α-Anomeric Deoxynucleotides, Anoxic Products of Ionizing Radiation, Are Substrates for the Endonuclease IV-Type AP Endonucleases†

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ABSTRACT: α-Anomeric 2′-deoxynucleosides (αdN) are one of the products formed by ionizing radiation (IR) in DNA under anoxic conditions. α-2′-Deoxyadenosine (αdA) and α-thymidine (αT) are not recognized by DNA glycosylases, and are likely removed by the alternative nucleotide incision repair (NIR) pathway. Indeed, it has been shown that αdA is a substrate for the *Escherichia coli* Nfo and human Ape1 proteins. However, the repair pathway for removal of αdA and other αdN in yeast is unknown. Here we report that αdA when present in DNA is recognized by the *Saccharomyces cerevisiae* Apn1 protein, a homologue of Nfo. Furthermore, αT is a substrate for Nfo and Apn1. Kinetic constants indicate that αdA and αT are equally good substrates, as a tetrahydrofuranyl (THF) residue, for Nfo and Apn1. Using *E. coli* and *S. cerevisiae* cell-free extracts, we have further substantiated the role of the *nfo* and *apn1* gene products in the repair of αdN. Surprisingly, we found that bacteria and yeast NIR-deficient mutants are not sensitive to IR, suggesting that DNA strand breaks with terminal 3′-blocking groups rather than αdN might contribute to cell survival. We propose that the novel substrate specificities of Nfo and Apn1 play an important role in counteracting oxidative DNA base damage.

Ionizing radiations (IRs)[1] induce a broad spectrum of base and sugar modifications and strand breaks in DNA (*1*, *2*). Importantly, even low doses of IR are likely to produce clustered lesions and double-strand breaks that disturb the genome functions (*3*). The α-anomeric 2′-deoxynucleosides (αdN) such as αdA and αT (Figure 1) are produced by abstraction of the anomeric hydrogen atom at C1′ by hydroxyl radicals (*4*). The intermediate radicals lead to epimerization at the C1′ atom by reduction with other molecules present in the vicinity followed by protonation (*5*). αdA constitutes ∼1.3% of all adenosine residues in salmon testis DNA exposed to γ-irradiation with a dose of 500 Gy under anoxic conditions (*6*). At this dose, several other DNA modifications are also generated.

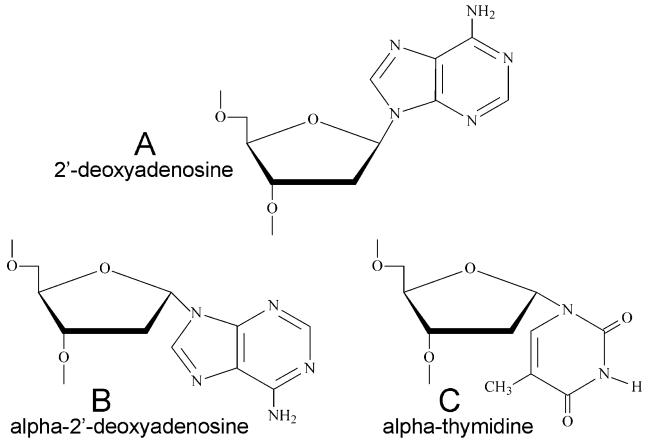

FIGURE 1: Natural and α-anomeric 2′-deoxynucleosides: dA (A), αdA (B), and αT (C).

Although the basal and induced levels of αdA and αT in cellular DNA are not known, several lines of evidence imply that these lesions, if left unrepaired, might have biological consequences. For example, it was shown *in vitro* that αdA constitutes a moderate replication block and directs the misincorporation of nucleotides, dCMP and dAMP, as well as the incorporation of the correct nucleotide, TMP, opposite the lesion in the template (*7*). Moreover, *in vivo* studies showed that a single-stranded M13 vector containing αdA at a defined position generates exclusively a single-nucleotide deletion (*8*), and the flanking sequence of αdA affects the deletion and bypass efficiencies. αdA is an unique lesion due to its strict requirement for anoxia during irradiation,

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[1] Abbreviations: IR, ionizing radiation; αdN, α-anomeric 2′-deoxynucleotides; Nfo, endonuclease IV; Apn1, yeast AP endonuclease 1; NIR, nucleotide incision repair; THF, tetrahydrofuranyl.

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similar to 8,5′-cyclopurine 2′-deoxynucleosides and 5,6-dihydropyrimidines (*6*, *9*, *10*). Thus, cell hypoxia could be an important factor in the DNA damage spectrum generated by IR. Interestingly, cancer cells have adapted to grow under hypoxic conditions, and tumor hypoxia is associated with poor prognosis and resistance to radiation therapy (*11*).

