Excision of 5,6-Dihydroxy-5,6-dihydrothymine, 5,6-Dihydrothymine, and 5-Hydroxycytosine from Defined Sequence Oligonucleotides by *Escherichia coli* Endonuclease III and Fpg Proteins: Kinetic and Mechanistic Aspects†

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ABSTRACT: Oligonucleotides that contain a single modified pyrimidine, i.e., thymine glycol (Tg), 5,6-dihydrothymine (DHT), and 5-hydroxycytosine (5-OHC) were synthesized in order to investigate the substrate specificity and the excision mechanism of two *Escherichia coli* repair enzymes: endonuclease III and formamidopyrimidine DNA glycosylase (Fpg). Three techniques of analysis were employed. A gas chromatography−mass spectrometry (GC−MS) assay with HPLC prepurification was used to quantify the release of the modified bases, while polyacrylamide gel electrophoresis and matrix-assisted laser-desorption ionization-mass spectrometry (MALDI-MS) provided insights into the mechanism of oligonucleotide cleavage. Values of V_m/K_m constants lead to the conclusion that the substrates are processed by endonuclease III with the following preference: Tg ≫ 5-OHC > DHT. This confirms that Tg is an excellent substrate for endonuclease III. Fpg-mediated cleavage of the 5-OHC-containing oligonucleotide is processed at the same rate than endonuclease III. Furthermore, Fpg was found to have a little but relevant activity on DHT-containing oligonucleotide, thus broadening the substrate specificity of this enzyme to a new modified pyrimidine. While 5-OHC-containing oligonucleotides are cleaved by the two enzymes, no or a small amount of the modified base was found to be released, as determined by GC−MS. From these data it may be suggested that 5-OHC could be modified during its enzymatic excision. Finally, MALDI-MS analyses shed new light on the mechanism of action of endonuclease III: the molecular masses of the repaired fragments of 5-OHC- and DHT-containing oligonucleotides showed that endonuclease III cleaves the DNA backbone mainly through a hydrolytic process and that no β-elimination product was detected.

Oxidation reactions of DNA, a critical cellular target, arise continuously in cells. They are likely to be induced by either endogenously generated oxidants or following exposure to physical exogenous agents such as ionizing and solar radiations (*1*−*3*). Reactive oxygen species, including $OH^{\bullet}$ radical and singlet oxygen, can give rise to the formation of several classes of DNA damage. The latter lesions may have deleterious consequences for the cell, such as mutagenesis, carcinogenesis, aging, and lethality (*4*−*6*). To prevent biological expression of injuries to DNA, cells are equipped with repair enzymes which take care of both damaged bases and sugar residues. One of these, the base excision repair (BER)[1] mechanism, accounts for by the replacement of most of the oxidative base damage (*7*, *8*).

A broad spectrum of lesions can be repaired by the BER pathway. This involves several types of glycosylases for the initial excision step of the modified bases. In *Escherichia coli*, uracil DNA glycosylase (Ung), endonuclease III (Nth), endonuclease VIII (Nei), formamidopyrimidine DNA glycosylase (Fpg), and alkyl DNA glycosylases (Tag and AlkA) are the main glycosylases involved in the BER pathway. Among these enzymes, Fpg, endonuclease III, and endonuclease VIII account for the excision of the major oxidative base lesions.

Endonuclease III (endo III) from *E. coli* is the product of the *nth* gene. It is a monomeric protein of 23 kDa which contains an Fe−S cluster (*9*, *10*). The enzyme possesses both glycosylase and 3′-apurinic/apyrimidinic (AP) endonuclease activities, and the removal of a lesion is believed to be followed by a β-elimination step catalyzed by the enzyme (*11*−*15*). Previous studies have shown that endonuclease III recognizes modified thymines and cytosines, including 5,6-

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[1] Abbreviations: 5-OHC, 5-hydroxycytosine; 5-OHU, 5-hydroxyuracil; AP site, apurinic/apyrimidinic site; BER, base excision repair; DHT, 5,6-dihydrothymine; endo III, endonuclease III; Fpg, formamidopyrimidine DNA glycosylase; GC−MS, gas chromatography−mass spectrometry; MALDI-MS, matrix-assisted laser-desorption ionization−mass spectrometry; PAGE, polyacrylamide gel electrophoresis; tBDMS, substitution of an hydrogen by a *tert*-butyldimethylsilyl group; Tg, 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol); TMS, substitution of an hydrogen by a trimethylsilyl group; Ug, 5,6-dihydroxy-5,6-dihydrouracil (uracil glycol).

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Table 1: Sequences of Oligonucleotides Used in the Study

name	sequence (5′ to 3′)
33-T	GCT ATG GTT TCG GAG **T**CA GCC AGA TAC GAG AGA
33-DHT	GCT ATG GTT TCG GAG (**DHT**)CA GCC AGA TAC GAG AGA
33−5-OHC	GCT ATG GTT TCG GAG (**5-OHC**)CA GCC AGA TAC GAG AGA
33-Tg	GCT ATG GTT TCG GAG (**Tg**)CA GCC AGA TAC GAG AGA
33-A	TCT CTC GTA TCT GGC TG**A** CTC CGA AAC CAT AGC
33-G	TCT CTC GTA TCT GGC TG**G** CTC CGA AAC CAT AGC
10	GCT ATG GTT T
11-p-T	pCGG AG**T** CAG CC
12-p	pAGA TAC GAG AGA

dihydrothymine (DHT), 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol = Tg), 5-hydroxy-5,6-dihydrothymine, 5,6-dihydrouracil, 5,6-dihydroxy-5,6-dihydrouracil (uracil glycol), 5-hydroxy-5,6-dihydrouracil, 5-hydroxyuracil, 6-hydroxy-5,6-dihydrocytosine, 5-hydroxycytosine (5-OHC), urea, methyltartronyl-*N*-urea, 5-hydroxy-5-methylhydantoin, and alloxan (*16*−*21*). Strains lacking the *nth* gene showed a weak mutator phenotype. However, they were not found to be unusually sensitive to oxidative stress conditions. It should be noted that endonuclease VIII is believed to be a back-up enzyme for endonuclease III (*22*−*24*). Functionnal and structural analogues of endonuclease III have been cloned in yeast and mammals (*25*−*27*).

