Recognition of oxidized abasic sites by repair endonucleases

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ABSTRACT

The recognition of 'regular' and 'oxidized' sites of base loss (AP sites) in DNA by various AP endonucleases was compared. Model substrates with regular AP sites (resulting from mere hydrolysis of the glycosylic bond) were produced by damaging bacteriophage PM2 DNA by exposure to low pH; those with AP sites oxidized at the C-4' and C-1'-position of the sugar moiety by exposure to Fe(III)-bleomycin in the presence of H₂O₂ and to Cu(II)-phenanthroline in the presence of H₂O₂ and ethanol, respectively. The results confirmed that AP sites—together with single-strand breaks—are indeed the predominant type of DNA modification in all three cases. For the recognition of 4'-oxidized AP sites, a 400-fold higher concentration of *Escherichia coli* exonuclease III and between 5-fold and 50-fold higher concentrations of bacteriophage T4 endonuclease V, *E. coli* endonuclease III and *E. coli* FPG protein were required than for the recognition of regular AP sites. In contrast, the recognition of 4'-oxidized AP sites by *E. coli* endonuclease IV was effected by 4-fold lower concentrations than needed for regular AP sites. 1'-oxidized AP sites (generated by activated Cu(II)-phenanthroline) were recognized by endonuclease IV and exonuclease III only slightly (3-fold and 13-fold, respectively) less efficiently than regular AP sites. In contrast, there was virtually no recognition of 1'-oxidized AP sites by the enzymes which cleave at the 3' side of AP sites (T4 endonuclease V, endonuclease III and FPG protein). The described differences were exploited for the analysis of the DNA damage induced by hydroxyl radicals, generated by ionizing radiation or Fe(III)-nitritotriacetate in the presence of H₂O₂. The results indicate that both regular and 1'-oxidized AP sites represent only minor fractions of the AP sites induced by hydroxyl radicals.

INTRODUCTION

Hydroxyl radicals and related radical species are known to induce a broad spectrum of DNA modifications including sites of base loss (AP sites) (1–4). Depending on the structure of the sugar moiety, 'regular' and 'oxidized' AP sites can be distinguished: a mere hydrolysis of the N-glycosylic bond, which frequently occurs as a result of certain base modifications, gives rise to regular AP sites, while radical attack at the positions 1', 2' or 4' of the sugar residue gives rise to oxidized AP sites, in which the desoxyribose moiety is modified (Fig. 1). (The term AP site is used here for all types of sites of base loss, not only regular sites.)

After exposure of DNA to ionizing radiation, AP sites oxidized at C-1', C-2' and C-4' are formed, in accordance with the suggestion that free hydroxyl radicals are able to abstract hydrogen atoms from all possible sugar positions (1, 2). A much higher selectivity is observed with certain complexes of transition metals which bind non-covalently to DNA. Thus, Fe(III)-bleomycin, after 'activation' by H₂O₂ or reducing agents, reacts with the 4' position very selectively, generating 4'-oxidized AP sites, in addition to strand breaks (5, 6). Cu(I)-phenanthroline in the presence of H₂O₂ gives rise to products consistent with a predominant attack at the C-1' hydrogen (7–9).

In all organisms, AP sites seem to be recognized by a set of specific endonucleases, which incise the DNA at the modification and thereby initiate the repair (10–14). Some of these repair endonucleases recognize certain base modifications as well, i.e., they are also damage-specific N-glycosylases involved in base excision repair, while others lack this type of activity (Table I). The majority of the simple AP endonucleases (in *Escherichia coli* exonuclease III and endonuclease IV) cleave the DNA at the 5' side of the AP site. In contrast, many of the N-glycosylases generate a strand break at the 3' side of the AP site by a β -elimination mechanism (11, 15).

While all repair endonucleases incise at regular AP sites, there is evidence that they may differ greatly in the recognition of

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Figure 1. Chemical structures of regular and oxidized sites of base loss (AP sites). In some cases, several isomeric acetal and hemiacetal forms are supposed to exist in equilibrium; they are not shown.

oxidized AP sites (16). However, detailed information about the AP substrate specificities of the various enzymes is still not available. Better knowledge of these specificities could not only help to elucidate the mutagenic potential of the various types of AP sites, but would also improve the use of repair endonucleases as probes to characterize oxidative DNA damage induced by various agents (17–20).

