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In vitro detection of endonuclease IV-specific DNA damage formed by bleomycin *in vivo*

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ABSTRACT

Endonuclease IV of *Escherichia coli* has been implicated by genetic studies in the repair of DNA damage caused by the antitumor drug bleomycin, but the lesion(s) recognized by this enzyme *in vivo* have not been identified. We used the sensitive primer activation assay, which monitors the formation of 3′-OH groups that support *in vitro* synthesis by *E.coli* DNA polymerase I, to determine whether endonuclease IV-specific damage could be detected in the chromosomal DNA of cells lacking the enzyme after *in vivo* treatment with bleomycin. Chromosomal DNA isolated after a 1 h bleomycin treatment from wild-type, endonuclease IV-deficient (*nfo*⁻) and endonuclease IV-overproducing (p-nfo; ~10-fold) strains all supported modest polymerase activity. However, *in vitro* treatment with purified endonuclease IV activated subsequent DNA synthesis with samples from the *nfo*⁻ strain (an average of 2.6-fold), to a lesser extent for samples from wild-type cells (2.1-fold), and still less for the p-nfo samples (1.5-fold). This pattern is consistent with the presence of unrepaired damage that correlates inversely with the *in vivo* activity of endonuclease IV. Incubation of the DNA from bleomycin-treated *nfo*⁻ cells with polymerase and dideoxynucleoside triphosphates lowered the endonuclease IV-independent priming activity, but did not affect the amount of activation seen after endonuclease IV treatment. Primer activation with DNA from the *nfo*⁻ strain could also be obtained with purified *E.coli* exonuclease III *in vitro*, but a quantitative comparison demonstrated that endonuclease IV was ≥5-fold more active in this assay. Thus, endonuclease IV-specific damage can be detected after *in vivo* exposure to bleomycin. These may be 2-deoxypentos-4-ulose residues, but other possibilities are discussed.

INTRODUCTION

Bleomycin (BLM) is a glycopeptide antibiotic that is a clinically important antitumor agent (1). The cytostasis and cytotoxicity caused by BLM is thought to be due to base elimination and DNA strand scission by BLM (2). In the presence of Fe(II), BLM initiates DNA degradation *in vitro* by abstracting a hydrogen atom from C-4′ of DNA deoxyribose (3), in a manner analogous to the action of hydroxyl radicals formed by ionizing radiation or chemical oxidation (4,5). The products of this reaction are base propenals, DNA-5′-phosphates, and DNA-3′-phosphoglycolate esters (3,6). BLM also forms alkali-labile sites in DNA that are reminiscent of conventional apurinic/apyrimidinic (AP) sites, but have the deoxyribose C-4 oxidized to form 2-deoxypentos-4-ulose (7,8). In addition, the degradation of DNA–RNA hybrids by BLM leads to alkali-labile sites that are oxidized at deoxyribose C-1 (9).

In crude extracts of *Escherichia coli*, exonuclease III accounts for most (85–90%) of the total DNA-3′-repair diesterase/AP endonuclease activity, and endonuclease IV accounts for about half of the remainder (10–12). Endonuclease IV activity is induced by O_2^- as part of the *soxRS* regulon for defense against oxygen radicals (13,14). Cells lacking endonuclease IV (*nfo* mutants) are clearly hypersensitive to BLM and *t*-butylhydroperoxide, modestly hypersensitive to methyl methane sulfonate and mitomycin C, but not to H_2O_2, UV light or γ-rays (15). The hypersensitivity of exonuclease III-deficient cells (*xth* mutants) to many of these agents is further exaggerated by the introduction of an *nfo* mutation (15), which suggests a ‘back-up’ function for endonuclease IV in the repair of DNA damage formed by H_2O_2, X-rays, methyl methane sulfonate or mitomycin C. In contrast, BLM and *t*-butylhydroperoxide evidently generate DNA damages that specifically require endonuclease IV, rather than exonuclease III, for their efficient repair (15). Endonuclease IV-specific damage is also formed by nitric oxide exposure of *E.coli*, as shown by the hypersensitivity of *nfo* single mutants to killing by macrophages that generate this free radical gas (16).

