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3′-Phosphodiesterase activity of human apurinic/apyrimidinic endonuclease at DNA double-strand break ends

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ABSTRACT

In order to assess the possible role of human apurinic/apyrimidinic endonuclease (Ape) in double-strand break repair, the substrate specificity of this enzyme was investigated using short DNA duplexes and partial duplexes, each having a single 3′-phosphoglycolate terminus. Phosphoglycolate removal by Ape was detected as a shift in mobility of 5′-end-labeled DNA strands on polyacrylamide sequencing gels, and was quantified by phosphorimaging. Recombinant Ape efficiently removed phosphoglycolates from the 3′-terminus of an internal 1 base gap in a 38mer duplex, but acted more slowly on 3′-phosphoglycolates at a 19 base-recessed 3′-terminus, at an internal nick with no missing bases, and at a double-strand break end with either blunt or 2 base-recessed 3′-termini. There was no detectable activity of Ape toward 3′-phosphoglycolates on 1 or 2 base protruding single-stranded 3′-overhangs. The results suggest that both a single-base internal gap, and duplex DNA on each side of the gap are important binding/recognition determinants for Ape. While Ape may play a role in repair of terminally blocked double-strand breaks, there must also be additional factors involved in removal of at least some damaged 3′-termini, particularly those on 3′-overhangs.

INTRODUCTION

Oxidative damage to DNA in living cells is induced by ionizing radiation (1), by reactive oxygen species produced by aerobic metabolism (2,3), and by some antitumor agents such as neocarzinostatin and bleomycin (4). Repair of oxidative damage to both the bases and the backbone of DNA requires several distinct repair pathways incorporating numerous enzymatic activities (4–6). Oxidative damage to the sugar moiety of DNA often results in DNA breaks with damaged ends, and removal of terminal 3′-blocking groups is an essential early step in repair of these lesions. However, while several apurinic/apyrimidinic (AP) endonucleases have been shown to remove 3′-phosphoglycolates (PGs) from single-strand break termini (5,7), their activity toward 3′-blocks on double-strand break ends has not been carefully examined. Ape, also called Hap1 (9), is the major AP endonuclease of human cells (5,7,8). In order to determine whether Ape can remove 3′-blocks from double-strand break ends, a series of defined DNA substrates bearing PGs at blunt, recessed and overhanging 3′-termini were designed and constructed. In addition to assessing its possible role in double-strand break repair, the results help to define crucial factors involved in substrate recognition by Ape.

MATERIALS AND METHODS

Materials

Bleomycin was obtained from Sigma (St. Louis, MO), and was dissolved in distilled water at a concentration of 2 mM as determined from the A_{295} (ε_{295} = 14 100) (10). Fe(III)·Bleomycin was prepared by adding an equal amount of 2 mM ferric ammonium sulfate (dissolved in 0.5 mM HCl to prevent ferric hydroxide precipitation), and was stored at –20°C. Ferric ammonium sulfate, magnesium chloride, potassium chloride, boric acid, Tris base, and ammonium peroxydisulfate were purchased from Sigma Chemical Company (St. Louis, MO). Urea, acrylamide, and bis-acrylamide were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Depurinated pUC19 plasmid was prepared by heat treatment of the plasmid at pH 4 as described (11).

Preparation of DNA substrates

A 25mer oligonucleotide (Fig. 1) was phosphorylated at the 5′ end with [γ-^{32}P]ATP (5500 Ci/mmol, Du Pont Chemical Co., Wilmington, DE) and subsequently with an excess of unlabeled ATP using T4 polynucleotide kinase according to standard procedures (12). The radiolabeled 25mer was annealed to the complementary 10mer oligonucleotide by incubating at 70°C for 10 min followed by slow cooling, over 2 h, to 15°C. Reaction mixtures for bleomycin-induced cleavage contained 25 mM HEPES-NaOH, pH 7.5, 2 μM 5′-^{32}P-end-labeled duplex, 0–100 μM Fe(III)·bleomycin, and 1 mM H_2O_2. Bleomycin was added last, and the mixture was incubated at 0°C for 1 h. After ethanol precipitation, DNA samples were mixed with an equal volume of formamide containing 20 mM EDTA, and then subjected to electrophoresis on a 24% polyacrylamide denaturing

