The Ring Fragmentation Product of Thymidine C5-Hydrate When Present in DNA Is Repaired by the *Escherichia coli* Fpg and Nth Proteins†

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ABSTRACT: Various forms of oxidative stress, including γ-radiolysis and UV irradiation, result in the formation of damaged bases. (5*R*)-Thymidine C5-hydrate is one of several modified nucleosides produced from thymidine under these conditions. *N*-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-*N*-3-[(2*R*)-hydroxyisobutyric acid]urea or αRT is the respective fragmentation product formed from (5*R*)-thymidine C5-hydrate upon hydrolysis. This modified nucleoside has potential mutagenic or lethal properties. No enzymatic activity responsible for the removal of αRT has been identified. We report here that when present in DNA, αRT is a substrate for two purified enzymes from *Escherichia coli* involved in the repair of oxidized bases: the Nth and the Fpg proteins. The Fpg protein removes the αRT lesion more efficiently than the Nth protein. This is the first example of efficient excision of a ring-opened form of a pyrimidine by the Fpg protein. The high efficacy of the Fpg protein suggests that it is likely to be involved in vivo in the excision of αRT. The kinetics of the reaction of the Fpg protein with DNA containing αRT suggest substrate inhibition. Duplex oligodeoxynucleotides containing αRT positioned opposite T, dG, dC, and dA were cleaved efficiently by both enzymes, although the profiles of activity of the two enzymes were different. The Nth enzyme preferentially excises αRT when opposite a dG, followed by αRT·dA, αRT·T, and αRT·dC. For the Fpg protein, the order is αRT·dC ≥ αRT·dG ∼ αRT·T > αRT·dA. Moreover, we show that human cell extract exhibits an activity that excises αRT from an oligonucleotide, suggesting that human homologues of the Nth and/or Fpg proteins could be involved in repair of this lesion in human cells.

The base modifications induced in DNA by endogeneous or exogeneous factors lead to adverse effects in cells. These modified residues are subject to cellular repair processes in vivo and are removed from DNA by specific repair enzymes. In *Escherichia coli*, several proteins that are involved in the repair of oxidative base damages have been identified (*1*−*3*). These enzymes include endonucleases III (Nth protein[1]), IV (Nfo protein), and VIII, exonuclease III (Xth protein), formamidopyrimidine DNA glycosylase (Fpg protein), and 3-methyladenine DNA glycosylase II (AlkA protein).

The Nth protein has previously been identified as an enzyme involved in the repair of DNA damage induced by X-rays, UV irradiation, and free radicals (*1*). The spectrum of modified bases recognized and excised in vitro by the *E. coli* Nth protein includes thymine glycol, 5,6-dihydrothymine, the fragmentation product of 5,6-dihydrothymine (β-ureidoisobutyric acid), urea, 5-hydroxy-5,6-dihydrothymine, 5,6-dihydrouracil, and 5-hydroxy-2′-deoxyuridine. This enzyme releases the damaged bases by *N*-glycosylase activity, followed by incision of the 3′-phosphodiester bond of the abasic site by syn β elimination reactions (*4*, *5*). The *E.coli nth* mutant deficient in Nth protein exhibits no apparent phenotype. They are not sensitive to X-rays, H_2O_2, or other agents which produce ring saturation and fragmentation products in DNA (*6*).

The DNA *N*-glycosylase activity associated with the Fpg protein also exhibits a broad substrate specificity. It excises damaged purine nucleosides, including 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) (*7*, *8*), which is highly mutagenic in vitro and in vivo (*9*), as well as 8-oxo-2′-deoxyadenosine, and the imidazole ring-opened purines that are commonly referred to as formamidopyrimidines (Fapy-G and Fapy-A) (*10*−*12*). The role of the Fpg protein in protecting *E. coli* from oxidative stress is shown by the observation that the *fpg*$^-$ mutant (*13*), which is the same as the *mutM* mutant, displays a G·C to T·A spontaneous mutator phenotype (*9*). Initially, it was believed that the Fpg protein

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[1] Abbreviations: αRT, *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-*N*-3-[(2*R*)-hydroxyisobutyric acid]urea; ANPG40 protein, human alkylpurine DNA glycosylase-truncated form; dsUDG protein, *E. coli* double-stranded uracil-DNA glycosylase; Fpg protein, *E. coli* formamidopyrimidine DNA glycosylase; hTDG protein, human thymine DNA glycosylase; Nfo protein, *E. coli* endonuclease IV; Nth protein, *E. coli* endonuclease III; TagI protein, *E. coli* 3-methyladenine DNA glycosylase I; UNG protein, *E. coli* uracil-DNA glycosylase; Xth protein, *E. coli* exonuclease III.

