoisobutyric acid product as was found for endonuclease VIII.

Endonuclease VIII Is DNA N-Glycosylase. Endonuclease III was shown to act first as an *N*-glycosylase by releasing

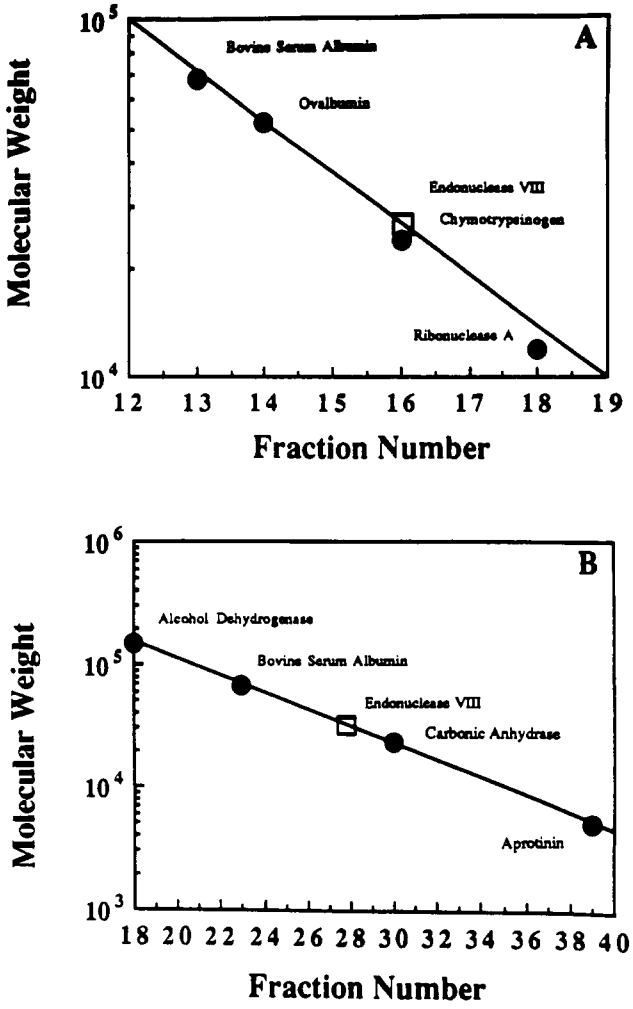

FIGURE 5: Gel permeation chromatography of endonuclease VIII using Superose 12 FPLC (A) and Sephadex G100 (B). Endonuclease VIII (0.5 mL of Mono S fraction) was injected (0.5 mL) onto the Superose column, and a total of ~30 units were loaded onto the Sephadex column. Both columns were eluted with a flow rate of 1 mL/min. The columns were calibrated using the standards indicated in the figure. 1-mL fractions were collected, and endonuclease activity (5-min incubation, 37 °C) was determined by fluorometry using OsO_4-treated PM2 DNA as described under the Materials and Methods.