Here, we report on a comparison of the recognition potential of several AP endonucleases for regular AP sites and for oxidized AP sites induced by (i) Fe(III)-bleomycin in the presence of H_2O_2 and (ii) Cu(II)-phenanthroline in the presence of H_2O_2 and ethanol. The results indicate that 1'-oxidized and 4'-oxidized AP sites are each recognized by only some of the AP endonucleases. The enzymes therefore can be used to differentiate between the various types of AP site.

MATERIALS AND METHODS

Materials

DNA from bacteriophage PM2 (PM2 DNA) was prepared according to the method of Salditt *et al.* (21). *E. coli* formamidopyrimidine-DNA glycosylase (FPG protein) was prepared as reported earlier (22). Endonuclease III was purified from the overproducing *E. coli* strain BH410 (*fpg-1*) (23) harboring the pNTH10 plasmid using a procedure adapted from Asahara *et al.* (24). Plasmid pNTH10 contains the *nth* gene of *E. coli* placed under the control of the *lac*-promoter and derived from the pRPC53 plasmid (25). T4 endonuclease V was partially purified by the method described by Nakabeppu *et al.* (26) from the *E. coli* strain A 32480 (*uvrA*, *recA*, *F'* *lac* IQ1) carrying the plasmid *ptac*-denV (kindly provided by L. Mullenders, Leiden) after induction with isopropyl- β -D-thiogalactopyranoside. Endonuclease IV was prepared essentially as described earlier (27). Exonuclease III was purchased from Boehringer, Mannheim, (Germany). All repair endonucleases were tested for their incision at reference modifications (i.e. thymine glycols induced by OsO_4 , AP sites induced by low pH, pyrimidine dimers induced by UV²⁵⁴) under the applied assay conditions (see below) to ensure that the correct substrate modifications were fully recognized and no incision at non-substrate modifications took place (see Ref. 28).

DNA modification

The exposure of PM2 DNA (final concentration 15 μ g/ml unless otherwise stated) to all agents was carried out in phosphate buffer (5 mM KH_2PO_4 , 50 mM NaCl, pH 7.4). To generate regular

AP sites, PM2 DNA was incubated for 2 min (70°C) at pH 4.5 according to Lindahl and Nyberg (29). A Fe(III)-bleomycin stock solution was prepared by mixing a solution of $FeCl_3$ (50 μ M) in citric acid (100 μ M) with a three-fold excess of bleomycin in phosphate buffer. The reaction with DNA (at 90 nM bleomycin/30 nM Fe) was started by the addition of H_2O_2 (500 μ M) and allowed to proceed for 30 min at 25°C. A Cu(II)-phenanthroline stock solution was prepared by dissolving 200 μ mole $CuSO_4$ and 800 μ mole 1,10-phenanthroline in 10 ml H_2O . The reaction with DNA in the presence of 5% ethanol (final Cu(II) concentration 1 μ M) was started by the addition of H_2O_2 (500 μ M) and allowed to proceed for 30 min at 25°C. A stock solution of iron-nitritotriacetate (Fe(III)-NTA) contained 2.5 mM $FeCl_3$, 5 mM citric acid and 10 mM nitritotriacetate. The reaction with DNA and H_2O_2 (2 mM) was carried out at 30 μ M Fe(III) and allowed to proceed for 30 min at 20°C. γ -Irradiation (20 Gy) was carried out on ice with a Cobalt-60 unit at a DNA concentration of 50 μ g/ml and a dose rate of 2 Gy/min.

In all cases, the DNA was precipitated by ethanol/sodium acetate and redissolved in BE₁-buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA) for damage analysis by the relaxation assay (see below).

Quantification of DNA modifications

The assay employed makes use of the fact that supercoiled PM2 DNA is converted by either a single-strand break (SSB) or the incision of a repair endonuclease into a relaxed (nicked) form which migrates separately from the supercoiled form in agarose gel electrophoresis (see Ref. 17).