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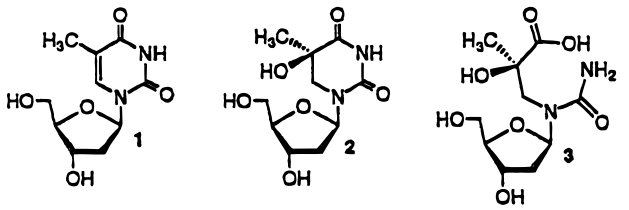

FIGURE 1: Chemical structures of thymine ring saturation and fragmentation products: thymidine (**1**), (5*R*)-5,6-dihydro-5-hydroxythymidine (**2**), and *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-*N*-3-[(2*R*)-hydroxyisobutyric acid]urea or αRT (**3**).

excises only damaged forms of purines. However, it was shown that 5-hydroxy-2′-deoxycytidine (5-OHdC), 5-hydroxy-2′-deoxyuridine (5-OHdU), and 5,6-dihydrothymidine are also substrates (*14*).

In addition to possessing DNA glycosylase activity, the Fpg protein which is a zinc finger protein has two other enzymatic activities. (i) It cleaves DNA at both phosphodiester bonds on the 3′- and 5′-side of the abasic site by efficient $\beta-\delta$ elimination reactions (*15*). (ii) It removes deoxyribose 5′-terminal deoxyribose phosphates from DNA which are formed following hydrolytic cleavage at abasic sites (*16*).

Aside from the limited examples mentioned above, the activity of the Fpg protein on modified pyrimidines has not been probed. This is surprising considering that the fragmentation and other modifications of thymine induced by the action of ionizing radiation have been extensively studied (*17*−*19*). Thymidine glycol and the fragmentation products of dihydrothymidine such as *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-*N*-3-(isobutyric acid)urea have been shown to be the cause of lethal lesions in vivo (*20*−*22*). *N*-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-*N*-3-[(2*R*)-hydroxyisobutyric acid]-urea or αRT (**3**) is the respective fragmentation product formed from (5*R*)-thymidine C5-hydrate (**2**) upon hydrolysis (Figure 1). When present in DNA, αRT inhibits snake venom phosphodiesterase, λ exonuclease, and the Klenow (exo$^-$) fragment. Kinetic measurements for insertion of nucleotides opposite αRT by the Klenow (exo$^-$) fragment suggest that this lesion base pairs with native nucleobases (*23*). Such properties imply that the αRT lesion cannot be tolerated by cells, and investigating its repair was of interest. We report data showing the removal in vitro of αRT by two *E. coli* base excision repair enzymes involved in repair of oxidative damage of DNA: the Nth and Fpg proteins. The efficient kinetic constants for the excision of αRT suggest that repair of this lesion by the Fpg protein could be of biological importance.

MATERIALS AND METHODS

Oligonucleotides. The 30mer oligonucleotides containing αRT at position 11 were synthesized as described (*23*, *24*). The sequence used in this work was 5′-d(TGA CTG CAT AαRTG CAT GTA GAC GAT GTG CAT). Four complementary oligonucleotides, containing dA, dG, dC, or T at the position opposite αRT, were synthesized using standard methods.

^{32}P Labeling of Oligonucleotides. The 30mer containing αRT at position 11 (10 pmol) was 5′-end labeled by T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-^{32}P]ATP (4000 Ci/mmol, ICN Pharmaceuticals, Inc.) or 3′-end labeled with [α-^{32}P]dATP in the presence of terminal transferase (Boehringer Mannhein) following the instructions of the supplier. Unincorporated radioactivity was removed using Sephadex G-50 spin column chromatography (*25*).

Duplex oligonucleotides were obtained by annealing to the respective oligonucleotide by heating to 60 °C for 3 min and cooling to room temperature in 0.5× SSC buffer [75 mM NaCl and 7.5 mM sodium citrate (pH 7.0)]. The ^{32}P-labeled oligonucleotide containing the αRT residue was hybridized with the respective oligonucleotide containing one of the four naturally occurring deoxynucleotides opposite αRT at a 1:2 molar ratio. Unlabeled duplexes were prepared using a ratio of 1:1. Nondenaturing 15% PAGE of the products of the annealing reaction indicated that more than 95% of the labeled strand was duplexed (*26*).