The αdA lesion is not repaired by DNA glycosylases/AP lyases, and instead, *Escherichia coli* endonuclease IV (Nfo) directly incises the phosphodiester bond 5′ to the lesion in DNA (*12*). Nfo is a monomer of 30 kDa displaying several activities: AP endonuclease, 3′-phosphatase, 3′ → 5′ exonuclease, and 3′-phosphoglycoaldehyde diesterase (*13*−*15*). The *nfo* gene is under the control of the soxRS system and can be induced by oxidative stress (*16*−*19*). Mutants devoid of Nfo are hypersensitive to oxidative agents such as bleomycin and *tert*-butyl hydroperoxide (*20*).

Homologues of *E. coli* Nfo have been identified in eukaryotes, such as *Saccharomyces cerevisiae* AP endonuclease 1 (Apn1), accounting for >90% of the total AP endonuclease activity in cells (*21*). Like Nfo, Apn1 is a metalloenzyme that has AP endonuclease, 3′-diesterase, 3′ → 5′ exonuclease, and 3′-phosphatase activities (*22*−*24*). Yeast mutants lacking Apn1 (*apn1*Δ) are hypersensitive to both oxidative (H_2O_2 and *tert*-butyl hydroperoxide) and alkylating (methyl- and ethylmethane sulfonate) agents, and have 6−12-fold higher rates of spontaneous mutation than the wild type (*25*). At present, it is not known whether Apn1 can repair αdA and αT.

Recently, we have shown that Nfo, Apn1, and the human major AP endonuclease 1 (Ape1) are involved in the alternative nucleotide incision repair (NIR) pathway and that Ape1 incises duplex DNA at the αdA and αT nucleosides (*26*, *27*). In the NIR pathway, a damage-specific endonuclease nicks oxidative DNA base damage in a DNA glycosylase-independent manner, providing the correct ends for DNA synthesis coupled to the repair of the remaining 5′-dangling nucleotide (*26*). Since the NIR pathway is evolutionarily conserved from bacteria to humans, it has been proposed that NIR may serve as a backup pathway for the DNA glycosylase-mediated BER pathway. In this study, we show that αdA and αT when present in DNA are recognized by Nfo and Apn1. Furthermore, the physiological relevance of αdN in the absence of the NIR pathway was investigated.

EXPERIMENTAL PROCEDURES

Oligonucleotides and Strains. Oligonucleotides (17-mers) containing single αdA and αT lesions were prepared as described previously (*12*, *28*). The oligonucleotide sequence was d(AGCATTCG**X**GACTGGGT), where X is αdA, αT, or tetrahydrofuranyl (THF). This sequence has been previously used to study the repair of αdA in *E. coli* (*12*). Complementary oligonucleotides, containing either dA, dG, dC, or T opposite αdN, were purchased from Eurogentec (Seraing, Belgium). Oligonucleotides were end-labeled and annealed as previously described (*29*). The resulting duplex oligonucleotides are termed X·C (G, A, or T), where X is THF, αdA, or αT.

E. coli Strains. AB1157 [*IeuB6 thr-1 Δ(gpt-proA2) hisG4 argE3 lacY1 galK2 ara-14 mtl-1 xyl-5 thi-1 tsx-33 rpsL31 supE44 rac*] (wild type) and its isogenic derivative BH130 (*nfo*::kan^R) were from the laboratory stock. *S. cerevisiae* strains FF18733 (*MAT*a *his*7−3 *leu*2−1,112 *lys*1−1 *trp*1−289 *ura*3−52) (wild type) and its isogenic derivative BG1 (*apn1*Δ::*HIS*3) were kindly provided by S. Boiteux (CEA, Fontenay aux Roses, France). YW465 (*MAT*α *ade2−0 his3−200 leu2−1 met15−0 trp1−63 ura3−0*) was kindly provided by T. Wilson (University of Michigan Medical School). YW465 isogenic derivatives YW605 (*apn1*Δ*::HIS3*), YW774 (*apn2*Δ*::kanMX4*), and YW781 (*apn1*Δ*::HIS3 apn2*Δ*::kanMX4*) were from the laboratory stock. Treatment with paraquat was performed as described previously (*17*). The whole cell-free extracts were prepared as described previously (*26*).