An aliquot of 0.3 μ g PM2 DNA in 20 μ l BE₁ buffer was incubated for 30 min at 37°C with 10 μ l of BE₁ buffer (for the determination of direct strand breaks) or of one of the following repair endonuclease preparations: (i) FPG protein (0–3 μ g/ml) in BE₁ buffer, (ii) endonuclease III (0–30 ng/ml) in BE₁ buffer and (iii) T4 endonuclease V (0–3 μ g/ml) in BE₁ buffer, (iv) endonuclease IV (0–4 μ g/ml) in BE₁ buffer or (v) exonuclease III (0–3 μ g/ml) in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 15 mM $CaCl_2$. The reactions were stopped by addition of 3 μ l 10% sodium dodecyl sulfate and the DNA applied to an agarose slab electrophoresis gel. After electrophoresis and staining with ethidium bromide, the relative amounts of the supercoiled and the relaxed (nicked) forms of the DNA were determined using a fluorescence scanner (FTR20, Sigma Instruments, Berlin). From these values, the average number of direct single-strand breaks (SSB) plus endonuclease-sensitive modifications (ESS) per PM2 DNA molecule was calculated according to Eq. 1, which corrects for the 1.4-fold higher fluorescence of the nicked forms than the supercoiled forms and for the fact that only the first strand break (or endonuclease incision) in each molecule induces a relaxation.

$$SSB + ESS = -\ln [1.4 * I / (1.4 * I + II)] \text{ (Eq. 1)}$$

where I: fluorescence of supercoiled form

II: fluorescence of relaxed form

Values obtained were corrected for the numbers of sites observed in unmodified PM2 DNA, which did not exceed 0.15 sites/10⁴ bp. To obtain the numbers of ESS, the numbers of SSB (observed without endonuclease) were subtracted.

The number of DNA double-strand breaks (DSB) was calculated from the relative amount of linear DNA (III), which migrates as a third band during gel electrophoresis (Eq. 2).

Table I. Repair endonucleases used in this study^a

Repair endonuclease	Gene locus	Base modifications recognized by intrinsic DNA glycosylase	Reference
FPG protein ^b	<i>fpg</i>	8-oxo-Gua ^c , Fapy ^d	22
Endonuclease III	<i>nth</i>	5,6-dihydropyrimidines	24
T4 endonuclease V	<i>denV</i>	Py < > Py ^e	26
Endonuclease IV	<i>nfo</i>	—	27
Exonuclease III	<i>xth</i>	—	36

^afor reviews, see Ref. 10–14.^bformamidopyrimidine-DNA glycosylase^c7,8-dihydro-8-oxoguanine (8-hydroxyguanine)^dformamidopyrimidines (imidazole ring-opened purines)^ecyclobutane pyrimidine photodimers**Table II.** Half-maximal recognition by various AP endonucleases of AP sites induced by various agents

Repair endonuclease	Protein concentration (ng/ml) required for the incision of 50% of the AP sites induced by ^a		
	H ⁺ -DNA	Bleo-DNA	CuPhe-DNA
FPG protein	2	10	>300 ^b
Endonuclease III	0.007	0.1	>10 ^b
T4 endonuclease V	2	100	>2000
Endonuclease IV	2	< 0.5	7
Exonuclease III	0.4	150	5

^aValues are taken from the saturation curves shown in Fig. 2.^bLower limit since endonuclease-sensitive base modifications may be present

$$\text{DSB} = \text{III} / (1.4 \cdot \text{I} + \text{II} + \text{III}) \quad (\text{Eq. 2})$$

In this case no correction for multiple breaks can be made; therefore, the number of double-strand breaks per DNA molecule must be kept low.

RESULTS

Recognition of AP sites induced by low pH

PM2 DNA depurinated by exposure to pH 4.5 (H⁺-DNA) served as a model substrate with regular AP sites. The incision at these AP sites by various AP endonucleases (listed in Table I) was quantified with the relaxation assay as described in Materials and Methods and the results are shown in Fig. 2 (upper panels). With increasing enzyme concentration, saturation curves were obtained in all cases. The endonuclease concentrations at which 50% of the AP sites are incised under the assay conditions were determined from these data and are listed in Table II.