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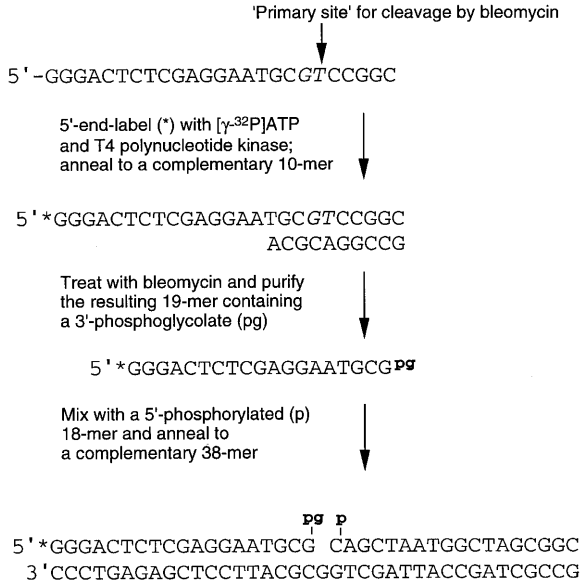

Figure 1. Scheme for the construction of a 38mer duplex oligomer containing a 1 base gap with a 3′-PG.

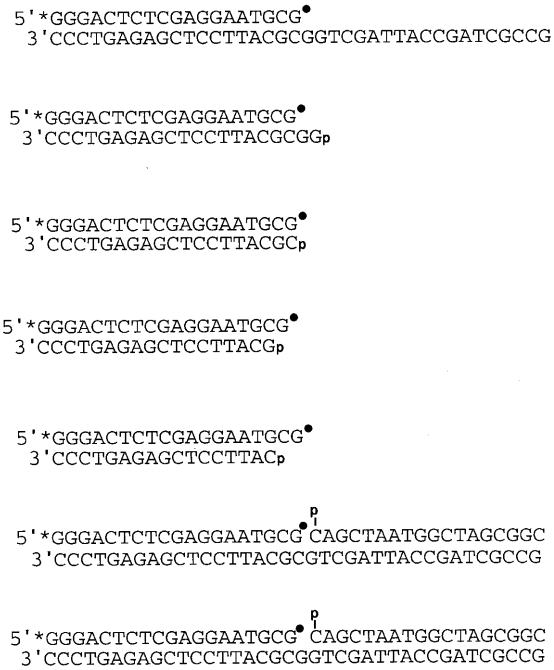

Figure 2. Double-stranded DNA constructs containing 3′-PG termini (indicated by •) at a 19 base-recessed 3′ end, a 2 base-recessed 3′ end, a blunt end, a 1 base 3′ overhang, a 2 base 3′ overhang, an internal nick in a 37mer duplex and an internal gap in a 38mer duplex, from the top to the bottom, respectively.

gel. The band corresponding to the 3′-PG-terminated 19mer was located by autoradiography, cut from the gel and eluted in 1 mM EDTA. The eluate was filtered (0.2 µm Gelman Acrodisc) and evaporated to a volume of 1 ml, and the 19mer was further purified by reverse-phase HPLC (13). Using the same slow cooling procedure, the purified 3′-PG 19mer and a 5′-phosphorylated 18mer were annealed to a complementary 38mer, as shown in the last step in Figure 1. Other DNA substrates were constructed in a similar manner by annealing the 5′-phosphorylated 19mer to oligomers of different lengths, to give structures with 3′-blocked ends in various configurations, as shown in Figure 2.

To obtain an oligomeric substrate with an AP site, a 38mer duplex containing a single uracil (5′-end-labeled GGGACTCTC-GAGGAATGCGUCAGCTAATGGCTAGCGGC annealed to a complementary 38mer with G opposite the U) was treated with 2 U uracil DNA glycosylase (14) (provided by D. W. Mosbaugh, Oregon State University) for 20 min at 37°C, and placed on ice until treatment with Ape.