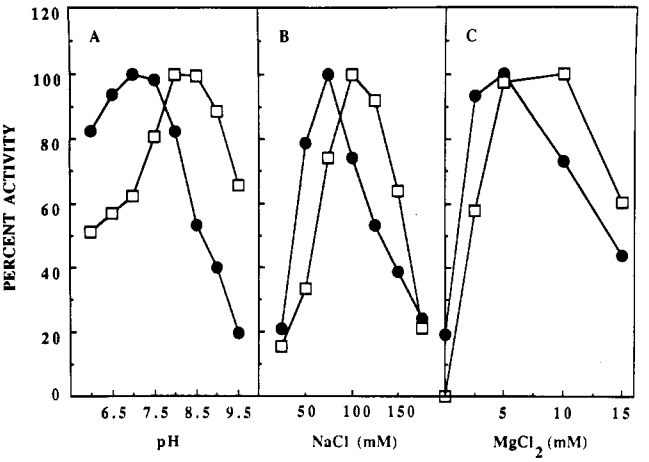

FIGURE 6: (Panel A) Effect of pH on the endonuclease activity of endonuclease VIII (●) and endonuclease III (□). PM2 DNA containing thymine glycols was treated with subsaturating amounts of endonuclease VIII (Mono Q fraction, ~0.05 unit, 2 min incubation, 37 °C) or endonuclease III (~0.05 unit, 2-min incubation, 37 °C) at the indicated pH. (Panel B) Effect of NaCl concentration on the endonuclease activities of endonuclease VIII (●) and endonuclease III (□). PM2 DNA containing thymine glycols was treated with subsaturating amounts of endonuclease VIII or endonuclease III activity as above at the indicated NaCl concentration. (Panel C) Effect of $MgCl_2$ concentration on the endonuclease activities of endonuclease VIII (●) and endonuclease III (□). PM2 DNA containing thymine glycol was treated with subsaturating amounts of endonuclease VIII or endonuclease III as above at the indicated $MgCl_2$ concentration. Endonuclease activity was measured by fluorometry as described under Materials and Methods.

thymine glycol as a free base (Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1985) and then to incise at remaining AP sites by an enzyme-catalyzed β-elimination (Kow & Wallace, 1987; Bailly & Verly, 1987; Kim & Linn, 1988; Mazumder *et al.*, 1991). The results shown in Figure 10 demonstrate that endonuclease VIII released thymine glycol as a free base from OsO_4-oxidized DNA and dihydrothymine from dihydrothymine-containing DNA. HPLC analysis (Whatman ODS-3 column) of the tritiated products released showed them to co-migrate with authentic thymine glycol and dihydrothymine markers. Since dTMP was not observed after endonuclease VIII digestion of thymine glycol-containing DNA, the enzyme does not appear to have exonuclease activity.

Endonuclease VIII Has an Associated Class I AP Endonuclease Activity. The ability of *E. coli* DNA polymerase I to utilize endonucleolytically nicked DNA as a substrate for polymerization serves as a basis for classifying endonuclease activity (Mosbaugh & Linn, 1980). Class I endonucleases (AP lyases), such as endonuclease III, make incisions 3′ to a damage and do not produce substrates for polymerization. Class II endonucleases, such as exonuclease III and endonuclease IV, incise 5′ to the damage producing sites for DNA polymerase activity. Figure 11 shows that PM2 DNA containing AP sites or thymine glycols and incised by endonuclease VIII or endonuclease III was not a good substrate for DNA polymerase I. However, the addition of endonuclease IV to the nicked damaged DNA produced a substrate for DNA polymerase I.

Endonuclease VIII, like endonuclease III, did not nick DNA containing reduced AP sites (Table 2). In contrast, PM2 DNA containing reduced AP sites was a substrate for the class II endonucleolytic activity of exonuclease III (Kow, 1989) and endonuclease IV (Takeshita *et al.*, 1987). These observations suggest that, in contrast to the *E. coli* class II AP endonucleases, endonuclease VIII may act like endonuclease III via an enzyme-catalyzed β-elimination.

Table 1: Purification of Endonuclease VIII

	vol (mL)[a]	total protein	total act. (unit)	specific act. (unit/mg)	recovery (%)	purification (x-fold)
PEG-precipitated crude	3000	1.5×10^4 mg	1.7×10^5	11.3	100	
radial flow S Sepharose	300	138 mg	2.2×10^5	1.6×10^3	129	145
Mono S FPLC	72	13.4 mg	63 300	4.7×10^3	37.2	429
phenyl Superose	4.1	2.84 mg	51 200	1.8×10^4	30.1	1593
Superose 12	17	<17 μg	47 400	$>10^5$	27.9	>10 000

[a] Entire pooled fractions were not loaded at each step. Volume numbers were normalized to complete loads.