Alkali-labile sites induced in DNA by BLM *in vitro* are cleaved by endonuclease IV at least five times more efficiently than by exonuclease III (17–19). These sites are distinct from the C-1-oxidized residues formed in DNA by Cu(II)-phenanthroline (18). Indeed, *E.coli* endonuclease IV was independently identified in a search for nuclease activities specific for BLM-damaged DNA (19). Thus, both genetic and *in vitro* biochemical data suggest a key role for endonuclease IV in the repair of specific DNA damage not effectively handled by other enzymes. In the present work, we demonstrate directly that endonuclease IV is specifically required for the repair of certain types of BLM-induced DNA damage formed *in vivo*.

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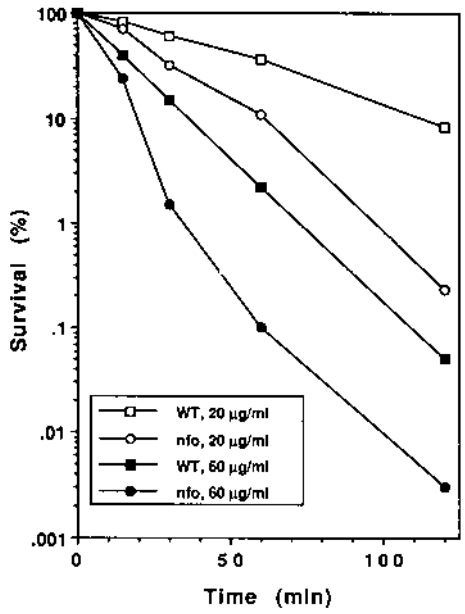

Figure 1. Cultures of *E.coli* strains AB1157 and BW527 were grown to exponential phase as described in the text and treated for 60 min with the indicated amount of BLM. For each point, an aliquot of cells was diluted into M9 salts (21) and plated to determine colony-forming units. Squares, wild-type strain; circles, *nfo*⁻ strain; open symbols, 20 µg/ml BLM; filled symbols, 60 µg/ml BLM.

MATERIALS AND METHODS

Strains

BW527 (*nfo::kan*; ref. 15) was kindly provided by Dr Bernard Weiss (University of Michigan). Strain AB1157 was a stock in our laboratory. Plasmid pKC7, containing the wild-type *nfo* gene expressed under its own promoter (20), was generously supplied by R. P. Cunningham (State University of New York, Albany).

Enzymes and chemicals

Endonuclease IV was purified as previously described (11) with appropriate modifications (12). Exonuclease III was purchased from New England BioLabs and DNA polymerase I was from Life Technologies Inc. (Gaithersburg, MD). Bleomycin sulfate (Blenoxane) was either purchased from Sigma or was a gift from the Bristol-Myers Co. BLM was dissolved in 10 mM HEPES–KOH, pH 7.6, at a concentration of 10 mg/ml, and the concentration of drug was checked using ε_{290} = 14 000 M^{-1} and a molecular weight for BLM of 1440 g/mol (2). Hydrogen peroxide was from Fluka, and paraquat, menadione, *t*-butylhydroperoxide and cumene hydroperoxide were from Sigma.

Growth of cells and drug treatment

Cells were grown in overnight cultures at 37°C in M9 medium (21) supplemented with 1% glucose, 1% casamino acids, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 µg/ml thiamine and 100 µg/ml of the appropriate antibiotic (streptomycin for AB1157, kanamycin for BW527, and ampicillin for AB1157 pKC7). Cells were diluted into fresh medium the next day, and grown to exponential phase (OD_{600} = 0.2–0.6), then treated with drug (typically 50 µg/µl BLM in 10 ml cultures for 1 h at 37°C). Survivals were obtained by diluting cells into M9 medium, followed by plating onto LB plates to determine colony-forming units (21,22). Cells were pelleted, washed with M9 salts, and frozen at –80°C.

DNA isolation

Cell pellets were thawed on ice, suspended in 1 ml HE (10 mM HEPES–KOH, pH 7.6, 1 mM EDTA), followed by the addition of 200 µg chick egg lysozyme and 0.34 µg phage P22 lysozyme (a gift of Dr L. Hardy, University of Massachusetts Medical Center), and continued incubation for 1 h on ice. Sodium dodecyl sulfate was then added to a final concentration (w/v) of 0.5% and incubation on ice was continued for another hour. The DNA was extracted with phenol, then dialyzed against 100–1000 volumes of HE containing 1 M NaCl, followed by HE containing 10 mM NaCl. The dialyzed DNA was treated with 2 µg RNase A (freshly boiled) and extracted first with phenol, and then with water-saturated ether. NaCl was added to a final concentration of 0.5 M, and the DNA was precipitated by the addition of 2 vol cold absolute ethanol. The DNA pellet after centrifugation was resuspended in a minimal volume (50 µl/10 ml culture) of HE. DNA concentrations were determined using ε_{260} = 7500 M^{-1}.

