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Mechanism of Action of *Escherichia coli* Endonuclease III†

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ABSTRACT: Endonuclease III isolated from *Escherichia coli* has been shown to have both *N*-glycosylase and apurinic/apyrimidinic (AP) endonuclease activities. A nicking assay was used to show that the enzyme exhibited a preference for form I DNA when DNA containing thymine glycol was used as a substrate. This preference was reduced or eliminated either when the DNA was relaxed or when the type of damage was altered to urea residues or AP sites. The combined *N*-glycosylase/AP endonuclease activity was at least 10-fold higher than the AP endonuclease activity alone when urea-containing DNA was used as a substrate as compared to AP DNA. When DNA containing thymine glycol was used as a substrate, the combined *N*-glycosylase/AP endonuclease activity was about 2-fold higher than the AP endonuclease activity. Yet, when DNA containing thymine glycol or urea was used as substrate, no apurinic sites remained. Furthermore, magnesium selectively inhibited endonuclease III activity when AP DNA was used as a substrate but had no effect when DNA containing either urea or thymine glycol was used as substrate. These data suggest that both the *N*-glycosylase and AP endonuclease activities of endonuclease III reside on the same molecule or are in very tight association and that these activities act in concert, with the *N*-glycosylase reaction preceding the AP endonuclease reaction.

The base excision repair pathway for the removal of modified DNA bases involves two types of enzymes, DNA *N*-glycosylases and apurinic/apyrimidinic (AP) endonucleases. The sequential action of these enzymatic activities removes the modified base, leaving a nick adjacent to an abasic site on the DNA molecule.

Endonuclease III from *Escherichia coli* has been shown to be an AP endonuclease with an associated *N*-glycosylase activity (Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984). Although it has not been unambiguously established, both activities appear to reside on the same molecule having a native molecular weight of about 25 000 (Katcher & Wallace, 1983; Breimer & Lindahl, 1984). The associated glycosylase activity recognizes a spectrum of thymine ring saturation or fragmentation products including thymine glycols (Armel et al., 1977; Gates & Linn, 1977; Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984), 5,6-dihydrothymine (Demple & Linn, 1980; Ide, Kow, and Wallace, unpublished data), urea (Breimer & Lindahl, 1980; Katcher & Wallace, 1983), 5-hydroxy-5-methylhydantoin (Breimer & Lindahl, 1984, 1985), and methyltartronylurea (Breimer & Lindahl, 1984). The enzyme also recognizes some unknown cytosine radiolysis product(s) (Doetsch et al., 1986; Weiss & Duker, 1986).

Most studies undertaken with repair endonucleases and glycosylases have focused on substrate specificity, subunit structure, the nature of the nick produced, and other enzymatic reaction parameters. With the exception of the uracil DNA

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N-glycosylase (Carodonna & Cheng, 1980) and the T4 *denV* gene product (Nakabeppu et al., 1982; Seawell et al., 1981), little is known about the kinetic parameters of these enzymes. In this paper we report that endonuclease III exhibits a preference for supercoiled DNA containing thymine glycol. Further, the kinetic data support the idea that the glycosylase and AP endonuclease activities reside in the same protein and act in concert.

Materials and Methods

Bacteria and Bacteriophages. *Escherichia coli* AB3027 and AB1157 were obtained from the *E. coli* Genetic Stock Center (Yale University). PM2 bacteriophage and host *Alteromonas espejiana* (obtained from H. Gray) were used for the preparation of PM2 DNA. *E. coli* strains BW410, BW415, and BW372 were obtained from B. Weiss (Weiss & Cunningham, 1985).

Materials. Agarose, ultrapure grade, was obtained from Bio-Rad Laboratories. 2-Mercaptoethanol, poly(ethylene glycol) (PEG) 6000, *N,N,N',N'*-tetramethylethylenediamine (Temed), acrylamide, and *N,N'*-bis(acrylamide) were obtained from Sigma Chemical Co. Phosphocellulose (P-11) and (diethylaminoethyl)cellulose (DE-52) were obtained from Whatman. Sephadex G-75 was obtained from Pharmacia Fine Chemicals.

Nucleic Acids. ^{3}H-Labeled PM2 phage was prepared as previously described (Wallace et al., 1981). Unlabeled PM2 phage was prepared by infecting host *A. espejiana* grown in Bal broth at a multiplicity of infection of 10 as previously described (Wallace et al., 1981). Phage lysates were centrifuged at 6000 rpm (GSA rotor, Sorvall RC5B) for 20 min to remove unlysed cells and debris, and PM2 phage was then precipitated with 8% PEG 6000 and purified by sucrose gradient sedimentation (8–20%). Sucrose-purified phage was then extracted 6–10 times with equal volumes of chloroform/1-butanol (5:1), and the DNA obtained was dialyzed extensively against 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, and 1 mM ethylenediaminetetraacetic acid (EDTA). The preparation usually gave 90–95% form I PM2 DNA with an $A_{260/280}$ of 1.6–1.8.

