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Micrococcus luteus Endonucleases for Apurinic/Apyrimidinic Sites in Deoxyribonucleic Acid. 2. Further Studies on the Substrate Specificity and Mechanism of Action†

Josiane Pierre and Jacques Laval*

ABSTRACT: Two endonucleases specific for D -containing apurinic or apyrimidinic sites (AP-endonucl s A and B) have been isolated from *Micrococcus luteus* d highly purified. These enzymes have no exonuclease tivity. Both AP-endonucleases hydrolyze DNA-containi apurinic or apyrimidinic sites at the 5′ end of the lesion, t s generating 3′-hydroxyl and 5′-phosphoryl end groups. DNA-containing pyrimidine dimers, introduced at low doses of UV, are not hydrolyzed, whereas DNA-containing lesions, introduced at high doses of UV or by γ irradiation are nicked by either AP-endonuclease. During hydrolysis of apurinic DNA, neither of the AP-endonucleases acts as a processive enzyme.

Two endonucleases, which act specifically n DNA containing apurinic or apyrimidinic sites, have bee isolated from *Micrococcus luteus* and designated AP-endo leases A and B. In the previous paper (Pierre & Laval,)), the purification and the physical and catalytical pro rties of these enzymes have been described. They have no det ctable activity against native DNA or modified DNA conta ining uracil or 3-methyladenine.

The experiments reported in this paper indi ate the mechanism of action of the two enzymes. Measu ments of the length of the oligonucleotides produced by tre ment of apurinic DNA with AP-endonucleases show that th enzymes have neither intrinsic nor contaminating exonuc ase activity. Scission of the DNA backbone takes place a he 5′ ends of the apurinic sites and generates 3′-hydroxyl an 5′-phosphoryl end groups. The action of the AP-endonucle ases has been studied on UV- and γ-ray-irradiated DNA. I is also shown that AP-endonucleases are not processive e zymes: they dissociate from the apurinic DNA after each catalytic event.

Materials and Methods

Enzymes. Snake venom phosphodiesterase (EC 3.1.4.1) from *Crotalus adamentus*, spleen phosphodiesterase (EC 3.1.4.18), pancreatic DNase (grade I) (EC 3.1.4.5), and bacterial alkaline phosphatase (EC 3.1.3.1) were purchased from Worthington Biochemicals. Alkaline phosphatase, which was further purified according to Weiss et al. (1968), was a gift from Dr. H. Jacquemin-Sablon. *M. luteus* DNA polymerase (EC 2.7.7.7) was a gift from Dr. L. Grossman. Uracil–DNA glycosylase was prepared as described by Laval & Pierre (1978). Endonuclease from *Helix aspersa* was prepared according to Laval & Paoletti (1972a).

Nucleic Acids and Oligonucleotides. [α-^{32}P]dUTP was prepared by alkaline deamination of [α-^{32}P]dCTP (Amersham), as described by Clements et al. (1978). Poly[d(A-T)] (Miles Laboratories) containing [5′-^{32}P]dUMP residues was synthetized by *M. luteus* DNA polymerase, according to Clements et al. (1978).

T5 [^{3}H]DNA was prepared, alkylated, and depurinated as previously described (Pierre & Laval, 1980). Its specific activity was 2562 cpm/nmol. ^{14}C-Labeled λ DNA was obtained as described by Saucier & Wang (1973), using

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[^{14}C]thymine (CEA, Saclay). Its specific activity was 3478 cpm/nmol. ^{32}P-Labeled λ DNA was prepared as the λ [^{14}C]DNA, except that phages were grown in a low phosphate medium containing 5 μCi/mL inorganic ^{32}P (CEA, Saclay), as described by Rubin (1975). Its specific activity was 3785 cpm/nmol. Depurination of the λ DNAs was performed as described for T5 [^{3}H]DNA. PM_2 DNA and PM_2 [^{3}H]DNA were prepared as described by Laval (1974). The specific activity of the latter DNA was 14 440 cpm/nmol.

UV Irradiation. PM_2 DNA at a concentration of 11.8 nmol, in 50 mM Hepes–KOH, pH 7.5, was irradiated in a 0.2 cm path length quartz cell by using a UV lamp, UV S-11. A black ray ultraviolet monitor (Ultra-Violet Products, Inc.) was used to measure the UV dose.

γ-ray Irradiation. For γ irradiation, 11.8 nmol of PM_2 DNA in 50 mM Hepes–KOH, pH 7.5, was irradiated at room temperature with a ^{137}Cs source, at a dose rate of 768 rad/min.