The Fpg protein of *E. coli* is a 30.2 kDa zinc-finger protein which exhibits both a glycosylase and an AP-endonuclease activities (*28*, *29*). In contrast to endonuclease III, Fpg cleaves the DNA backbone through a $\beta-\delta$ reaction, thus leading to the release of the sugar residue from the DNA strand (*30*). Substrates recognized and excised by Fpg include 8-oxo-7,8-dihydroguanine (8-oxoG), the predominant physiological substrate of the enzyme, together with 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-guanine), 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (Me-Fapy-guanine), and 4,6-diamino-5-formamidopyridine (Fapy-adenine) (*31*−*36*). It was shown that 5-OHC is also recognized and excised by Fpg (*21*). Strains lacking the *fpg* gene show a mutator phenotype. Interestingly, the latter phenotype is strongly enhanced by the inactivation of the *mutY* gene which takes part in the repair of 8-oxoG (*37*). Genes for a functional analogue of Fpg, namely Ogg1 have been cloned in yeast and mammals (*38*−*44*).

To assess the substrate specificity of the repair enzymes, K_m and V_m constants of the Michaelis−Menten kinetics have to be determined. Comparison of the values of the V_m/K_m ratio permits the determination of the order of preference for the enzymes toward their different substrates at low concentration. In this respect, kinetic constants for *E. coli* endonuclease III are available for uracil glycol, 5-OHC, and 5-hydroxyuracil (*45*). For Fpg, they have been determined for some purine lesions (*33*, *34*), but they are lacking for pyrimidine lesions, such as 5-hydroxycytosine. We synthesized 33-mer-long oligonucleotides that contain single modified bases, i.e. thymine glycol, DHT, and 5-OHC, with the aim to assess the substrate specificity of endonuclease III anf Fpg proteins. Thus, the kinetic constants were determined either by using a gas chromatography−mass spectrometry (GC−MS) assay or by measuring the extent of strand breakage of the oligonucleotides with polyacrylamide gel electrophoresis (PAGE). Analysis of the oligonucleotides by matrix-assisted laser-desorption ionization−mass spectrometry (MALDI-MS) was also applied in order to gain insights into mechanistic aspects of oligonucleotide cleavage by the two repair enzymes.

EXPERIMENTAL PROCEDURES

Repair Enzymes. Purified endonuclease III and Fpg protein of *E. coli* were gifts of Dr. Serge Boiteux, CEA Fontenay-aux-Roses, France. Fpg protein was obtained and purified according to ref 29. Endonuclease III was overproduced from a bacterial strain harboring a disrupted *fpg* gene. The purification of endonuclease III is described in ref 20. Apparent homogeneity of the preparations was assessed by the presence of a single polypeptide band on SDS−polyacrylamide gel electrophoresis.

Oligonucleotides Synthesis. Unmodified oligonucleotides were synthesized by standard phosphoramidite chemistry using an Applied Biosystems 392 DNA/RNA synthesizer (*46*). The standard 1 μmol synthesis scale with retention of the 5′-terminal DMTr group was used. Modified 33-mer long oligonucleotides containing DHT and 5-OHC were prepared as described elsewhere (*47*, *48*). After deprotection with aqueous ammonia, the 5′-DMTr-oligomers were purified and detritylated on-line by reversed-phase HPLC as previously described (*48*). All 33-mer long oligonucleotides were subsequently purified by preparative PAGE, extracted by the "crush and soak" method, and then desalted on NAP-25 sephadex columns (Pharmacia).

All prepared oligonucleotides are listed in Table 1. Quantification was made by UV absorption at 260 nm with the extinction coefficient calculated using the "oligo 4.0" program.

The preparation of a Tg-containing oligonucleotide was achieved by potassium permanganate oxidation of a 11-mer which exhibits a single thymine (11-p-T). The conditions of oxidation were adapted from the protocols described by Basu et al. (*49*) and Kao et al. (*50*). Typically, 200 nmol of oligonucleotide (20 μM) was dissolved in a 200 mM phosphate buffer (pH 8.6), and the resulting solution was cooled in an ice bath. $KMnO_4$ was added to a final concentration of 4 μM, and the reaction was stopped after 5 min by adding 200 μL of allyl alcohol. The solution was left at 4 °C for, at least, 12 h in order to achieve MnO_2 precipitation. The solid residue was then removed by centrifugation. After NAP-25 desalting, the oxidized oligonucleotide was purified by reversed-phase HPLC on a Ultremex 5 C18 250 × 10 mm column (Phenomenex) using an acetonitrile gradient in 25 mM triethyl ammonium acetate (pH 7) buffer as follows: total flow, 2.5 mL/min; 0 min, 0% acetonitrile; 8 min, 0%; 12 min, 6%; 45 min, 10%. A typical chromatogram is shown in Figure 1. The oligonucle-

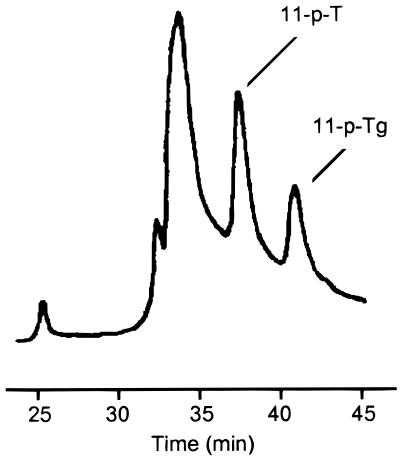

FIGURE 1: HPLC elution profile (Ultremex 5 C18 250 × 10 mm column; acetonitrile gradient in 25 mM TEAA, pH 7) of the $KMnO_4$ oxidized 11-p-T. Detection by UV absorbance at $\lambda = 260$ nm.

otide (11-p-Tg) collected within the third HPLC peak (41 min) showed an increase of 34 amu by comparison to the mass of 11-p-T, as inferred from electrospray mass spectrum analyses (Platform II, Fisons). This was attributed to the oxidation of thymine into thymine glycol. About 8% of the 11-p-T was oxidized into 11-p-Tg. Furthermore, to confirm the presence of thymine glycol in the oligonucleotide, 0.3 OD (260 nm) of 11-p-Tg was digested using a mixture of 10 units of nuclease P1 (Pharmacia) and 7 units of sweet potato acid phosphatase (Sigma) in 30 mM sodium acetate (pH 5.5) buffer together with 100 μM $ZnSO_4$, for 2.5 h at 37 °C. The resulting solution was analyzed by HPLC with a photodiode array UV detector.