Enzymes. X-ray endonuclease, endonuclease III, was prepared according to the procedure of Katcher and Wallace (1983). The specific activity ranged from 10^7 to 10^8 units/mg of protein. One unit of activity is defined as 1 fmol of enzyme-induced nicks/min at 30 °C. Calf thymus topoisomerase I was obtained from Bethesda Research Laboratory. Exonuclease III was obtained from P-L Biochemicals.

Substrates. Supercoiled PM2 DNA containing thymine glycol was prepared as previously described except that the temperature used was 65 °C and the ether extraction step was omitted (Katcher & Wallace, 1983). A solution of 0.04% OsO_4 produced about 1 thymine glycol/DNA molecule in 5 min. Very few apurinic sites or strand breaks were produced under these conditions, and the DNA treated with OsO_4 was about 85–90% form I.

Apurinic DNA was prepared according to the method of Lindahl and Andersson (1972) by heat/acid depurination. The number of apurinic sites was determined by alkaline fluorometry (Futcher & Morgan, 1978; Kowalski, 1979) using Fluorometer-A4 (Farrand Optical Co., Inc., New York). Depurinated PM2 DNA (500–1000 ng) was mixed with 1.5 mL of buffer containing 20 mM KH_2PO_4–KOH, pH 11.8–12.0, 0.5 mM EDTA, and 0.5 μg of ethidium bromide/mL. The fluorescence of the mixture was taken (before), and the mixture was then heated at 90–95 °C for 15 min. Heating for 5–10 min was enough to cleave all the AP sites at this pH. The heated mixture was quickly cooled in a slushed ice bath for 2–3 min and then allowed to warm to room temperature. The fluorescence was then measured again (after). The number of AP sites was estimated by the formula −ln (after/before).

Urea-containing PM2 DNA was prepared by dialyzing DNA containing thymine glycol for 24 h at 25 °C against 20 mM KH_2PO_4–KOH, pH 12.0, and 1 mM EDTA followed by dialyzing overnight at 4 °C against 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. The number of urea residues was equal to the original number of thymine glycol sites (Ide et al., 1985; Kow & Wallace, 1985).

Relaxed DNA containing thymine glycol was prepared by treating supercoiled DNA containing thymine glycol with calf thymus topoisomerase I. Supercoiled DNA, 10 μg, was incubated with 20 units of topoisomerase I in 200 μL of a buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 5 mM mercaptoethanol, 1 mM EDTA, and 30 μg BSA/mL for 30–60 min. Complete conversion of form I to form I′ was monitored by agarose gel electrophoresis and fluorometry. The commercially obtained topoisomerase I sometimes contained a contaminating nuclease activity that degraded damaged DNA only.

Endonuclease III Assays. The activity of the enzyme was based on the rate of conversion of form I (or form I′) to form II. When the fluorometric method of Kowalski (1979) was used for the determination of form I DNA remaining, the reaction was performed in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM mercaptoethanol, and 0.1 M KCl at 30 °C. In order to minimize any systematic error from the instrument and maintain a similar fluorescence unit for each experimental point, the DNA concentration was changed by varying the volume of the reaction mixtures and the amount of enzyme, while the absolute amount of DNA was kept constant. Reactions were terminated by adding 1.5 mL of buffer containing 20 mM KH_2PO_4, pH 11.8–12.0, 0.5 mM EDTA, and 0.5 μg of ethidium bromide/mL. Fluorescence readings were determined before and after heating at 90 °C for 5–10 min as described above. The amount of form I DNA remaining was calculated by the following formula: for supercoiled substrates (form I), % form I remaining = 130/[0.3 + (before/after)]; for relaxed substrates (form I′), % form I′ remaining = 190/[0.9 + (before/after)].

For the determination of the amount of form I and form II PM2 DNA, electrophoresis was performed with 0.8% agarose gels made with TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). The gels were electrophoresed in TBE buffer at 10 V/cm for 2–3 h for ^{3}H-labeled DNA or for 4–5 h for unlabeled DNA. The gels were stained with 0.5 μg/mL ethidium bromide for 30 min. For radioactive DNA, DNA bands were cut out, melted in 2.0 mL of hot 1 N HCl, and cooled. To each was added 20 mL of Liquiscint. Radioactivity was counted in a liquid scintillation counter. For nonradioactive DNA, DNA bands were scanned in a Zeineh soft laser scanning densitometer (Model SL-TRFF). The intensity of each of the forms of DNA was corrected for the 30% enhancement of the form II DNA.