Enzymatic assays of Ape

Recombinant human AP endonuclease (Ape) was isolated as a glutathione *S*-transferase fusion protein following biosynthesis in an overproducing *Escherichia coli* strain. Following cleavage by factor Xa, the ‘clipped’ Ape protein was purified to apparent homogeneity as described in detail previously (15). This preparation gave a single band on silver-stained polyacrylamide gels and typically had a specific AP endonuclease activity of 500–750 U/µg, where one unit is defined as the amount of enzyme required to cleave one pmol of AP sites per min at 37°C under standard assay conditions (16). This specific activity is comparable to that of highly purified native Ape from HeLa cells (15). Each reaction mixture contained 50 mM HEPES-NaOH, pH 7.5, 50 mM KCl, 100 µg/ml bovine serum albumin, 10 mM $MgCl_2$ and DNA substrate in a volume of 25 µl. Following enzyme addition, the reaction mixtures were incubated for 10 min at 37°C and then the reaction was stopped by addition of 0.1 M EDTA followed by immersion in an ice bath.

Polyacrylamide gel electrophoresis

Following Ape treatment, a 5 µl aliquot of each sample was added to 5 µl formamide, loaded onto a 24% polyacrylamide sequencing gel (30 × 40 × 0.8 cm) and electrophoresed for 6 h at 50 W in a buffer composed of 8 M urea, 90 mM tris-borate, 2.5 mM EDTA, pH 8.3. Gels were covered with cellophane wrap, placed on a phosphor screen overnight at 4°C and analyzed quantitatively by phosphorimaging. Removal of 3′-PG from the 5′-end-labeled 19mer to produce a 3′-hydroxyl terminus was detected as a shift to slower electrophoretic mobility. AP site cleavage was detected as conversion of the full-length 38mer to a 3′-hydroxyl 19mer (15).

Agarose gel analysis

Following depurination and Ape treatment, pUC19 plasmid was electrophoresed on horizontal 1.6% agarose gels containing 90 mM tris-borate, pH 8.3, 2.5 mM EDTA, and 0.5 µg/ml ethidium bromide. Electrophoresis was for 2 h at 12 V/cm. Bands representing the supercoiled and nicked circular topological forms were visualized under long-wavelength ultraviolet light and were quantified by transferring a digital image of the gel from a Stratagene Eagleeye video system to the Molecular Dynamics ImageQuant program. The intensity of bands corresponding to the supercoiled form was multiplied by 1.25 to correct for the decreased binding of ethidium bromide to supercoiled molecules (17). The formation of nicked circular molecules by cleavage of AP sites was measured and used for estimation of AP endonuclease

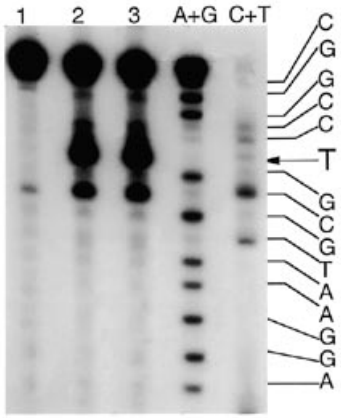

Figure 3. Isolation of a 5′-end-labeled 19mer with a PG at the 3′-end by denaturing polyacrylamide gel electrophoresis of a bleomycin-treated duplex. A+G and C+T markers represent chemical cleavage at sites of purines and pyrimidines in the initial 25 base labeled oligonucleotide. Lanes 1–3 show DNA fragments generated by treatment with 0, 50, and 100 μM Fe(III)·bleomycin in the presence of 1 mM H_2O_2.

activity. The details of strand break calculations were described previously (11).