The 33-mer long oligonucleotide that contained a Tg residue was obtained by enzymatic ligation of 11-p-Tg with 10- and 12-mer oligonucleotides as follows: hybridization against the complementary strand 33-A was achieved by heating 10 nmol of each oligonucleotide at 60 °C for 3 min in 100 μL of 250 mM Tris-HCl (pH 7.5) buffer with 5 mM $MgCl_2$, 1 mM dithioerythritol, and 1 mM ATP, followed by a slow cooling to 4 °C overnight. The ligation reaction was carried out during 24 h at 4 °C in the same buffer with 20 units of T4 DNA ligase (Boehringer Mannheim). The resulting double-stranded Tg-containing oligonucleotide was then purified using nondenaturing conditions PAGE and desalted on NAP-10 column. Thus, 7.5 nmol of the double-stranded 33-mer long oligonucleotide was obtained.

Construction of Duplex DHT- and 5-OHC-Containing Oligonucleotides and Enzymatic Reactions. The 33-mer long oligonucleotides 33-DHT and 33-5-OHC (10 μM final concentration) were hybridized against 13 μM of the complementary strand, 33-A and 33-G, respectively, in 40 mM Tris-HCl (pH 7.5) buffer with 200 mM KCl and 2 mM EDTA. This was achieved by heating at 85 °C for 3 min, followed by slow cooling to 4 °C overnight.

All enzymatic reactions were carried out on duplex oligonucleotides at 37 °C in 20 mM Tris-HCl (pH 7.5) buffer to which 100 mM KCl and 1 mM EDTA were added. Reactions were stopped either by liquid nitrogen freezing followed by ethanol precipitation for GC−MS analysis or by adding an equivalent volume of formamide for subsequent PAGE analysis.

Enzymatic Assay with GC−MS Detection. All enzymatic reactions (total volume of 50 μL) were carried out in microtubes that contain the stable isotope-labeled analogues of the lesion to be excised. After ethanol precipitation of the remaining oligonucleotides, the supernatant and the pellet were separated. Conditions of acid hydrolysis of the pellets and HPLC prepurification of the lesions prior to GC−MS analysis are described elsewhere (*51*). The collection time intervals for the blind HPLC prepurification were the following: 3.4−5.2 min for 5-OHC, 3.7−5.8 min for Tg, and 13.1−15.7 min for DHT.

GC−MS analysis of the product is adapted from the method described previously (*51*): the temperature of the injection port was set at 250 °C and the products were derivatized with either *N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for Tg or *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) for both DHT and 5-OHC. The characteristic recorded ions were, respectively, the following: $m/z = 299.1$ (DHT + 2 tBDMS − 57); $m/z = 303.1$ ([α,α,α,6-d_4]DHT + 2 tBDMS − 57); $m/z = 259.1$ (cis or trans Tg + 4 TMS − 189); $m/z = 262.1$ (cis or trans [α,α,α,6-d_4]Tg + 4 TMS − 190); $m/z = 412.3$ (5-OHC + 3 tBDMS − 57); $m/z = 415.3$ ([2-^{13}C,1,3-$^{15}N_2$]5-OHC + 3 tBDMS − 57). The quantitative measurement of Tg was made by adding the areas of the two peaks corresponding to the cis and trans isomers of Tg.

The internal standards were obtained as described elsewhere, from [α,α,α,6-d_4]thymine for DHT (*51*) and Tg (*52*) and from [^{13}C,$^{15}N_2$]urea for 5-OHC (*53*). The quantification of the products for the GC−MS calibration curves was made by UV absorption at $\lambda = 286$ nm for 5-OHC and by NMR analysis with respect to a known amount of thymine for Tg and DHT.

Enzymatic Assay with PAGE Analysis. Modified single-stranded oligonucleotides were 5′-end-labeled with [γ-^{32}P]-ATP before hybridization and subsequent enzymatic assay. Typically, 10 pmol of the oligonucleotide was mixed with 2 pmol of [γ-^{32}P]ATP and 10 units of polynucleotide kinase (Pharmacia) in 10 μL of the supplied buffer. Reaction which was held for 30 min at 37 °C, was stopped by adding 1 μL of 0.5 M EDTA solution (pH 8), and the oligonucleotide was subsequently purified on a Microspin G-25 column (Pharmacia). Then, to the labeled oligonucleotide, 90 pmol of the same unlabeled oligonucleotide was added in order to define more precisely the concentration, together with 130 pmol of the complementary strand. Hybridization was done as described previously, and subsequent enzymatic reactions were carried out at 37 °C in a total volume of 10 μL. Analyses were performed on 16% polyacrylamide gels (0.4 mm thickness) under denaturing conditions (8 M urea). The gels were electrophoresed in 50 mM Tris borate and 1 mM EDTA buffer (pH 8) for 40 min at 1200 V. The radioactivity in the bands was counted with the "Image Quant" program using a Molecular Dynamics Phosphorimager after exposure of the gel for 1 h on the storage phosphor screen.

Determination of K_m and V_m. Quantitation of the enzymatic activity was achieved using GC−MS analysis by integration of the area of the selected ion peak and further comparison with the area of the internal standard correponding peak. An alternative approach involved the determination of the extent of cleaved oligonucleotide using the PAGE analysis. The velocity was expressed as picomoles of substrate excised per

minute and per nanogram of enzyme. The ranges of substrate concentration were determined in order that each of the Michaelis−Menten curves reaches a plateau. They were the following: 0.2−4 μM for Tg, 0.23−11.4 μM for DHT, and 0.2−20 μM for 5-OHC. The concentrations of endo III were 1, 20, and 3 ng/μL for Tg, DHT, and 5-OHC, respectively. Fpg concentrations of 6 and 20 ng/μL were used for 5-OHC and DHT, respectively. Reactions, carried out at 37 °C, were stopped after 15 min of incubation in the case of further GC−MS measurement and after 10 min when PAGE analysis was performed. K_m and V_m constants were calculated using nonlinear least-squares fitting of the data points, on the basis of, at least, three separate experiments.

MALDI-MS Analysis of Repaired Oligonucleotides. Enzymatic reactions were carried out on 20 pmol of duplex oligonucleotides in 20 μL of standard buffer. Endo III or Fpg was added at a final concentration of 75 ng/μL, and solutions were let at 37 °C for 1 h. The oligonucleotides were subsequently precipitated three times with 5 M ammonium acetate and cold ethanol. MALDI mass spectra were recorded on a time-of-flight instrument (Perseptive Biosystems) equipped with a pulsed delay source extraction. Spectra were recorded from 256 laser shots (nitrogen laser, 337 nm) with an accelerating voltage of 25 kV in the linear and positive modes. For the matrix, a mixture of 3-hydroxypicolinic acid and picolinic acid in a 4/1 (w/w) ratio was dissolved in aqueous acetonitrile solution (50%) containing 0.1% TFA and a small amount of cation-exchange resin Dowex-50X50 × 8-200 (Sigma). One microliter of the sample was added to 1 μL of the matrix, and the resulting solution was stirred. The resulting sample was then placed on the top of the target plate and allowed to dry by itself. The spectra were calibrated with 1 pmol/μL solution of myoglobin (*m*/*z* 16 952), using the same conditions of assay that were described for the oligonucleotides.