Results

Activities of Endonuclease III in E. coli Extracts. Endonuclease III has been shown to have *N*-glycosylase activity against a variety of thymine derivatives (Gates & Linn, 1977; Katcher & Wallace, 1983; Breimer & Lindahl, 1984), as well as an AP endonuclease activity (Radman, 1976; Katcher & Wallace, 1983). It has never been unambiguously established that these activities reside on the same molecule. Table I shows

Table I: Activities of Endonuclease III of *E. coli*[a]

strain	plasmid or mutation	relative activity: apurinic DNA	relative activity: urea-containing DNA	relative activity: DNA containing thymine glycol
AB1157	none	1.0	1.0	1.0
BW410	*pLC*9-9	6.7	7.5	6.2
BW415	(*manA-nth*)	0.4	0.08	0.1
BW372	*nth1*::*Kan*	0.3	0.1	0.08

[a] Relative activity of endonuclease III in each extract was normalized to that of strain AB1157. The endonuclease III activities of AB1157 against apurinic DNA, urea-containing DNA, and thymine glycol containing DNA were 0.30, 0.84, and 0.48 nicks (DNA molecule)$^{-1}$ min^{-1} (10 μg of protein)$^{-1}$.

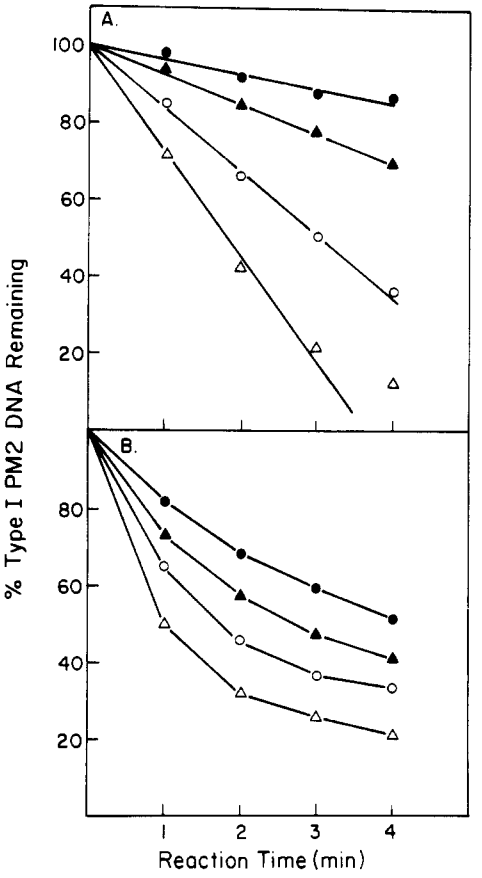

FIGURE 1: Rate of nicking by endonuclease III of OsO_4-treated PM2 DNA (4 sites/molecule). Supercoiled (A) and closed circular relaxed (B) OsO_4-treated PM2 DNA molecules were used as substrates for endonuclease III. The closed circular relaxed OsO_4-treated PM2 DNA was derived from supercoiled OsO_4-treated PM2 DNA by treatment with calf thymus topoisomerase I. (A) DNA concentrations were 3.2 (●), 1.6 (▲), 0.8 (○), and 0.4 nM (△). (B) DNA concentrations were 0.8 (●), 0.6 (▲), 0.4 (○), and 0.2 nM (△).

that when the wild type *nth* was inactivated either by kanamycin insertion or deletion, all three activities were reduced compared to that of the wild type. When the *nth* gene was present on a multicopy plasmid, a 7-fold increase in all three activities was observed. These data strongly support the notion that the product of the *nth* gene codes for both the *N*-glycosylase and AP endonuclease activities.

Time Course for DNA Containing Thymine Glycol and Urea. Figure 1A illustrates the time course for endonuclease III activity on supercoiled (form I) DNA containing thymine glycol. The extent of reaction (% form I remaining) was observed to be linear with reaction time until about 80% of the form I DNA was converted to form II, at which time the

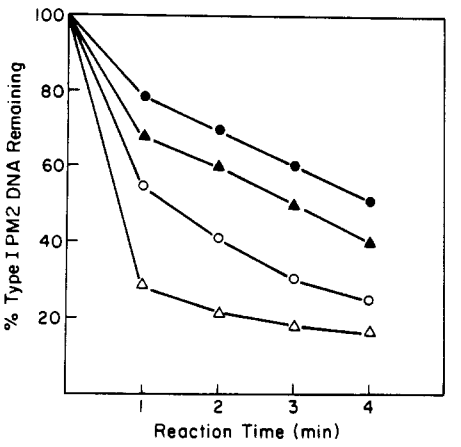

FIGURE 2: Rate of nicking by endonuclease III of supercoiled PM2 DNA containing urea residues. PM2 DNA containing urea residues (4 sites/molecule) was prepared by alkaline hydrolysis of supercoiled OsO_4-treated PM2 DNA. DNA concentrations used were 1.6 (●), 0.8 (▲), 0.4 (○), and 0.2 nM (△).