For treatment of DNA with dideoxynucleoside triphosphates (ddNTPs), DNA samples were split immediately after the second dialysis and before the RNase treatment. The DNA was incubated in 20 mM HEPES–KOH pH 7.6, 10 mM $MgCl_2$, 20 µM of each of the four ddNTPs and 1 U *E.coli* DNA polymerase I; polymerase was not added to control samples. The reactions were incubated for 18 h at 37°C and DNA isolation was completed as described above.

Treatment of DNA with repair enzymes

Reactions (25 µl) containing 50 mM HEPES–KOH, pH 7.6, 50 mM KCl, 50 µg/ml bovine serum albumin, 1.55 nmol DNA and either 1 mM EDTA (for endonuclease IV reactions) or 1 mM $MgCl_2$ (for reactions containing exonuclease III), and the indicated amounts of enzyme, were incubated at 37°C for 30 min. Endonuclease IV was inactivated by heating the reaction at 70°C for 3 min, then addition of $MgCl_2$ to 1 mM. Exonuclease III was inactivated by the addition of EDTA to 1 mM followed by incubation of the reaction at 70°C for 3 min. DNA synthesis reactions were performed essentially as described (10), except that thiols were omitted from the polymerase reaction. For each reaction, 1 U *E.coli* DNA polymerase I was used, and the specific radioactivity of [^{3}H]dTTP was typically ~1000 c.p.m./pmol in the polymerase reactions. Endonuclease IV and exonuclease III were assayed for DNA-3′-phosphoglycolaldehyde (PGA) diesterase activity as described (10,11), except that the buffers described above were used for enzyme incubations in order to allow a direct comparison between the two assays.

RESULTS

Cunningham *et al*. (15) demonstrated that a disruption of the *nfo* gene in *E.coli* confers hypersensitivity to BLM and to *t*-butyl hydroperoxide, under conditions of chronic exposure to the drugs. *E.coli nfo::kan* mutants also exhibited hypersensitivity to BLM during a transient liquid challenge (Fig. 1), although the effect (~2-fold greater killing rate in the *nfo*⁻ strain) was not as substantial as that observed during the chronic exposure assay (15). Using a transient challenge assay, an *nfo::kan* strain showed the same sensitivity as wild-type *E.coli* to *t*-butyl hydroperoxide, paraquat, menadione, H_2O_2 or cumene hydroperoxide (data not shown). Thus,

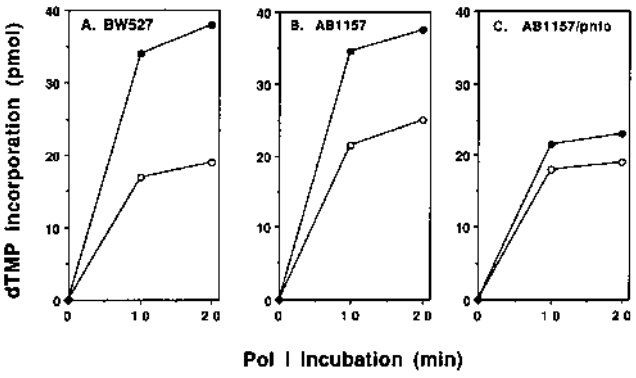

Figure 2. Chromosomal DNA was isolated from BLM-treated cells: (**A**) BW527 (*nfo*⁻); (**B**) AB1157 (*nfo*⁺); (**C**) AB1157/pKC7 (AB1157/p-nfo). After incubation with 0.5 U endonuclease IV for 30 min where indicated, the DNA was assayed for the ability to support DNA repair synthesis as described in Materials and Methods. (●), *in vitro* endonuclease IV treatment; (○), not treated with endonuclease IV *in vitro*.