RESULTS

Synthesis and Characterization of the 33-mer-Long Tg-Containing Oligonucleotide. Tg is a lesion that presents notable instability in alkaline conditions, and thus, its incorporation into oligonucleotides by the phosphoramidite chemistry is not appropriate. Therefore, the preparation of Tg-containing oligonucleotide was achieved by oxidation of a 11-mer that exhibits a single thymine as it has been previously described (*49, 50*). $KMnO_4$ oxidation of 11-p-T at pH 8.6 led to the HPLC profile presented in Figure 1. Electrospray ionization MS analysis of the main HPLC fractions revealed that the third major peak, eluting after the 11-p-T-containing peak, showed a single product with the expected mass for 11-p-Tg (*m*/*z* = 3456.2). It should be noted that Tg-containing oligonucleotides were reported to elute earlier than unmodified oligonucleotides on reversed-phase HPLC columns. The mass spectrum of the products eluting in the first major peak revealed the presence of compounds with mass differences of +4 amu and +50 amu, respectively, by comparison with the molecular weight of 11-p-T. These masses were, respectively, assigned to oligonucleotides that contain 5-hydroxy-5-methylhydantoin and methyltartronyl-*N*-urea residues in place of the thymine residue. This is in agreement with previous findings on the potassium permanganate oxidation of thymidine (*54*). Confirmation of the presence of Tg in the oligonucleotide was provided by HPLC analysis of enzymatic hydrolysates of the oligonucleotide. The HPLC elution profile revealed a complete lack of the thymidine residue ($k' = 11.5$) for absorbance set at $\lambda = 260$ nm, and the presence of a peak corresponding to *cis* (*5R-6S*)-thymidine glycol ($k' = 3.6$) at $\lambda = 210$ nm. UV spectrum of the product eluting in the latter peak was identical to that of thymidine glycol.

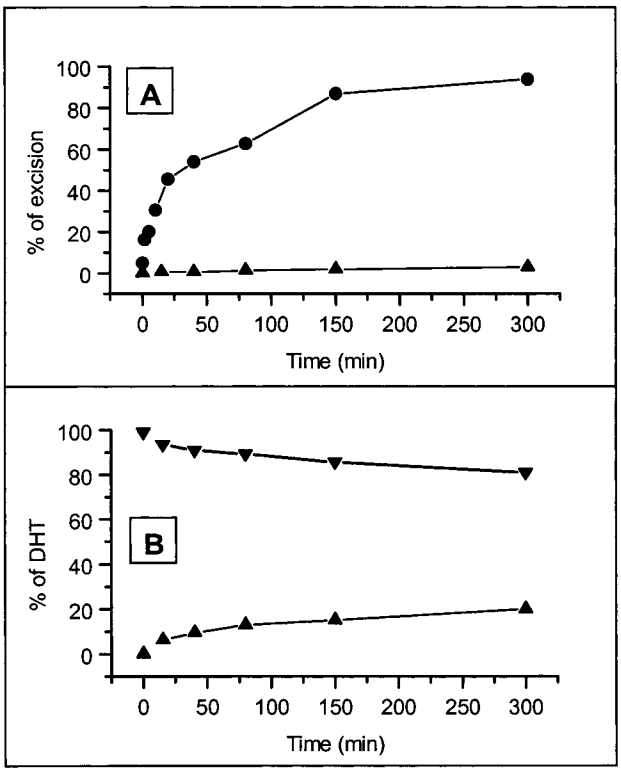

FIGURE 2: Time course experiments. (A) Percentages of Tg (circles) and DHT (up triangles) released by endo III; 4 μM oligonucleotide/endo III (4 ng/μL). (B) Percentages of DHT excised by endo III (up triangles) and remaining in oligonucleotide as determined by acid hydrolysis (down triangles); 4 μM oligonucleotide/endo III (20 ng/μL).

To obtain a Tg-containing oligonucleotide of sufficient length to assess substrate specificity of the repair enzymes, enzymatic ligation with appropriate oligonucleotides against a 33-mer long complementary strand was processed. Ligation efficiency was high (more than 90%) as revealed by PAGE analysis under denaturating conditions. The oligonucleotide was then purified in nondenaturating PAGE conditions, to obtain the double-stranded 33-mer long Tg-containing oligonucleotide. Final characterization of the integrity of the Tg in the oligonucleotide was provided by endo III excision of 100% of Tg as determined by the GC−MS assay (vide infra).

Excision of Tg, DHT, and 5-OHC from Modified Oligonucleotides. A GC−MS assay, previously developped (*51*), was applied in order to determine the kinetic parameters of the endonuclease III-mediated excision of the modified bases from the synthesized oligonucleotides. Time course kinetics were carried out on Tg- and DHT-containing oligonucleotides (Figure 2). Noteworthy differences were found between enzymatic processing of Tg and DHT. When endo III was used at a concentration of 4 ng/μL, DHT excision was almost not observed for a 4 μM oligonucleotide concentration. On