Preparation of Oligonucleotides Bearing Specific Termini. (*1*) *Apurinic DNA Cleaved by AP-endonuclease A or B.* The incubation mixture (0.1 mL) contained 0.8 nmol of depurinated T5 [^{3}H]DNA in 50 mM Hepes–KOH, pH 7.5, 25 mM NaCl, 2 mM dithiothreitol, and 0.035 unit of AP-endonuclease A or 0.03 unit of AP-endonuclease B. Incubation was done for 30 min at 37 °C. Under these conditions, 11 and 15% of the DNA treated with AP-endonucleases A and B, respectively, became acid soluble.

(*2*) *Apurinic DNA Cleaved by Pancreatic DNase I.* Depurinated T5 [^{3}H]DNA (0.8 nmol) was incubated in 0.1 mL of 50 mM Tris-HCl, pH 8.3, and 10 mM $MgCl_2$ with 0.002 unit of pancreatic DNase for 10 min at 37 °C. Under these conditions, 6% of the DNA became acid soluble.

(*3*) *Apurinic DNA Cleaved by DNase from H. aspersa.* Depurinated T5 [^{3}H]DNA (0.8 nmol) in 0.1 mL of 50 mM acetate buffer, pH 5.6, was incubated with 0.145 unit of DNase for 10 min at 37 °C. Under these conditions, 15% of the DNA became acid soluble.

(*4*) *Removal of Terminal Phosphorus by Phosphatase.* Internal and external phosphate ends of the oligonucleotides produced by the different endonucleases were removed by bacterial alkaline phosphatase. DNA (1 nmol) in 50 mM Tris-HCl, pH 8.3, and 10 mM $MgCl_2$ (0.1 mL) was incubated with 0.0015 unit of alkaline phosphatase for 20 min at 65 °C.

Assays. (*1*) *AP-endonuclease Assay.* (*a*) *Standard Assay.* AP-endonucleases were tested by using the standard assay described in the preceding paper, with alkylated depurinated T5 [^{3}H]DNA as the substrate (Pierre & Laval, 1980).

(*b*) *Nicking Assay.* This assay measures the cleavage of supercoiled PM_2 DNA (form I), giving rise to an open circular form (form II). The substrate was prepared by heating at 70 °C PM_2 [^{3}H]DNA form I in 0.15 M NaCl and 15 mM sodium citrate, pH 4.8. Under these conditions, an average of one apurinic site per molecule was produced in 2 min. For the nicking assay, the reaction mixture (0.1 mL) contained 0.15 nmol of substrate, 2.5 mM $MgCl_2$, 50 mM Hepes–KOH, pH 7.5, 25 mM NaCl, and the enzyme. After 20 min at 37 °C, the reaction was stopped by denaturing the DNA at room temperature for 30 s with 1 mL of alkaline denaturation buffer (0.9 M NaCl, 25 mM EDTA, and 0.1 M PO_4K_2H, pH 12.1). To allow renaturation of the unnicked molecules, we lowered the pH to 8.0 by adding 0.2 mL of 2 M Tris-HCl, pH 2.0. After the addition of 2 mL of 2 × SSC,[1] the solution was filtered through a nitrocellulose membrane filter (Schleicher & Schuell, B-6, 0.45 μm). The reaction tube and the filter were rinsed 3 times with 2 × SSC. Under these conditions form I DNA renatured rapidly and was not retained by the filter, while form II DNA was adsorbed by the filter (Center & Richardson, 1970). The filters were dried and the retained radioactivity was measured by liquid scintillation counting.

The average number of DNA apurinic sites was chemically determined as described by Lindahl & Andersson (1972). Aliquots of 0.05 mL of 2 M glycine–NaOH and 5 mM Na_2EDTA, pH 12.8, were added to 0.05 mL of enzyme-free DNA in 5 mM Na_2EDTA, pH 8.6, for 150 min at 25 °C. At the end of incubation, the number of nicks per molecule was measured by filtration, as described above.

(*2*) *Endonuclease Assay.* Endonucleolytic activity on native and on UV- or γ-irradiated PM_2 DNA was measured by agarose gel electrophoresis as previously described (Pierre & Laval, 1980).