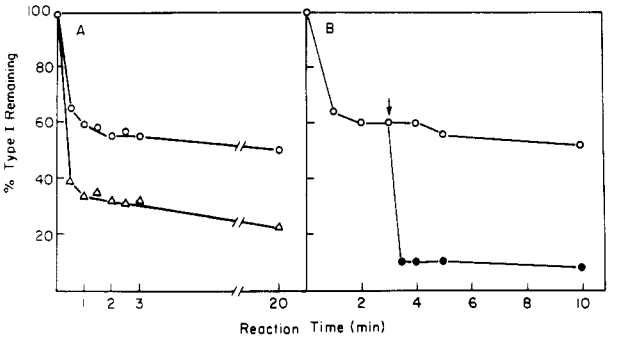

FIGURE 3: Rate of nicking by endonuclease III of apurinic PM2 DNA. Supercoiled apurinic PM2 DNA, 0.8 (○) or 0.4 nM (△), containing 2.7 apurinic sites/molecule was incubated with endonuclease III (A). After 3 min of incubation (B), the reaction mixture was divided in two. To one was added another 300 units of endonuclease III (○), and to the other was added 2 Weiss units of exonuclease III (●). A Weiss unit is the amount of enzyme required to release 1 nmol of acid-soluble product from ^{3}H–DNA duplex in 30 min at 37 °C.

reaction appeared to slow down and the points deviated from a straight line. The linearity of the reaction time course was independent of the concentration of the DNA substrate used; this is, DNA concentrations from 0.4 to 3.2 nM all gave a linear time course. Furthermore, when supercoiled (form I) PM2 DNA containing different numbers of thymine glycols (between 1 and 10 per molecule) was used, the linear reaction time course was also observed (data not shown). However, when the same supercoiled substrate was converted by topoisomerase I to the relaxed form (form I′), the pattern of the time course was dramatically changed (Figure 1B). The rate of conversion of form I DNA to form II DNA decreased with time at all DNA substrate concentrations used, indicating that the mode of action of the enzyme is affected by the topological structure of the substrate.

The time course for urea-containing supercoiled DNA resembled the time course observed for relaxed DNA containing thymine glycol (Figure 2). These data indicate that the reaction time course is affected not only by the topological structure of the substrate DNA but also by the nature of the damage residing on the DNA backbone.

Time Course for Apurinic DNA. Apurinic DNA, prepared by acid/heat depurination, showed biphasic kinetics with endonuclease III (Figure 3A). There was an initial rapid nicking of the substrate followed by a much slower rate of nicking. The extent of the initial rapid reaction was inversely proportional to the initial substrate DNA concentration. When the

Table II: V_{max}^{app} and K_m^{app} of Endonuclease III

av no. of damages per DNA molecule	V_{max}^{app} [nmol of nicks min^{-1} (mg of protein)$^{-1}$]	K_m^{app} (nM DNA)
2, DNA containing thymine glycol	4.8	0.2
2, urea-containing DNA	28.3	0.56
1.5, apurinic DNA	1.5	0.52

Table III: Activities of Endonuclease III[a]

substrate	rate [nmol of nicks min^{-1} (mg of protein)$^{-1}$]	
	alkali fluorometry	agarose gel electrophoresis
DNA containing thymine glycol	2.5	2.1
urea-containing DNA	8.6	9.2
apurinic DNA	*b*	1.1

[a] The rate of nicking on different substrates was measured both by alkali fluorometry and neutral agarose gel electrophoresis. One microgram of substrate DNA was incubated with 300 units of endonuclease III in standard reaction buffer (see Methods and Materials). [b] Due to the alkali lability of apurinic sites, endonuclease III activity on apurinic sites was not measurable by alkali fluorometry.

DNA concentration was decreased from 0.8 to 0.4 nM, only 25% of form I DNA remained as compared to 57% when the substrate concentration was 0.8 nM. Furthermore, only about 50% of the total available apurinic sites were nicked (1.5 of a total of 3 apurinic sites were detected by the enzyme after 20 min at a DNA concentration of 0.4 nM). To account for these data, a number of alternatives can be proposed: The enzyme might bind to the substrate and, after nicking it, it may remain bound or be released very slowly. The enzyme might be strongly inhibited by the reaction product. The enzyme might be detecting two different types of apurinic sites generated by heat/acid depurination, one which it prefers, or the enzyme activity might be sequence dependent.

