Excision of C-4'-oxidized Deoxyribose Lesions from Double-stranded DNA by Human Apurinic/Apyrimidinic Endonuclease (Ape1 Protein) and DNA Polymerase β*

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Oxidative damage to DNA deoxyribose generates oxidized abasic sites (OAS) that may constitute one-third of ionizing radiation damage. The antitumor drug bleomycin produces exclusively OAS in the form of C-4-keto-C-1-aldehydes in unbroken DNA strands and 3'-phosphohydroxypyruvate esters terminating strand breaks. We investigated whether two human DNA repair enzymes can mediate OAS excision in vitro: Ape1 protein (the main human apurinic endonuclease (also called Hap1, Apex, or Ref1)) and DNA polymerase β, which carries out both the abasic excision and the resynthesis steps. We used a duplex oligonucleotide substrate with one main target for bleomycin-induced damage. Ape1 catalyzed effective excision at the C-4-keto-C-1-aldehyde sites at a rate that may be only a few-fold lower than incision of hydrolytic abasic sites at the same location. Consistent with several previous studies, Ape1 hydrolyzed 3'-phosphohydroxypyruvate 25-fold more slowly than C-4-keto-C-1-aldehydes. DNA polymerase β excised the 5'-terminal OAS formed by Ape1 excision at a rate similar to its removal of unmodified abasic residues. Polymerase β-mediated excision of 5'-terminal OAS was stimulated by Ape1 as it is for unmodified abasic sites. Escherichia coli Fpg (MutM) protein also excised 5'-terminal OAS, but in our hands, the Rec3 protein did not. These observations help define mammalian pathways of OAS repair, point to interactions that might coordinate functional steps, and suggest that still unknown factors may contribute to removal of 3'-phosphohydroxypyruvate esters.

Under physiological conditions, the human genome is continuously damaged by hydrolysis; reactive metabolic by-products, such as oxygen radicals; exogenous agents that penetrate cells; and radiation (1, 2). The tremendous sizes of chromosomal DNA molecules render them susceptible to even infrequent events that covalently alter their structure. For example, all of the hydrogen atoms of DNA deoxyribose can potentially be abstracted by free radicals. Four of the seven sugar hydrogens in B-form duplex DNA, i.e. C-1′-H, C-2′-H(R), C-4′-H, and C-5′-H(S) are exposed to free radicals through the minor groove and the others through the major groove (3, 4). γ-Radiation initiates oxidative DNA damage on sugar moieties by hydrogen abstraction from the C-1′, C-2′, and C-4′ positions (5). Hydrogen abstractions from minor groove-accessible C-1′, C-4′, or C-5′ positions by some antitumor drugs, such as the enediyne agents and bleomycins (6–9), result in modified abasic residues and in strand breaks terminated with different damaged sugar residues at either the 3′- or the 5′-end of the breaks. Such oxidized abasic sites (OAS) may comprise up to one-third of total oxidative damage (2, 10). It is expected that the abasic sites and DNA fragmentation resulting from oxidative agents would block DNA replication and be cytotoxic or mutagenic (1, 2). However, little is known about the biochemistry of repair of these naturally occurring and environmentally induced lesions that may threaten genetic stability.

Genetic instability can be counteracted by various pathways of DNA repair (1, 2, 11, 12). Nucleotide excision repair, mediated by ~30 proteins in mammalian cells, acts on a wide variety of DNA lesions (11, 12). Base excision repair handles altered bases through the action of DNA glycosylases that produce apurinic/apyrimidinic (AP) sites. The AP sites generated by glycosylases can be incised at the 5′-side by a number of AP endonucleases, such as the mammalian Ape1 protein (13). Ape1 protein2 (also called Ref1 (14), Hap1 (15), or Apex (16)) is the major AP endonuclease found in human cells and belongs to a large family of nucleases homologous to exonuclease III of Escherichia coli (2, 17). Ape1 has a broad specificity for AP sites and incises them to generate a 3′-OH and a 5′-terminal deoxyribose-5-phosphate (5′-dRpy) residue (17–19). The resulting 5′-dRpy can be removed by an intrinsic activity of human DNA polymerase β (Polβ) (20, 21), followed by DNA repair synthesis to fill the gap and ligate to complete the repair process (2). Recently, human Ape1 protein and Polβ were found to act in an orchestrated manner mediated by protein–protein interactions (22). An alternative base excision repair pathway is used when strand displacement repair synthesis generates a single-stranded 5′-end that must be removed by a “FLAP” endonuclease (23).