(*3*) *Hydrolysis of Nicked DNA by Snake Venom Exonuclease.* The substrates used were oligonucleotides bearing the specific termini described above. The pH of the solutions was adjusted to 8.3 with NaOH, and denaturation of the DNA was achieved by heating it at 100 °C for 5 min. After addition of 0.006 unit of venom phosphodiesterase, hydrolysis was performed at 37 °C for varying lengths of time. The reaction was stopped by cooling the mixtures to 0 °C and adding 0.05 mL of calf thymus DNA (0.5 mg/mL). After 2 min at 0 °C, 0.2 mL of 0.8 N perchloric acid was added. After 5 min at 0 °C, the tubes were centrifuged for 10 min at 6000*g*. Radioactivity of the supernatant was determined.

(*4*) *Hydrolysis of Nicked DNA by Spleen Exonuclease.* The samples were treated as described for the snake venom phosphodiesterase, except that the pH was adjusted to 5.6 and that 0.005 unit of spleen phosphodiesterase was used.

(*5*) *Hydrolysis of Poly*$[d[(A\text{-}[^{3}H]T)_n(A^{32}pU)_{n'}]$. The preparation was made and analyzed as described by Clements et al. (1978). An alternative copolymer $d(A\text{-}T)_n$, containing a low amount of [5′-^{32}P]dUMP, was synthetized by using *M. luteus* DNA polymerase. When the copolymer was hydrolyzed with micrococcal DNase and spleen phosphodiesterase to obtain 3′-P mononucleotides, more than 95% of the ^{32}P radioactivity was found in the 3′-dAMP. Hydrolysis of the copolymer with pancreatic DNase I and snake venom phosphodiesterase to obtain 5′-P mononucleotides showed that more than 95% of the ^{32}P radioactivity was associated with dUMP and less than 2% was associated with dCTP. In order to prepare a copolymer containing apyrimidinic sites, we made a reaction mixture (0.05-mL initial volume) containing 1 nmol of polymer (40 000 ^{3}H cpm; 1500 ^{32}P cpm) in 50 mM Tris-HCl, pH 8.2, and 0.08 mL (20 units) of uracil–DNA glycosylase from *M. luteus*. Incubation was carried out for 5 min at 37 °C. $MgCl_2$ was then added to a final concentration of 10 mM, followed by the addition of 0.012 mL (0.09 unit) of AP-endonuclease A or by 0.005 mL (0.08 unit) of AP-endonuclease B. Incubation was carried out for 15 min at 37 °C. The presence of terminal ^{32}P was shown by incubation with 0.5 unit of *Escherichia coli* alkaline phosphatase for 15 min at 65 °C. In order to obtain the chemical breakage of DNA by β elimination of the AP site after incision by AP-endonuclease, we made the polymer 0.015 M in Na_3EDTA and 0.2 M in NaOH and incubated it for 30 min at 60 °C. Aliquots of the solution were used to determine the acid-soluble radioactivity and Norit-nonadsorbable radioactivity (Clements et al., 1978).

(*6*) *Size Determination of Oligonucleotides Produced by AP-endonucleases.* The size of the oligonucleotides produced

[1] Abbreviations used: SSC, 0.15 M NaCl and 15 mM sodium citrate, pH 7.0; AP site, apurinic/apyrimidinic site.

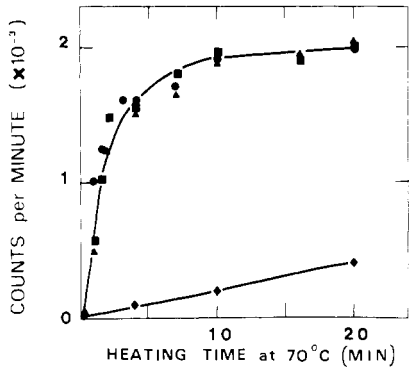

FIGURE 1: Enzymatic cleavage of PM_2 [^{3}H]DNA by AP-endonuclease A or B. PM_2 [^{3}H]DNA was heated at 70 °C at pH 4.8 for different lengths of time in order to produce AP sites. Aliquots (0.15 nmol) were treated with 0.035 unit of AP-endonuclease A (▲) or 0.03 unit of AP-endonuclease B (●) for 30 min or glycine–NaOH (■). Control: DNA heated at 70 °C for different lengths of time without further treatment (♦). Nicks in PM_2 DNA were measured by retention of single-stranded DNA on filters after denaturation and renaturation of the samples. For details, see Assays.

by treating an apurinic DNA with AP-endonucleases was determined by gel filtration. A Sephadex G-50 column (0.78 cm^2 × 30 cm) was equilibrated in 40 mM Tris-HCl, pH 7.2, and 6 M urea. The same buffer was used for elution (Müller et al., 1976). A set of markers, $d(A)_n$ (Miles Laboratories), was added to the column, and the void volume was determined with blue Dextran 2000. Depurinated T5 [^{3}H]DNA (4 nmol) was hydrolyzed for different lengths of time (1–4 h) by AP-endonuclease A or B or glycine–NaOH. At the end of the hydrolysis periods, solid urea was added (final concentration 6 M), and the hydrolysate was analyzed in a Sephadex–urea column. Fractions of 1 mL were collected and the radioactivity was determined.