In order to differentiate among these possibilities, apurinic DNA containing about 3 apurinic sites/molecule was incubated with endonuclease III. After 3 min, the reaction was split into two, and additional endonuclease III or exonuclease III was added. In addition to having a 3′–5′ exonuclease activity, exonuclease III is also a class II apurinic/apyrimidinic endonuclease. Only the addition of exonuclease III caused the conversion of all the AP sites to single-strand breaks (Figure 3B). Additional endonuclease III had no effect. Furthermore, the original endonuclease III in the reaction mixture was still active since the addition of OsO_4-treated form I DNA resulted in its conversion to form II (data not shown). It appears, therefore, that either heat/acid treatment might have generated two classes of apurinic sites, one of which was preferred, or the AP activity of endonuclease III exhibited sequence dependency.

Apparent Kinetic Constants for Endonuclease III. The apparent V_{max} and K_m for endonuclease III were measured with different substrates by using nicking as an end point (Table II). The apparent V_{max} for endonuclease III was more than 15-fold higher when DNA containing urea was used as a substrate and 3-fold higher when DNA containing thymine glycol was used as a substrate as compared to AP DNA. Yet, under these conditions, no unnicked AP sites were observed when DNA containing either urea or thymine glycol was used as substrates (Table III). This is deduced from the observation that the number of strand breaks determined by alkali fluorometry, that measures both endonuclease III induced nicks and any AP sites generated by glycosylase action, is the same as the number determined by agarose gel electrophoresis. The latter measures only endonuclease III induced nicks since latent AP sites are stable under these conditions. This is in contrast to endonuclease V of T4 were unnicked AP sites were observed when DNA containing pyrimidine dimer was used as a substrate (Seawell et al., 1980). Despite the large differences in the V_{max}, the apparent K_m of these three substrates only varied within 2-fold.

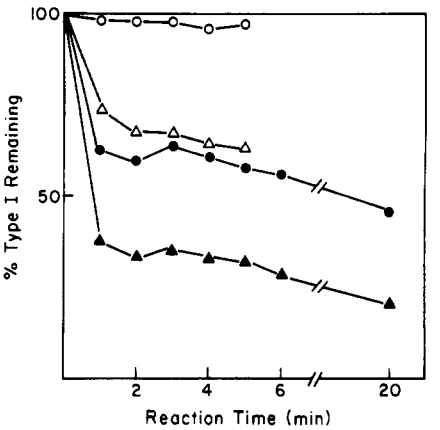

FIGURE 4: Effect of $MgCl_2$ on the reaction of endonuclease III with supercoiled apurinic PM2 DNA containing three sites per molecule. Reactions were performed with (○, ●) or without (△, ▲) 20 mM $MgCl_2$. Open symbols were reactions performed with 30 units of endonuclease III, while filled symbols were reactions performed with 300 units of enzyme.

Table IV: Effect of Pyrimidine Analogues on Endonuclease III Activity

inhibitor	concn (mM)	inhibition (%)		
		DNA containing thymine glycol	urea-containing DNA	apurinic DNA
thymine glycol	2.5	53	40	0
thymine glycol monophosphate	0.5	50	47	0
5-bromo-6-hydroxy-5,6-dihydro-thymine	20	30	20	0
barbituric acid	50	0	0	0
diethylbarbituric acid	50	0	0	0
5-hydroxy-5-methyl-hydantoin	50	0	0	0
thymine	50	0	0	0
cytosine	50	0	0	0
uracil	50	0	0	0
urea	1000	0	0	0

Effect of Magnesium on Endonuclease III Activity. Magnesium is not required for endonuclease III activity on DNA containing thymine glycol (Gates & Linn, 1977; Katcher & Wallace, 1983; Breimer & Lindahl, 1984) or urea (Breimer & Lindahl, 1980; Katcher & Wallace, 1983). Further, it was not inhibitory even up to 50 mM when either of these substrates was used. However, when apurinic DNA was used as a substrate for endonuclease III, magnesium was a very effective inhibitor of the reaction (Figure 4). These data show that magnesium was inhibitory to the endonucleolytic reaction of endonuclease III only when it was uncoupled from the *N*-glycosylase reaction; when the reaction was coupled, magnesium had no effect on the endonucleolytic reaction.

Effect of Pyrimidine Analogues on Endonuclease III Activity. Table IV shows the effect of some of the pyrimidine analogues on endonuclease III activity. Of all the analogues

examined, only thymine glycol monophosphate and 5-bromo-6-hydroxy-5,6-dihydrothymine showed appreciable inhibition of endonuclease III activity. Further, inhibition was only observed when DNA containing thymine glycol and urea was used as substrate; when AP DNA was used as substrate, no inhibition was observed.