The C-4′ oxidized deoxyribose damage initiated by bleomycin is a mixture of oxidized abasic lesions and strand breaks (6, 7, 9, 24). It is proposed (6, 7) that the activated bleomycin, after binding into the DNA minor groove, position-specifically abstracts a hydrogen atom from the C-4′ position of the deoxyribose of the pyrimidine (Py) in a d(GpPy) sequence to initiate the degradation process. One pathway (Fig. 1, lower branch) leads to the formation of a C-4′-hydroxylated abasic site (2-deoxypentose-4-ulose), which can be chemically cleaved by hydrazine treatment to form a strand break with a 3′-pyridazine

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The abbreviations used are: OAS, oxidized abasic site; AP, apurinic/apyrimidinic; Polβ, DNA polymerase β, 5′-dRpy, 5′-terminal deoxyribose-5-phosphate; Endo IV, endonuclease IV; 3′-PG, 3′-phosphohydroxypyruvate; BSA, bovine serum albumin.

1 We have modified the previous name for this protein by adding a number, which will accommodate naming of new enzymes of this type. Accordingly, the gene should be called APE1.
(25). Strand breaks with 3'-phosphoglycolate (3'-PG) can be generated directly (Fig. 1, upper branch) with the concomitant release of base-propenal. Although the mechanisms are different, C-4' hydrogen abstraction by enediyne antitumor antibiotics, such as neocarzinostatin (8, 26) and C1027 (27), results in the same DNA damage products in either the presence or the absence of thiols. Since C-4'-oxidized abasic sites constitute as much as 10% of the damage formed by ionizing radiation (10), they are also likely to be significant products of the chemical oxidation that occurs in vivo as a by-product of aerobic metabolism (2).

Here we address whether these C-4'-oxidized damage products can be enzymatically excised in vitro and investigate the efficiency with which these products are processed by known enzymes. We have designed a fragment of duplex DNA (Fig. 2, duplex D1-D2) that contains one predominant attack site for bleomycin, which exhibits a limited sequence dependence for its reactions (6, 7). The results show that the C-4'-oxidized abasic residues are readily excised by the human enzymes and confirm that 3'-PG lesions are poor substrates for excision by Ape1, consistent with previous studies of randomly introduced 3'-PG lesions (see “Discussion”). As they do acting on hydrolytic AP sites, Ape1 and Polβ can work cooperatively in the excision of OAS.

**EXPERIMENTAL PROCEDURES**

**Materials**—High pressure liquid chromatography-purified DNA oligonucleotides were commercial products from Operon Technologies (Alameda, CA). Radiolabeled nucleotides and the enzymes used for labeling were from NEN Life Science Products and New England Biolabs, Inc. (Beverly, MA), respectively. Bleomycin (1.2–1.7 units/mg) was purified from E. coli was purified (95% homogeneity as judged by silver staining of polyacrylamide-SDS gels) as described by Wilson et al. (19). Endonuclease IV (Endo IV) was purified (>98% purity) by D. M. Wilson III using a method described previously (28). Recombinant human Polβ protein was purified by R. A. O. Bennett as described previously (22) using an expression vector generously provided by Drs. J. Carney and S. Linn (University of California, Berkeley). E. coli uracil-DNA glycosylase and Fpg protein were kindly provided by Dr. D. Mosbaugh (Oregon State University) and Dr. A. Grollman (State University of New York, Stony Brook), respectively.

**Terminal Labeling of Oligonucleotides with 32P**—Single-stranded DNA oligomers were end-labeled with [γ-32P]ATP and T4 polymerase kinase (29) and annealed to the complementary strands. For 3'-end labeling, the oligonucleotides were first annealed and then incubated with [α-32P]dATP (for D1 and D3) or [α-32P]dCTP (for D2) and the Klenow fragment of E. coli DNA polymerase I (29).