Results

Action of AP-endonucleases at Apurinic Sites. In order to establish that the AP-endonucleases hydrolyze at apurinic sites, it is first necessary to show (a) that the enzymes are inactive on native DNA, (b) that they can act on DNA bearing no lesions other than AP sites,[1] and (c) that an independent and well-characterized agent nicking at an AP site will produce the same effect as the enzymes. These three prerequisites were met in the following way. Heat treatment, under carefully controlled conditions, resulted in a limited depurination of DNA and the number of lost bases being a function of the heating time (Lindahl & Andersson, 1972). These authors have also shown that an alkaline treatment of DNA-bearing AP sites nicks the DNA at these sites without affecting native DNA. As shown in Figure 1, heat treatment produces in supertwisted DNA an increasing number of AP sites as a function of time, the sites being revealed by glycine–NaOH treatment. Exposure of native and heat-treated DNA to a large excess of AP-endonuclease A or B shows that (a) both enzymes imitate the action of the chemical treatment and (b) the enzymes are inactive on untreated DNA, thus ruling out the possibility of a contaminating nuclease. Hence, the endonucleases are specific for AP sites.

AP-endonucleases Are Devoid of Exonuclease Activity. No exonucleolytic activity of the AP-endonuclease A or B on native double-stranded DNA or on heat-denaturated DNA could be observed by using the standard assay (data not shown). When the products obtained by hydrolysis of depurinated DNA by AP-endonuclease were analyzed according to their lengths, only oligonucleotides were formed without any

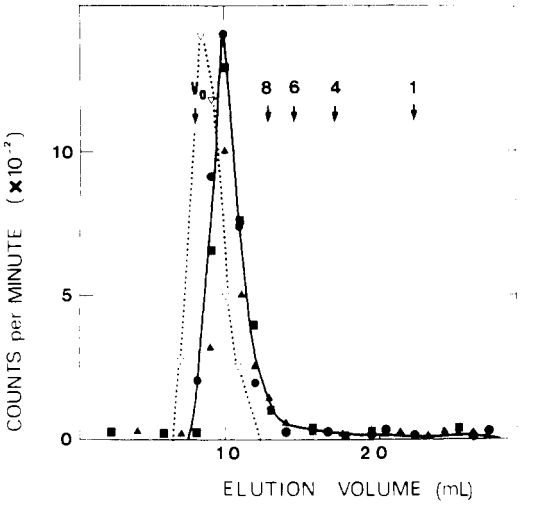

FIGURE 2: Lengths of oligonucleotides produced by AP-endonuclease A or B. The reaction mixture containing depurinated T5 [^{3}H]DNA treated with either AP-endonuclease A (▲), AP-endonuclease B (●), or glycine–NaOH (■) was analyzed by chromatography on Sephadex G-50 in the presence of 6 M urea. Radioactivity of each fraction was measured. The numbers indicate the length of oligonucleotides markers; V_0 = elution of blue Dextran. (▽) Untreated DNA.

detectable mononucleotides (Figure 2). When the length of oligonucleotides produced by the action of AP-endonucleases was compared to the length of oligonucleotides produced by chemical hydrolysis with glycine–NaOH, it was found that the breakage of the DNA backbone by AP-endonucleases was endonucleolytic and similar to the chemical action of glycine–NaOH. This experiment confirmed the previous results above described.

Site of Endonucleolytic Cleavage. (1) Incision by AP-endonuclease A or B Generated 3′-Hydroxyl, 5′-Phosphate Termini. In order to determine the nature of the termini formed by AP-endonucleases and at which side of the AP site the breakage of the phosphodiester bond occurs, we made use of different specificities of spleen and snake exonucleases. The spleen exonuclease initiates hydrolysis of oligonucleotides at a 5′-hydroxyl and yields 3′-P mononucleotides. The venom exonuclease initiates hydrolysis of oligonucleotides at a 3′-hydroxyl and yields 5′-P mononucleotides. As shown in Figure 3A, oligonucleotides produced by either AP-endonuclease A or B were substrates for snake venom exonuclease. This indicates that the termini produced by AP-endonucleases were 5′-phosphorus and 3′-hydroxyl. A control experiment shows that DNA-carrying apurinic sites nicked by pancreatic DNase yielded oligonucleotides which were also substrates for the venom enzyme.