Enzymes. *E. coli* TagI and Fpg proteins were purified as previously described (*27*; B. Tudek and J. Laval unpublished results). The uracil-DNA glycosylase and Nth proteins of *E. coli* were purified from overproducing strains as previously described (*16*). The ANPG40 protein was purified to apparent homogeneity from the extract of *E. coli* strain BH290 (*tag*, *alkA*) harboring the plasmid containing the ANPG40 gene (*28*). The Nfo protein of *E. coli* was prepared from an overproducing strain as previously described (*29*). Human thymine DNA glycosylase (hTDG) (*30*) was purified from extracts of *E. coli* strain BL21 (DE3) harboring plasmid DNA pT7-hTDG (this plasmid was kindly provided by J. Jiricny, Institute for Medical Radiobiology, Zùrich, Switzerland). Double-stranded uracil-DNA glycosylase (dsUDG) (*31*) was purified to apparent homogeneity from extracts of *E. coli* strain RZ1032 (*ung*, *dut*) (M. Saparbaev and J. Laval, unpublished results). Xth protein, BSA (Molecular Biology Grade), and molecular biology products were purchased from Boehringer Mannheim.

Assays for Enzymatic Activity. The release of αRT was measured by the cleavage of the oligonucleotide containing a single lesion at a defined position. The standard assay for Fpg protein activity (50 μL final volume) contained the 5′-[^{32}P]αRT·dA duplex (0.5 pmol) in 70 mM Hepes-KOH (pH 7.6), 100 mM KCl, 1 mM EDTA, 5 mM β-mercaptoethanol, 5% glycerol, and 100 μg/mL BSA. The standard assay for UNG, Nth, TagI, and ANPG40 proteins (50 μL final volume) contained the 5′-[^{32}P]αRT·dA duplex (0.5 pmol) in 70 mM Hepes-KOH (pH 7.8), 100 mM KCl, 1 mM EDTA, 5 mM β-mercaptoethanol, and 100 μg/mL BSA. The standard assay for dsUDG and hTDG proteins was the same as that for the ANPG40 protein, but without KCl. The standard assay for the Nfo protein (50 μL final volume) contained the 5′-[^{32}P]αRT·dA duplex (0.5 pmol) in 50 mM Hepes-KOH (pH 8.2), 200 mM NaCl, 5 mM β-mercaptoethanol, and 100 μg/mL BSA. The standard assay for the Xth protein (50 μL final volume) contained the 5′-[^{32}P]αRT·dA duplex (0.5 pmol) in 66 mM Tris-HCl, 0.66 mM $MgCl_2$, and 1 mM β-mercaptoethanol (pH 7.6) as previously described (*32*). After incubation at 37 °C (unless otherwise stated) for a given time in the presence of the various DNA repair proteins, the products of the reaction were analyzed as previously described (*33*). The gels were exposed to a Storm 840 Phosphor Screen, and the amounts of radioactivity in the bands were quantified using the ImageQuaNT Software. The ^{32}P-labeled 10mer generated by sequential action of the DNA

glycosylase and the associated β-lyase activity corresponds to the amount of αRT excised by the DNA glycosylase.

Formation of Enzyme−DNA Covalent Complexes. The Nth and Fpg proteins were incubated with 0.2 pmol of the 5′-[^{32}P]αRT·dA duplex at 37 °C for 20 min in 20 μL of a solution containing 25 mM sodium phosphate (pH 7.8), 1 mM Na_2EDTA, 100 μg/mL bovine serum albumin, and 100 mM $NaBH_4$ or 100 mM NaCl (a 2 M $NaBH_4$ stock solution in water was prepared inmediately prior to use). The reaction was stopped by addition of SDS (0.5% final concentration) and heating for 10 min at 60 °C. The reaction products were separated by 15% SDS−PAGE. The gels were exposed to a Storm 840 Phosphor Screen and analyzed using the ImageQuaNT Software.

Preparation of Cell Extracts. Lich cells are derived from a human hepatoma (*34*)**.** The cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% horse serum, 5% fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) in a 5% CO_2 humidified atmosphere. The cells were recovered by centrifugation, washed with Earle's balanced salts, resuspended in 70 mM Hepes-KOH (pH 7.5), 100 mM KCl, 2 mM EDTA, 5% glycerol, and 5 mM mercaptoethanol, and sonicated. The cell extract was then centrifuged, and the enzymatic activity was determined as described above (*34*).