Recognition of AP sites induced by activated bleomycin

PM2 DNA modified by Fe(III)-bleomycin in the presence of H₂O₂ (Bleo-DNA) served as a model substrate with AP sites oxidized at the C-4' position. When Bleo-DNA was reacted with various concentrations of the endonucleases, saturation curves were observed that were quite different from those obtained with H⁺-DNA (Fig. 2, central panels): while endonuclease IV recognized the bleomycin-induced AP sites even better than the regular AP sites, the capacity of FPG protein, endonuclease III, T4 endonuclease V and exonuclease III to recognize the bleomycin-induced AP sites was 5- to 400-fold less than their capacity to recognize the regular AP sites (Table II). The number of modifications recognized at endonuclease concentrations

sufficiently high to saturate the incision reaction was similar for all endonucleases tested. This observation is consistent with the assumption that the same AP sites in Bleo-DNA are recognized by all enzymes and that base modifications sensitive to endonuclease III, FPG protein or T4 endonuclease V (see Table I) are rare or absent in Bleo-DNA (30).

DNA double-strand breaks were found to constitute $12 \pm 2\%$ of the single-strand breaks in Bleo-DNA. The incision by the various repair endonucleases, however, increased the number of double-strand breaks by less than 30% (data not shown). Therefore, the AP sites incised are rarely closely opposed to direct single-strand breaks or to other AP sites.

Recognition of AP sites induced by activated Cu(II)-phenanthroline

PM2 DNA modified by Cu(II)-phenanthroline in the presence of H₂O₂ and ethanol (CuPhe-DNA) was used as a model substrate with 1'-oxidized AP sites. (The presence of ethanol was found to increase the extent of DNA damage several-fold. It probably serves as a mild reducing agent for Cu(II), like thiols which have been frequently added for that purpose.)

The recognition by the various AP endonucleases of DNA modifications in CuPhe-DNA (Fig. 2, lower panels) differs from both that observed with the regular AP sites (Fig. 2, upper panels) and that observed with AP sites generated by bleomycin (Fig. 2, central panels): endonuclease IV and exonuclease III, which cleave DNA at the 5' side of an AP site, recognize the AP sites induced by Cu(II)-phenanthroline reasonably well [only three to tenfold less efficiently than regular AP sites (Table II)]. In contrast, T4 endonuclease V, endonuclease III and FPG protein, which catalyze a β -elimination reaction at the 3' position of an AP site, recognize these modifications not at all or with a much reduced efficiency (Fig. 2, Table II).

Figure 2. Recognition by various concentrations of repair endonucleases of modifications induced in PM2 DNA by exposure to (i) low pH (H^+ -DNA), (ii) Fe(III)-bleomycin plus H_2O_2 (Bleo-DNA) and (iii) Cu(II)-phenanthroline (CuPhe-DNA). Data points give the sum of single-strand breaks (generated directly by the damaging agent) and DNA modifications incised by the endonucleases at various concentrations. The number of direct single-strand breaks is given by the values on the ordinate axis (at endonuclease concentration zero). The number of sites observed in unmodified PM2 DNA has been subtracted in all cases. For DNA damaging conditions, see Materials and Methods.

As in the case of Bleo-DNA, the number of base modifications sensitive to FPG protein and endonuclease III must be low. The exact number cannot be determined since no saturation of the incision reaction is observed with these enzymes (Fig. 2). The absence of modifications sensitive to even high concentrations of T4 endonuclease V (Fig. 2) indicates that both regular AP sites and AP sites of the type predominantly induced by bleomycin (4'-oxidized sites) are virtually absent in the CuPhe-DNA.

To exclude the possibility that traces of (unremoved) copper ions are inhibiting the repair endonucleases, the incision of regular AP sites (H^+ -DNA) by T4 endonuclease V in the presence of Cu(II)-phenanthroline was tested. The recognition of these sites was found not to be impaired (data not shown).

Comparison with the DNA damage induced by hydroxyl radicals

In Fig. 3, the results of the enzymatic damage analysis are shown in the form of normalized damage profiles. In these presentations,

Figure 3. Normalized DNA damage profiles: Columns indicate the relative number of various endonuclease-sensitive modifications (ESS) and 'direct' strand breaks (SSB) induced in PM2 DNA by (a) Fe(III)-bleomycin in the presence of H_2O_2 (b) Cu(II)-phenanthroline in the presence of H_2O_2 (c) ionizing radiation and (d) Fe(III)-NTA in the presence of H_2O_2 . In all cases, the number of single-strand breaks is normalized to 10 units. Values represent means of 3–10 independent experiments (including those shown in Fig. 2 and 4) $\pm$ S.D. For DNA damaging conditions, see Materials and Methods.