(2) Incision Was Produced on the 5′ Side of the Apurinic Site. Next we determine whether the incision of the phosphodiester chain occurred on the 5′ or 3′ side of the apurinic site. The answer to this question will indicate the direction for the excision of the lesion by appropriate exonucleases. Spleen exonuclease initiated the hydrolysis of oligonucleotides from the 5′ end, thus producing deoxyribonucleosides 3′-P. It is inactive on oligonucleotides with a 5′-phosphomonoester end group or an apurinic site end group (Margison et al., 1975). As shown in Figure 3B, oligonucleotides produced by AP-endonuclease A or B or by pancreatic DNase were not substrates for the spleen exonuclease, as might be expected from the result obtained with the snake venom enzyme. Control experiments, using oligonucleotides produced by *Helix* enzyme, showed that the exonuclease was fully active: the *Helix* DNase produced 3′-phosphate termini (Laval & Paoletti, 1972b).

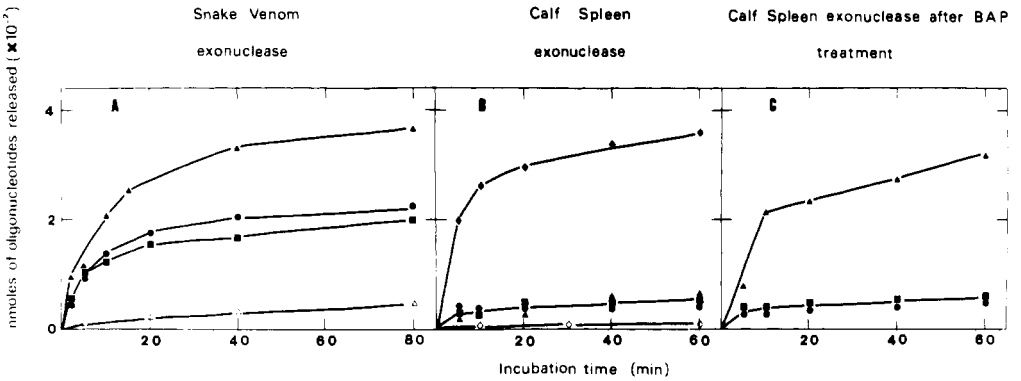

FIGURE 3: Digestion of incised apurinic T5 [^{3}H]DNA with different exonucleases. (A) Digestion by snake venom exonuclease of apurinic T5 [^{3}H]DNA treated by AP-endonuclease A (■) or AP-endonuclease B (●). As the control, apurinic T5 [^{3}H]DNA untreated (△) or nicked by pancreatic DNase (▲) was used. (B) Digestion by calf spleen exonuclease of control apurinic T_5 [^{3}H]DNA (◇), apurinic T_5 [^{3}H]DNA nicked by *Helix* DNase (◆), and apurinic T_5 [^{3}H]DNA treated by AP-endonuclease A (■) or B (●) or by pancreatic DNase (▲). (C) Digestion by calf spleen exonuclease after phosphatase (BAP) treatment of apurinic T_5 [^{3}H]DNA previously treated with pancreatic DNase (▲) and of apurinic T_5 [^{3}H]DNA previously treated with AP-endonuclease A (■) or B (●). For details see Materials and Methods.

After removal of terminal phosphorus by phosphatase (Figure 3C), oligonucleotides produced by pancreatic DNase became substrates for spleen enzyme. However, under the same condition oligonucleotides produced by AP-endonuclease A or B were not hydrolyzed by spleen enzyme, suggesting that the initial break due to either AP-endonuclease A or B was at the 5′ side of the lesion.