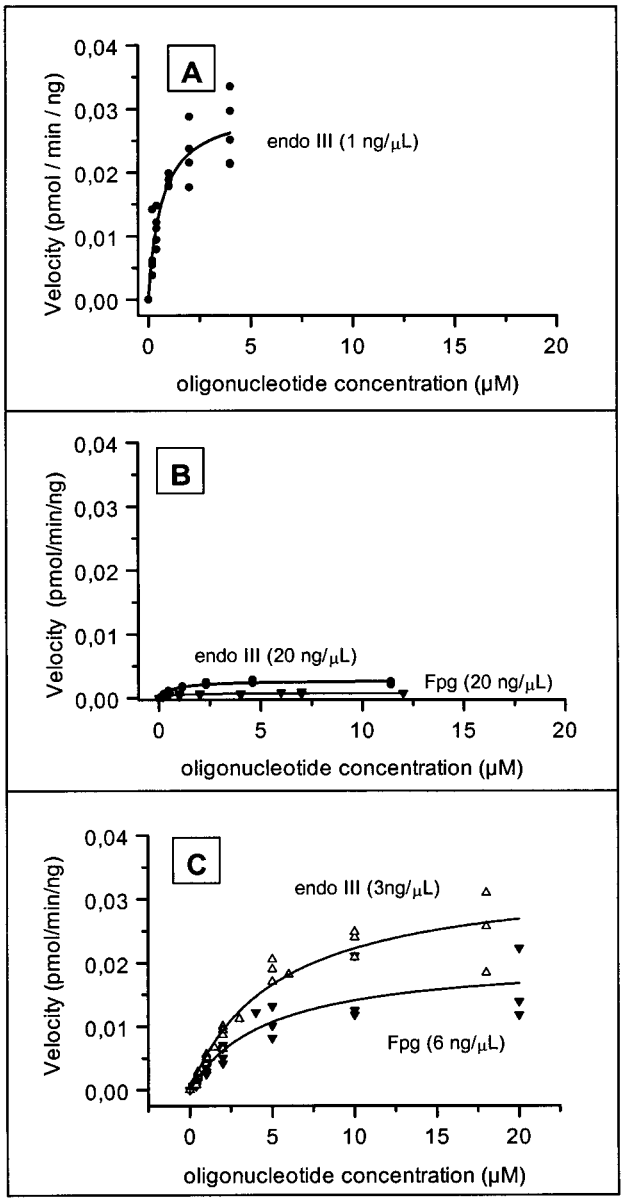

FIGURE 3: Michaelis−Menten kinetics. (A) Digestion of Tg-containing oligonucleotide by endo III (1 ng/μL). (B) Digestion of DHT-containing oligonucleotide by endo III (20 ng/μL) (circles) Fpg (20 ng/μL) (triangles). (C) Digestion of 5-OHC-containing oligonucleotide by endo III (3 ng/μL) (up triangles) and Fpg (6 ng/μL) (down triangles). Results reported as circles were obtained by the GC−MS assay, while those reported as triangles were inferred from PAGE analyses.

the other hand, almost 100% of Tg was excised from the oligonucleotide under the same conditions (Figure 2, panel A). It should be pointed out that DHT could not be totally excised, even at concentration of endo III up to 20 ng/μL. To confirm the presence of DHT in the remaining oligonucleotide, pellets were submitted to acid hydrolysis. An almost complete balance of DHT still present in DNA fragment and released by endo III was observed using this analytical method (Figure 2, panel B).

K_m and V_m values which were calculated for enzymatic excision of DHT and Tg by endo III (Figure 3, panels A and B), are reported in Table 2. The Michaelis−Menten kinetic constants definitively assess that Tg is a better substrate than DHT for endo III. The V_m value was found to be 10 times higher for Tg in comparison with DHT, while

Table 2: Values of V_m and K_m

enzyme	substrate	V_m (pmol/min/ng)	K_m (μM)	rel V_m/K_m
endo III	Tg	0.028 (0.002)	0.66 (0.16)	7.0
endo III	5-OHC	0.034 (0.003)	5.2 (0.9)	**1 (ref)**
Fpg	5-OHC	0.021 (0.003)	4.7 (1.6)	0.68
endo III	DHT	0.0028 (2×10^{-4})	0.68 (0.13)	0.63
Fpg	DHT	7.8×10^{-4} (7×10^{-5})	0.45 (0.17)	0.26

Numbers in parentheses indicate standard errors.

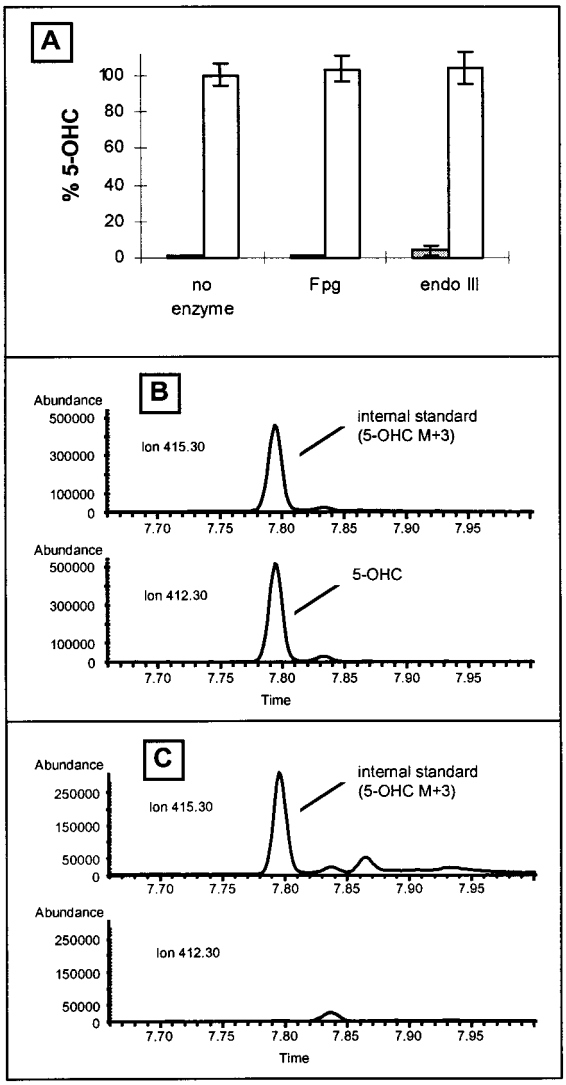

FIGURE 4: GC−MS analysis of excised 5-OHC upon endo III and Fpg treatment: 4 μM oligonucleotide incubated with no enzyme, endo III (40 ng/μL) or Fpg (30 ng/μL) during 1 h. (A) percentage of 5-OHC in the supernatant (grey bars) or in the pellet (white bars). (B) GC−MS chromatogram of the formic acid hydrolysate of Fpg treated oligonucleotide. Panel C: GC−MS chromatogram of the supernatant after Fpg treatment of the oligonucleotide.

K_m values are almost the same for the two 5,6-saturated base lesions.

Enzymatic release of 5-OHC from modified oligonucleotides was not detected upon Fpg treatment and only to a small extent after endo III incubation. The results reported in Figure 4, panel A, were obtained for a 4 μM oligonucleotide incubated for 1 h with either 30 ng/μL Fpg or 40 ng/μL endo III. The GC−MS chromatograms corresponding to the amount of 5-OHC measured in the supernatant and in the pellet after Fpg treatment are shown in Figure 4, panels

B and C, respectively. As determined by PAGE analyses, the experimental conditions used for the GC−MS assay allowed cleavage of 35−40% of the oligonucleotide with either Fpg or endo III. On the other hand, only 5% of 5-OHC residues were released upon endo III treatment, whereas no excision was detected after Fpg incubation. The discrepancies between these results cannot be attributed to a possible trapping of released 5-OHC, as known amounts of 5-OHC added before the enzymatic reaction were found to be present in the supernatant (data not shown).