**Internal Labeling of Oligonucleotides with 3H or 35P**—For 32P internal labeling, oligomer D5 (which corresponds to the 3' half of D1; Fig. 2) was first 5'-32P-end labeled as described above and then gel-purified. Oligomer D4 (the 5'-half of D1; Fig. 2) was placed adjacent to the 5'-labeled D5 by annealing both oligonucleotides to a third oligomer, D6 (Fig. 2). The nick between D4 and D5 was sealed by T4 DNA ligase to produce an internally 32P-labeled D1. 3H internal labeling at the bleomycin target site was done following the method of T. A. Winters et al. (30) with some modifications. Briefly, the deoxyribose of the target T nucleotide in D1 was labeled with 3H at the C-5'- and C-2'-positions by incorporation of [methyl-1-3H]dthdTPP and duplex D4-D6 with the Klenow fragment of DNA polymerase I in the presence of dGTP and dATP. Similarly, the C-5'-position was labeled with 3H by incubation of [5,5',-3H]dCTP with Klenow fragment in the presence of duplex D4-D7, dGTP, and dATP. The internally 3H-labeled D4 oligonucleotide was then gel-purified and annealed to oligonucleotide D8 (Fig. 2). The 3' portion of D1 was then synthesized by Klenow fragment in the presence of all four dNTPs. When [5,5',-3H]dCTP was incorporated, which substitutes the target T nucleotide on D1, a complementary strand containing a G on the opposite position was annealed. The DNA duplex with C replacing T is still expected to provide a good target for bleomycin reaction, but more double-stranded lesions are expected to be generated (24). However, these double-stranded lesions should not interfere with the analysis conducted here. All labeled oligonucleotides were purified using electrophoresis in denaturing polyacrylamide gels (National Diagnostics) and then annealed to their complementary strands at a molar ratio of 1:1.

**Bleomycin Reactions with DNA**—Bleomycin reactions with DNA were carried out according to published protocols (6, 7, 24). In brief, bleomycin was first mixed with an equal amount of ferrous ammonium sulfate at 4 °C for exactly 1 min to make “activated bleomycin.” The activated bleomycin was then added to the annealed DNA oligonucleotides in Hepes-KOH buffer (50 mM, pH 7.5) to initiate the drug-DNA reactions (6, 7). Alternatively, the drug-DNA reactions were started by adding ferrous ammonium sulfate to a mixture of DNA and bleomycin (24). The reactions were allowed to proceed for 1–2 h at room temperature and then stopped by the addition of sodium acetate to 0.3 M (from a pH 7.0 stock) and ethanol precipitation or by washing with 20 volumes of 5 mM Hepes-KOH buffer (pH 7.6) in an ultrafiltration apparatus (Centricon 10, Amicon, Beverly, MA). These procedures remove most of the free bleomycin.

**Repair Enzyme Reactions**—The freshly prepared, bleomycin-treated DNA substrates (0.25 μM final concentration) with position-specific, C-4'-oxidized deoxyribose lesions were incubated with the indicated amounts of Ape1, Endo IV, Fpg, or Polβ protein in 50 mM Hepes-KOH buffer (pH 7.6) containing different enzyme-specific components. For Ape1 reactions, 50 mM KCl, 100 μg/ml BSA, 0.05% Triton X-100, and 10 μM MgCl2 were included (18). The Endo IV reactions contained 50 mM KCl, 50 μg/ml BSA, and 1 mM EDTA (28). The Ape1 or Endo IV reactions were stopped by flash freezing and drying in a Speed-Vac concentrator or further incubated with Fpg or Polβ in Hepes-KOH buffer containing 2 mM dithiothreitol, 50 mM NaCl, 2.5% glycerol, and 100 μg/ml BSA (22). The repair enzyme reactions were assembled at 0–4 °C and incubated at room temperature. In order to stabilize the C-4'-oxidized abasic sites and unexcised 5'-dRP, Polβ reaction mixtures were terminated by treatment with 300 mM NaBH4 for 1 h at room temperature.
temperature. The DNA samples were then precipitated in ice-cold 75% ethanol containing 0.3 M sodium acetate and pelleted by centrifugation.

High Resolution Sequencing Gel Analysis—After the DNA-bleomycin or DNA-enzyme reactions, the samples were either directly frozen and dried in a Speed-Vac concentrator or precipitated with ethanol and pelleted by centrifugation. The DNA samples were then taken up in a loading buffer containing 80% formamide and dyes, and then separated on 15 or 20% polyacrylamide sequencing gels as described previously (27). The gels were visualized by autoradiography, and the intensities of the bands were quantitated using a phosphor imager (Bio-Rad). When needed, the bands were excised, the materials inside the gel slices were eluted with distilled H2O, and the amount of radioactivity was determined by liquid scintillation using an LS 1801 counter (Beckman Instruments, Fullerton, CA).