Discussion

Endonuclease III Prefers Supercoiled DNA Containing Thymine Glycol over the Relaxed Form. When supercoiled PM2 DNA containing thymine glycol was used as the substrate, there was a time-dependent linear decrease (Figure 1A) of the conversion of form I DNA to form II. Since the method we use only only detects the first nicking event on a supercoiled molecule, the linear time dependency suggests that once the enzyme removed the damage and nicked the DNA, it dissociated from the the form II molecule and proceeded to another supercoiled substrate. If there were no preference for a supercoiled substrate, one would have expected that as the reaction proceeded, the form II molecules formed during the reaction would have accumulated and competed with the supercoiled molecules in a time-dependent manner, thus slowing down the apparent rate with time. The preference for form I PM2 DNA containing thymine glycol may reflect the lower apparent K_m of endonuclease III for form I PM2 DNA containing thymine glycol as compared to that for form I′ PM2 DNA containing thymine glycol [K_m^{app} for form I PM2 DNA containing thymine glycol $\sim$ 0.2 nM (DNA), and K_m^{app} for form I′ PM2 DNA containing thymine glycol $\simeq$ 1 nM (DNA) (Y. Kow and S. Wallace, unpublished data)].

The preference for form I was dependent on the topology of the DNA molecule since no preference was observed for form I′ (Figure 1B); that is, a time-dependent linearity of the loss of form I′ was not observed. Furthermore, the preference for form I was affected by the nature of the damage; when urea-containing or apurinic supercoiled DNA was used as substrate, no preference for supercoiled molecules was observed (Figures 2 and 3). It is possible that the microenvironment around the damage may play a role in determining the preference of recognition by endonuclease III. When thymine glycols are present on form I DNA as compared to form I′, the negative supercoiling might help to present the damages, thus allowing for easier detection by the enzyme, whereas when the damage is either a urea residue or an apurinic site, there are no bases present to expose and the microenvironment around the damage might be similar in both the supercoiled and relaxed forms.

N-Glycosylase and Apurinic Activities of Endonuclease III Reside on the Same Molecule and Act in Concert. Endonuclease III is the gene product of *nth*, which lies at 36.8 min on the *E. coli* chromosome map (Cunningham & Weiss, 1985; Weiss & Cunningham, 1985). Table I shows that the endonuclease activity against DNA containing thymine glycol or urea and against apurinic DNA paralleled the status of the structural gene product. A deletion in *nth* led to the reduction of these three activities simultaneously, while a strain carrying pLC9-9, a multicopy plasmid containing *nth*, showed a 7-fold increase in all three activities over those of the wild type (Table I). Thus, *nth* appears to code for the activity against all three substrates.

The AP endonuclease and *N*-glycosylase activities of endonuclease III appear to be concerted. When PM2 DNA containing either urea or thymine glycol was used as a substrate for endonuclease III, no unnicked apurinic sites remained (Table III). Furthermore, the *N*-glycosylase activity was 5–10 times higher than the apurinic endonuclease activity when urea-containing DNA was used as a substrate and about 2-fold higher when DNA containing thymine glycol was used as a substrate (Table II). Breimer and Lindahl (1984) obtained similar results with urea-containing DNA. A similar preference for urea-containing DNA has also been observed for *Micrococcus luteus* γ-endonuclease (Jorgenson et al., 1987), which has properties very similar to those of endonuclease III. These data suggest that the endonuclease action follows immediately after the damaged base has been removed by the *N*-glycosylase reaction. Further evidence to substantiate this mode of action comes from the study with magnesium (Figure 4). Here, apurinic activity was inhibited by magnesium when apurinic DNA was used as a substrate; however, when DNA containing either urea or thymine glycol was used, no magnesium inhibition of endonuclease activity was observed. This suggests that magnesium might bind to the apurinic site, preventing access to the enzyme. However, when endonuclease III catalysis involved a concerted *N*-glycosylase/AP endonuclease reaction, such as when DNA containing thymine glycol or urea was used as a substrate, the intermediary apyrimidinic site was probably enzyme bound, and magnesium was not able to exert its inhibitory effect. Mg^{2+} has been shown to catalyze the cleavage of apurinic sites in DNA (Lindahl & Andersson, 1972), indicating that it might bind to these sites (Felsenfeld & Huang, 1959). The selective inhibition by magnesium ions suggests not only that the activity of the glycosylase/endonuclease of endonuclease III is concerted but also that these two activities probably reside on the same molecule or are very closely associated. If these activities represented two different enzymes, one would have expected magnesium to inhibit the endonuclease activity independently of the substrate used.

Kinetics and Mechanism of Endonuclease III Reactions. Table II shows that the V_{max}^{app} for endonuclease activity was fastest for urea-containing DNA, followed by thymine glycol containing DNA, and the V_{max}^{app} was slowest for apurinic DNA. Since cleaving of the phosphodiester bond is common to all three substrates, these data suggest that the rate-limiting step for the endonuclease III reaction is not the breaking of the phosphodiester bond of the reaction intermediates. Endonuclease III is considered to be a class I AP endonuclease (Warner et al., 1980; Mosbaugh & Linn, 1982; Katcher & Wallace, 1983) that leaves a baseless deoxyribose residue. However, when the apurinic deoxyribose residue of apurinic DNA was reduced with sodium cyanoborohydride (which reduces the aldehyde of the deoxyribose at the apurinic site to an alcohol), it was no longer a substrate for endonuclease III (Jorgenson et al., 1987). Further, endonuclease III reactions with DNA containing urea or thymine glycol and with apurinic DNA were sensitive to pyridoxal 5′-phosphate (unpublished data), indicating the possible involvement of an active amino group in the active site of endonuclease III. On the basis of the above considerations, it appears that one of the intermediate steps of the endonuclease III reaction may involve the formation of a Schiff base between the enzyme amino group (possibly a lysine residue) and the aldehyde group of the ring open form of apurinic deoxyribose residue or the imine group of the ring open form of thymine glycol or urea residues as shown in Figure 5.