Enzymatic Cleavage of 5-OHC- and DHT-Containing Oligonucleotides. K_m and V_m values were calculated for 5-OHC removal by endo III and Fpg using the PAGE assay (Figure 3, panel C), since the release of the modified base was not detected by GC−MS. The K_m and V_m constants were found to be in a similar range, respectively. The V_m values are close to that observed for Tg; however, the K_m for 5-OHC is much higher than for either Tg or DHT.

DHT-containing oligonucleotides were also cleaved upon Fpg treatment and Michaelis constants were determined (Figure 3, panel B). The values of K_m were very close for Fpg and endo III. However, the V_m value was lower for Fpg than for endo III, suggesting that DHT is a poor substrate for Fpg.

MALDI-MS Analysis of the Enzyme-Mediated Cleavage of the Oligonucleotides. MALDI-MS analysis of the 5-OHC-containing oligonucleotide with its complementary strand is shown in Figure 5, panel A. Masses at $m/z = 10\,041$ and 10 243 are from the protonated oligomers (33-G, calculated $M + H^+ = 10\,041$ and 33-5-OHC, calculated $M + H^+ = 10\,244$). The oligonucleotides are present in the protonated form, but also with K^+ or NH_4^+ as counterions of the phosphate groups. This explains why all oligonucleotides are represented by a distribution of peaks (unmarked peaks have been attributed to such compounds). Badly resolved peaks with masses between 5000 and 5200 are from the doubly charged form of the 33-mer long oligonucleotides (33-G, calculated $(M + 2H^+)/2 = 5021$ and 33-5-OHC, calculated $(M + 2H^+)/2 = 5128$).

Panel B of Figure 5 shows the mass spectrum of the products resulting from the cleavage of 33-5-OHC by Fpg. Masses between 5000 and 5200 are from the doubly charged 33-mer long oligonucleotides, as already observed. Fragments at $m/z = 4719$ and 5317 arise from the cleavage of the 33-5-OHC as follows: the fragment at $m/z = 5317$ is accounted for by the 17-mer oligonucleotide released 3′ to the lesion (5′ pCA GCC AGA TAC GAG AGA 3′, calculated $M + H^+ = 5318$) and $m/z = 4719$ is the molecular weight of the 15-mer oligonucleotide released 5′ to the lesion (5′ GCT ATG GTT TCG GAGp 3′, calculated $M + H^+ = 4720$). Altogether this is consistent with the expected β-δ-elimination mechanism of Fpg (Figure 6).

Panel C of Figure 5 shows the mass spectrum of the products obtained after cleavage of 33-5-OHC by endo III. The fragment at $m/z = 5320$ corresponds to the 3′-released oligonucleotide as for the Fpg-mediated cleavage. The compound with $m/z = 4722$ was assigned to the oligonucleotide released in 5′ position by the splitting of the phosphodiester bond 5′ to the lesion, as observed for Fpg. The fragment at $m/z = 4838$ may be accounted for by the main 5′-product of cleavage by endo III. Interestingly, the mass corresponds to that of an oligonucleotide released by a hydrolysis process rather than by a β-elimination mechanism (hydrolysis product, 5′ GCT ATG GTT TCG GAGp-deoxyribose 3′, calculated $M + H^+ = 4836$; β-elimination product, 5′ GCT ATG GTT TCG GAGp-α,β-unsaturated aldose 3′, calculated $M + H^+ = 4818$). It should be noted that the minor peak at $m/z = 4904$ has not been attributed to any product, while present in most of the spectra of endo III digested oligonucleotides.

The results obtained by MALDI-MS are consistent with the PAGE analysis (Figure 7): the oligonucleotide cleaved by Fpg at the 5′ position was revealed on the gel as a single band, while for endo III repair, two bands appeared, corresponding to, at least, two cleavage products. One of these DNA fragments that was detected only for important concentrations of enzyme comigrated with the product of excision by Fpg (β-δ-elimination). However, the other main oligonucleotide migrated slightly slower than the β-δ-elimination product.

Similar results were obtained, with either MALDI-MS or PAGE analyses, for DHT-containing oligonucleotide cleaved upon incubation with either Fpg or endo III (Figure 5, panels D and E).

DISCUSSION

Substrate Specificity of Endo III and Fpg Proteins. The synthesis of 33-mer-long oligonucleotides that contain a single lesion has allowed detailed studies of the repair mode of both endo III and Fpg proteins. Thus, comparable kinetic parameters were obtained in order to better determine the substrate specificity of these enzymes toward the targeted base lesions.

A very low amount of released 5-OHC was measured by GC−MS with respect to the extent of the oligonucleotide cleavage, upon either endo III or Fpg incubation. This is in agreement with previous repair studies involving GC−MS analysis of the excised base products from oxidized DNA. Fpg protein was not able to release any 5-OHC (*36*), whereas contradictory results were obtained for the excision of 5-OHC by endo III (*20*, *55*). The presence of 5-OHC in the supernatant upon endo III treatment of oxidized DNA, could be rationalized in terms of initial excision of cytosine glycol, an unstable product that undergoes subsequent dehydratation into 5-OHC (*56*). Furthermore, in the present study, small amount of 5-OHC was found to be released by endo III, and this could be related to what was previously observed by other groups. However, the main part of the endo III-mediated cleavage of the oligonucleotide was not correlated with the yield of 5-OHC released in the supernatant. This suggests that excision of the lesion from the oligonucleotide is accompanied by a chemical change of the modified base. Further work is required to confirm this hypothesis. Nevertheless, MALDI-MS experiments revealed that when the 5-OHC-containing oligonucleotide was cleaved, 5-OHC was no more present in the fragmented oligonucleotides. Thus, whatever the mechanism of cleavage of the *N*-glycosidic bond would be, 5-OHC appears to be a substrate for both endo III and Fpg proteins. Michaelis constants were determined on the basis of the data provided by the PAGE assay.