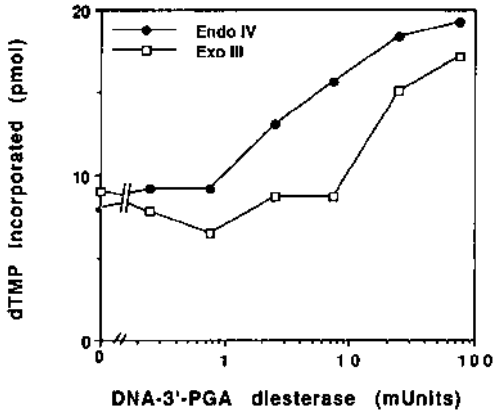

Figure 3. Chromosomal DNA was isolated from BLM-treated BW527, incubated with the indicated amounts of endonuclease IV or exonuclease III, and then assayed for the ability to support DNA repair synthesis. (●), endonuclease IV treatment (Endo IV); (□), exonuclease III treatment (Exo III).

the hypersensitivity of *nfo::kan* mutants to BLM and to *t*-butyl hydroperoxide is more apparent under chronic than under transient exposure.

Chromosomal DNA was isolated from wild-type and endonuclease IV-deficient cells that had been treated with 50 µg/ml BLM *in vivo*. The ability of purified endonuclease IV to activate primers for DNA repair synthesis (10) on this DNA was measured. Endonuclease IV treatment led to a 2- to 3-fold activation of priming ability for DNA isolated from both endonuclease IV-deficient cells (Fig. 2A) and wild-type *E.coli* (Fig. 2B). This result suggested that, under these conditions, endonuclease IV might be limiting *in vivo* even in the wild-type case. We therefore introduced a multicopy plasmid (pKC7) containing the *nfo* gene (20) into wild-type AB1157, generating an isogenic strain that overproduces endonuclease IV ~10-fold over wild-type (data not shown), and the chromosomal DNA was isolated from this strain following *in vivo* BLM treatment. Endonuclease IV treatment of this DNA (Fig. 2C) led to significantly lower primer activation for repair synthesis than for the DNA isolated from BLM-treated *nfo*⁻ cells (Fig. 2A). Little or no DNA synthesis was observed with DNA isolated from untreated cells, and purified endonuclease IV had little or no effect on this DNA (data not shown). The greater activating effect of endonuclease IV on the DNA from BLM-treated *nfo*⁻ cells compared to DNA from BLM-treated cells with multicopy *nfo*⁺ was observed consistently over many independent DNA preparations and assays (Table 1). Although the activation pattern was consistent (compare last two columns of Table 1), there was significant variation between sets of DNA preparations in both the level of DNA synthesis observed for DNA not treated with endonuclease IV, and in the absolute amount of activation produced by the enzyme treatment (Table 1). Such variation could result from differences in the amount of DNA damage caused by BLM or the amount of repair that occurred during the 1 h exposure.

The results shown in Figure 2 and Table 1 indicate that, under the conditions of our experiments, modest overproduction of endonuclease IV *in vivo* serves to eliminate BLM-generated DNA damage sites that are substrates for endonuclease IV in the *in vitro* primer activation assay. This suggests that endonuclease IV, rather than exo–nuclease III, is responsible for the repair of this damage *in vivo*, since both the *nfo::kan* strain and the endonuclease IV-overproducing strain presumably have similar levels of exonuclease III activity. It seems possible that the level of endonuclease IV in wild-type cells might also be sufficient to correct the BLM-generated damage detected in the primer activation assay, if enough time is allowed for repair. This possibility has not been explored.

The repair specificity of endonuclease IV *in vivo* should be reflected in the ability of the enzyme *in vitro* to activate primers for repair synthesis more efficiently than exonuclease III. Chromosomal DNA was isolated from endonuclease IV-deficient cells that had been treated with 50 µg/ml BLM *in vivo*, and this DNA was treated with a range of endonuclease IV and exonuclease III concentrations. The amounts of endonuclease IV and exonuclease III used in these experiments was quantitated by measuring DNA-3′-PGA diesterase activity (10,11). In a typical experiment, endonuclease IV was clearly more efficient in activating primers for repair synthesis of BLM-damaged DNA from the *nfo*⁻ strain than was exonuclease III (Fig. 3). As a quantitative measure of this activating ability, the concentration of enzyme that activated 50% of the new primers for repair synthesis in this assay was ~5-fold higher for exonuclease III than for endonuclease IV. However, this type of comparison may underestimate the difference, because 2.5 mU endonuclease IV (3′-PGA diesterase) gave ~50% of maximal activation, while 8 mU (3′-PGA diesterase) of exonuclease III was without apparent effect (Fig. 3).