Enzymes. The Xth protein was purchased from Roche Diagnostics (Meylan Cedex, France). Purification of *E. coli* Ung, TagI, AlkA, Mug, Fpg, and Nth and human Ape1, ANPG70, hOGG1, and hTDG proteins was performed as described previously (*30*). Human NTH1 protein was generously provided by R. Roy (American Health Foundation, Valhalla, NY). Purification of Nfo was performed as described previously (*14*). Apn1-cDNA was cloned into plasmid pET11a (Novagen, VWR International S.A.S., Fontenay-sous-Bois, France), and the protein was overexpressed overnight at 30 °C in *E. coli* BL21(DE3) Origami cells (Novagen, VWR International S.A.S.) after addition of 0.2 mM isopropyl 1-β-D-thio-1-galactopyranoside and 30 μM $ZnCl_2$ to the growing culture in LB. Purification of Apn1 was achieved using four chromatographic steps as follows. Cells (4 g) were lysed in buffer A [20 mM Hepes-KOH (pH 7.6), 1 mM dithiothreitol, and 5% glycerol] contaning 500 mM KCl, 0.1 mM EDTA, and Complete protease inhibitor cocktail (Roche Diagnostics), using a French press device at 18 000 psi. The homogenate was centrifuged at 40000*g* for 20 min, and the supernatant was adjusted to 30 mM KCl in buffer A and passed through a column packed with 40 mL of QMA anion-exchange resins (Waters S.A.S., Saint-Quentin-en-Yvelines Cedex, France) pre-equilibrated in the same buffer. The flow-through fraction was applied to a 1 mL HiTrap-Heparin column (Amersham Biosciences, Orsay, France). Proteins bound to the column were eluted by a 30 to 800 mM NaCl gradient in buffer A. Apn1 was eluted at 400−500 mM NaCl. Fractions containing Apn1 were pooled and passed through a 1 mL HiTrap-Ni^{2+} ion chelating column (Amersham Biosciences). Finally, using an FPLC Superose 12 gel filtration column (Amersham Biosciences), a homogeneous preparation of Apn1 was obtained. The homogeneity of protein preparations was verified by SDS−PAGE. The specific activities of various DNA repair enzymes were tested on appropriate DNA substrates as previously described (*27*, *29*−*32*).

Incision Assays. The standard assay mixture for DNA damage-specific incision activity (final volume of 20 μL) contained 0.1 pmol of the 5′-[^{32}P]- or 3′-[^{32}P]dCMP-end-labeled 17-mer oligonucleotide duplex in 20 mM Hepes-KOH (pH 7.6), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 100 μg/mL bovine serum albumin, and limiting amounts of purified enzymes or 6 μg of cell-free extracts, unless otherwise stated. Either 5 mM $CaCl_2$ or $MgCl_2$ was included in the reaction mixtures when using the Xth or Apn1 and Ape1 proteins, respectively. The reaction buffer was supplemented with 1 or 0.1 mM EDTA when using bacteria or yeast cell-free extracts, respectively, unless otherwise stated. Note that in the cell-free extracts Apn1 has no absolute

requirement for divalent cations (*22*). Incubations were carried out at 37 °C for 10 min, or 30 min for cell-free extracts, unless otherwise stated. Reaction products were analyzed by electrophoresis in denaturing 20% (w/v) polyacrylamide gels (7 M urea and 0.5× TBE), visualized with a PhosphorImager Storm 840 instrument (Molecular Dynamics, Sunnyvale, CA), and quantified using ImageQuant software.

Survival Curves. Sensitivity to γ-rays was measured with cell suspensions of *E. coli* AB1157 (wild type) and BH130 (*nfo*::kan^R) under either anoxic (N_2) or aerobic conditions (air). Irradiation was performed as previously described (*20*, *33*). Briefly, for γ-irradiation, fresh cultures, grown to an OD_{600} of 0.6 at 37 °C with aeration, were kept in an ice bath during treatment. Prior to irradiation, the cultures were diluted 1:10000 in 50 mM potassium phosphate (pH 7.6) saturated with N_2 or air. For saturation with nitrogen, the buffer was degassed and then bubbled with N_2 for 1 h. The tubes were sealed under air or N_2, irradiated with ^{137}Cs γ-rays (1.7 Gy/min), and removed at 30 min intervals. Diluted samples were plated in duplicate on LB agar at 37 °C to determine survival.

For yeast cell irradiation, the following strains were used: YW465 (wild type), YW605 (*apn1*Δ*::HIS3*), YW774 (*apn2*Δ*::KanMX4*), and YW781 (*apn1*Δ*::HIS3 apn2*Δ*::KanMX4*). An exponentially growing cell culture with an OD_{600} of 1.0 was washed twice with 20 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer. Cells were diluted 1:50000 and irradiated with ^{60}Co γ-rays (2.9 Gy/min). Cells were diluted and plated onto solid YPD (yeast peptone dextrose), and colonies were scored after incubation at 30 °C for 2 days. The results for bacteria and yeast cultures were obtained from three independent experiments.