RESULTS

Site-specific Bleomycin Damage in Oligonucleotides—Based on the reported sequence specificity of bleomycin (6, 7, 24), a 36-mer DNA oligonucleotide was designed and 5'-32P-end-labeled for in vitro studies. When this duplex DNA fragment (duplex of oligomers D1 and D2) was reacted with different amounts of bleomycin, one major damage site was found on strand D1 (Fig. 3, lanes 4–9) and two minor sites on D2 (lanes 13–18), respectively. The band in Fig. 3, lane 4 (indicated by an arrow) that moves slightly faster than the Maxam-Gilbert “T” sequencing marker corresponds to a DNA fragment bearing a 3'-PG terminus, which is diagnostic for C-4' chemistry (27). To reveal additional OAS, samples were treated with hydrazine, which specifically cleaves C-4'-oxidized abasic sites to generate strand breaks terminated with 3'-pyridazine (Fig. 1). As shown in Fig. 3, lane 5, the band (marked by an asterisk) that was generated by hydrazine treatment and moves more slowly than the “T” sequencing marker corresponds to a DNA cleavage product containing a 3'-pyridazine terminus (27). Such a band was not observed without bleomycin treatment (Fig. 3, lanes 2 and 11). Quantitation by phosphor imaging of the bleomycin damage products showed that, under our conditions, C-4'-oxidized abasic sites and direct strand breaks (3'-PG) were generated at a ratio of ~1:5.1. Comparison of lanes 5 and 14 of Fig. 3 shows that, when almost all of the D1 strand had been modified, 35% of oligomer D2 remained without detectable bleomycin damage. Thus, the central T nucleotide within oligonucleotide D1 was indeed the major site for bleomycin attack in this DNA fragment. However, we also note that, at the highest level of bleomycin treatment, considerable damage at secondary sites was observed, which was accompanied by some loss of material from the gel analysis. Therefore, to ensure that the bleomycin-treated DNA fragments were prepared for later enzymatic treatment would contain only one damaged nucleotide, the extent of damage in the D1 oligomer was limited to 5–7% of the total D1 oligomer by using bleomycin concentrations of 3–5 μM.

Excision of 3'-PG Residues and 5' Incision of the C-4'-oxidized Abasic Sites by Human AP Endonuclease Ape1—The abundance of Ape1 protein in human cells (13, 18) and its ability to cleave DNA at diverse types of abasic sites (19) prompted us to test the enzyme's ability to act on OAS. Incubation of the bleomycin-treated, 5'-labeled D1-D2 duplex with Ape1 initially generated a single cleavage product (band labeled b in Fig. 4), which remained as the main product at the highest level of enzyme treatment (Fig. 4, lane 6). For comparison, the 5'-labeled D2-D3 DNA duplex, treated with uracil-DNA glycosylase to generate a single AP site, was cleaved by Ape1 protein to generate a single product of the same mobility (Fig. 4, lanes 7–10). As expected, the mobility of this product (faster than the G (not shown) and slower than the T Maxam-Gilbert markers with 3'-phosphate ends generated by piperidine treatment) was consistent with cleavage by Ape1 at the single AP site to form a DNA fragment containing a 3'-OH end. Thus, Ape1 cleaves C-4'-oxidized abasic lesions in the same way. Because only some of the DNA in the bleomycin-treated
samples contained OAS at the target site, and allowing for possible inhibitory effects of other damage in these samples, we cannot make an accurate quantitative comparison of Ape1 activity on C-4'-oxidized versus hydrolytic abasic sites. However, we note that 54 nM Ape1 acting on C-4'-oxidized abasic sites generated product at \( \approx 10\% \) of the amount generated from hydrolytic abasic sites (Fig. 4, lane 5 versus lane 10). Since other lesions in the bleomycin-treated DNA probably compete for Ape1 with the C-4'-oxidized abasic residues at the target site, the relative efficiency of the enzyme on this type of OAS may be within a few-fold of the activity on regular AP sites.