RESULTS

Preparation of DNA constructs containing 3′-PGs

Bleomycin treatment of DNA produces two predominant lesions, 4′-oxidized AP sites and strand breaks with 3′-PG termini (4,18), both of which are selectively formed at G-Py sequences (19,20). We designed a 25mer containing a potential cleavage site for bleomycin and annealed it to a complementary 10mer to form a partial duplex with a 5′ overhang. When this DNA was treated with Fe(III)·bleomycin, in the presence of hydrogen peroxide to activate the drug, a faster moving DNA fragment was produced whose mobility was that expected for a 19mer bearing a 3′-PG terminus (migrating just ahead of the corresponding Maxam–Gilbert marker; refs 19,20) (Fig. 3). At the appropriate bleomycin concentration, this 3′-PG fragment was formed as the main cleavage product, and was isolated by excising the band from a preparative gel. Following further purification by HPLC (not shown) the 3′-PG-terminated 19mer was annealed to various complementary oligonucleotides, to generate the DNA constructs shown in Figure 2. In addition to a DNA duplex with 3′-PGs at an internal 1-base gap and at an internal nick, there are duplexes bearing PGs at recessed or overhanging 3′ termini of various lengths, as well as at a blunt end. These DNA oligomers were intended to represent a variety of different structures of both single- and double-strand DNA breaks.

Ape releases 3′-PGs from the ends of DNA strand breaks

Since a free 3′-hydroxyl terminus is essential for gap-filling by DNA polymerase or joining by DNA ligase, the removal of any blocked 3′ termini should be an early step in repair of both single- and double-strand DNA breaks. The efficiency of 3′-PG removal from the various substrates by Ape was therefore assessed by monitoring the conversion of the 5′-end-labeled, 3′-PG 19mer to the corresponding 3′-hydroxyl form. These two species are known to migrate just ahead of (3′-PG) and just behind (3′-hydroxyl) the

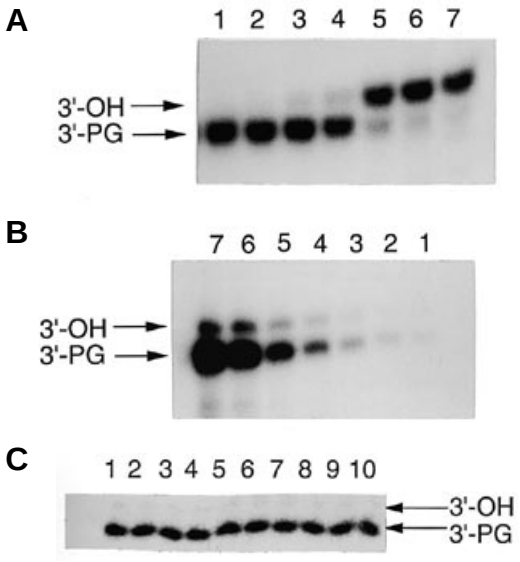

Figure 4. Electrophoretic mobility assay for PG removal. The lower and upper bands represent the 3′-PG 19mer substrate and the 3′-hydroxyl (OH) 19mer product, respectively. (**A**) 3′-PG removal from 1 base internal gap in a 38mer (0.1 μM) was determined following 10 min incubation with 0.002, 0.007, 0.02, 0.06, 0.18, 0.6 and 1.7 μg/ml Ape (lanes 1–7). (**B**) The same gapped substrate at concentrations of 0.004, 0.008, 0.016, 0.031, 0.0625, 0.125 and 0.25 μM (lanes 1–7) was treated with 0.03 μg/ml Ape. (**C**) 3′-PG release from a 2 base 3′ overhang on a 17mer duplex (0.08 μM) was assessed following treatment with 0, 6, 12, 20 or 40 μg/ml Ape protein (lanes 1–5). Removal from a 1-base 3′ overhang was similarly assessed (lanes 6–10). There was no detectable removal in either case.

corresponding 3′-phosphate Maxam–Gilbert markers (13,19–21); presumably, this shift is due to the loss of two negative charges when the PG is removed.

When the labeled 19mer was incorporated into a 38mer duplex such that the 3′-PG was positioned at an internal 1 base gap, treatment with Ape resulted in a mobility shift on a denaturing gel that was consistent with its conversion to a 3′-hydroxyl 19mer, with half-maximal conversion at ~0.1 μg/ml (3.5 nM) Ape (Fig. 4A). Significantly higher Ape concentrations were required to remove 3′-PGs at an internal nick, at a blunt end, or at recessed 3′ ends, but in all cases treatment with a sufficient excess of enzyme resulted in nearly complete conversion (data not shown). However, no conversion of 3′-PG to 3′-hydroxyl was detected at ends with 3′-overhangs, even when treated with 40 μg/ml (1100 nM) Ape (Fig. 4C). Since PG removal from the gapped substrate could be detected at an Ape concentration of 0.02μg/ml, the overhanging termini were at least 1000-fold more resistant to PG removal by Ape than was the 1 base gap.