RESULTS

Activity of Various E. coli, Yeast, and Human DNA Repair Proteins on Oligodeoxynucleotides Containing α*dA and* α*T Lesions.* To study the repair of αT in DNA, we investigated whether this lesion was a substrate for previously characterized base excision repair (BER) enzymes. For this purpose, the 5′-^{32}P-labeled αT·A duplex oligonucleotide was incubated with a variety of purified BER enzymes. Since not all DNA glycosylases possess AP site-nicking activity, the samples were treated with piperidine after incubation with the enzymes. This step allows chemical cleavage of the DNA at the potential abasic site generated by base excision. When the various *E. coli*, *S. cerevisiae*, and human enzymes were tested on αT·A, only Nfo and Apn1 cleaved the labeled oligonucleotide at the 5′-position of the modified nucleoside (Figure 2A, lanes 9 and 11). Despite being used in molar excess (10:1 enzyme:substrate ratio), Ung, TagI, AlkA, Mug, Nth, Fpg, Xth, GST−hTDG, ANPG70, hNTH1, and hOGG1 proteins did not act on αT·A (Figure 2A, lanes 3−8, 10, and 13−16). Similar results were obtained with the αdA·T oligonucleotide (data not shown), indicating that Nfo and Apn1 are enzymes that process αdN DNA lesions in yeast.

Under the reaction conditions that are optimal for AP endonuclease activity and an excess amount of the enzyme, Ape1 completely degraded the αT-containing oligonucleotide possibly due to the 3′-exonuclease activity (Figure 2A, lane 12). Therefore, it was not possible to determine whether Ape1 is active toward αT and αdA. To characterize the incision efficiency of Nfo, Apn1, and Ape1 toward αdA·T and αT·A, we used low enzyme concentrations (0.2−1.0 nM). As a control substrate, we used THF·A and A·A duplex oligonucleotides containing a model abasic site and a mismatch, respectively. All three AP endonucleases cleaved THF·A with high efficiencies (Figure 2B, lanes 2−4, and Figure 2C, lanes 6−8). As previously shown, Ape1 was not able to incise αdA·T and αT·A in the presence of 5 mM $MgCl_2$ (Figure 2B, lanes 6 and 10), although it incised these lesions at low $MgCl_2$ concentrations and at pH 6.8 (*27*).