Kinetic parameters for endo III excision of uracil glycol (Ug), 5-hydroxyuracil (5-OHU), and 5-OHC have been already determined using modified oligonucleotides as the

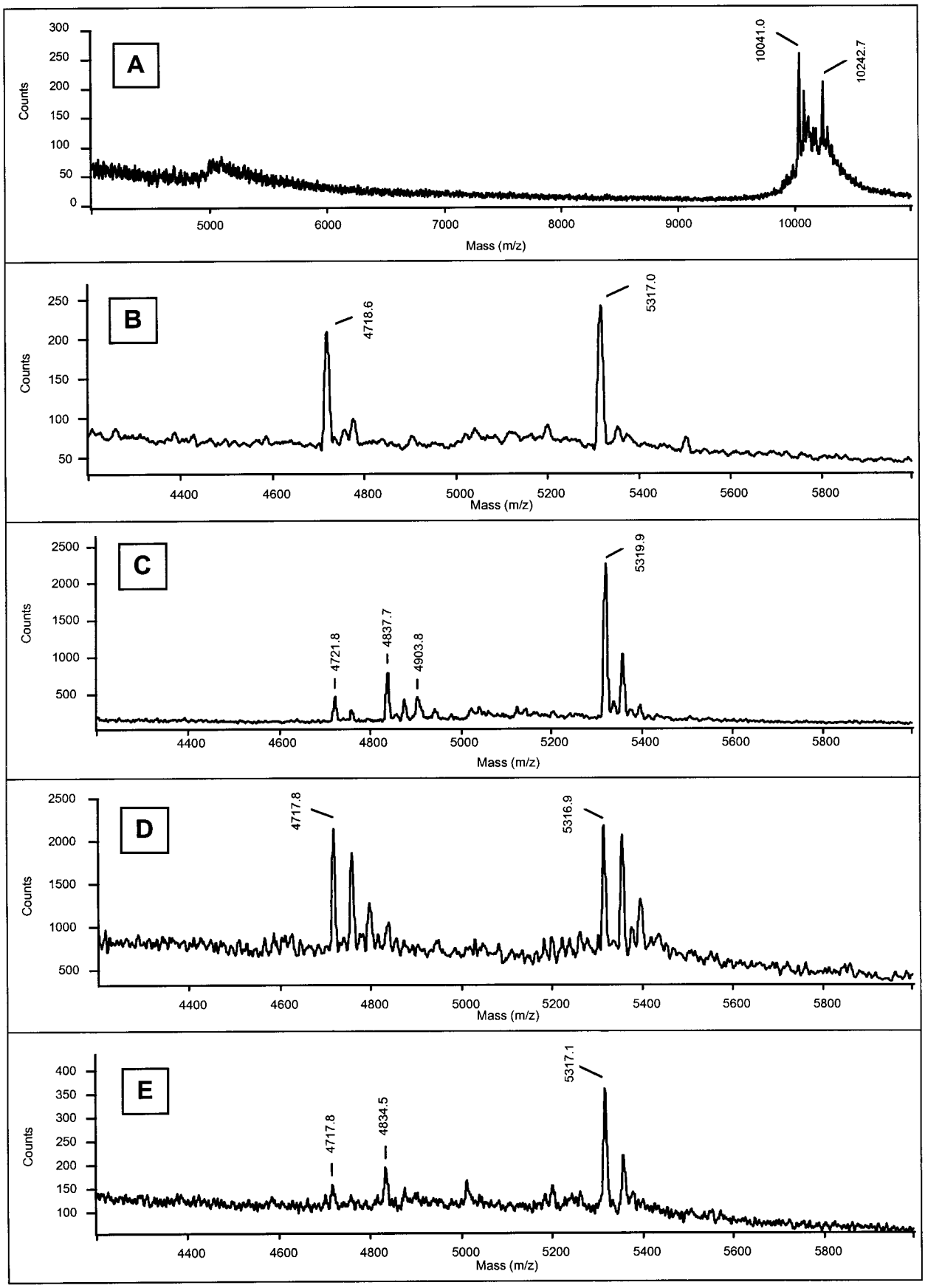

FIGURE 5: MALDI-MS analyses. (A) mass spectrum of 33-5-OHC and its complementary strand; *m*/*z* from 4000 to 11 000. (B−E) Mass spectra of the cleaved oligonucleotides (1 μM) upon incubation with either Fpg or endo III (75 ng/μL); *m*/*z* from 4200 to 6000. (B) 33-5-OHC + Fpg. (C) 33-5-OHC + endo III. (D) 33-DHT + Fpg. (E) 33-DHT + endo III.

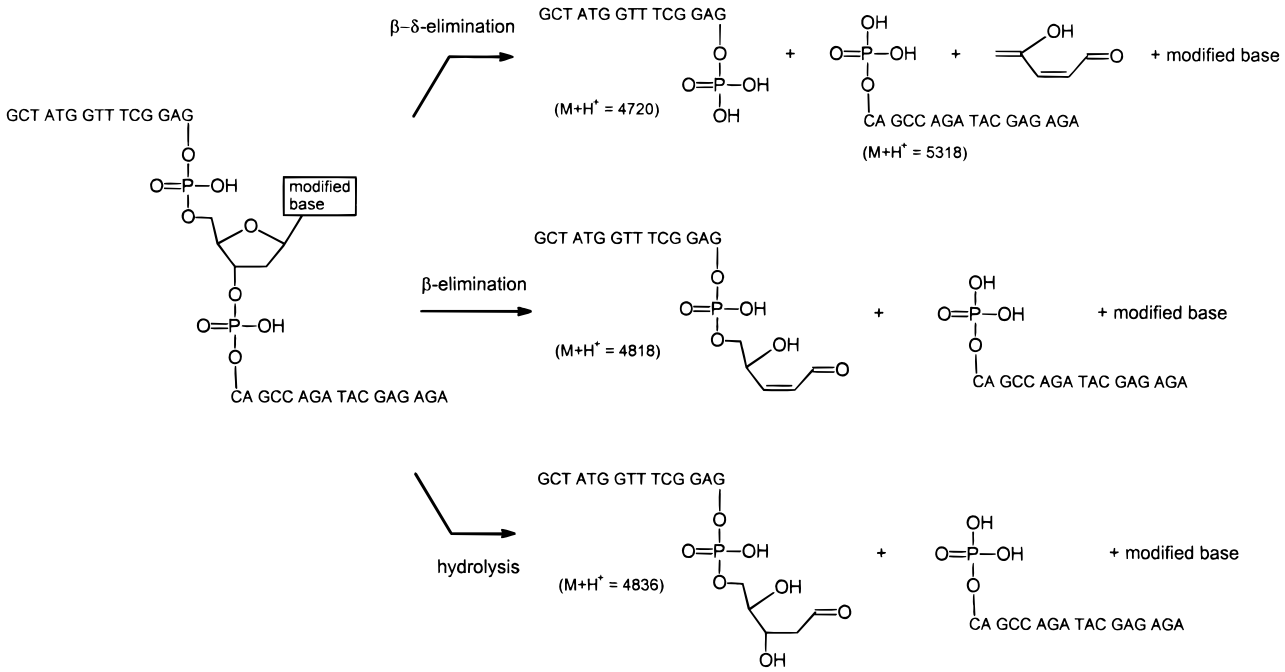

FIGURE 6: Expected released oligonucleotide products mediated by the enzymes, according to the different possible mechanisms of phosphodiester bond cleavage.