If this scheme is correct, we would expect that the breaking of the phosphodiester bond by endonuclease III would not be a hydrolytic process, but rather an enzyme-catalyzed β-elimination that leaves a baseless 2,3-didehydro-2,3-dideoxyribose residue (Bailly & Verly, 1984; Grossman & Grafstrom, 1982) and not a 2-deoxyribose residue as proposed for class I AP endonuclease activities (Warner et al., 1980; Mosbaugh &

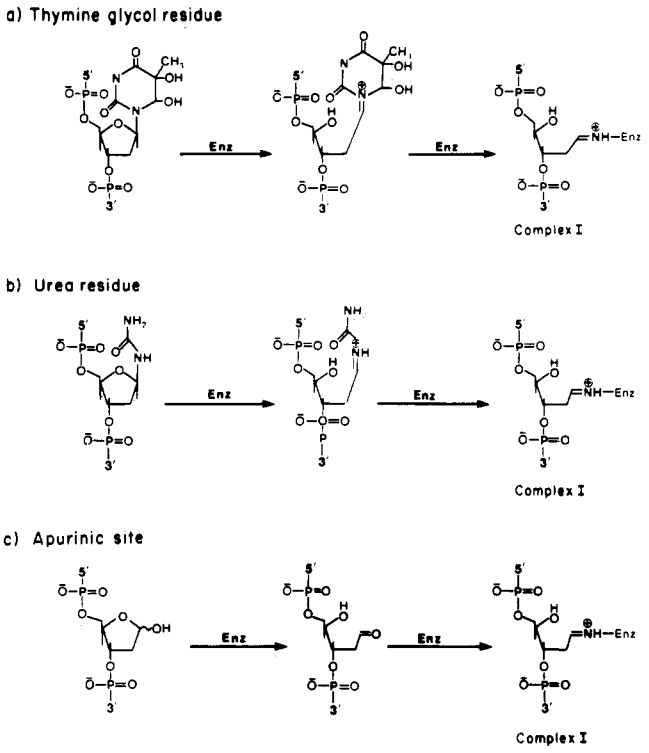

FIGURE 5: Proposed sequential reaction sequence of endonuclease III with apurinic DNA substrates (c) and DNA substrates containing thymine glycol (a) or urea (b).

Linn, 1982; Katcher & Wallace, 1983). Bailly and Verly (1984) noted that apurinic/apyrimidinic sites are easily removed by the 3′–5′ exonuclease activity of Pol I or Pol I Klenow fragment, yet endonuclease III generated nicked apurinic sites provide a poor substrate for both Pol I and Pol I Klenow fragment (Mosbaugh & Linn, 1982; Katcher & Wallace, 1983), suggesting that endonuclease III might not leave a clean 2-deoxyribose. This is consistent with the fact that the product of β-elimination, 2,3-didehydro-2,3-dideoxyribose 5-phosphate residue, is a poor primer binding site for Pol I (Huberman & Kornberg, 1970). Grafstrom et al. (1982) have demonstrated that the α,β-unsaturated deoxyribose 5-phosphate can be readily separated from deoxyribose 5-phosphate by descending paper chromatography. Recently, the α,β-unsaturated compound was shown to be the product of endonuclease III reaction with AP DNA (S. Linn, personal communication), supporting the idea that the endonucleolytic reaction of endonuclease III is through enzyme-catalyzed β-elimination. Further, a recent report by Grondal-Zocchi and Verly (1985) showed that the product of DNase IV (from liver chromatin) digestion of apurinic DNA is not 2-deoxyribose 5-phosphate but a related unidentified compound believed to be a product of β-elimination by the 5′–3′ exonuclease activity of DNase IV.