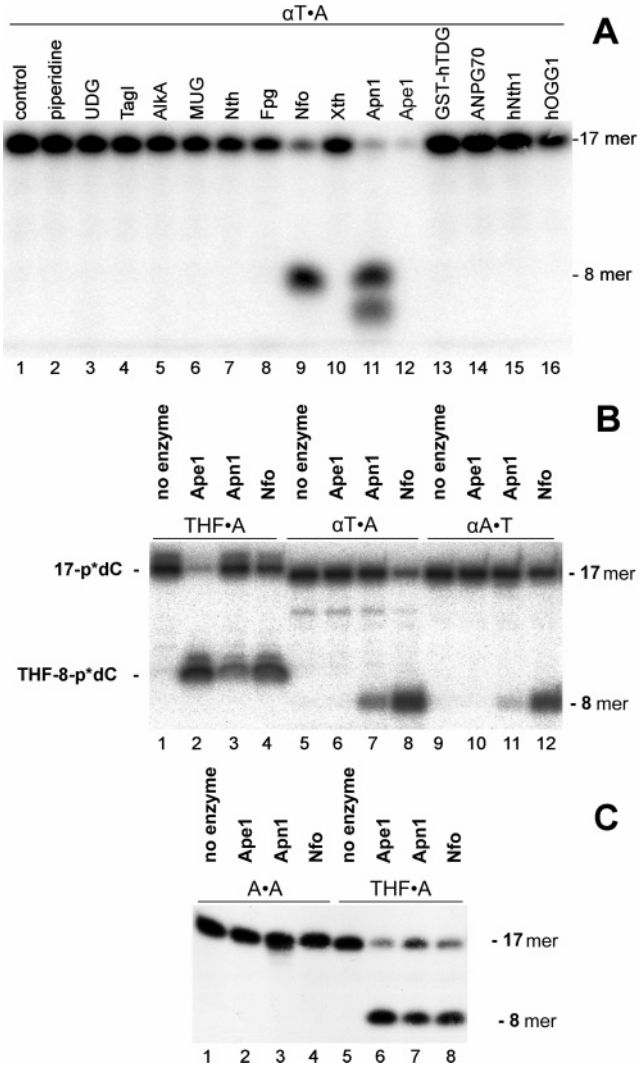

FIGURE 2: Activities of various *E. coli* and human DNA repair proteins on the duplex αT·A and αdA·T. (A) Activities of Ung, TagI, AlkA, Mug, Nth, Fpg, Xth, and Nfo proteins of *E. coli*, the Apn1 protein of *S. cerevisiae*, *and* human Ape1, GST−hTDG, ANPG70, hNTH1, and hOGG1 proteins toward αT·A. The 5′-^{32}P-labeled αT·A (10 nM) was incubated with an excess of a given repair protein (100 nM) at 30 °C for 30 min. To avoid degradation of the substrate by exonuclease activities of Xth and Ape1 proteins, 0.1 and 0.5 μM nonspecific G·C 17-mer duplexes were added, respectively. In addition, $MgCl_2$ was replaced with 0.1 mM EDTA in the reaction buffer for Apn1. (B and C) Comparison of the activities of Ape1, Apn1, and Nfo proteins on A·A, THF·A, αdA·T, and αT·A duplexes. five nanomolar 5′-[^{32}P]- (c) or 3′-[^{32}P]dCMP-labeled (B) THF·A duplex oligonucleotide was incubated with a given enzyme at 0.2 (B) or 1 nM (C) at 37 °C for 5 min. The reaction products were analyzed as described in Experimental Procedures.

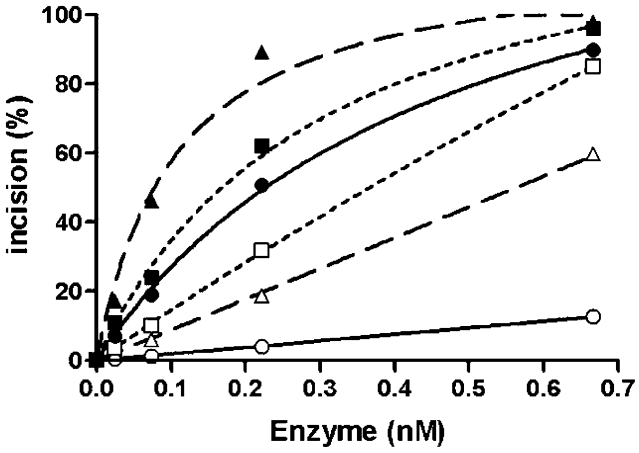

FIGURE 3: Activities of Nfo (filled symbols) and Apn1 (empty symbols) proteins toward oligonucleotides containing α-anomeric 2′-deoxynucleosides and tetrahydrofuranyl, as a function of enzyme concentration. A 5′-^{32}P-labeled (5 nM) αdA·T (circles), αT·A (triangles), or THF·G (squares) duplex oligonucleotides were incubated with the indicated amount of a given enzyme at 37 °C for 10 min. The products of the reaction were analyzed as described in Experimental Procedures.

Interestingly, at the low enzyme concentration, Apn1 was less efficient at cleaving αdA·T and αT·A than Nfo (Figure 2B, lanes 7, 8, 11, and 12). None of the enzymes showed any detectable cleavage of the oligonucleotide containing the A·A mismatch (Figure 2C, lanes 2−4). Taken together, the results demonstrate for the first time that (i) Nfo recognizes αT when present in DNA and (ii) yeast Apn1 can also incise αdA·T and αT·A.

Kinetic Parameters and Base Pair Specificity of Nfo and Apn1. For quantitative evaluation of the substrate specificity of Nfo and Apn1, the amount of incised oligonucleotides containing THF, αdA, and αT was measured. As shown in Figure 3, protein concentration-dependent product formation reveals the difference between Nfo and Apn1. The preferred substrate for Nfo was αT, while it was THF for Apn1. The relative order of substrate preference was as follows: αT·A > THF·A ≥ αdA·T for Nfo and THF·A > αT·A > αdA·T for Apn1. Nfo and Apn1 recognize THF, αdA, and αT lesions only when they are present in duplex DNA, and incision was not observed for single-stranded DNA (data not shown). Interestingly, Nfo was slightly more efficient than Apn1 on αdN substrates (Figure 3).