Lane 3 of Fig. 4 shows the direct cleavage product of bleomycin acting on the 3'-PG labeled D1-D2 duplex to generate a strand break with 3'-PG (indicated by an arrow). Incubation with increasing amounts of Ape1 protein led to the disappearance of the 3'-PG band, with nearly complete excision at the highest Ape1 level (Fig. 4, lane 6). Band b contains the common product of Ape1 3'-PG excision and abasic site incision activities. However, at an intermediate Ape1 concentration, most of the 3'-PG substrate remained intact in the same sample in which there was extensive incision at the C-4'-oxidized abasic site (Fig. 4, lane 5). This result suggested that Ape1 acts more readily on the intact abasic residues formed by bleomycin than it does on the 3'-PG sites, and this conclusion was verified by a kinetic analysis (see below).

The same products were formed by Endo IV (Fig. 4, lanes 11–14), in keeping with earlier reports of this enzyme’s activity upon bleomycin cleavage (28, 31). However, Endo IV excised 3'-PG as efficiently as it incised C-4'-oxidized abasic sites (compare lanes 12 and 13 of Fig. 4). In addition, the action of the bacterial enzyme on the OAS was nearly as robust as its activity on hydrolytic AP sites (see lanes 15–18 of Fig. 4). Band c in Fig. 4 is probably generated by a trace amount of contaminating exonuclease in this particular Endo IV preparation (data not shown).

To verify the incision sites of Ape1 and Endo IV at bleomycin damages, the D1 oligomer was annealed with D2 and 32P-labeled at its 3'-end before treatment with bleomycin. Ape1 or Endo IV treatment of the 3'-labeled substrate generated a single cleavage product (Fig. 5, lanes 2 and 7, labeled with an asterisk) of slower mobility than the 5'-phosphate produced by the direct action of bleomycin (arrow in Fig. 5). The slower mobility of this product is consistent with the presence of an abasic residue at the 5' terminus. Thus, Ape1 protein recognizes and incises the C-4'-oxidized abasic residues on their immediate 5’ side.

Kinetik of the 3’-Diesterase and 5’-Incision Activities of Ape1 on C-4’-oxidized Deoxyribose Lesions—Ape1 protein can remove 3’-PG residues from DNA single-stranded breaks, one-base gaps, and from double-stranded breaks with either blunt or two-base recessed 3’-termini (Refs. 30 and 32 and this work). Since we found that Ape1 can also incise on the 5’ side of C-4’-oxidized abasic sites, it was of interest to compare the excision and incision activities of Ape1 in the same reaction system. For this purpose, oligomers were 32P-labeled at either the 5’-end or internally in the phosphodiester at the immediate 5’ side of the target T nucleotide in sequence D1 (Fig. 2). With the internal-labeled oligomers, the DNA fragments with 3’-PG residues (direct damage products) and the fragments with 5’-terminal oxidized abasic residues (Ape1 dependent products) can be well separated as two bands on 15% sequencing gels (see Fig. 7). Quantitation by phosphor imaging showed that the incision activity of Ape1 on C-4’-oxidized AP sites was much more active (>25-fold) than its 3’-diesterase activity (Fig. 6). After a 2-h incubation with Ape1 protein at a Ape1:DNA molar ratio of 1:1.1, there was almost no diesterase activity detected, while >50% of the C-4’-oxidized abasic sites had been incised.
Repair of C-4’-oxidized DNA Lesions by Human Enzymes

**A** Kinetics of 3’-PG excision and C-4’-oxidized abasic site incision by Ape1 protein. The DNA (D1-D2) substrates treated with bleomycin to contain 3’-PG and abasic sites at the target T were incubated with Ape1 protein at room temperature at Ape1:DNA molar ratios of 1.11 (A) or 2.21 (B). Samples were removed at the indicated times, mixed with an equal volume of 10% SDS, and flash-frozen in liquid nitrogen. Samples were then thawed and treated with NaBH₄ to stabilize the unincised abasic lesions. The bands containing DNA fragments with 3’-PG or 3’-OH ends were quantified by phosphor imaging. The 5’-incision activity of Ape1 was calculated by subtracting the amount of 3’-PG excised from the amount of 3’-OH generated; 3’-OH is the common product of the two enzymatic activities of Ape1 protein acting on 3’-PG and abasic sites. ●, abasic site incision activity; ○, 3’-diesterase activity. There was no detectable spontaneous decomposition (<5%) of the C-4’-oxidized abasic lesions under the reaction and analysis conditions used here.