(3) Incision by AP-endonuclease A or B of Depyrimidinated Copolymers. We have used the copolymer described by Clements et al. (1978) in order to confirm the specificity of AP-endonucleases. It is an alternating copolymer d(A-[^{3}H]T)$_n$ in which some dTMP residues are substituted by [5′-^{32}P]-dUMP. Uracil residues were removed with *M. luteus* uracil–DNA glycosylase, leaving all labeled phosphate in 5′ linked to apyrimidinic deoxyribose residues. As shown in Table I (A), when uracil–DNA glycosylase, AP-endonuclease A or B, and phosphatase act together on the copolymer, Norit-nonadsorbable ^{32}P was released, and no acid-soluble tritium was released. This result implies that nicks occurred only at the 5′ side of the apyrimidinic site and that there was no exonuclease activity.

Other experiments showed that such an activity was not observed if one of the three enzymes was omitted. These results do not enable us to determine whether the termini produced has a 3′- or 5′-phosphoryl end group. If the AP-endonucleases produced a 5′-^{32}P end group, this group will be linked to the DNA by an alkali-labile bond (Lindahl & Andersson, 1972).

Therefore, we tested the copolymer nicked by the sequential action of uracil–DNA glycosylase and AP-endonucleases under alkaline conditions and monitored the fate of Norit-nonadsorbable ^{32}P (base-free material). As shown in Table I (B), NaOH acted like phosphatase, releasing ^{32}P as Norit-nonadsorbable material. These two experiments confirmed the specificity of the enzymes. AP-endonucleases have been shown to (a) incise the phosphodiester on the 5′ side of the apurinic site and (b) yield 3′-hydroxyl and 5′-phosphorus termini. The results also show that the enzyme preparations were free of phosphatase and exonuclease activities.

Activity against UV-Irradiated and γ-Irradiated DNA. Experiments were performed to determine whether AP-endonucleases A and B can recognize and hydrolyze DNA-containing lesions introduced at low or high UV doses. As shown in Table II, PM_2 DNA irradiated with a low dose of UV (10 J/m^2) is not a substrate for the enzymes. This result shows that AP-endonuclease A or B was completely free of UV endonuclease or pyrimidine–pyrimidine glycosylase activities. Other experiments showed that UV-irradiated DNA was nicked by *M. luteus* extracts, whereas native DNA was not. It was also shown that low doses of UV did not produce AP sites in DNA, in good agreement with the results of Ljungquist et al. (1974).

When DNA was irradiated at higher UV doses (500 J/m^2), it became slightly sensitive to both AP-endonucleases. The two enzymes introduced ~0.2 nick per molecule of PM_2 DNA (20% conversion to nicked molecules after treatment with

Table I: Selective Release of ^{32}P and ^{3}H from d[(A-[^{3}H]T)$_n$(A^{32}pU)$_{n'}$] by the Combined Action of Different Enzymes

	^{3}H acid-soluble radioact (%)	^{32}P Norit-nonadsorbable radioact (%)
(A) Uracil-DNA Glycosylase, AP-endonuclease A or B, and Alkaline Phosphatase (Complete Reaction Mixture)		
uracil-DNA glycosylase + AP-endonuclease A + phosphatase	1.2	17
uracil-DNA glycosylase + AP-endonuclease B + phosphatase	1.2	36
AP-endonuclease A + phosphatase	<0.5	1.5
AP-endonuclease B + phosphatase	<0.5	2
uracil-DNA glycosylase + phosphatase	<0.5	<0.5
uracil-DNA glycosylase + AP-endonuclease A	0.9	<0.5
uracil-DNA glycosylase + AP-endonuclease B	1	<0.5
(B) Uracil-DNA Glycosylase, AP-endonuclease A or B, and NaOH		
uracil-DNA glycosylase + AP-endonuclease A + NaOH	1.2	25
uracil-DNA glycosylase + AP-endonuclease B + NaOH	1.0	36
AP-endonuclease A + NaOH	<0.5	1.3
AP-endonuclease B + NaOH	<0.5	3.2
uracil-DNA glycosylase + NaOH	<0.5	<0.5
uracil-DNA glycosylase + AP-endonuclease A	0.9	<0.5
uracil-DNA glycosylase + AP endonuclease B	1.1	<0.5

Table II: Average Number of Nicks Introduced by AP-endonuclease A or B, by *M. luteus* Extracts on Native PM_2 DNA and on UV- or γ-ray-Irradiated PM_2 DNA

	av no. of nicks when treated with					
			AP-endonuclease A		AP-endonuclease B	
	no enzyme	*M. luteus* extract, 10 μg of protein	7.5×10^{-3} unit	37.5×10^{-3} unit	16×10^{-3} unit	80×10^{-3} unit
native DNA	0.07	0.094	ND[a]	0.09	ND	0.06
UV-irradiated DNA (10 J/m²)	0.15	1.96	ND	0.12	0.15	0.17
UV-irradiated DNA (500 J/m²)	0.094	3.05	ND	0.41	0.25	0.31
γ-irradiated DNA (2700 rad)	0.19[b]	0.69	0.39	0.38	0.21	0.31