RESULTS

Action of Various E. coli and Human DNA Repair Proteins on Oligodeoxynucleotides Containing the αRT Lesion. To study the repair of αRT in DNA, we investigated whether this lesion was a substrate for a previously characterized DNA repair enzyme. We challenged a 5′-^{32}P-labeled oligonucleotide duplex containing αRT with a variety of base excision DNA repair enzymes that had been purified to homogeneity. A 30mer oligonucleotide containing αRT at position 11 which was hybridized to a complementary oligonucleotide was employed as the substrate. Since not all of the base excision repair proteins possess AP-nicking activity, the samples were treated with hot piperidine after the DNA glycosylase reaction to cleave DNA at the potential abasic sites generated by the reaction with the various DNA glycosylases. When various homogeneous *E. coli* and human DNA glycosylases and AP-nicking enzymes were tested on 5′-^{32}P-labeled oligonucleotide containing αRT (Figure 2), only incubation with the Fpg or the Nth proteins led to the cleavage of the duplex at the position of the modified base. Despite being used in very large excess, TagI, ANPG40, UNG, dsUDG, hTDG, Nfo, and Xth proteins did not act on the αRT·dA 30mer duplex. However, there is a small amount of scission (less than 3%) of the duplex containing αRT after treatment with hot piperidine or the Nfo protein (Figure 2). Since the presence of abasic site in DNA could be revealed by treatment with hot piperidine, or by action of abasic-site nicking enzymes such as Nfo and Xth, the data suggest that the slight amount of the 10mer band could indicate the presence of a small amount of oligonucleotide containing an abasic site due to adventitious hydrolysis of αRT. However, treatment of the αRT duplex with the Xth protein (known as abasic-site specific endonuclease) does not lead to cleavage, raising the possibility that a small amount of a derivative of αRT is present in the oligonucleotide. It should be noted that various DNA glycosylases possess different catalytic mechanisms (*35*) and the products of enzymatic incision of the αRT duplex oligonucleotide migrate differently. The Fpg enzyme and hot piperidine treatment cleave the αRT·A oligomer, generating a ^{32}P-labeled fragment carrying a phosphate at the 3′-terminus (lanes 2 and 6−11). The Nfo protein, which incises on the 5′-side of the AP-site by a hydrolytic mechanism, generates a fragment which has a 3′-hydroxy terminus and thus migrates slower than the fragment carrying a phosphate at the 3′-terminus (lane 5) (*15*). The Nth protein incises at the abasic site by a β elimination mechanism, leaving on the 3′-end of the labeled 10mer an α,β-unsaturated aldehyde which migrates slightly slower than the product of hydrolysis by Nfo (*15*).

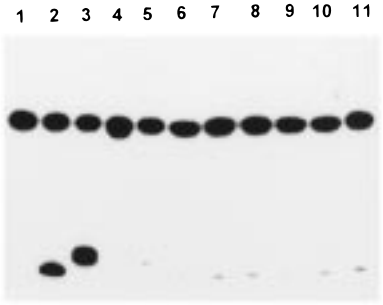

FIGURE 2: Action of various *E. coli* and human DNA repair proteins on the 30mer duplex αRT·dA oligonucleotide. The 5′-^{32}P-labeled 30mer αRT·dA was incubated with an excess of the given repair protein at 37 °C for 30 min (unless otherwise stated) and then treated with 10% piperidine for 30 min at 90 °C, except for control αRT·dA-oligo and αRT·dA treated with Fpg, Nth, Nfo, and Xth proteins: lane 1, 30mer αRT·dA; lane 2, like lane 1, but treated with the Fpg protein (25 ng); lane 3, Nth protein (100 ng); lane 4, Xth protein (4 nM, 10 min, 23 °C); lane 5, Nfo protein (630 ng); lane 6, like lane 1, but treated with piperidine; lane 7, TagI (350 ng); lane 8, ANPG40 (1.3 μg); lane 9, dsUDG (5 ng); lane 10, UDG (85 ng); and lane 11, hTDG (250 ng, 30 °C). The products of the reaction were analyzed by electrophoresis on a denaturing polyacrylamide gel and visualized by phosphorimaging. For details, see Materials and Methods.

Mechanism of Action of Fpg and Nth Proteins. It is not possible to show the direct release of the αRT residues by the Fpg and Nth proteins. The modified base does not possess a diagnostic chromophore to assist in its identification by UV spectroscopy. Moreover, the αRT residue cannot be analyzed by gas chromatography−mass spectrometry, due to its reactivity. However, it is possible to demonstrate the occurrence of an AP-site during the enzymatic reaction by trapping the enzyme−AP-site complex. Both the Fpg and Nth proteins excise the modified base and nick DNA in a concerted manner. The mechanism involves a Shiff base intermediate that can be characterized by the covalent trapping of the enzyme−substrate complex by reduction with $NaBH_4$. Indeed, treatment of both the Fpg and Nth protein reaction mixtures with $NaBH_4$ results in formation of a covalent complex (Figure 3). Since the Shiff base intermediate is a complex between the enzyme and the AP-site present in DNA, the observations in Figure 3 support the occurrence of an AP-site during the enzymatic reactions with αRT.