Kinetics of PG removal by Ape for various DNA constructs

Ape was found to be active only on certain double-stranded DNA substrates, not on single-stranded DNA; furthermore, the reaction rate was constant for at least 30 min and was linearly dependent on enzyme concentration (data not shown). For kinetic anaysis, the concentration of each substrate was varied at a fixed enzyme concentration, chosen such that conversion of the 19mer from 3′-PG to 3′-hydroxyl was <30% even at the lowest substrate concentration; thus, all kinetic data were determined from initial reaction rates. Lineweaver–Burk plots of these data were

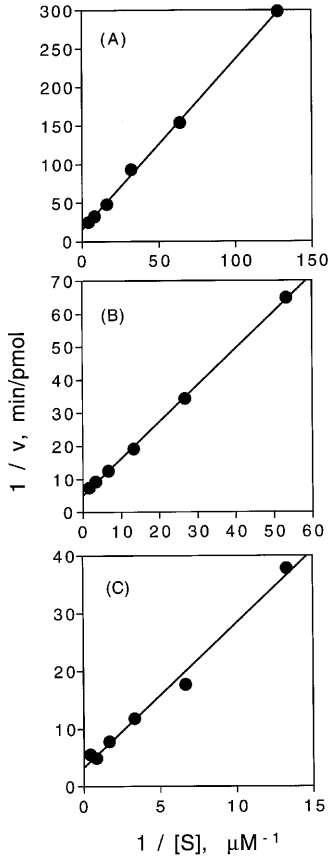

Figure 5. Lineweaver–Burk plots for 3′-PG removal from various substrates, determined from data similar to those presented in Figure 4B. The substrates were: (**A**) a 38mer duplex with a 1 base internal gap; (**B**) a 37mer duplex with an internal nick; and (**C**) a 19mer duplex with a blunt end. The Ape concentration was 0.03 μg/ml (0.8 nM) in (A), and 0.53 μg/ml (15 nM) in (B) and (C).

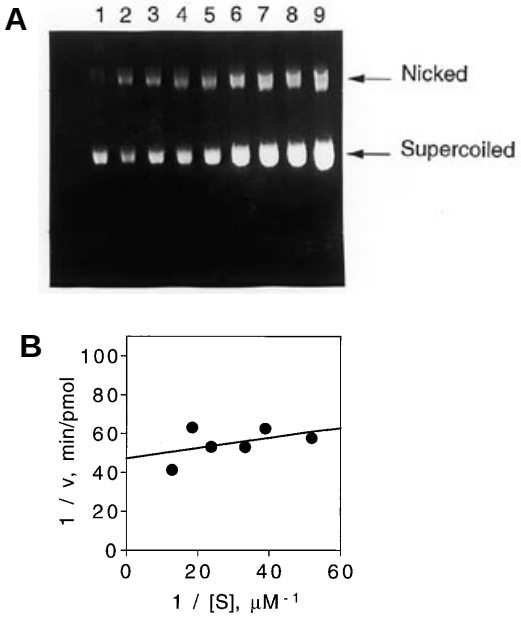

Figure 6. AP endonuclease activity of Ape. (**A**) Agarose gel assay for AP site cleavage. Lower bands represent the supercoiled, partially depurinated plasmid substrate, while upper bands represent nicked circular plasmid. Lane 1 shows the background level of strand breaks (0.08 breaks per molecule) for depurinated plasmid incubated in the absence of enzyme. Lanes 2–7 show the cleavage induced in 0.02, 0.026, 0.03, 0.042, 0.054, 0.078, 0.102, and 0.14μM depurinated plasmid by 4 ng/ml (0.12 nM) Ape protein. In plasmid treated with a large excess of Ape, there were 1.06 breaks per molecule; thus, there were 0.98 Ape-sensitive sites per molecule. (**B**) Kinetic analysis of the data. [S] is the concentration of AP sites, and V is the rate of strand break formation.