[a] ND = not determined. [b] This sample of DNA was freshly prepared and contained before treatment less than 0.01 nick per molecule. PM_2 DNA was irradiated either with UV or with γ-rays and then treated with different amounts of AP-endonuclease A or B or by *M. luteus* extract. Supertwisted and nicked DNA molecules were analyzed by agarose gels. The number of breaks (n) per molecule was calculated by Poisson's law, $n = -\ln e$, e being the fraction of remaining supertwisted molecules. For details, see Materials and Methods.

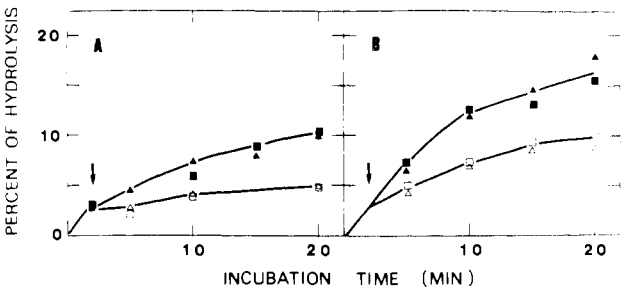

FIGURE 4: Processivity of the two AP-endonucleases. Depurinated λ [^{32}P]DNA (1 nmol) was treated with AP-endonuclease A (0.08 unit) (A) or with AP-endonuclease B (0.16 unit) (B) in the standard AP-endonuclease assay medium. Three minutes after the beginning of the reaction, 1 nmol of depurinated λ [^{14}C]DNA was added, and the release of ^{32}P (□) and ^{14}C (△) as acid-soluble material was measured as previously described. The arrow indicates the time when the second substrate was added. Control experiments show the hydrolysis of depurinated λ [^{32}P]DNA (■) or depurinated λ [^{14}C]DNA (▲) with AP-endonucleases under standard conditions.

AP-endonucleases), whereas *M. luteus* extract introduced 3 nicks. This result was expected since it had already been shown that a dose of 270 J/m² introduces 0.1–0.2 apyrimidinic site in DNA (Ljungquist et al., 1974).

γ-rays, at a dose of 2700 rad, introduced 0.2 nick per molecule of PM_2 DNA (Table II). AP-endonucleases A and B nicked the γ-irradiated DNA, and the number of nicks reached a plateau which is the same for either enzyme. An extract of *M. luteus* produced twice as many nicks as were produced by the purified AP-endonucleases.

Are AP-endonucleases A and B Processive Enzymes? Hydrolysis of AP sites by AP-endonucleases can occur by two different mechanisms. They can dissociate from the DNA molecules after each catalytic event (distributive enzymes) or remain bound to the DNA molecules until complete hydrolysis of all phosphodiester bonds adjacent to the lesion (processive enzymes).

We have investigated these possibilities in a DNA challenge experiment. As shown in Figure 4, ^{14}C- or ^{32}P-labeled λ DNA is an equally good substrate for both AP-endonucleases. AP-endonuclease A or B was first incubated with depurinated λ [^{32}P]DNA. After 3 min of incubation, λ [^{14}C]DNA was added. The addition of the second substrate induced an immediate decrease in hydrolysis of the first substrate. The sum of the ^{14}C- and ^{32}P-labeled acid-soluble oligonucleotides liberated was equal to the amount of acid-soluble oligonucleotides liberated when the enzymes act on only one substrate. In the case of a processive enzyme, we should not have noticed a change in the hydrolysis rate of the first substrate. Thus, AP-endonucleases A and B acted similarly on DNA and were not processive enzymes.