Kinetic Parameters of Excision of the αRT Residue from Duplex DNA by Nth and Fpg Proteins. On the basis of the above qualitative observations, we further investigated the efficiencies of the Fpg and Nth proteins at recognizing αRT

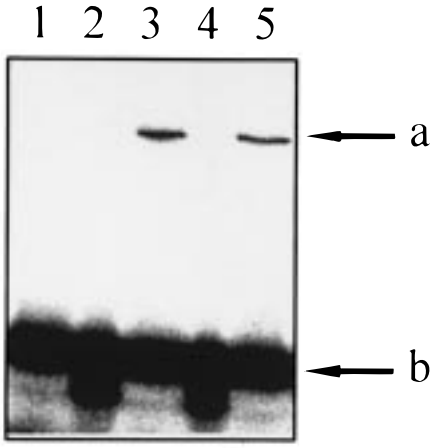

FIGURE 3: Probing for an enzyme−substrate covalent complex by $NaBH_4$ reduction. The substrate (0.2 pmol) was reacted with Nth and Fpg proteins in the presence of 100 mM NaCl or $NaBH_4$, and the products were subjected to 15% SDS−PAGE: lane 1, substrate alone; lanes 2 and 3, reactions of the substrate with the Nth protein in the presence of NaCl and $NaBH_4$, respectively; and lanes 4 and 5, reactions of the substrate with the Fpg protein in the presence of NaCl and $NaBH_4$, respectively. Arrow a points to the enzyme−substrate covalent complex and arrow b to the substrate.

Table 1: Kinetic Constants for the Nth Protein and for the Fpg Protein for the Excision of αRT and Various Other Lesions from Oligonucleotides

protein	lesion recognized	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} nM^{-1})
Nth	αRT	84	0.18	0.0022
	AP-site (*37*)	61	1.10	0.018
Fpg	αRT	7.8	0.40	0.052
	Fapy (*44*)	10	0.50	0.05
	8-oxo-*d*G[a]	4	0.43	0.11
	AP-site (*16*)	4	2.5	0.60

[a] O. Sidorkina and J. Laval, unpublished experiments.

in DNA. Both enzymes incise the oligonucleotide containing the lesion in an enzyme concentration- and time-dependent manner (data not shown). However, the Fpg protein is much more active than the Nth protein when acting on αRT residues. Excision of αRT from the duplex oligonucleotide containing this lesion by the Nth protein exhibits Michaelis−Menten kinetics (data not shown and Table 1). However, during excision of αRT by the Fpg protein, the reaction seems to be inhibited by an excess of substrate (Figure 4). The apparent K_m for the Fpg protein was calculated from the Lineweaver−Burk plot (7.8 nM) and is approximately 1 order of magnitude stronger than the affinity of the Nth protein (Table 1). Table 1 presents the K_m, k_{cat}, and k_{cat}/K_m values for the Fpg and Nth proteins acting on αRT. For comparison, the respective kinetic constants for occasions when these two proteins act on their classical substrates are also presented. The comparison of the kinetic constants of the two enzymes suggests that the biologically active protein for the excision of αRT residues in vivo is most probably the Fpg protein. It should be stressed that the kinetic constants of the Fpg protein for excision of the fragmented pyrimidine or purine are very similar.

Base Pair Specificity of the Nth and Fpg Proteins. We investigated the specificity of the Nth and Fpg proteins when they were acting on various αRT oligonucleotides containing each of the four naturally occurring nucleotides opposite

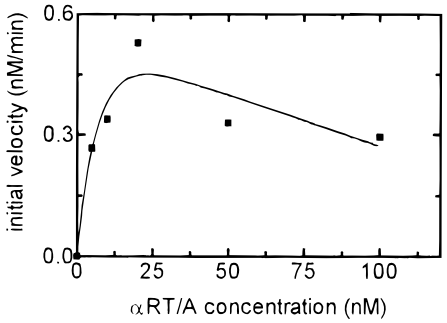

FIGURE 4: Plot of the rate against substrate concentration for the excision of the αRT lesion in DNA by the *E. coli* Fpg protein. Standard enzymatic assays were carried out by incubating the Fpg protein with αRT·dA oligonucleotides at the indicated concentrations of αRT residues ([S]) expressed in nanomolar. The initial velocity (*V*) is expressed as nanomolar per minute.