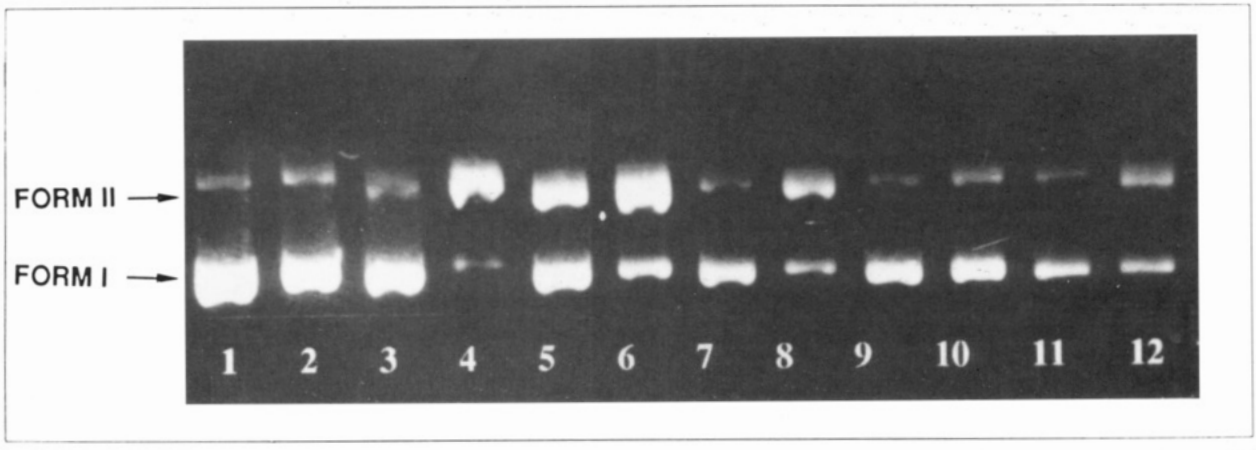

FIGURE 7: Double-stranded DNA substrate specificity of endonuclease VIII nicking activity. PM2 DNA containing the indicated DNA damages was treated with saturating amounts of endonuclease VIII (Mono Q fraction, 0.5 units, 5-min incubation, 37 °C) and run on agarose gels as described under Materials and Methods: lanes 1 and 2, undamaged DNA; lanes 3 and 4, AP DNA; lanes 5 and 6, thymine glycol-containing DNA; lanes 7 and 8, urea-containing DNA; lanes 9 and 10, UV-irradiated DNA; lanes 11 and 12, dihydrothymine-containing DNA. Lanes 1, 3, 5, 7, 9, and 11 contained DNA that had not been incubated with enzyme. Lanes 2, 4, 6, 8, 10, and 12 contained DNA that was incubated with endonuclease VIII.

Table 2: Reduced AP Sites Are Not Substrates for Endonuclease VIII

	activity: nicks/PM2 DNA molecule substrate[a]	
enzyme	reduced AP site	urea
endonuclease VIII	0.01	0.7
endonuclease III	0.05	0.8
exonuclease III	0.4	0.9

[a] PM2 DNA containing urea glycosides or reduced AP sites was treated with nonsaturating amounts (~0.05 unit, 2 min, 37 °C) of endonuclease VIII (Mono Q fraction), endonuclease III, or exonuclease III activity. Nicking activity was measured fluorometrically as described under Materials and Methods, using PM2 DNA containing the above damages.

DISCUSSION

Endonuclease VIII appears to be very similar to endonuclease III. The two enzymes have both *N*-glycolyase (Figure 10) and class I AP endonuclease (lyase) activities (Figure 11), as well as similar substrate specificities. The salt requirements of the two enzymes are close (Figure 6B); endonuclease VIII has a salt optimum of 75 mM NaCl whereas the optimum for endonuclease III is 100 mM. Endonuclease VIII appears to have a lower pH optimum than does endonuclease III (Figure 6A). Clearly, endonuclease VIII is not a modified form of endonuclease III since it is readily isolated from an *E. coli* strain (BW435) than contains an *nth* deletion.