approximately linear, suggesting that the reaction followed simple Michaelis–Menton kinetics (Fig. 5). The kinetic parameters (Table 1) indicated that the efficiency of 3′-PG removal from different DNA constructs by Ape was strongly dependent on the secondary structure of the substrates. The highest catalytic efficiency (k_{cat}/K_m) for 3′-PG removal was from an internal 1 base gap in DNA. For the other PG substrates, the efficiency was 6–18-fold lower, with a 19 base-recessed 3′ end, an internal nick, a 2 base-recessed 3′ end, and a blunt end being progressively less favorable substrates. In most cases, k_{cat} and K_m contributed about equally to the differences between the substrates, but the difference between the nicked and gapped substrates was largely in the k_{cat}. The k_{cat} for the gapped substrate was similar to that reported previously for a similar lesion in a 39mer duplex (3.4 min^{-1}; see Fig. 7 of ref. 7).

AP endonuclease kinetics of Ape

To compare the relative potencies of the 3′-phosphodiesterase and AP endonuclease activities of Ape, the endonuclease activity was estimated using as substrates both a duplex oligonucleotide with a single AP site, and a randomly depurinated plasmid. AP site cleavage in the 38mer duplex was determined by electrophoresis on sequencing gels, as described (15). AP site cleavage in the plasmid was determined as the conversion of supercoiled to nicked circular DNA, as shown in Figure 6; bands corresponding to each of topological forms were quantitatively analyzed by fluorescence imaging, and the number of enzyme-induced breaks was calculated (11). As expected (7,16), the k_{cat} for AP site cleavage in the oligomeric duplex (205 min^{-1}) was between 70- and 400-fold greater than the k_{cat} for PG removal from the various oligomeric PG substrates. The depurinated plasmid showed a much lower k_{cat} (7 min^{-1}), but also a significantly lower K_m (0.006 μM) than the oligomeric AP substrate. Similar differences in the kinetic constants for these two types of AP substrates have been reported previously (7,22).

DISCUSSION

AP endonucleases catalyze the cleavage of phosphodiester bonds at AP sites in duplex DNA. Type I AP endonucleases (AP lyases) catalyze β-elimination at the 3′ side of the AP site (23), while type II (or class II) enzymes catalyze hydrolysis at the 5′ side of the AP site, leaving a 3′-hydroxyl terminus. Type II enzymes include exonuclease III and endonuclease IV of *E.coli*, Apn1 of *Saccharomyces cerevisiae*, Rrp1 of *Drosophila* and the major mammalian AP endonuclease, variously designated Ape, Hap1, Apex, REF1 or fibroblast AP endonuclease II (5,8,24–32,34). Several of the these type II enzymes possess 3′→5′ exonuclease activity, but all possess 3′-phosphodiesterase activity and have been shown to remove PGs, phosphoglycoaldehydes, phosphates and/or terminal AP sites from 3′ DNA termini. Ape can remove such blocks at a gap in duplex DNA but does not act on single-stranded DNA (7); thus, its activity toward structures with partial double-strand character is difficult to predict *a priori*.

Table 1. Kinetic parameters for 3′-PG removal from various DNA constructs by Ape

Position of 3′-PG	[Ape] nM	K_m μM	V_{max} pmol/min[a]	k_{cat} min^{-1}	k_{cat}/K_m $min^{-1}\mu M^{-1}$
Internal gap in a 38mer duplex	0.8	0.13	0.060	3.0	22.0
internal nick in a 37mer duplex	15	0.21	0.176	0.47	2.2
19 base-recessed 3′ terminus	4	0.42	0.160	1.6	3.8
2 base-recessed 3′ terminus	6	0.56	0.125	0.83	1.5
blunt end on a 19mer duplex	15	0.53	0.228	0.61	1.2
1 base 3′ overhang on an 18mer duplex	1100	–	<0.004[b]	–	–
2 base 3′ overhang on a 17mer duplex	1100	–	<0.004[b]	–	–
AP site in a 38mer duplex	0.07	0.092	0.36	205	2230
AP site in a supercoiled plasmid[c]	0.12	0.006	0.021	7.0	1200

[a]In a vol of 25 μl.
[b]No detectable removal.
[c]AP site cleavage was measured as conversion of the supercoiled to the nicked circular form.