Figure 4. Recognition by various concentrations of repair endonucleases of modifications induced in PM2 DNA by exposure to ionizing radiation (20 Gy). For details, see legend to Fig. 2.

the relative numbers of DNA strand breaks (generated directly by the damaging agents) and of modifications incised by given (high) concentrations of the various endonucleases are indicated. Damage profiles induced by activated Fe(III)-bleomycin and Cu(II)-phenanthroline are compared with those induced by ionizing radiation and Fe(III)-nitrilotriacetate (Fe(III)-NTA) in the presence of H_2O_2 . The latter types of damage can probably be attributed to a reaction of DNA with free hydroxyl radicals (1, 31, 32).

The similarity of the damage profiles induced by ionizing radiation and by Fe(III)-nitrilotriacetate in the presence of H_2O_2 (Fig. 3) is in accordance with the suggestion that the damage is induced ultimately by the same agent, i.e., hydroxyl radicals. In both cases, the number of modifications sensitive to FPG protein exceeds that of AP sites (sensitive to endonuclease IV, exonuclease III and T4 endonuclease V) by a factor of 1.5–2.0,

Table III. Relative recognition of various AP sites by low concentrations of exonuclease III

Damaging agent	% Recognition ^a by exonuclease III at		type of AP site
	5 ng/ml	50 ng/ml	
pH 4.5 (70°C)	88 ± 7	95 ± 5	regular
Cu(II)-phen/H ₂ O ₂	54 ± 6	80 ± 3	1' -oxidized
Fe(III)-BLM/H ₂ O ₂	14 ± 10	39 ± 8	4' -oxidized
ionizing radiation	9 ± 9	49 ± 10	

^aThe number of sites incised at saturating concentrations (500–2000 ng/ml) of exonuclease III is assumed to represent 100% recognition. Values are means of 3–10 independent experiments (including those shown in Fig. 2 and 4) ± S.D.

in contrast to the situation observed with Bleo-DNA or CuPhe-DNA. This is best explained by the presence of FPG-sensitive base modifications, e.g. 7,8-dihydro-8-oxoguanine. In contrast, the smaller analogous excess in the case of endonuclease III indicates that base modifications sensitive to this enzyme (e.g. 5,6-dihydropyrimidines) are less frequent in DNA modified by hydroxyl radicals.

Information about the AP sites induced by ionizing radiation is obtained from experiments in which the incisions by the AP endonucleases at various enzyme concentrations were quantified. The results (Fig. 4 and Table III) indicate that the number of regular AP sites does not exceed ~10% of the total number of AP sites since low concentrations (5 ng/ml) of exonuclease III, which incise 90% of the regular AP sites, recognize only 10% of the AP sites detected by endonuclease IV (and by high concentrations of exonuclease III) (Table III). Only a minor fraction of the remaining AP sites is oxidized in the 1' position, as concluded from the relatively low number of modifications that are recognized by endonuclease IV, but not by T4 endonuclease V. This leaves the possibility that the majority of the AP sites generated by hydroxyl radicals are oxidized in the 4' position or—less likely—in the 2' position. The same conclusion may be drawn from the obvious similarities of the incision curves observed with DNA modified by ionizing radiation (Fig. 4) and activated bleomycin (Fig. 2, central panel).

Thermal lability of oxidized AP sites

DNA modifications induced by oxidizing agents may be chemically labile and readily suffer secondary modifications. To test the lability of the modifications described above, Bleo-DNA, CuPhe-DNA and DNA modified by ionizing radiation were incubated for 1 h at 60°C in Tris buffer and subsequently analysed again for strand breaks and endonuclease-sensitive modifications.

The results (Fig. 5) indicate that the number of direct strand breaks (observed without endonuclease treatment) is only slightly increased after incubation at 60°C in all cases. Therefore, hydrolysis of the sugar phosphate bonds is not facilitated at the majority of the AP sites and base modifications present.