The only other known endonuclease found in *E. coli* that acts on an osmium tetroxide-treated DNA is endonuclease V (Gates & Linn, 1977b). However, a number of reported properties of endonuclease V distinguish it from endonuclease VIII. First, endonuclease V does not have *N*-glycosylase activity (Gates & Linn, 1977b), although only uracil- and 7-bromomethylbenz[α]anthracene-containing DNA substrates were tested. In contrast, endonuclease VIII exhibits glycosylase activity, although only urea-, thymine glycol-, and dihydrothymine-containing DNA substrates were tested. Secondly, endonuclease V nicks single-stranded DNA (Gates & Linn, 1977b) wherease endonuclease VIII does not (Figure 8). Furthermore, endonuclease V nicks DNA containing uracil (Gates & Linn, 1977b), in particular DNA that is heavily substituted with uracils (Demple & Linn, 1982), and endonuclease VIII did not nick single-stranded f1 DNA that had 5% of its thymine replaced with uracil (Figure 8). Also, $NaHSO_4$-treated PM2 DNA containing a small number of uracils was not a substrate for endonuclease V (Demple & Linn, 1982). However, $NaHSO_4$ treatment of PM2 DNA has been shown to produce not only uracil sites (deamination of cytosine), but an equal number of sites, presumably thymine glycols, that are recognized by both endonuclease III and endonuclease VIII (data not shown). In this case, $NaHSO_4$-treated DNA is a substrate for endonuclease VIII but not endonuclease V. Taken together, the data suggest that endonuclease VIII and endonuclease V are different enzymes.

The existence in *E. coli* of functional duplication of DNA repair enzymes having similar substrate specificities has been documented in a number of cases. For example, the products of the *tag* and *alkA* genes are both 3-methyladenine DNA glycosylates [for a review, see Lindahl and Sedgewick (1988)]; the products of the *ada* and *ogt* genes are DNA methyltransferases (Lindahl & Sedgewick, 1988; Potter *et al.*, 1987), and the products of the *xth* and *nfo* genes are apurinic endonucleases [for a review, see Wallace (1988)]. Interestingly, one enzyme of each pair is constitutive (the products of *tag*, *ogt*, and *xth*) and the other is inducible (the products of *alkA*, *ada*, and *nfo*). By analogy, since endonuclease III is constitutive, one might expect endonuclease VIII to be inducible. However, no increase in endonuclease VIII levels were observed (data not shown) after the administration of hydrogen peroxide, paraquat, agents that induce the SOS response or in *oxyR* or *soxR* mutants constitutive for the hydrogen peroxide (Christman *et al.* (1989) and superoxide-inducible (Farr *et al.*, 1985; Walkup & Kogoma, 1989; Greenberg *et al.*, 1989) responses, respectively.

E. coli cells lacking endonuclease III (*nth*) have no known phenotype (other than a slight mutator effect), that is, they are not hypersensitive to X-rays or oxidizing agents known to produce substrates for the enzyme (Cunningham & Weiss, 1985). Thus, it would appear that in these cases, endonuclease VIII is fully substituting for endonuclease III in the repair of lethal damages. This explanation is complicated by the fact that duplex ϕX-174 DNA containing thymine glycols exhibits a 3-fold decrease in survival when transfected into *nth* mutants compared to wild-type hosts (Laspia & Wallace, 1988). In this latter case, endonuclease VIII appears not to fully substitute for endonuclease III. Furthermore, the UvrABC complex also repairs thymine glycol (Lin & Sancar, 1989; Kow *et al.*, 1990). It could be that the residual repair of cellular DNA in *nth* mutants may reflect different damage