To further characterize the substrate specificities of Nfo and Apn1, we measured the K_M, k_{cat}, and k_{cat}/K_M values using duplex oligonucleotides containing one of the four possible bases opposite THF, αdA, and αT. As shown in Table 1, the apparent k_{cat}/K_M values measured for Apn1 indicate that the most preferred substrate for this enzyme was THF, whereas αT and αdA were repaired less efficiently. In contrast, the preferred substrate for Nfo was αT rather than THF and αdA. Interestingly, the apparent k_{cat}/K_M values for Apn1 (70 min^{-1} nM^{-1}) and Nfo (580 min^{-1} nM^{-1}) toward αdA·T were 4 and 40 times higher, respectively, than that of Ape1 (16 min^{-1} nM^{-1}) (*27*). Nfo and Apn1 cleaved THF, αdA, and αT when these lesions were placed opposite any of the four bases. However, the incision efficiency (k_{cat}/K_M) of Apn1 toward THF and αdA exhibited 4-fold (THF·C vs THF·G) and 11-fold (αdA·A vs αdA·T) variations, respectively, depending on the paired base (Table 1). For Apn1,

Table 1: Kinetic Constants for the Incision of Duplex Oligonucleotides Containing Various Lesions Paired with Different Bases by the *E. coli* Nfo *and S. cerevisiae* Apn1 Proteins[a]

	Apn1			Nfo		
substrate	K_M (nM)[b]	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} nM^{-1})	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} nM^{-1})
αdA·A	18 ± 3.0	14 ± 0.7	**0.76**	21 ± 2.0	32 ± 2.2	**1.5**
αdA·T	17 ± 2.1	1.2 ± 0.1	**0.07**	24 ± 2.1	14 ± 0.3	**0.58**
αdA·G	30 ± 4.2	11 ± 1.2	**0.37**	31 ± 5.2	24 ± 2.5	**0.79**
αdA·C	12 ± 1.5	5.4 ± 0.3	**0.46**	13 ± 3.2	16 ± 1.0	**1.3**
αT·A	6.5 ± 1.7	3.1 ± 0.2	**0.48**	7.5 ± 3.0	11.0 ± 0.2	**1.5**
αT·T	17 ± 1.5	7.7 ± 0.2	**0.45**	8.0 ± 1.9	14 ± 1.5	**1.8**
αT·G	29 ± 1.9	7.8 ± 0.2	**0.27**	8.8 ± 2.1	9.4 ± 0.6	**1.1**
αT·C	14 ± 2.0	3.5 ± 0.1	**0.25**	3.8 ± 1.1	7.7 ± 0.9	**2.1**
THF·A	5.8 ± 0.8	4.6 ± 0.3	**0.80**	3.8 ± 0.8	4.5 ± 0.2	**1.2**
THF·T	24 ± 4.1	25 ± 3.0	**1.0**	16.2 ± 2.0	9.3 ± 1.2	**0.58**
THF·G	24 ± 4.4	11 ± 1.0	**0.48**	10.0 ± 2.2	5.7 ± 0.5	**0.57**
THF·C	13 ± 1.2	26 ± 2.0	**2.0**	4.8 ± 1.0	4.5 ± 0.2	**0.9**

[a] A 17-mer duplex oligonucleotide substrate (1−200 nM) was incubated under standard reaction conditions (see Experimental Procedures). For K_M and k_{cat} determination, the linear velocity was measured and the constants were determined from Lineweaver−Burk plots. [b] Kinetic constants values are shown with the standard deviation.

the relative order for opposite base-dependent incision of αdA·dN oligonucleotide duplexes was as follows: dA > dC ≥ dG ≫ T; that of THF oligonucleotides was as follows: dC > T ≥ dA > dG (Table 1). However, the incision efficiency of Apn1 on αT·dN oligonucleotide duplexes varied only slightly depending on the paired base. In contrast to Apn1, the incision efficiency of Nfo did not depend significantly upon the base opposite THF, αdA, and αT (Table 1). The relative order of the opposite base preference for Nfo in THF·dN was as follows: dA > dC > T ≈ dG. In αdA·dN, it was as follows: dA ≥ dC > dG ≥ T. In αT·dN, it was as follows: dC ≥ T > dA > dG (Table 1).

Nucleotide Incision Activity in E. coli and S. cerevisiae Cell-Free Extracts. Data obtained with the purified proteins suggest that the incision of αdN-containing oligonucleotides depends on the *nfo* and *apn1* genes in *E. coli* and *S. cerevisiae*, respectively. Therefore, we assessed the incision activity for 5′-labeled αdA·T and αT·A in the cell-free extracts of various *E. coli* and *S. cerevisiae* strains. As shown in Figure 4, specific incision products of αdA·T and αT·A were detected with wild-type strains but not with the *nfo* and *apn1* mutants (lanes 3−6 and 10−13). In agreement with kinetic parameters, the incision efficiency of αdA·T in the extracts was lower than that of αT·A (Figure 4, lanes 3 and 5 vs lanes 10 and 12). As expected from the inducible nature of Nfo by redox cycling agents (*17*), extracts derived from growing cultures of the wild-type *E. coli* strain treated with paraquat had greatly enhanced the incision of both αdA·T and αT·A lesions (lanes 15 and 17 compared to lanes 3 and 10). These results show that Nfo and Apn1 are indeed the enzymes that incise αdN in bacteria and yeast.