(Fig. 6A). Doubling the relative amount of Ape1 protein allowed some 3’-PG excision to be detected (Fig. 6B) at a rate >15-fold lower than the enzyme’s activity on the oxidized abasic residue. This result is consistent with the relative 3’-diesterase and AP incision activities of Ape1 determined using a synthetic substrate (18). The bacterial protein Endo IV, however, showed a similar efficiency for both activities (Fig. 4), consistent with published data (33).

5’-Excision Activity of Polβ for C-4’-oxidized Abasic Residues—The foregoing experiments show that the C-4’-oxidized abasic sites are effectively incised on the 5’ side by Ape1 or Endo IV to generate strand breaks with 3’-OH and 5’-abasic phosphate residues. For repair to proceed, the 5’-terminal abasic residues need to be removed. Mammalian DNA polymerase β has this ability for the 5’-dRp generated by AP endonucleases at a glycosylase-generated AP site (11, 20, 22). Since the excision occurs by β-elimination (20, 21), chemically reduced AP sites and some OAS are resistant to Polβ (23). To determine whether C-4’-oxidized residues specifically are substrates for excision by Polβ, the 3’-32P-labeled duplex was treated with bleomycin and then with an excess of Ape1 protein or Endo IV to generate an incised substrate. This material contained direct strand breaks with 5’-phosphate ends (Fig. 5, lanes 1 and 6 indicated by an arrow) accompanying 3’-PG formation, and 5’-terminal C-4’-oxidized abasic residues (Fig. 5, lanes 2 and 7, denoted by asterisk) generated by the endonucleases. After additional incubation with different amounts of Polβ, the intensities of the bands corresponding to the endonuclease products were decreased, while the amount of the DNA fragment with a 5’-phosphate end was correspondingly increased (Fig. 5, lanes 3–5 and 8–10). Thus, C-4’-oxidized abasic residues can be excised by human Polβ to generate a one-nucleotide gap with normal 3’-OH and 5’-phosphate ends. Chemical reduction of the 5’-terminal oxidized abasic residues with NaBH₄ before incubation with Polβ prevented the excision (results not shown), consistent with their removal by β-elimination. As for Ape1 incision at C-4’-oxidized abasic sites, it is difficult to make an accurate quantitative comparison of Polβ excision activity on the hydrolytic versus oxidized abasic residues using the bleomycin-treated substrate.

A careful analysis of the Polβ excision reactions shows that the polymerase was more efficient in the presence of Ape1 protein than when Endo IV was present. Approximately 3-fold more Polβ was required for extensive removal of the abasic residue when only the bacterial enzyme was present than when the cognate enzyme Ape1 was available (Fig. 5, compare lanes 3–5 and 8–10). This activation by Ape1 protein is consistent with its stimulation of 5’-dRp excision by Polβ (22). A quantitative analysis showed that >95% of the 5’-terminal C-4’-oxidized abasic residues were removed by Polβ in a reaction containing Ape1 protein in 30 min at a DNA:Polβ molar ratio of 5:3:1 (data not shown).

The Fpg protein of E. coli can excise 5’-dRp by a β-elimination reaction analogous to that of Polβ (34). In a separate experiment with internally 32P-labeled D1 oligomer in a bleomycin-treated D1-D2 duplex, Fpg protein was tested for exci-
To analyze the products released by Polβ, the samples were electrophoresed under the same conditions but for a much shorter time (45 versus ~200 min) after loading the samples in the same order (Fig. 8B). Because the D1 oligomer was internally labeled with $^{32}$P in its center, only the released products and DNA fragments longer than a 17-mer were visualized on the gel under these conditions. Fig. 8B shows the results of combined Endo IV and Polβ treatment. There are three released products, which are denoted by PG, PO$_4$, and an asterisk, respectively (lanes 2–5). The same products were released by a combined incubation with Ape1 and Polβ (results not shown). To identify these enzymatically released products, the bands were excised, the materials inside the gel were eluted, and the amounts of $^3$H and $^{32}$P were determined by liquid scintillation counting. The band denoted by PG in Fig. 8B was found to contain $^3$H when C-5'-$^{3}$H-labeled D1 was used in the reactions. When C-1$^{3}$H- and C-2'-$^{3}$H-labeled oligomer was used, no $^3$H was found in the PG band (Table I). Since C-5'- but not C-1' and C-2' are retained in PG (Fig. 1), these results are consistent with the identification of this band as PG. Quantitation by phosphor imaging of the radioactivity in the PG bands in Fig. 8B showed an amount of $^{32}$P almost equal to that of the 3'-PG band in lane 1, Fig. 8A, consistent with the origin of the excised PG from the 3'-PG formed by bleomycin.