Discussion

Apurinic and apyrimidinic sites can be introduced in DNA by chemical depurination, X-rays, or UV irradiation or enzymatically by specific DNA glycosylases. They are actively repaired through excision or insertion mechanisms. Specific endonucleases, acting at AP sites, seem to be good candidates for the initial step of excision repair. A requirement for such an activity is an action on the DNA backbone at or near AP sites. In this paper we show that two AP-endonucleases from *M. luteus*, inactive on native double-stranded PM_2 DNA, mimic the specific chemical action of glycine–NaOH known to break DNA at AP sites (Lindahl & Andersson, 1972). *M. luteus* AP-endonucleases recognize AP sites created by heat depurination (nicking assay), loss of alkylated bases (standard assay), enzymatic removal of 3-methyladenine (Laval, 1977), and depyrimidinization (as shown with the synthetic polymer). Furthermore, *M. luteus* AP-endonucleases recognize DNA treated with high doses of UV or γ-rays. These treatments are known to introduce AP sites in the DNA (Ljungquist et al., 1974). Other activities are observed in *M. luteus* crude extracts, possibly specific DNA glycosylase(s) or endonuclease(s) which recognize other lesions, introduced by UV (Grossmann et al., 1978) or X-rays.

It should be noticed that both AP-endonucleases are free of exonuclease activity, which is the only case described for a single organism. In contrast, *E. coli* has one AP-endonuclease (Verly & Rassart, 1975), which is the same enzyme as exonuclease III (Weiss, 1976), while another endonuclease is free of exonuclease activity (Ljungquist, 1977).

Both AP-endonucleases generate 3′-hydroxyl and 5′-phosphoryl termini, located on the 5′ side of the lesion. These properties are shared by the AP-endonucleases activity of exonuclease III of *E. coli* (Gossard & Verly, 1978) and *Hemophilus influenzae* (Clements et al., 1978). We have examined the possibilities that the *M. luteus* AP-endonucleases act on the substrate as processive or distributive enzymes. Both enzymes act as a distributive enzyme. Another distributive repair enzyme is the uracil–DNA glycosylase from *M. luteus* (J. P. Leblanc, J. Pierre, B. Martin, and J. Laval, unpublished experiments).

It seems likely that the two AP-endonucleases play an important role in the first step leading to the repair of AP sites in conjunction with the insertion mechanism described by Livneh et al. (1979). However, the implication of the mode of action of the two enzymes in vitro will be better understood when mutants in the genes coding for the enzymes are isolated.

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Effects of Growth at Different Temperatures on the Physical State of Lipids in Native Microsomal Membranes from *Tetrahymena*†

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ABSTRACT: Fluorescence measurements of the probe 1,6-diphenyl-1,3,5-hexatriene in native *Tetrahymena pyriformis* microsomal membranes revealed characteristic "break points" in curves of polarization vs. temperature. In the 5–35 °C range, membranes from cells grown at 39 °C exhibited two break points, one at 11.6 ± 0.6 °C and another at 23.1 ± 1.6 °C. Membranes from 15 °C grown cells also showed two break points, one at 8.0 ± 1.7 °C and another at 17.7 ± 1.7 °C. Complementary measurements of turbidity (absorbance at 360 nm) vs. temperature revealed break points at approximately the same temperatures as observed with the fluorescent probe, thus strengthening the likelihood that the break points signify the onset or termination of lipid phase separations or some other significant structural alteration of lipids. In general, break points measured in the native membrane samples occurred at slightly lower temperatures than did break points in lipids extracted from comparable membranes. This suggests two possible types of protein–lipid interaction. First, there may be a selective withdrawal of relatively highly saturated phospholipid molecular species from the bulk lipid phase and into the protein annulus regions. Alternatively, the configuration of the hydrophobic core of certain key membrane proteins may be such that nonspecific interactions with the lipids stabilize the liquid-crystalline phase.

The protozoan *Tetrahymena pyriformis* is widely used as a model system for investigating molecular mechanisms involved in cellular acclimation to environmental temperature extremes (Thompson & Nozawa, 1977; Thompson, 1980). It is now well established that *T. pyriformis*, like many other plant and animal cells, is capable of adapting to temperature changes by rapidly adjusting certain metabolic pathways. What is perhaps the key metabolic alteration results in a modification of the membrane lipid composition, which in turn offsets the pronounced rigidifying or fluidizing effects of sudden exposure to low or high temperatures, respectively.

One of the most important of these lipid changes associated with temperature acclimation involves fatty acid desaturase enzymes that occur as integral membrane proteins of the endoplasmic reticulum. We have postulated (Kasai et al., 1976) that one or more of the desaturases can in some fashion sense a change in the fluidity of its own immediate membrane environment and respond by increasing or decreasing its activity relative to the rates of other lipid-forming enzymes. Such a self-regulating mechanism could maintain membrane fluidity relatively constant at an optimal level despite sharp temperature fluctuations.

Recent work in this laboratory showed that temperature-induced changes in the physical properties of *T. pyriformis*

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