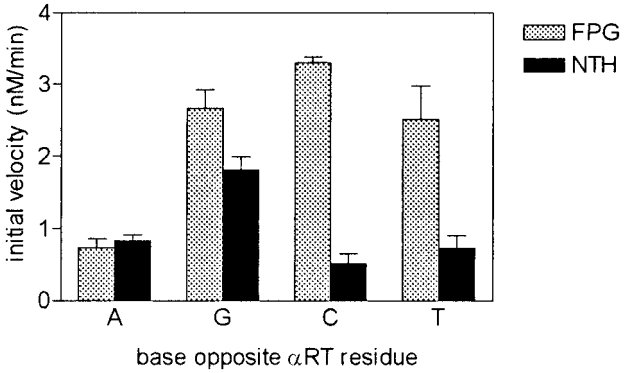

FIGURE 5: Cleavage of oligonucleotide duplexes containing different bases opposite the αRT residue by the *E. coli* Nth protein or Fpg protein. The oligonucleotide containing αRT was annealed with the complementary 30mer oligonucleotide to generate the following mismatches αRT·dA, αRT·dG, αRT·dC, and αRT·T, which were used as substrates for Nth or Fpg proteins. The initial velocity of the excision of αRT was measured by determining the amount of oligomer migrating at the position of the 10mer. The initial velocity for the Nth protein was measured using 80 ng of protein and 10 nM oligonucleotide in 100 μL of reaction mixture. The initial velocity for Fpg was measured using 5 ng of protein and 5 nM oligonucleotide in 100 μL of reaction mixture. For details, see Materials and Methods.

αRT. Figure 5 presents the initial velocity of the two enzymes when they were acting upon the four different mismatches. All of the duplexes containing αRT·dN base pairs are substrates for the Nth and Fpg proteins. The Nth protein preferentially excises the αRT lesion when it is opposite a dG, followed by dA, T, and dC (dG > dA ≥ T ∼ dC). For the Fpg protein, the αRT·dC base pairs were recognized most readily, followed by αRT·dG, αRT·T, and αRT·dA (dC ≥ dG ∼ T > dA).

An Activity Present in Human Cell Extracts Excises αRT from Duplex DNA. Since we have identified *E. coli* enzymes repairing αRT in DNA, investigating if such activities exist in human cells was of interest. We have already shown (Figure 2) that human 3meAde-DNA glycosylase (ANPG40 protein) and G·T mismatch specific glycosylase (hTDG) do not act upon an oligonucleotide containing αRT. Using a hepatoma cell line (Lich cells) to prepare crude cell extracts, we show, indeed, that αRT·dA is a substrate for enzymes present in this extract, and the extent of excision depends on the quantity of extract used (Figure 6A): 12 ± 3 and 27 ± 5% of the oligonucleotide was cleaved by 25 and 50 μg