There was a large apparent background of free 3′-OH termini (active primers) in the DNA isolated from BLM-treated cells for all three genotypes tested here. This was likely due to *in vivo* cleavage at some sites of damage by other enzymes prior to DNA isolation. The build-up of unblocked 3′-ends indicates that repair synthesis or ligation are limiting steps in DNA repair (10). We tested whether this high background of repair synthesis might be reduced or eliminated by incubation with polymerase and ddNTPs to block active primers prior to incubation with repair endonucleases. Such an experiment with the DNA from BLM-treated *nfo*⁻ cells demonstrated nearly complete blockage of background priming activity for DNA synthesis after incubation with polymerase and ddNTPs, but still allowed endonuclease IV-specific activation to be detected (Fig. 4). The amount of activation due to *in vitro* treatment with the repair enzyme was unchanged by the prior treatment with polymerase and dideoxynucleotides, but the fold activation by endonuclease IV was ~9-fold after 5 min of polymerase incubation.

Table 1. Primer activation by endonuclease IV on DNA isolated from BLM-treated *E.coli*

DNA preparation	No. of determinations	Control dTMP incorp. (pmol ± stand. dev.)	Mean primer activation ratio ± standard deviation AB1157/pnfo	BW527 (*nfo*⁻)
I	2	17.2 ± 0.9	1.1 ± 0.14	1.9 ± 0.14
II	5	4.2 ± 1.5	1.6 ± 0.17	2.3 ± 0.19
III	5	17.4 ± 6.9	1.2 ± 0.11	2.6 ± 0.34
IV	2	7.3 ± 4.6	2.3 ± 0.21	4.7 ± 0.21
V	2	12.9 ± 5.1	1.3 ± 0.29	2.2 ± 0.64

Primer activation assays were performed by treatment of chromosomal DNA isolated from BLM-treated cells as described in Materials and Methods. The activation values were determined after 10 min of incubation with 1 U DNA polymerase I per sample. 'Control dTMP incorp.' gives values for the amount of synthesis that occurred with DNA from BLM-treated AB1157/pnfo cells without *in vitro* endonuclease IV incubation, for the number of determinations shown; similar values were obtained for the DNA from BLM-treated *nfo*⁻ cells prepared in parallel. A more limited number of experiments with strain AB1157 not overexpressing endonuclease IV gave mean primer activation of 2.2-fold (± 0.72) over three determinations with two independent samples.

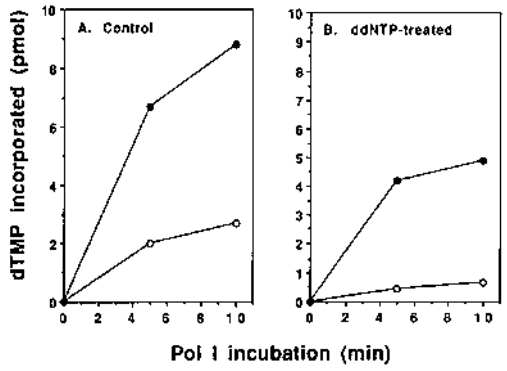

Figure 4. Blocking of 'background' primers in DNA with ddNTPs. Chromosomal DNA from BLM-treated BW527 was pre-incubated without (left panel) or with (right panel) DNA polymerase I in the presence of all four ddNTPs. After further purification (see Materials and Methods), the DNA samples were incubated where indicated with 0.5 U endonuclease IV for 30 min and assayed for the ability to support DNA repair synthesis. (●), treated with endonuclease IV; (○) , not treated with endonuclease IV.

DISCUSSION

Bacterial mutants deficient in endonuclease IV activity are hypersensitive to BLM under a variety of conditions (15,22; Fig. 1). Chromosomal DNA isolated from enzyme-deficient mutants treated with BLM contains sites that can be activated as primers for repair synthesis *in vitro* by treatment of the DNA with purified endonuclease IV, and the content of these activatable primer sites was substantially lower in a strain overproducing endonuclease IV. Moreover, endonuclease IV *in vitro* was ~5-fold more efficient in this primer activation than was exonuclease III. These results indicate that BLM generates *in vivo* DNA damage in *E.coli* that specifically requires endonuclease IV for its efficient repair and which can be recovered from the cells for analysis. These results and various published studies (15,22) do not exclude the possibility that exonuclease III might substitute inefficiently *in vivo* for the loss of endonuclease IV.