The band denoted in Fig. 8 by PO$_4$ contained no detectable $^3$H for either the C-5'-labeled or the C-1'- and C-2'-labeled substrate (Table I), consistent with its identification as free HPO$_4^{2-}$, which may arise as a decomposition product of incised or released lesions. The intensities of the bands marked by an asterisk, which retained the C-5$^{3}$H label in the same ratio as PG (Table I), were much less than that of band a in Fig. 8A. However, the combined total $^{32}$P radioactivity (determined by phosphor imaging) of this band and that of the PO$_4$ band was almost the same as that of band a, which suggests that the released C-4'-oxidized abasic residue was unstable and decomposed to HPO$_4^{2-}$ and an unknown product during the work-up or gel analysis. The PO$_4$ band and a small amount of the released abasic residue in lane 2 of Fig. 8B are probably decomposition products of the 5'-terminal C-4'-oxidized abasic residues formed by Endo IV incision. The excised products from uracil-DNA glycosylase-produced AP sites, which have a similar migration rate on the gel as that of the released C-4'-oxidized abasic residues, were found to be relatively stable, and no detectable HPO$_4^{2-}$ was found in these reactions under the same analysis conditions (data not shown).

**DISCUSSION**

In the present study, we have shown that Ape1 protein, the major human AP endonuclease, can recognize the C-4'-oxidized abasic site and efficiently incise on the 5' side to generate a nick bounded by a 3'-OH end and a 5'-terminal oxidized abasic residue. We confirmed that the bacterial enzyme endonuclease IV incises C-4'-oxidized abasic sites in the same way. The 5'-terminal abasic residue resulting from cleavage can then be excised by human Polβ or bacterial Fpg protein to leave a one-nucleotide gap with a 5'-phosphate and a 3'-OH end (Fig. 9). Further, Ape1 stimulates Polβ excision activity on the 5'-terminal oxidized abasic residue, consistent with the recent finding that Ape1 and Polβ act on an AP site in an orchestrated manner (22). Completion of the repair of these lesions in vivo probably involves repair synthesis by Polβ and ligation by DNA ligase I involved in the Xrc1 scaffold protein (36) or possibly by DNA ligase I in association with Polβ (37).

In addition to its incision activity on the C-4'-oxidized abasic sites, we found that Ape1 protein can also catalyze the hydrolysis of 3'-PG residues, consistent with other reports (18, 30, 32, 38). However, our work also demonstrates directly, by analysis...
of lesions at a single site, that Ape1’s incision activity for C-4’-oxidized abasic sites is much more efficient than its 3’-PG excision function (Fig. 6). This conclusion is consistent with the previous studies that used randomly generated 3’-PG (30, 38) or a structural analog of this lesion (18). In contrast, the Endo IV protein showed similar efficiencies in acting on the two lesions. The heterogeneous nature of the DNA substrate (major and minor damage sites, a mixture of strand breaks and abasic lesions) prevented assigning precise catalytic constants for the Ape1 acting on these oxidative lesions. However, our current and previous (18) results suggest that the release of 3’-damaged residues in human cells might involve other proteins, as noted by others (39). Indeed, earlier studies have shown that, in addition to Ape1, at least one (18) and possibly two (39) 3’-repair phosphodiesterase activities can be detected in mammalian cell extracts. The lability of these putative repair enzymes has hindered further characterization of their enzymatic and physical properties. However, our previous study does indicate that a second 3’-repair phosphodiesterase of HeLa cells has a higher ratio of 3’-phosphodiesterase to AP endonuclease activity than does Ape1 protein (18), consistent with a possible role in effecting excision of 3’-PG residues. Alternatively, other proteins could interact with Ape1 to stimulate some of its repair activities. The recent observation that Ape1 is inducible in response to bleomycin (40) underscores its likely importance in repair of oxidative DNA damage. The response to bleomycin may also involve novel inducible activities that participate in repair of OAS.