The schematic reaction (Figure 5) requires the *N*-glycosylase reaction of endonuclease III to proceed before the apurinic endonuclease reaction. This means that any inhibitor that inhibits the *N*-glycosylase reaction should inhibit the nicking event on the same substrate. Katcher and Wallace (1983) showed that when DNA containing urea or thymine glycol was used as substrate, the release of urea or thymine glycol as well as the nicking of these substrates was inhibited by *N*-ethylmaleimide. *N*-Ethylmaleimide had no effect, however, on the nicking of AP DNA. This suggests that *N*-ethylmaleimide only inhibits the *N*-glycosylase reaction which proceeds before the AP endonuclease reaction. In contrast, magnesium was inhibitory to the endonuclease III reaction only when apurinic DNA was used as a substrate (Figure 4). Apparently, magnesium binds only to apurinic sites and does not affect the *N*-glycosylase reaction. Another example of a selective inhibition of the *N*-glycosylase activity of endonuclease III is illustrated in Table III, which shows that thymine glycol and thymine glycol monophosphate as well as 5-bromo-6-hydroxy-5,6-dihydrothymine were only inhibitory to endonuclease III induced nicking when DNA containing thymine glycol or urea was used as substrate, but not apurinic DNA.

To explain the observed differences among the V_{max}^{app} of the various substrates, we suggest that the rate of formation of complex I (Figure 5) is greater with urea-containing DNA than with DNA containing thymine glycol, which is in turn greater than with apurinic DNA. There are several lines of evidence to support this idea. Deoxyribosylurea has a secondary amine and deoxyribosylthymine glycol has a tertiary amine at the glycosylic bond, thus the rate constant for the ring opening of deoxyribosylurea should be much greater than that of deoxyribosylthymine glycol. This is supported by the fact that ribosylurea can easily reach an equilibrium to form a mixture of α,β-ribopyranosylurea, while deoxyribosylthymine glycol remains as the β-furanoside (Miller & Cerutti, 1968). Further, even though deoxyribose is in rapid equilibrium between the furanoside and the open form (Bayley et al., 1961), the rate of formation of a Schiff base intermediate is slow due to the fact that the reaction of the carbonyl group with the amino group is slow. Since at pH 7.0 the pK_b for the carbonyl is about 21 while for the corresponding imine it is about 7, the fraction of protonated carbonyl is about 10^{-14} while half of the imine is protonated. Since the protonated form of the imine is at least 10^4–10^5 times more reactive toward the nucleophile than both the protonated and unprotonated carbonyl group (Hupe, 1984; Page, 1984), the transiminization step between the ring open form of deoxyribosylurea residue or deoxyribosylthymine glycol with the enzyme amino group would be much faster than the formation of Schiff base between the aldehyde group of the apurinic deoxyribose and the enzyme amino group. Therefore, during endonuclease III catalysis, the observed rate of cleavage of the phosphodiester bond would be determined in part by the rate of formation of complex I. This predicts that the rate of formation of complex I would be greatest for urea-containing DNA, followed by DNA containing thymine glycol and then apurinic DNA. This is actually the order of reactivity observed (Table II).

In summary, it appears that endonuclease III catalysis proceeds by a *N*-glycosylase reaction that releases the damaged base through a transiminization of the damaged base with the enzyme followed by an AP endonuclease activity, which is achieved by enzyme-catalyzed β-elimination of the 3′-phosphate.

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Registry No. Mg, 7439-95-4; endonuclease III, 60184-90-9; AP endonuclease, 65742-70-3; *N*-glycosylase, 9032-92-2; thymine glycol monophosphate, 6168-31-6; 5-bromo-6-hydroxy-5,6-dihydrothymine, 1195-73-9.

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Effect of the δ Subunit of *Bacillus subtilis* RNA Polymerase on Initiation of RNA Synthesis at Two Bacteriophage ϕ29 Promoters†

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ABSTRACT: Initiation of RNA synthesis by *Bacillus subtilis* RNA polymerase (σ-43) has been examined at two early promoters of phage ϕ29: the A2 promoter, which is a weak promoter, and the G2 promoter, which is a strong promoter. The δ subunit of the polymerase inhibits the rate of initiation at A2, but not G2. In addition, formation of stable complexes by the polymerase at A2, but not at G2, requires the presence of the first two nucleotides of the A2 transcript.

The predominant form of RNA polymerase isolated from vegetative *Bacillus subtilis* cells is associated with a 43 000-dalton σ subunit, formerly designated σ-55 (Gitt et al., 1985), and a 21 000-dalton δ subunit (Doi, 1982). Although the σ factor confers initiation activity upon the enzyme, the δ subunit, which has no known counterpart in *Escherichia coli*, also influences transcriptional specificity. Pero and her colleagues observed that specific in vitro transcription of phage SP01 middle genes required the host δ protein (Pero et al., 1975) and similar data have been reported for transcription from SP82 DNA by phage-modified and host cell RNA polymerase (Spiegelman et al., 1978; Achberger & Whiteley, 1981). It has also been demonstrated that δ limits σ-43 RNA polymerase/DNA complex formation to restriction fragments of phage ϕ29 and SP82 DNA that carry promoters recognized by the σ-43 enzyme (Dickel et al., 1980; Achberger &

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