The observations presented here are consistent with previous *in vitro* experiments (17–19), although the preference for endonuclease IV over exonuclease III in the repair of BLM-induced DNA damage was less dramatic in the present studies. There are at least three factors in our *in vivo* experiments which could mask a stronger preference for endonuclease IV over other enzymes in cleaving at BLM-induced DNA damage sites. First, endonuclease IV, even when overproduced 10-fold, may not completely repair DNA damage *in vivo* during incubation with BLM, because some further activation of primers by endonuclease IV was observed (Fig. 2B). Secondly, BLM *in vivo* may form a different distribution or collection of DNA damages than *in vitro* treatment, and some of these may be substrates for exonuclease III *in vitro*. Thirdly, the DNA extraction procedures might have generated substrates for exonuclease III that were not present *in vivo*, a possibility that could be addressed by exploring different extraction procedures.

A limited number of structures have been identified as BLM-induced damages in DNA (Fig. 5), including DNA-3′-phosphoglycolates (3), 4′-oxidized AP sites (7,8), and 1′-oxidized AP sites which may form under specialized conditions (9). The primer-activation assay for endonuclease IV and exonuclease III does not distinguish between 3′-blocking groups and abasic sites in DNA. However, DNA-3′-phosphoglycolates are cleaved at the same rate as DNA-3′- PGA by both endonuclease IV and exonuclease III *in vitro* (J. Dorfman, J. Levin and B. Demple, unpublished results), and so are unlikely to be the endonuclease IV-specific DNA damage suggested by the experiments of Figure 3. We also attempted to address this point by estimating DNA strand breakage using gel electrophoresis as performed for H_2O_2-treated *xth* strains (10), but we did not observe a significant difference in the fragmentation of the chromosomal DNA between the multicopy *nfo*⁺ and the *nfo*⁻ strains after BLM treatment (data not shown). The smaller effect of *nfo* on BLM sensitivity (~2-fold) than of *xth* on H_2O_2 sensitivity (~20-fold) suggests that such a difference might be difficult to observe.

Häring *et al.* (18) observed that the 1′-oxidized AP sites produced by Cu(II)-phenanthroline are not preferentially cleaved *in vitro* by endonuclease IV compared to exonuclease III. Therefore, a good candidate for the preferential endonuclease IV substrate produced by BLM is the 4′-oxidized AP site, 2-deoxy-pentos-4-ulose (7,8). It is not yet clear from our results or those of others how much the relative efficiency of endonuclease IV and exonuclease III for the various BLM-induced damage sites is influenced by the presence of other damage sites nearby (23) or by the local DNA structure. This determination awaits the development of synthetic DNA substrates containing site-specific 4′-oxidized abasic lesions.

The biological consequences of 2-deoxypentos-4-ulose residues in DNA have been only partially explored. This lesion is formed by

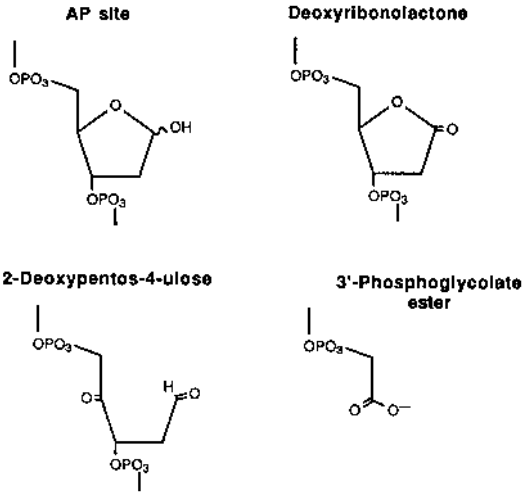

Figure 5. Structures of hydrolytic and oxidized abasic sites. See text and refs 5, 7–9 and 23 for discussion.

ionizing radiation (5), and mutants lacking both exonuclease III and endonuclease IV are hypersensitive to X-rays (15). The wild-type X-ray resistance displayed by *nfo*⁻ single mutants suggests that exonuclease III can replace endonuclease IV for repair of 2-deoxypentos-4-ulose in irradiated DNA, or that another radiation-induced lethal lesion predominates that is not differentially recognized by these enzymes. BLM treatment produces complex damage sites with 2-deoxypentos-4-ulose residues juxtaposed to oxidative strand breaks in the opposite strand (17,23), which might well have a differential effect on cleavage by endonuclease IV and exonuclease III. It is unknown whether the same structures are formed by ionizing radiation (5). Precise and sensitive methods to quantitate 2-deoxypentos-4-ulose and other oxidized abasic sites are needed to define the biological effects of the damage.

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