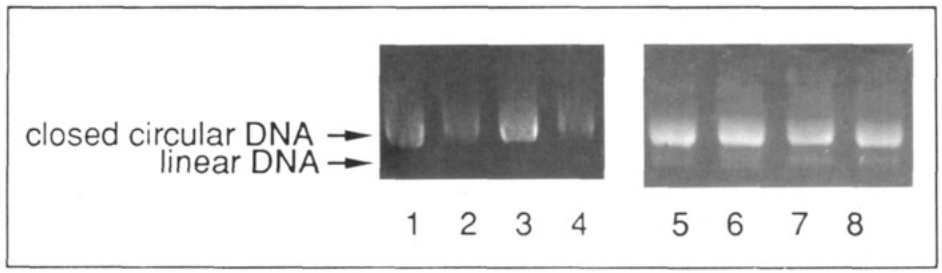

FIGURE 8: Single-stranded DNA substrate specificity of endonuclease VIII nicking activity. Unsubstituted f1 DNA (lanes 1 and 3) or f1 DNA containing 5% of its thymine substituted with uracil (lanes 2 and 4) was treated with saturating amounts of endonuclease VIII (lanes 3 and 4) (Superose fraction, 2 unit, 5-min incubation, 37 °C) using the reaction conditions for endonuclease V as described in the text. f1 DNA that contained 2 thymine glycols/DNA molecule (lanes 5 and 6) or uracil (lanes 7 and 8), as described above, was treated with endonuclease VIII (Superose fraction, 2 units, 5-min incubation, 37 °C, pH 7.5, 100 mM NaCl) (lanes 6 and 8).

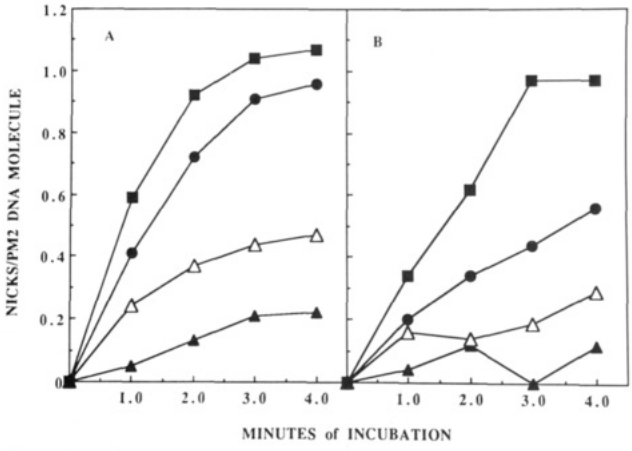

FIGURE 9: Substrate specificity of endonuclease VIII (panel A) and endonuclease III (panel B). PM2 DNA substrates that contained either urea glycoside (■), thymine glycol (●), β-ureidoisobutyric acid (△), or dihydrothymine (▲) were treated with subsaturating amounts of endonuclease (0.5 unit, 2-min incubation, 37 °C; Mono Q fraction for endonuclease VIII) for the indicated times. Nicking activity was measured by fluorometry as described under Materials and Methods.

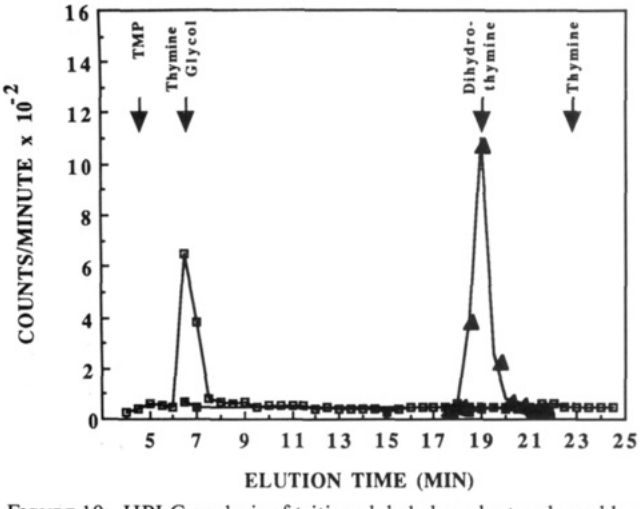

FIGURE 10: HPLC analysis of tritium-labeled products released by endonuclease VIII (Mono Q fraction) from tritium-labeled OsO_4-treated, calf thymus DNA (□) or calf thymus DNA that was nick-translated with [^{3}H]dihydrothymidine triphosphate (▲). The digestion products were injected onto a Whatman ODS column and isocratically eluted with 0.5% methanol at a flow rate of 0.5 mL/min as described under Materials and Methods. No tritium-labeled products were released from control undamaged DNA (■).

