Covalent Trapping of Human DNA Polymerase β by the Oxidative DNA Lesion 2-Deoxyribononolactone

Received for publication, October 5, 2001, and in revised form, January 7, 2002
Published, JBC Papers in Press, January 22, 2002,
DOI 10.1074/jbc.C100577200

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Oxidized abasic residues in DNA constitute a major class of radiation and oxidative damage. Free radical attack on the nucleotidyl C-1′ carbon yields 2-deoxyribononolactone (dL) as a significant lesion. Although dL residues are efficiently incised by the major human abasic endonuclease enzyme Ape1, we show here that subsequent excision by human DNA polymerase β is impaired at dL compared with unmodified abasic sites. This inhibition is accompanied by accumulation of a protein-DNA cross-link not observed in reactions of polymerase β with unmodified abasic sites, although a similar form can be trapped by reduction with sodium borohydride. The formation of the stably cross-linked species with dL depends on the polymerase lysine 72 residue, which forms a Schiff base with the C-1 aldehyde during excision of an unmodified abasic site. In the case of a dL residue, attack on the lactone C-1 by lysine 72 proceeds more slowly and evidently produces an amide linkage, which resists further processing. Consequently dL residues may not be readily repaired by “short-patch” base excision repair but instead function as suicide substrates in the formation of protein-DNA cross-links that may require alternative modes of repair.

Mutagenesis and disruption of the cell cycle caused by DNA damage is counteracted by DNA repair systems. In the base excision repair pathway (1–3), DNA glycosylases eliminate damaged bases to generate abasic (AP) sites, which are also formed in large numbers by spontaneous depurination (2). In either case, AP sites are incised by an AP endonuclease to allow subsequent DNA repair synthesis and excision of the abasic residue. In mammalian cells, incision is carried out by the major AP endonuclease Ape1 protein (also called Apex, Hap1, or Ref1), while the excision step for regular abasic residues is thought to be mainly carried out by DNA polymerase β (Polβ) using a β-elimination mechanism. A distinct branch of the base excision pathway involves strand displacement repair synthesis and excision of the displaced, damaged strand by the FEN1 nuclease (4–6). Still another variation is potentiated by the initial DNA glycosylase (7) because some of these enzymes carry out a second reaction to cleave at the abasic site by β-elimination (1, 3). The resulting 3′-blocked products must then be removed by an enzyme such as Ape1 before repair synthesis can proceed (1). Base excision repair acts on a wide variety of deaminated, alkylated, or oxidized bases (2, 3). However, oxidative damage to DNA also produces various modified abasic residues that may complicate the repair scenario (1). For example, free radical attack forms strand breaks with fragmentary or oxidized products of deoxyribose; when these are present at the 3′ terminus, removal by Ape1 may be the rate-limiting repair step (8, 9). Oxidized abasic residues without direct strand breakage (10) include 2-deoxyxypentos-4-ulosol residues (a major lesion produced by the antitumor drug bleomycin) and 2-deoxyribononolactone (dL) residues (formed by diverse oxidative agents). 2-Deoxyxypentos-4-ulosol residues are processed rather efficiently in vitro by the central base excision enzymes Ape1 and Polβ (11). However, the effectiveness of repair on dL was unknown, and the irreversible cross-linking of dL observed for Escherichia coli endonuclease III (12) suggested that Polβ might encounter the same fate. We have shown that Ape1 acts effectively on dL residues in DNA.2 We show here that excision of Ape1-incised dL residues by Polβ is hampered by the formation of a stable covalent cross-link between the Polβ and the dL site in the DNA.

EXPERIMENTAL PROCEDURES

Materials—Except as noted, reagents were from Sigma/Aldrich. Acrylamide/bisacrylamide (a 29:1 mixture) was purchased from Bio-Rad. Urea was from American Bioanalytical (Natick, MA). Radionuclides were from PerkinElmer Life Sciences. Microcentrifugal filters were from Millipore Corp. (Bedford, MA). Enzymes—Polydeoxynucleotide kinase, Klenow fragment DNA polymerase, and uracil-DNA glycosylase were from New England Biolabs, Inc. (Beverly, MA). DNase I was from Ambion, Inc. (Austin, TX). Proteinase K was from Sigma/Aldrich. Recombinant human Ape1 was purified as described previously (13). Hexahistidine-tagged human DNA Polβ was >95% pure as determined following SDS-PAGE and silver staining. For the experiment depicted in Fig. 4B, both wild-type and lysine 72 to alanine (K72A) mutant Polβ (14) were provided by Drs. R. Prasad and S. H. Wilson (NIEHS, National Institutes of Health, Research Triangle Park, NC).