Radiation Sensitivity of E. coli nfo and S. cerevisiae apn1 Mutants. To study the physiological relevance of the αdN-specific incision activity, we assessed the radiosensitivity of the mutant strains. Irradiation experiments were performed under N_2 so anoxic conditions could be achieved. The *E. coli nfo* mutant was not particularly sensitive to γ-rays and survived slightly better than the wild type under N_2 (see Figure 1A of the Supporting Information). In agreement with a previous observation, the *nfo* mutant was not sensitive to

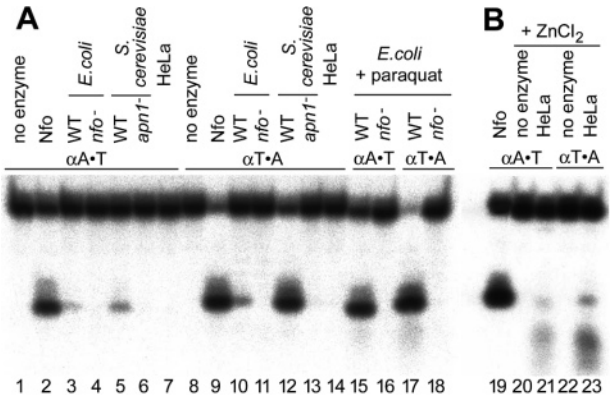

FIGURE 4: Cleavage of oligonucleotides containing α-anomeric deoxynucleotides by whole-cell extracts from *E. coli* and *S. cerevisiae* cells. Assays were carried out with the 5′-^{32}P-labeled duplex oligonucleotides containing αdA·T or αT·A. A duplex oligonucleotide (10 nM) was incubated with 10 nM Nfo protein or 6 μg of cell-free extracts from *E. coli* [wild-type (WT) and mutant strains without or with paraquat induction] and *S. cerevisiae* under standard nicking assay conditions. For details, see Experimental Procedures.

γ-rays under aerated conditions (*20*). The *S. cerevisiae apn1* single and *apn1 apn2* double mutants were also not sensitive to IR as compared to wild-type cells (see Figure 1B of the Supporting Information). *APN2* encodes a second AP endonuclease that is related to the human Ape1 and *E. coli* Xth proteins (*34*, *35*).

Nature of DNA Lesions Induced by IR under Anoxic Conditions. To generate a natural DNA substrate containing αdN, a purified supercoiled plasmid DNA was irradiated under anoxic conditions. As expected, IR produced single-stranded breaks in circular duplex DNA (see Figure 2 of the Supporting Information). The irradiated plasmid DNA was further nicked by the Nth, Fpg, Nfo, and Apn1 proteins, generating double-strand breaks. This result indicates that irradiation of DNA under anoxic conditions generates multiple types of lesions which are substrates for various DNA glycosylase/AP lyases and AP endonucleases (see Figure 2 of the Supporting Information). Since the DNA glycosylases are also active on DNA irradiated under N_2, it is difficult to conclude whether αdN, AP sites, or 5,6-dihydropyrimidines in plasmid DNA were incised by Nfo and Apn1.

DISCUSSION

The occurrence of αdA and αT nucleosides might have important biological consequences since the eukaryotic nucleus is a very poorly oxygenated cellular compartment due to the fact that the oxidative metabolism in eukaryotes has been confined to mitochondria (*36*, *37*). Previously, we have demonstrated that αdA is repaired by Nfo in *E. coli* and by Ape1 in human cells via the NIR pathway (*12*, *27*). However, the yeast enzyme which participates in the repair of αdN had not previously been identified. In this work, we investigate the repair of αdA and αT in *S. cerevisiae* and whether known DNA glycosylases are able to recognize αdN.

