Volume 16 Number 3 1988 Nucleic Acids Research

The mechanisms of action of *E.coli* endonuclease III and T4 UV endonuclease (endonuclease V) at AP sites

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Received October 13, 1987; Revised and Accepted January 6, 1988

ABSTRACT

Treatment of DNA containing AP sites with either T4 UV endonuclease or with *E. coli* endonuclease III followed by a human class II AP endonuclease releases a putative β-elimination product. This result suggests that both the T4 endonuclease and *E. coli* endonuclease III class I AP endonucleases catalyze phosphodiester bond cleavage *via* a lyase- rather than a hydrolase mechanism. Indeed, we have not detected a class I AP endonuclease which hydrolytically catalyzes phosphodiester bond cleavage. Whereas these enzymes use a lyase-like rather than a hydrolytic mechanism, they nonetheless catalyze phosphodiester bond cleavage. We suggest that the term endonuclease can be properly applied to them.

INTRODUCTION

Apurinic or apyrimidinic (AP) lesions in DNA may result from spontaneous depurination (1), exposure to alkylating agents (2) or the action of various DNA glycosylases upon abnormal DNA bases. Such lesions are recognized by AP endonucleases which have been isolated from numerous sources (3,4,5). While the majority of AP endonucleases are specific for AP sites, several are also associated with DNA glycosylase activities. For example, T4 UV endonuclease cleaves one glycosylic bond of a pyrimidine dimer to produce an apyrimidinic site (6) and *E. coli* endonuclease III is representative of a group of enzymes which cleave the glycosylic bond of thymine glycols and related damaged pyrimidine residues (7). According to the criteria defined by Linn *et al.* (8), these two enzymes act as class I AP endonucleases since they leave the baseless sugar residue on the 3'-terminus and a phosphomonoester on the 5'-terminus. Moreover, when these class I enzymes act in concert with class II enzymes (which normally cleave on the 5'-side of the AP site to produce 3'-hydroxyl nucleotide and sugar 5-phosphate termini), a baseless sugar phosphate is released *in vitro*. We report here that when T4 UV endonuclease or *E. coli* endonuclease III are used in conjunction with a class II enzyme to remove the baseless sugar, the resultant product suggests that the 3'-cleavage had been generated by a β-elimination process.

MATERIALS AND METHODS

Materials

Supercoiled [^{3}H]DNA (1.1 x 10^4 cpm/nmol) was isolated from the phage PM2 grown on the *Alteromonas espejiana* thymidine auxotroph, Bal 31-14, as described (9,10). [*Uracil*-^{3}H]DNA from the *Bacillus subtilis* phage PBS2 was prepared according to the method of Gates and Linn (11). Calf thymus DNA, deoxyribose-5-phosphate, and unlabeled deoxyribonucleoside triphosphates and monophosphates were purchased from Sigma. Poly(dA-dT) was obtained from Pharmacia P-L Biochemicals; Amersham was the source of deoxy[5-^{3}H]uridine 5'-triphosphate and [α-^{32}P]dCTP.

E. coli endonuclease III (11), uracil DNA glycosylase (11), T4 UV endonuclease (12), HeLa AP endonuclease II (4) and human fibroblast AP endonuclease II (13) were prepared as described previously. *E. coli* DNA polymerase I (10 U/µl) was purchased from BRL. One unit of polymerase activity catalyzes the incorporation of 10 nmol of total dNMP into DNA in 30 min at 37°C.