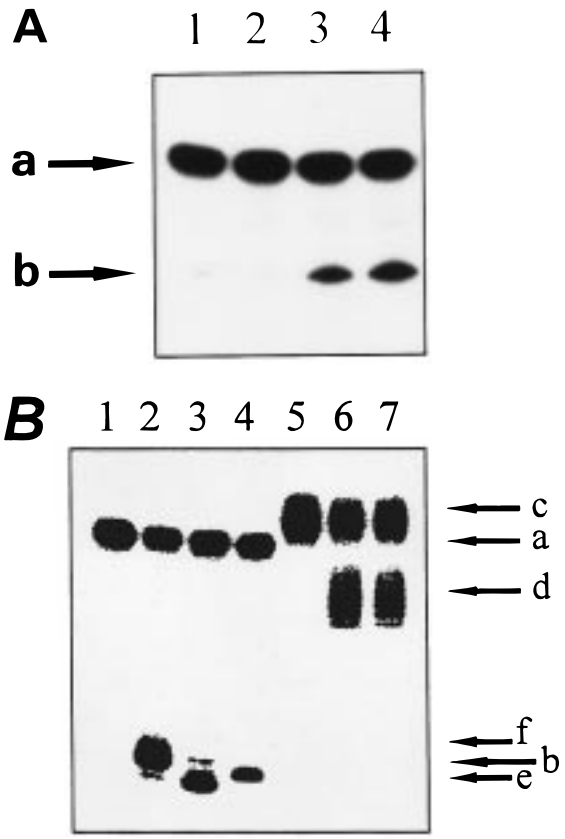

FIGURE 6: Cleavage of oligonucleotide containing αRT by human hepatoma cell extracts (Lich cells). (A) Lich cell extract was incubated with 5′-^{32}P-labeled oligonucleotides containing the αRT residue. The products of the reaction were separated on a 20% denaturing PAGE for 30 min: lane 1, duplex oligonucleotide αRT·dA; lane 2, single-stranded oligonucleotide αRT incubated for 30 min in the presence of 100 μg of Lich cell extract; lane 3, duplex oligonucleotide αRT·dA as in lane 2, but with 25 μg of Lich cell extract; and lane 4, like lane 3, but with 50 μg of Lich cells. The upper arrow **(**a**)** points to the αRT 30mer; the lower arrow **(**b**)** points to cleaved 10mer oligonucleotides. (B) Processing of αRT-containing oligonucleotides by Lich cell extracts and identification of the products. The oligonucleotides are either 5′-^{32}P-end labeled or 3′-^{32}P-end labeled and treated with Lich cell extracts. The products of the reaction are separated on a 20% denaturing PAGE for 2 h: lane 1, 5′-end labeled oligonucleotide αRT·dA; lane 2, like lane 1, but incubated with the Nth protein prior to analysis; lane 3, like lane 1, but incubated with the Fpg protein prior to analysis; lane 4, like lane 1, but incubated with Lich cell extracts as described above (lane 4 of panel A); lane 5, 3′-end labeled oligonucleotide αRT·dA; lane 6, like lane 5, but incubated with the Fpg protein; and lane 7, like lane 5, but incubated with Lich cell extracts. Arrow c indicates the 30mer oligonucleotide containing the [α-^{32}P]poly(A) tail. Arrow d points to the 19mer containing the [α-^{32}P]poly(A) tail. Arrow e points to the 10mer oligonucleotide with a phosphate group at the 3′-terminus. Arrow f points to the 10mer oligonucleotide with an α,β-unsaturated aldehyde at the 3′-terminus. For details, see Materials and Methods.

of the cell extract, respectively. One should note that excision is not detected when the oligonucleotide containing αRT is single-stranded. These results suggest that such activity in human cells could represent analogues of Fpg and Nth proteins. To rule out the possibility that the observed reaction products result from the possible activity of an unknown double-stranded 3′−5′ exonuclease that is blocked by the lesion, the oligonucleotide containing αRT was radioactively labeled on the 3′-side with [α-^{32}P]dATP using terminal transferase and was used as substrate. As shown in Figure 6B (lanes 6 and 7), the oligonucleotide is not degraded, showing that an enzyme(s) in human cells excises the αRT residues by, probably, a DNA glycosylase mechanism (Figure 6B, lane 4).

It should be noted (Figure 6B) that the termini generated by the human crude extract when it was acting upon αRT containing an oligonucleotide are peculiar. They are different from the end groups generated by the Nth protein (lane 2) which incises the DNA backbone at the abasic site by a β elimination mechanism or the Fpg protein (lane 3), with a $\beta-\delta$ elimination mechanism. The human crude extract generates products of the reaction that migrate at an intermediate rate. We can speculate that the cleavage product contains a 3′-hydroxyl group. This suggests either that the 3′-phosphate generated by a $\beta-\delta$ elimination mechanism is removed by a phosphatase or that the deoxyribose phosphate generated by a β elimination mechanism is removed by a 3′-phosphoglycoaldehyde diesterase activity. The details concerning the various termini identified are described in ref *15*.

DISCUSSION

We have shown that the Nth protein from *E. coli*, which is known to excise damaged pyrimidines, excises the αRT lesion. This observation adds a new substrate to the previously reported excision of the respective fragmentation product of dihydrothymidine by this repair enzyme (*1*). We also demonstrated that the Fpg protein from *E. coli* repairs αRT in vitro. This is something of an unexpected observation because the Fpg protein was originally identified as an enzyme that specifically recognizes purine oxidation products (e.g. 8-oxo-dG and formamidopyrimidines). The Fpg protein has been shown to repair pyrimidine oxidation products such as 5-hydroxy-2′-deoxycytidine and 5-hydroxy-2′-deoxyuridine. However, the kinetic constants were not determined (*14*). The authors proposed that the active site of the Fpg protein, which mainly recognized purines, can also accommodate the smaller pyrimidines. This study shows for the first time that a ring fragmentation product of thymidine is a substrate for the Fpg protein. We do not know at this time if the active site of the Fpg protein, which is involved in repair of the oxidized purines, is also responsible for the excision of αRT or if two separate active sites within the Fpg protein are responsible for excision of these structurally distinct families of lesions.