Our results show for the first time that Apn1 shares common substrate specificity with Nfo and Ape1 in processing αdA and αT lesions. In contrast, these lesions are not the substrates for any of the DNA glycosylases that have been tested. Characterization of the substrate specificity of Nfo and Apn1 showed that the relative order of the substrate preference was as follows: αT·A > THF·A > αdA·T for Nfo and THF·A > αT·A > αdA·T for Apn1, indicating that αdA·T is a less preferred substrate than αT and THF for both Nfo and Apn1. Interestingly, molecular modeling studies of a 9-mer duplex oligonucleotide containing a single αdA show that the αdA·T base pair is able to form two hydrogen bonds, one of which is not canonical (*38*). Recently, the NMR solution structure of the DNA decamer duplex showed that αdA is intrahelical and stacks in a reverse Watson−Crick fashion, which is consistent with a slight decrease in thermostability (*39*). This may suggest that recognition and catalysis of αdA by the repair protein could be less efficient than THF and αT. Indeed, we observed a significant decrease in the k_{cat}/K_M values for incision of the αdA·T duplex by Nfo and Apn1 relative to those for THF·A and αT·A.

The differential recognition of a modified residue is dependent upon the opposite base, and is an important characteristic of DNA repair enzymes. The incision efficiency of THF·N and αdA·N by Apn1 varied dramatically with the opposite base, whereas cleavage of these lesions by Nfo varied only slightly (∼2-fold) with all base pairs tested. The data obtained with Nfo acting upon THF and αdA were in good agreement with previous observations (*12*, *40*). The apparent preference of Apn1 and Nfo toward αdA·A compared to αdA·T may reflect the distinct structure and thermodynamic properties of the lesion-containing duplexes. Alternatively, we can speculate that the large decrease in the incision efficiency of αdA·T by Apn1 as compared to Nfo could be due to the damage recognition mechanism of Apn1 which may be somehow different from that of its bacterial homologue (*41*).

It has been shown that the nucleotide incision activity of Nfo and Apn1 requires the same catalytic active site as AP site cleavage (*41*, *42*). The Nfo protein detects AP sites by insertion of its side chains into the DNA minor groove (*43*). It then flips the target AP site and the opposite nucleotide out of the DNA base stack to produce a 90° bend in the duplex. Interestingly, normal β-nucleotides are sterically excluded from binding in the enzyme active site pocket. It seems that a nucleotide in the α-configuration, such as αdA, can be accommodated by placing it in the solvent-accessible pocket on the enzyme surface. However, the proposed model and other DNA glycosylase based-models are unable to explain the ability of Nfo and Apn1 to accommodate certain modified bases in the β-configuration such as 5,6-dihydropyrimidines (*26*) and 3,N^4-benzetheno-dC (*44*) in the same active site.

Using *E. coli* and *S. cerevisiae* cell-free extracts, we have shown that NIR is the major activity toward αdA and αT in bacteria and yeast. The observation that the NIR-deficient strains of *E. coli* and yeast were not sensitive to IR under both oxygen and anoxic conditions was unexpected since αdA is a moderately blocking lesion for a DNA polymerase, and one might expect some degree of sensitivity to αdA *in vivo* (*8*). However, IR generates a variety of types of DNA damage, including single-strand breaks with 3′-blocking moieties, double-strand breaks, and clustered lesions which are highly cytotoxic (*45*−*47*). Indeed, under anoxic conditions and at the relatively small doses of radiation corre-

sponding to 75% survival used in this study, 98% of the supercoiled DNA was converted to an open circular form, indicating efficient formation of single-strand breaks. The *E. coli xth nfo* double and *S. cerevisiae apn1 apn2 tpp1* triple mutants are extremely sensitive to IR, suggesting that DNA strand breaks with 3′-blocking moieties are likely the most lethal lesions generated by IR (*20*, *48*). Therefore, it is possible that 3′-blocking lesions might mask the cytotoxic effect of IR-induced αdA and αT in the NIR-deficient strains.

Since in *E. coli* and yeast there are no enzymes other than Nfo and Apn1 that recognize αdN, it is reasonable to speculate that the repair pathway for these lesions is conserved. It has been argued that bleomycin might also generate αdN in DNA (*49*). The *E. coli nfo* and *S. cerevisiae apn1* mutants are sensitive to oxidizing agents, such as bleomycin and *tert*-butyl hydroperoxide, indicating the biological relevance of αdN and possibly other oxidative DNA damage which are not substrates for DNA glycosylases. In summary, we have shown that Apn1 can incise DNA containing αdA and αT lesions in budding yeast. Kinetic parameters indicate that αdN and AP sites are incised by Nfo and Apn1 with similar efficiencies. The observation that enzymes are conserved from *E. coli* to yeast and humans to process αdA and αT lesions implies that base lesions with the α-configuration with respect to the *N*-glycosidic bond might be biologically significant.

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SUPPORTING INFORMATION AVAILABLE

Sensitivity of *E. coli* and *S. cerevisiae* mutants to γ-rays and incision of an irradiated plasmid DNA by various DNA repair proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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