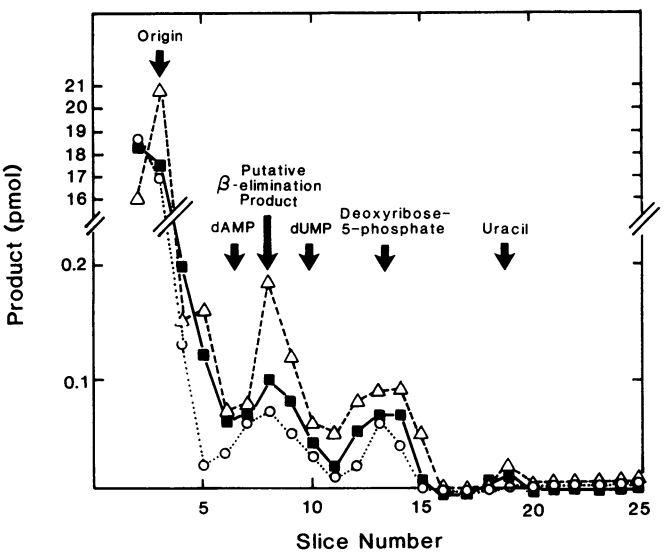


Figure 1. Paper chromatography of product of human fibroblast AP endonuclease II and alkaline β-elimination. After depyrimidination of [^{32}P, *uracil*-^{3}H]poly(dA-dT) with uracil DNA glycosylase, 3 reactions were incubated with 1 unit of AP endonuclease II for 10 min. Each reaction initially contained 0.5 nmol of total polymer nucleotide residues and 43 pmol of dUMP residues. Two of these were then adjusted to pH 11 by addition of 0.3M ammonium acetate buffer (pH 13.2) and incubated at 25°C for 5 hrs or 12 hrs, respectively. Δ-Δ [AP endonuclease II + alkali (12 hr)]; ■-■ [AP endonuclease II + alkali (5 hr)]; o-o [AP endonuclease only]. Products were then applied to Whatman 3 MM chromatographic paper, descending chromatography was performed as described in Materials and Methods, the paper sliced and radioactivity was determined. The origin was contained in slice 3. The specific activity of ^{32}P-dUMP residues was 2000 cpm/pmol. An impure fraction of AP endonuclease II which was contaminated with exonuclease activity was used and this resulted in the release of some deoxyribose-5-phosphate which served as an internal marker.

Methods

Preparation of [^{32}P,*uracil*-^{3}H]*poly(dA-dT)*

[α-^{32}P]dUTP was produced by alkaline deamination of [α-^{32}P]dCTP then [^{32}P,*uracil*-^{3}H]poly(dA-dT) containing uracil residues from the dUTP was synthesized with DNA polymerase I as described previously (13). The reaction (1.8 ml) contained 70 mM potassium phosphate buffer (pH 7.5), 1 mM 2-mercaptoethanol, 7 mM $MgCl_2$, 27 μM poly(dA-dT), 5 μM dATP, 5 μM [α-^{32}P]dUTP (2000-5000 cpm/pmol) plus [^{3}H]dUTP (5000 cpm/pmol), and 5 units of *E. coli* DNA polymerase I. After 30 min at 37°C, 50 μM each of dTTP and dATP were added and synthesis was continued for an additional 10 min. The reaction was stopped by adjustment to 200 mM NaCl and heating for 5 min at 70°C. Mononucleotides and nucleoside triphosphates were removed by extensive dialysis *vs.* 10 mM Tris-HCl (pH 7.5), 200 mM NaCl, then *vs.* 10 mM Tris-HCl (pH 7.5). Extensive treatment of the product with uracil DNA glycosylase removed 99% of the [^{3}H]uracil but less than 1% of the ^{32}P.

Enzyme Assays

Uracil DNA glycosylase was assayed as described previously (13); 1 unit of activity releases 1 pmol of uracil per min from DNA at 37°C. AP endonuclease activity was measured using nitrocellu-

Table 1. Products of *E. coli* endonuclease III or T4 UV endonuclease acting in concert with a class II AP endonuclease upon AP sites in DNA

Addition	fmol β-elimination product	fmol deoxyribose-5-phosphate
HeLa AP endonuclease II	8	2
E. coli endonuclease III	1	2
Endonuclease III then AP endonuclease II	32	7
T4 UV endonuclease	3	1
T4 UV endonuclease then AP endonuclease II	40	8

After removal of approximately 0.2 pmol of uracil from 0.5 nmol of [^{32}P, ^{3}H-*uracil*]poly(dA-dT) with uracil DNA glycosylase, the glycosylase was inactivated for 5 min at 70°C. Reactions were adjusted to 10 mM $MgCl_2$ and 0.005% Triton X-100 for AP endonuclease II (one unit), 200 mM NaCl and 0.005% Triton X-100 for T4 UV endonuclease (0.08 unit) and 0.005% Triton X-100 for *E. coli* endonuclease III (0.5 unit). Incubation times were 10 min for HeLa AP endonuclease II, 20 min for *E. coli* endonuclease III, and 60 min for T4 UV endonuclease. *E. coli* endonuclease III or T4 UV endonuclease were inactivated for 5 min at 70°C before the second reactions. The specific activity of the ^{32}P-dUMP residues was 5000 cpm/pmol.

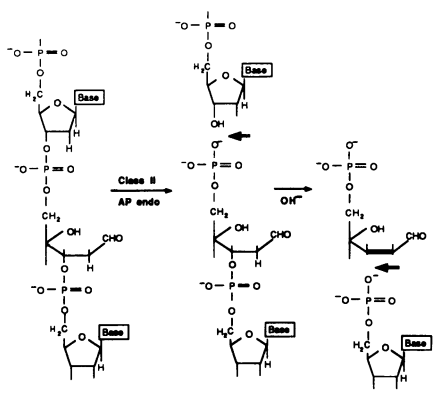
lose filters to selectively bind incised duplex circular DNA as described previously (13); 1 unit of activity produces 1 pmol of nicks per min specifically into AP DNA at 37°C.