Preparation of DNA Substrates—Oligonucleotides containing a 1′-butylcarbonyl-uridylicate residue (indicated by X in the 30-mer 5′-GTC-ACGTGTCGCAAXACGACGCTGAGCCT or the 17-mer 5′-XACGAC-GTCGCTGCC) were prepared as described previously (15). Other oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, CA). Using standard methods (16), the DNA substrates were

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labeled at the 5'-end and hybridized to a complementary strand or hybridized first and labeled at the 3'-end. To generate a site-specific dL lesion, 2–10 pmol of radiolabeled duplex DNA containing the modified uracil were diluted with water to a volume of 30 μl, transferred to a glass tube, and subjected to photolysis in a Photochemical Reactor (RPR-100, Rayonet Corp., Branford, CT) at 350 nm, 900 micro-watts/cm² for 150 min (15). This material was then used immediately in enzyme reactions. To generate unmodified abasic sites, radiolabeled duplex DNA containing a uracil residue was treated with 2–3 units of uracil-DNA glycosylase for 60 min and used at once.

**Assays—**Reactions (12.5 μl in 50 mM HEPES-KOH, pH 7.5, 5% glycerol, 8 mM MgCl₂, 0.5 mg/ml bovine serum albumin) contained 40–50 nm radiolabeled DNA substrate and the enzyme concentrations indicated in the figure legends. After incubation at 25 °C for the specified times, samples were either directly analyzed by electrophoresis (see below) or subjected to further chemical or enzymatic treatment. Subsequent incubations with DNase I or proteinase K were performed at 37 °C prior to SDS-PAGE. Treatment with 48–300 m U DNase I was carried out for >30 min at 25 °C followed by desalting where indicated using Microcon filters and analysis by SDS-PAGE.

Analysis—For analysis on 19% polyacrylamide, 8 μ urea gels (Fig. 1), samples were mixed 1:1 with a 2-fold concentrated formamide loading buffer (16). Samples for analysis by SDS-PAGE on 8% polyacrylamide gels (Figs. 2–4) were mixed 1:1 with 6-fold concentrated loading buffer containing SDS. Samples were heated for 4 min at 90 °C prior to electrophoresis. Following electrophoresis, SDS-PAGE gels were silver-stained and dried, while urea-containing gels were dried immediately. The dried gels were analyzed using a Molecular Imager System (model GS-525, Bio-Rad), and images were obtained by autoradiography.

**RESULTS**

The selective generation of dL via a photosensitive precursor enables analysis of the repair enzymology of this key oxidative lesion. Using this approach, a recent study showed that bacterial endonuclease III becomes cross-linked to dL during its β-elimination cleavage reaction (12). We have shown that the hydrolytic endonuclease Apel cleaves dL in DNA effectively. 2. Thus, although human cells contain enzymes similar to endonuclease III (2), the abundant Apel protein would likely convert much of dL in vivo to a cleaved form available to the downstream repair enzyme Polβ. Since excision of abasic sites by Polβ occurs through a β-elimination mechanism, the process might lead to cross-linking of the polymerase to dL during attempted repair.

Photoconversion of the precursor nucleotide to dL generated a site that was sensitive to Apel protein. Apel treatment yielded an oligonucleotide bearing a dL-5-phosphate residue at its 5’ terminus, which thus had slower mobility (Fig. 1, lane 2) than the 5’-phosphate product (Fig. 1, lane 1). The latter product accompanies the generation of 3’-phosphates by the same C-1’ radical that produces dL (15) and may also reflect instability of dL during electrophoresis (e.g. chemically induced β-elimination (15)). Treatment with piperidine to cleave at dL generated an adduct of slower mobility (Fig. 1, lane 3) due to addition of the amine to the dL aldehyde as previously noted (17). This procedure also demonstrated that the photoconversion to dL and 3’-phosphates was only partial (the top band in Fig. 1 represents noncleaved oligonucleotide).

A 25-min incubation of the Apel-cleaved dL product with increasing amounts of Polβ diminished the amount of the 5’-terminal dL substrate only partially (Fig. 1, lanes 4–6). In contrast, a 10-min incubation with Polβ sufficed to completely remove the unmodified 5’-terminal deoxyribose residues produced by Apel acting on a glycosylase-generated abasic site (Fig. 1, lanes 9 and 10). Thus, Polβ was clearly less effective against dL than at conventional abasic sites, although the nonenzymatic cleavage products noted above interfered with accurate quantitation using PAGE.

To test the possibility that the poor activity of Polβ in dL excision might be related to the cross-linking reaction proposed above, we turned to SDS-PAGE. This analysis revealed other products with Polβ that did not correspond to DNA trimmed of the dL residue. These experiments (Fig. 2) demonstrated the time-dependent formation of a major radiolabeled species with electrophoretic mobility (M_r 45,000) greatly shifted from the DNA substrate (bottom of the gel in the figure) and significantly slower than free Polβ (indicated just below the M_r 40,000 marker; silver-stained gel not shown). The formation of this new species depended on the prior reaction with Apel (Fig. 2). The amount of the M_r 45,000 product also increased as a function of the Polβ concentration (data not shown). These results are consistent with the formation of the hypothesized Polβ-dL cross-link.