Recently, a second pathway for mammalian base excision DNA repair was found that yields repair patches of several nucleotides (23, 36). This second pathway requires proliferating cell nuclear antigen and FEN1 (FLAP endonuclease), which removes a single-stranded region displaced by repair synthesis. This pathway seems to be determined in large part by modifications that prevent the excision of the abasic residue by the β-elimination mechanism of Polβ. For example, chemical reduction of an AP site directs its repair into the longer patch pathway, and some OAS may also be processed in this way (23). Consistent with this report, we found that Polβ cannot excise a 5’-terminal C-4’-oxidized abasic residue following chemical reduction (data not shown). However, our demonstration that Polβ can quite efficiently excise the 5’-terminal C-4’-oxidized abasic residues indicates that Polβ could participate in repair oxidative damage. Oxidation at positions other than C-4’ (C-1’, C-2’, or C-5’) might generate OAS that are refractory to Polβ, although only C-2’ oxidation seems an obvious candidate. Whether Polβ can excise other 5’-terminal OAS certainly merits further study.

In summary, in order to study the mammalian repair mechanism of defined oxidative damage to the sugar moiety of the DNA backbone, we have constructed a 36-base pair DNA fragment containing only one predominant site for attack by bleomycin, which generates two types of C-4’-oxidized deoxyribose lesions, i.e., single-stranded breaks with 3’-PG ends, and C-4’-oxidized abasic sites. These lesions can be effectively excised by the major human endonuclease Ape1 in combination with Polβ to generate one-nucleotide gaps bracketed by 3’-OH and 5’-phosphate termini (Fig. 9). Repair of the gaps thus generated could be finished by repair synthesis of a one-nucleotide patch, followed by ligation to seal the remaining nick.

| Band                  | 3H | 3H/32P | 32P |
|-----------------------|----|--------|-----|
| Asterisk              | 74 | 498    | ND  |
| PO₄                   | 0  | 827    | 2   |
| PG                    | 731| 3738   | 0   |
| Phosphomethylpyridazine | 637| 6224   | 412 |

*Corrected for spillover from 32P channel.

Asterisk, the band corresponding to the band marked by an asterisk in Fig. 8B.

ND, not determined.

3’-Pyridazine produced by hydrazine treatment (Fig. 1) can be excised by Ape1 or Endo IV, and the released product, which is not shown in Fig. 8B, moves between the PO₄ and the ATP bands.

**TABLE I**

**Analysis of excision products from double-labeled substrates**

The D1-D2 duplex, internally labeled with 32P and with 3H at the indicated positions on the deoxyribose of the target T nucleotide, was treated with bleomycin and incubated with Endo IV and Polβ as described under “Experimental Procedures.” The enzymatically released products were separated by gel electrophoresis and analyzed by phosphor imaging (Fig. 8B). The product bands were excised, eluted, and quantitated by liquid scintillation. The results are expressed in cpm.

**Fig. 9. Repair of OAS: proposed mechanism for enzymatic removal of C-4’-oxidized deoxyribose lesions.** In step one, 3’-PG residues and C-4’-oxidized abasic sites are removed or incised, respectively, by the 3’-diesterase or 5’-incision activities of Ape1 or Endo IV. These reactions generate 3’-OH ends and 5’-terminal C-4’-oxidized abasic residues. The 5’-terminal abasic residues are then excised in step two by Polβ or Fpg proteins by β-elimination or possibly by hydrolysis with other unknown proteins.

The heterogeneous nature of the DNA substrate (major and minor damage sites, a mixture of strand breaks and abasic lesions) prevented assigning precise catalytic constants for the Ape1 acting on these oxidative lesions. However, our current and previous (18) results suggest that the release of 3’-damaged residues in human cells might involve other proteins, as noted by others (39). Indeed, earlier studies have shown that, in addition to Ape1, at least one (18) and possibly two (39) 3’-repair phosphodiesterase activities can be detected in mammalian cell extracts. The lability of these putative repair enzymes has hindered further characterization of their enzymatic and physical properties. However, our previous study does indicate that a second 3’-repair diesterase of HeLa cells has a higher ratio of 3’-phosphodiesterase to AP endonuclease activity than does Ape1 protein (18), consistent with a possible role in effecting excision of 3’-PG residues. Alternatively, other proteins could interact with Ape1 to stimulate some of its repair activities. The recent observation that Ape1 is inducible in response to bleomycin (40) underscores its likely importance in repair of oxidative DNA damage. The response to bleomycin may also involve novel inducible activities that participate in repair of OAS.

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