In order to test whether Ape can remove 3′ blocks from double-strand break ends, a series of defined DNA substrates bearing PGs at blunt, recessed and overhanging 3′ termini was prepared, and their susceptibility to Ape was systematically examined. The kinetic data demonstrate that the enzymatic activity of Ape varies markedly depending on the secondary structure of the DNA constructs. The substrate most efficiently processed by the 3′-phosphodiesterase activity of Ape was a 3′-PG at a 1 base internal gap in a 38mer, which is the structure that would be formed by oxidative degradation of a single sugar moiety in duplex DNA. Absence of either the gap, as in the nicked 37mer, or of DNA 3′ to the gap, as in the substrate with a 3′-blocked blunt end, significantly reduced the efficiency of PG removal by Ape, suggesting that both these features are important for substrate binding or catalysis by the enzyme. Ape showed no detectable phosphodiesterase activity toward 3′-PGs on 3′-overhanging ends, even when the enzyme was present at high concentrations. Thus, the 3′-phosphodiesterase activity of Ape requires at least duplex DNA immediately 5′ to the terminal 3′-blocking group, and this activity appears to be enhanced by the presence of duplex DNA 3′ to the lesion. These results are consistent with X-ray crystallographic studies of exonuclease III [an Ape homologue (8,29)], which suggest that this enzyme may contact duplex DNA on both the 5′ side and the 3′ side of the AP site (33). Alkylation interference studies of Ape binding to an oligomeric duplex containing a site-specific synthetic AP site also indicate the presence of enzyme–DNA contacts on both sides of the AP site, and in both the major and minor grooves (35). The apparent requirement for an intact base pair immediately 5′ to the lesion is consistent with a previous finding that a mismatched bp immediately 5′ to an AP site reduces Ape-induced cleavage of the site by nearly 100-fold for some substrates (15). In general, the results are consistent with the view that the AP endonuclease and 3′-phosphodiesterase activities of Ape depend on similar stereochemical features in DNA for substrate recognition.

DNA double-strand breaks induced by certain radiomimetic antibiotics such as neocarzinostatin and calicheamicin, and probably a substantial fraction of radiation-induced double-strand breaks, have 3′-overhanging ends bearing terminal phosphates or PGs (1,4,5,36). Since these terminal blocking groups apparently cannot be removed by Ape, additional enzymes or protein factors must be invoked to account for the repair of these breaks. At least two 3′-phosphodiesterase activities in addition to Ape have been detected in mammalian cell extracts (16,37). The instability of these putative repair proteins has prevented detailed examination of their substrate specificities, but they are obvious candidates for effecting removal of 3′ blocks from overhanging termini; at least one of them has a higher ratio of 3′-phosphodiesterase to AP endonuclease activity than does Ape (16).

An alternative possibility is that there are accessory factors which interact with either DNA or Ape in such a way that the double-strand break ends are rendered suitable for processing by the 3′-phosphodiesterase activity of Ape. For example, several lines of indirect evidence suggest that there is in eukaryotes an end-joining pathway of double-strand break repair, in which any required trimming and patching of the termini is preceded by alignment of the two ends to be joined (38,39). This alignment is apparently facilitated by the annealing of any available complementary single-strand overhangs, even as little as 1–2 bp. Such an aligned structure might be similar enough to a fully double-stranded DNA molecule to be recognized and processed by Ape. One likely participant in such an end-alignment mechanism is DNA-dependent protein kinase (DNA–PK). Though the exact function of DNA–PK is not known, this kinase binds to and acts at double-strand break ends, and is required for efficient double-strand break repair in mammalian cells (review: ref. 40). It is thus intriguing that, in *Xenopus* egg extracts, removal of 3′-PGs from double-strand break ends is suppressed by DNA–PK inhibitors, at appropriate concentrations (41). This result suggests that, rather than being a simple function of a single enzyme, removal of damaged double-strand break termini may be a stringently controlled process effected by a multiprotein repair complex. Recent evidence for interaction between Ape and the Ku protein, a subunit of DNA–PK (42), raises the possibility that Ape may be a component of such a complex.

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