The number of AP sites recognized by virtually all endonucleases in Bleo-DNA (and—less markedly—those in DNA modified by ionizing radiation) is strongly reduced after the incubation. This indicates a chemical modification of the 4'-oxidized AP sites that makes them less sensitive or insensitive to the endonucleases. In contrast, the recognition of oxidized AP sites in CuPhe-DNA (Fig. 5) and of regular AP sites in H⁺-DNA (data not shown) is not or only slightly changed after incubation at 60°C. The observed decrease of recognizable AP

Figure 5. DNA damage profiles after 60°C incubation: Quantification of strand breaks and endonuclease-sensitive sites was carried out as for Fig. 3, but after incubation of the modified DNA for 1 h at 60°C in BE₁ buffer. The number of single-strand breaks observed without 60°C incubation (see Fig. 3) is normalized to 10 units.

sites is less pronounced when Tris buffer is replaced by HEPES buffer during the 60°C incubation (not shown).

DISCUSSION

The results show that AP sites generated by Fe(III)-bleomycin in the presence of H₂O₂, by Cu(II)-phenanthroline in the presence H₂O₂ and by low pH are of three different types that can be distinguished by repair endonucleases. Mechanistically, it is clear that depurination by low pH generates only regular AP sites. The mechanism of DNA damage by activated bleomycin is also well understood and there is little doubt that the AP sites recognized by repair endonucleases in Bleo-DNA are 4'-oxidized sites (Fig. 1). In the case of activated Cu(II)-phenanthroline, the knowledge of the spectrum of modifications is less complete, but there is good evidence for a preferential attack at the C-1' position, probably as a result of the noncovalent interaction of the complex with DNA (33). The results obtained in this study are in agreement with this proposal and also suggest that the majority of the AP sites generated by activated Cu(II)-phenanthroline are 1' -oxidized sites (Fig. 1). These AP sites are 1.5-fold more frequent than the direct single-strand breaks and at least 2.5-fold more frequent than oxidative base modifications such as 7,8-dihydro-8-oxoguanine, formamido-pyrimidines and 5,6-dihydropyrimidine derivatives, which are incised by FPG protein or endonuclease III at enzyme concentrations comparable with those required for the recognition of regular AP sites (Ref. 28 and unpublished results).

Provided that the structural assignments made above are correct, the data indicate that 1'-oxidized AP sites are recognized

by endonuclease IV and, less efficiently, by exonuclease III, but are poor substrates for those endonucleases that cleave at the 3' -site of an AP site by a β -elimination mechanism (T4 endonuclease V, endonuclease III, FPG protein). This is in accordance with earlier suggestions (16) and may be explained by the fact that the β -elimination involves the intermediate formation of a Schiff base at C-1', which is not possible after oxidation at C-1'. The 4'-oxidized AP sites are excellent substrates for endonuclease IV, but relatively poor substrates for exonuclease III, as has been described elsewhere (34). The recognition of such sites by the enzymes that cleave by a β -elimination mechanism (T4 endonuclease V, endonuclease III, FPG protein) is only moderately efficient.

The conclusions described above are based on the assumption that the sequence context of the AP sites has no major influence on their substrate properties, so that the sequence specificities of the damaging agents used to generate the model substrates are of minor importance. To our knowledge, there is no evidence for pronounced sequence specificities of the repair endonucleases employed here. In the case of FPG protein, the recognition of 7,8-dihydro-8-oxoguanine in various sequence contexts has been compared. No significant differences were observed (S.Boiteux, unpublished results).

It is interesting to note that the recognition of 4'-oxidized AP sites is impaired after incubation of the modified DNA at 60°C (Fig. 5). Possibly, autooxidation or Schiff base formation at the aldehyde function at C-1' is responsible. The reaction may be of biological relevance if it occurs *in vivo* as well.

Knowledge of the substrate specificities of the repair endonucleases allows us to use them as probes to analyse oxidative DNA damage induced by any agent, as exemplified above for the damage induced by ionizing radiation and by Fe(III)-nitritotriacetate. The results suggest that the majority of the AP sites induced by hydroxyl radicals are of the same type as those induced by activated bleomycin, i.e. oxidized at the 4' position. In contrast to other techniques (35), the endonuclease approach allows simultaneous quantification of base modifications, single-strand breaks and various types of AP sites. Major limitations arise from the fact that the substrate specificities of the enzymes are still not known in detail and may be relatively broad in some cases. In particular, the recognition by the enzymes used in this study of AP sites oxidized in the 2' position (Fig. 1) remains unknown.

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