Identification of Reaction Products.

Reaction mixtures containing 50 mM Tris-HCl (pH 8.2), 1 mM EDTA, 0.05 units of uracil DNA glycosylase and 0.35 nmol of [^{32}P,*uracil*-^{3}H]poly(dA-dT) were incubated for 20 min at 37°C to produce approximately 1 pmol of AP sites. After the uracil DNA glycosylase was inactivated for 5 min at 70°C, the reactions were placed on ice, and AP endonucleases were added as described (13). Reactions were also adjusted to 10 mM $MgCl_2$ for human fibroblast AP endonuclease II or HeLa AP endonuclease II and 200 mM NaCl for T4 UV endonuclease. After incubation, the reaction products were spotted onto Whatman 3 MM chromatography paper (27 x 45 cm) and descending chromatography was performed for 14 h at 25°C with 95% ethanol and 1 M ammonium acetate (pH 7.5) (70:30) as solvent. Uracil, dAMP and dUMP were located by absorbance of internal markers under a 254-nm UV light, and deoxyribose-5-phosphate was identified by molybdate staining with a Sigma kit. Finally, chromatograms were dried, cut into 2-cm slices and counted for radioactivity. Rf values for dAMP, dUMP, the putatitive β elimination product, and deoxyribose-5-phosphate were 0.36, 0.43, 0.40, and 0.56, respectively.

RESULTS

When originally analyzed by paper chromatography in an ammonium sulfate/sodium acetate/isopropyl alcohol system, the product of the combined actions of class I and class II AP endonucleases was observed to migrate like deoxyribose-5-phosphate (13). However, Grafstrom *et al.* (5) subsequently showed that while deoxyribose-5-phosphate and a putative β-elimination product co-migrated in that solvent system, they were separated in an ethanol/ammonium acetate system, an observation which we have reproduced (Fig. 1). In this case, the β-elimination product was generated by the concerted action of the class II AP endonuclease, human fibroblast AP endonuclease II and an alkali-catalyzed β-elimination reaction:



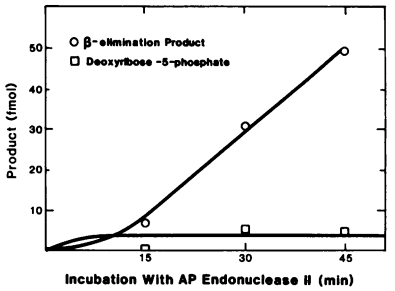


Figure 2. Product of combined action of *E. coli* endonuclease III and human fibroblast AP endonuclease II. After removal of approximately 1 pmol of uracil from 1.5 nmol [^{32}P, ^{3}H-*uracil*]poly(dA-dT) with uracil DNA glycosylase, the glycosylase was inactivated for 5 min at 70^{o}C and the reaction mixture was adjusted to 0.005% Triton X-100. *E. coli* endonuclease III (0.3 U) was added as indicated, and the reaction mixture was incubated for 10 min at 37^{o}C. After inactivation of *E. coli* endonuclease III for 5 min at 70^{o}C, the reaction mixture was adjusted to 10 mM $MgCl_2$, 0.3 units of human fibroblast AP endonuclease II was added as indicated. The reactions were incubated at 37^{o}C and aliquots containing 0.35 nmol of [dUMP-5'-^{32}P, Uracil-^{3}H]poly(dA-dT) were removed at times indicated and spotted onto Whatman 3 MM chromatographic paper. Descending chromatography was performed and radioactivity determined as described in Materials and Methods. The values for *E. coli* endonuclease III and AP endonuclease II which were incubated separately and chromatographed under the identical conditions were subtracted to give the final value. Each enzyme alone released 12 fmol or less of each product. The specific activity of the ^{32}P-dUMP residues was 3000 cpm/pmol. The delay in product release might have been due to preferential nicking by the fibroblast enzyme at sites unnicked by the *E. coli* enzyme.