Smaller amounts of a product of M_r >50,000 were sometimes observed (Fig. 2). Results to be detailed elsewhere3 indicate that this secondary species may represent the same product as the M_r 45,000 species but retaining the complementary DNA strand.

The nature of the hypothetical Polβ-dL cross-link was addressed by selective digestion of either the protein or the DNA component. A 32P-labeled 5’-terminal dL in a 17-mer oligonucleotide was annealed to the complementary and upstream strands, and reaction of this substrate with Polβ generated a substantial amount of the M_r 45,000 complex (Fig. 3). Treatment with DNase I did not eliminate the extra band but shifted its mobility by ~5 kDa, consistent with the removal of ~15 nucleotides. In the same sample, the residual unlabeled DNA (Fig. 3, bottom of gel) was completely destroyed. Treatment with proteinase K eliminated the M_r 45,000 band, (and the Polβ and bovine serum albumin bands observed on silver-stained gels) but did not remove the residual DNA. These features are consistent with the formation of a cross-link in which labeled dL was directly bonded to the Polβ protein and resistant to nuclease digestion.

Polβ excises unmodified, 5’-terminal abasic residues by the
Mechanism-based cross-linking of Polβ with dL. A, co-migration of Polβ-dL cross-link and reductively trapped intermediate at normal abasic sites. A duplex, 5'-end-labeled DNA substrate (50 nM) containing either dL (lane 1) or a regular AP site (lanes 2–4) was incubated with 5 nM Ape1 for 18 min. Polβ (300 nM) was then added with either fresh (fr) (lane 3), spent (sp) (lane 4), or no (lanes 1 and 2) 48 mM NaBH₄ in a 2.5-h incubation. All reactions were terminated by adding loading buffer. The samples were directly analyzed by SDS-PAGE and autoradiography. B, dependence of the cross-link with dL on lysine 72 of Polβ. Where indicated, the dL substrate was incubated with Ape1 as described for panel A. Wild-type (lanes 3 and 4) or K72A mutant Polβ (lanes 5 and 6) (600 nM) was then added, and the incubation continued for 30 min followed by SDS-PAGE and autoradiography. WT, wild type; ox., oxidized; Reg., regular.

FIG. 3. The cross-linked species contains both protein and DNA components. A duplex DNA substrate (50 nM) containing either a 5'-end-labeled precursor lesion (lanes 5 and 6) or a 5'-end-labeled, photochemically (UVA) generated dL site (lanes 1–4) was incubated without (lanes 1 and 5) or with 600 nM Polβ for 2 h (lanes 2–4 and 6). DNase I (0.15 units) (lane 3) or proteinase K (0.8 mg/μl) (lane 4) were then added, and the incubation continued for 45 min at 37 °C. Samples were analyzed by SDS-PAGE and autoradiography. ProK, proteinase K.

Discussion

The data presented here demonstrate a new complication upon processing an oxidative DNA lesion (12): formation of a covalent DNA-protein cross-link with Polβ. Consistent with this conclusion, phosphate label associated with the abasic dL residue itself became resistant to nuclease digestion upon reaction with Polβ. In its apparent irreversibility and dependence on the active-site nucleophile (lysine 72) of Polβ, this cross-linking corresponds to a mechanism-based suicide inhibition of the enzyme. Given the biological role of the abasic excision activity of Polβ (19), this inhibition may have important cellular consequences. The imino intermediate (Schiff base) formed by Polβ during excision of abasic residues (20, 21) is an Achilles heel in the repair of oxidative DNA damage since dL residues can covalently trap the enzyme. The nucleophilic attack by the Polβ lysine 72 on the lactone carbonyl of dL is typical of the reaction of alkylamines with esters and would be enhanced by the release of strain upon cleavage of the lactone. This trapping generates an amide linkage that resists further processing by Polβ (see Scheme 1 under “Results”).

The newly discovered DNA polymerases ɛ and θ also have intrinsic lyase activity for 5'-abasic residues (22, 23) and might be similarly trapped by dL. This lesion therefore constitutes a new covalent adduct that reverses the protein-DNA phosphodiester of aborted topoisomerase II complexes (24). Although the base excision repair-associated FEN1 endonuclease can excise displaced DNA “flaps” and generally enhances the repair of radiation-induced abasic sites in cell-free extracts (6), we have not detected cleavage of Polβ-dL cross-linked molecules even at high levels of FEN1 protein (data not shown). This observation is consistent with the inability of FEN1 to act on flaps containing a protein bound within the displaced flap (25, 26). Other enzymes must be tested for their ability to dispose of this unusual species.
Acknowledgements—We are grateful to Dr. Richard A. O. Bennett for numerous discussions and a critical reading of the manuscript. We thank Dr. Rajendra Prasad and Dr. Samuel H. Wilson for generously supplying samples of the K72A and corresponding wild-type forms of Polβ.

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Polymerase β Cross-linked at 2-Deoxyribonolactone in DNA