The crystal structure of the Nth protein reveals two domains. They form a deep cleft with a solvent-filed pocket (*36*). The crystallographic data and site-directed mutagenesis of the Nth protein show that highly conserved lysine 120 is a putative candidate for the active site nucleophile in the enzyme (*37*). The three-dimensional structure of the Fpg protein has not yet been resolved. However, recent studies demonstrated that the N-terminal proline residue of the Fpg protein, which is a highly conserved amino acid, acts as a nucleophile in the glycosylase/AP-lyase reaction using an 8-oxoG containing an oligonucleotide as a substrate (*38*). Sequence alignments of Fpg homologues in prokaryotes reveal two highly conserved lysines, Lys57 and Lys155. Site specific alteration of these lysines did not affect AP-lyase activity on preformed abasic sites but considerably diminished 8-oxoG-DNA glycosylase activity (O. Sidorkina and J. Laval, unpublished experiments; *39*).

The observations that the substrate specificities of Nth and Fpg proteins overlap and that the structure of the αRT residue is completely different from that of damaged purines (8-oxoG and Fapy) lead us to speculate that two active sites within the Fpg protein could be responsible for excision of these structurally distinct families of lesions. One can also speculate that the N-terminal part of the protein could be involved in the repair of fragmented and oxidized purines in DNA, whereas the region containing the conserved lysine could be responsible for the repair of fragmented pyrimidines.

Establishing that αRT is recognized by more than one enzyme in vitro does not necessarily indicate that all of these enzymes act on the lesion in vivo. In fact, there is a large difference between the kinetics of excision of αRT from DNA by the Fpg and Nth proteins. The ratio of k_{cat}/K_m for excision of αRT when the lesion is across from dA by the Fpg protein is about 24 times higher than that for excision by the Nth protein (Table 1). The kinetic constants for the excision of αRT by the Fpg protein are very similar to those for the Fapy lesion, and the k_{cat}/K_m is $^1/_2$ of that for excision of 8-oxo-dG. This suggests that lesion αRT could be repaired in vivo by the Fpg protein.

The possible apparent substrate inhibition of the Fpg protein could be explained by the inhibition of this protein by the highly reactive free base. Unfortunately, the previously reported chemical lability of the αRT prevents investigating whether the free nucleoside or the free base acts as an inhibitor (*23*). Considering that the source of the free nucleoside's instability is its propensity to oligomerize via esterification, one could speculate that a similar transformation (e.g. acylation of a lysine side chain) is responsible for the enzyme inhibition. However, this issue remains to be investigated.

Preferential recognition of a modified residue paired with one of the four natural bases in duplex DNA is an important feature of various DNA glycosylases and has been studied in detail for the excision of hypoxanthine (*40*), ethenoadenine (*33*), and 8-oxoG (*41*). Assuming that the Fpg protein's activity is associated with its ability to recognize distortions in the duplex DNA structure, the selectivity of the Fpg protein for excising αRT from the duplex oligonucleotides is consistent with the previously reported study on the effect of αRT on Klenow exo$^-$ fidelity (*23*). When faced with αRT in a DNA template across from a point of insertion, Klenow exo$^-$ preferentially incorporates dA. The other three naturally occurring nucleotides are incorporated significantly more slowly, in the relative order dG > T > dC. These observations can be tentatively interpreted to imply that the extent to which the structure of oligonucleotide duplexes containing αRT are perturbed varies depending upon the nucleotide across from the lesion. Qualitatively, the structural alterations of the duplexes vary in the following order: αRT·dA < αRT·dG < αRT·T < αRT·dC. This indeed parallels the order in which αRT is excised from the respective oligonucleotide duplex by the Fpg protein.

Although αRT has not been identified as a biologically relevant lesion yet, concerns regarding its reactivity may impact upon its detection in biological samples. αRT does not have a suitable chromophore for monitoring by UV spectroscopy. It also cannot be analyzed by GC−MS, which requires formic acid and persilylation treatments at high temperature before analysis, because these treatments will result in recyclization of the αRT residue (*42*, *43*). In conclusion, the potential biological relevance of these processes is supported by the fact that the excision of αRT by the Fpg protein is effected at rates comparable to that of the repair of the 8-oxo-dG and Fapy lesions and the observation that such activity is present in human cell extracts.

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