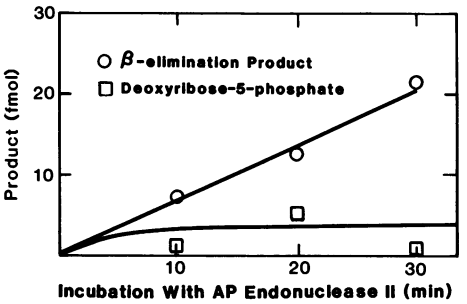


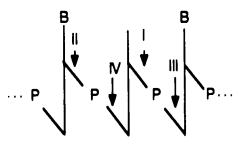
Figure 3. Product of combined action of phage T4 UV endonuclease and HeLa AP endonuclease II. After removal of approximately 0.2 pmol of uracil from 1.5 nmol [^{32}P, ^{3}H-*uracil*]poly(dA-dT) with uracil DNA glycosylase, the glycosylase was inactivated for 5 min at 70^{o}C and the reaction mixture was adjusted to 0.005% Triton X-100 and 200 mM NaCl. T4 UV endonuclease (0.12 U) was added as indicated, and the reaction mixture was incubated for 30 min at 37^{o}C. After inactivation of T4 UV endonuclease for 5 min at 70^{o}C, the reaction mixture was adjusted to 10 mM $MgCl_2$, 0.2 units of HeLa AP endonuclease II was added as indicated. The reactions were incubated at 37^{o}C and aliquots containing 0.35 nmol of [dUMP-5'-^{32}P, uracil-^{3}H]poly (dA-dT) were removed at times indicated and spotted onto Whatman 3 MM chromatographic paper. Descending chromatography was performed and radioactivity determined as described in Materials and Methods. The values of phage T4 UV endonuclease and AP endonuclease II which were incubated separately and chromatographed under the identical conditions were subtracted to give the final value. Each enzyme alone released 7 fmol or less of each product. The specific activity of ^{32}P-dUMP residues was 4300 cpm/pmol.

The products formed by the combined action of the class I AP endonucleases, *E. coli* endonuclease III or T4 UV endonuclease and the class II AP endonucleases, human fibroblast AP endonuclease or HeLa AP endonuclease II were then examined. In both the cases of *E. coli* endonuclease III (Table 1, Fig. 2), and T4 UV endonuclease (Table 1, Fig. 3), the putative β-elimination product which migrated between AMP and UMP in the ethanol and ammonium acetate system predominated.

DISCUSSION

AP endonuclease II alone released low levels of the β-elimination product, presumably because class II endonuclease cleavage of AP sites on DNA produces suitable sites for subsequent spontaneous β-elimination. On the other hand, the human fibroblast AP endonuclease II alone released deoxyribose-5-phosphate, possibly due to contamination of that enzyme with an exonuclease. Despite these background values, it appears both class I enzymes can act in concert with a class II AP endonuclease to catalyze the release of a sugar phosphate residue *in vitro* (presumably 2,3-didehydro-2,3-dideoxyribose-5-phosphate). To our knowledge there is no evidence as to whether either of these pairs of reactions occurs *in vivo*.

There are four conceivable points of cleavage by AP endonucleases, assuming that cleavage occurs next to the baseless site.



It had been assumed that by analogy to class II enzymes, class I AP endonuclease activities leave deoxyribose termini. However, Kow and Wallace (14) and Bailly and Verly (15) have recently suggested that endonuclease III catalyzes a β-elimination reaction, and Jorgensen *et al.* (16) have recently reached the same conclusion for *M. luteus* γ-endonuclease. We have also found that three mammalian class I AP endonucleases which we have characterized each acts by a β-elimination mechanism (to be published). Therefore, at this point it is a real possibility that all class I AP endonucleases act by this mechanism.

Should class I AP endonucleases act *via* a β-elimination mechanism, reduction of the C-1' aldehyde should make AP sites resistant to them. Indeed such resistance has been reported by Jorgensen *et al.* for *M. luteus* γ-endonuclease (16) and by Seawell *et al.* for T4 UV endonuclease (17).

Bailly and Verly (15) have stated that "*E. coli* endonuclease III is not an endonuclease but a β-elimination catalyst." We suggest instead that endonuclease is a proper term that ought to be maintained. The AP endonuclease activity is enzymic in that we find that it is relatively unstable to storage,

it is heat-labile and it follows Michaelis-Menten kinetics with an apparent K_m of 7.5 μM DNA-nucleotide for PM2 DNA containing 1.3 AP sites per molecule. This value is equivalent to 0.5 nM AP sites. Moreover, the term "nuclease" implies an enzyme which catalyzes phosphodiester bond cleavage--it does not formally suggest a hydrolytic mechanism. Thus, we see no reason to create an unnecessarily complex nomenclature by not including lyase-type phosphodiesterases under the generic term, nucleases.

ACKNOWLEDGEMENT

This work was supported by grant DE-FG03-86ER60395 from the Department of Energy.

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