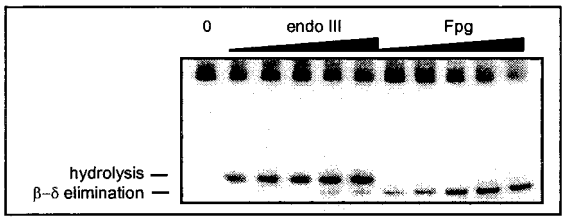

FIGURE 7: PAGE analysis of strand cleavage of 5-OHC-containing oligonucleotides (1 μM) treated by endo III (2−40 ng/μL) or Fpg (3−60 ng/μL) during 30 min.

substrates (*45*). In the present study, we report kinetic constants values for two other known substrates of endo III: Tg and DHT. Furthermore, the 5-OHC-containing oligonucleotide of the same sequence as Tg- and DHT-containing oligonucleotides was synthesized in order to enable comparison between values reported in the present study and those published by Wang and Essigman. From our results, the values of the relative V_m/K_m, as presented in Table 2, give the following preference for endo III reactivity at low concentrations of substrates: Tg ≫ 5-OHC > DHT. To our knowledge, the present work provides, for the first time, kinetic parameters for the endo III processing of Tg in comparison with other oxidized pyrimidine base (5-OHC) and a 5,6-saturated thymine that cannot undergo ring-chain tautomerism at 1−6 positions (DHT). The V_m for 5-OHC is in the same range than for Tg; however, the K_m value is about 8-fold higher for 5-OHC than for Tg. On the other hand, DHT shows about the same value of K_m as Tg. However, DHT is excised far less efficiently than Tg, as inferred from the V_m value, which is 10 times lower. To broaden the comparison between our data and those from Wang and Essigman, the value of the relative V_m/K_m constant for 5-OHC that was determined in both studies was arbitrary fixed at 1. In this respect, the relative V_m/K_m values for endo III give the following preferences at low concentration of substrate: Tg (rel. $V_m/K_m = 7.0$) > Ug (rel. $V_m/K_m = 1.3$) > 5-OHC (rel. $V_m/K_m = 1$) > DHT (rel. $V_m/K_m = 0.63$) > 5-OHU (rel. $V_m/K_m = 0.25$). Recently, a comparison was made between endo III cleavage efficiency of oligonucleotides containing either a Tg or an Ug residue (*57*). The kinetic parameters showed a higher activity of endo III for Ug than for Tg. These different conclusions for the preference of endo III toward Tg and Ug can be explained by the fact that our comparison is much more indirect, as the result of the use of a reference product (5-OHC) in attempts to correlate two studies. Nevertheless, it may be concluded that pyrimidine glycols appear to be the main substrates for endo III from comparison of the available data. However, other modified bases which exhibit a ring-chain tautomerism, such as 5-hydroxy-5-methylhydantoin, 5-hydroxyhydantoin, and imidazolinediol (*58*), may be also considered as potentially relevant substrates for endo III.

Attempts were made to compare the efficiency of 5-OHC excision by endo III and Fpg. Kinetic constants values were in the same range, thus showing that 5-OHC could be processed by the two enzymes at the same rates. Furthermore, Fpg was found to have an activity on DHT-containing oligonucleotide, however, with smaller efficiency as compared to endo III. In this case, the value of V_m was very low ($V_m = 7.8 \times 10^{-4}$ pmol/min/ng), suggesting that the Fpg activity on DHT could be not relevant under physiological conditions. Interestingly, Purmal et al. have recently shown that Tg is also substrate for Fpg at a reasonable rate (*57*). This received confirmation from the incubation of the Tg-containing oligonucleotide with Fpg (Figure 8). It appears therefore that the Fpg protein can excise a wide array of substrates, its activity not being restricted to modified purine bases and formamidopyrimidine derivatives.

Mechanistic Aspects of the Enzyme-Mediated Cleavage of the Oligonucleotides. MALDI-MS analyses were first carried out in order to confirm the removal of 5-OHC from the oligonucleotide by the repair enzymes. It turns out that the latter measurement was able to shed new light on the mechanism of the enzymatic action of endo III. Earlier

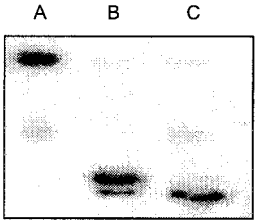

FIGURE 8: PAGE analysis of strand cleavage of Tg-containing oligonucleotide (0.1 μM): lane A, control with no enzyme; lane B, endo III (40 ng/μL), 30 min; lane C, Fpg (30 ng/μL), 30 min.

studies concluded to a β-δ-elimination mechanism for Fpg (*30*) whereas endo III is processing the base damage via a β-elimination mechanism (*11, 13*−*15*) (Figure 6). Under the present experimental conditions, Fpg, when acting on 5-OHC- or DHT-containing oligonucleotides, gave rise to fragments with molecular weights corresponding to the expected products of a β-δ-elimination mechanism. In contrast, the results of the endo III-mediated excision of modified oligonucleotides were not consistent with the previous proposed mechanism of action of the enzyme. As a striking feature, no peak corresponding to the molecular weight of the β-elimination product was observed. On the other hand, the main observed peak was assigned to the product of hydrolysis of the phosphodiester bond 3′ to the lesion. Interestingly, the detected product of hydrolysis is 18 amu higher than the β-elimination product, and thus cannot be a fragmentation of the latter oligonucleotide. A second peak in the mass spectrum was attributed to a fragment that may be accounted for by a β-δ-elimination reaction. The presence of this product was already observed in PAGE analysis at high concentrations of the enzyme. Thus, this path seems to be a secondary mode of cleavage of the oligonucleotide by the enzyme. Therefore, under our experimental conditions, within the substrates we assayed, endo III acts as a class I AP endonuclease cleaving the phosphodiester bond by a hydrolysis mechanism, as it was first believed for this type of enzyme (*59*). It turns out that MALDI-MS is a straightforward technique for assessing the mechanistic pathway of repair enzymes.

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