processing in cellular DNA compared to transfecting-phage DNA. For example, thymine glycol might be a substrate for both enzymes in cellular DNA but a poor substrate for endonuclease VIII in supercoiled transfecting DNA. In fact,

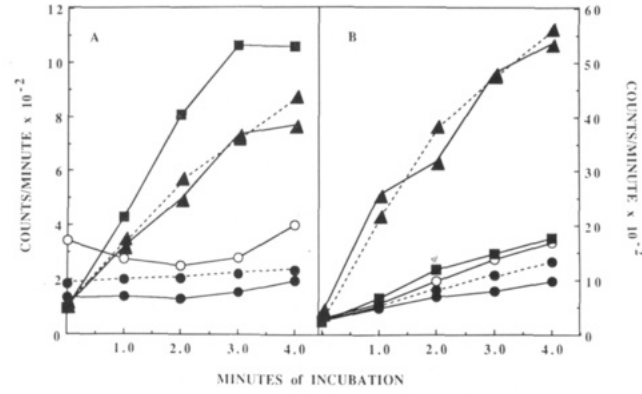

FIGURE 11: PM2 DNA containing urea (panel A) or thymine glycols (panel B) was treated with no enzyme (○); endonuclease III, dotted line (●); endonuclease IV (■); endonuclease VIII (0.5 unit of Superose fraction), solid line (●); endonuclease III plus endonuclease IV, dotted line (▲); endonuclease VIII plus endonuclease IV, solid line (▲). All endonuclease incubations were performed under saturating conditions of enzyme activity as determined by fluorometry. Substrate DNA (both urea-containing and thymine glycol-containing) had one damage per molecule. DNA polymerase I was then added as described under Materials and Methods. Incubations were continued for the indicated times at which the reactions were terminated with TCA, precipitated, and washed. The amount of radioactivity was determined by scintillation spectroscopy.

preliminary observations (data not shown) suggest that thymine glycol-containing supercoiled DNA is a better substrate for endonuclease III than for endonuclease VIII. Topological constraints might also affect repair enzyme activities (as opposed to the physical constraints of protein–protein interactions) potentially playing a role in functional compartmentalization of repair in transcribing versus non-transcribing regions or pre- versus post-replication forks. Differential repair in transcribing versus non-transcribing genes has been documented in eukaryotes (Bohr *et al.*, 1985; Mellon *et al.*, 1986) and in *E. coli* (Mellon & Hanawalt, 1989). Also, the effect of topology on repair rates has been implicated in several studies in eukaryotic cells where nucleosomal versus internucleosomal regions have been examined (Wilkins & Hart, 1974; Cleaver, 1977; Smerdon & Lieberman, 1978; Zolan *et al.*, 1982; Hunting *et al.*, 1984).

There is another possible explanation that could account for the apparent discrepancy between the lack of phenotype for *nth* cells exposed to agents that produce a spectrum of damages, including those recognized by endonuclease III, and the presence of a phenotype when DNA containing a unique lesion, thymine glycol, is transfected into *nth* mutants. The lesions recognized by endonucleases III (or VIII) may be produced by X-rays in quantitities too low to significantly contribute to lethality. This explanation does not seem likely since current measurements of the rate of production of

damages recognized by endonucleases III and VIII suggest that these should be present at levels sufficient to block replication in the absence of repair. Clearly, the elucidation of the role played by endonuclease VIII in the repair of oxidative DNA damage awaits cloning of the gene and isolation of mutants lacking the enzyme, a task we are currently pursuing.

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