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Kinetics of excision of purine lesions from DNA by *Escherichia coli* Fpg protein

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ABSTRACT

The kinetics of excision of damaged purine bases from oxidatively damaged DNA by *Escherichia coli* Fpg protein were investigated. DNA substrates, prepared by treatment with H_2O_2/Fe(III)-EDTA or by γ-irradiation under N_2O or air, were incubated with Fpg protein, followed by precipitation of DNA. Precipitated DNA and supernatant fractions were analyzed by gas chromatography/isotope-dilution mass spectrometry. Kinetic studies revealed efficient excision of 8-hydroxyguanine (8-OH-Gua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde). Thirteen other modified bases in the oxidized DNA substrates, including 5-hydroxycytosine and 5-hydroxyuracil, were not excised. Excision was measured as a function of enzyme concentration, substrate concentration, time and temperature. The rate of release of modified purine bases from the three damaged DNA substrates varied significantly even though each DNA substrate contained similar levels of oxidative damage. Specificity constants (k_{cat}/K_M) for the excision reaction indicated similar preferences of Fpg protein for excision of 8-OH-Gua, FapyGua and FapyAde from each DNA substrate. These findings suggest that, in addition to 8-OH-Gua, FapyGua and FapyAde may be primary substrates for this enzyme in cells.

INTRODUCTION

Oxidative DNA damage produced by endogenously- and exogenously-generated reactive oxygen species has been implicated in mutagenesis and carcinogenesis and may play an important role in the pathogenesis of aging (reviewed in 1). Among oxygen-derived species, the hydroxyl radical is highly reactive, producing a variety of lesions in DNA (reviewed in 2,3). Most of these lesions are substrates for enzymes engaged in DNA repair in bacteria and mammalian cells (reviewed in 4–7).

Fpg protein of *Escherichia coli* (7–10) has been purified to apparent homogeneity and shown to possess N-glycosylase and β-lyase activities (11–13). Several products of oxidative DNA damage, including 8-hydroxyguanine (8-OH-Gua) (14,15), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) (15) and 4,6-diamino-5-formamidopyrimidine (FapyAde) (9,15), are substrates for this enzyme. Fpg protein appears to act weakly on 8-hydroxyadenine (8-OH-Ade) (15), but several laboratories, using different analytical techniques, did not detect this activity (16,17). Recently, 5-hydroxyuracil (5-OH-Ura) and 5-hydroxycytosine (5-OH-Cyt) were reported to be efficiently excised from duplex oligonucleotides by Fpg protein (18). The specificity of Fpg protein has been examined in terms of its binding affinity and kinetic parameters for cleavage of defined DNA substrates (17). Duplex oligonucleotides containing a single lesion were used for this purpose and a catalytic mechanism was proposed to explain the facile excision of formamidopyrimidines and 8-OH-Gua by the enzyme (17).

Simultaneous measurement of the kinetics of excision of 8-OH-Gua, FapyGua and FapyAde by *E.coli* Fpg protein from oxidatively damaged DNA has not been reported. It is not known whether Fpg protein excises these purine lesions with similar specificity from oxidatively damaged DNA, and whether the kinetics of excision depends on the nature of DNA substrate. Oxidatively damaged DNA contains a variety of pyrimidine and purine lesions (2,3). The distribution and concentration of the lesions are affected by many factors including the radical environment of DNA, and the presence or absence of oxygen. In this context, the kinetics of excision may depend on the nature of DNA substrates, which may differ from one another in terms of types, distribution and quantities of lesions.

The objective of the present study was to simultaneously measure the kinetics of excision of 8-OH-Gua, FapyGua and FapyAde by *E.coli* Fpg protein from oxidatively damaged DNA, and thus to provide a quantitative comparison of the specificity of the enzyme for these lesions under similar conditions. Furthermore,

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we wished to determine if the rate of excision was affected by the nature of the DNA substrate, using several oxidatively damaged DNA substrates. Calf thymus DNA was used as a model for these experiments. We utilized the technique of gas chromatography/isotope-dilution mass spectrometry (GC/IDMS) to measure the excision rates of purine lesions. This technique permits precise identification and quantification of numerous base lesions in a given DNA sample (3,19), and is well suited for the determination of the substrate specificity of DNA repair enzymes (15,20–23).

MATERIALS AND METHODS

Materials

Modified DNA bases, their stable isotope-labeled analogues, and materials for gas chromatography/isotope-dilution mass spectrometry (GC/IDMS) were obtained as described previously (19). Calf thymus DNA and hydrogen peroxide solution were purchased from Sigma.

Preparation of DNA substrates

Calf thymus DNA was dissolved in 10 mM phosphate buffer (pH 7.4) (0.3 mg/ml), and then dialyzed against 10 mM phosphate buffer using dialysis membranes with a molecular weight cutoff of 6000–8000. Aliquots of the DNA solution were bubbled with N_2O or air for 30 min and subsequently irradiated with γ-rays in a ^{60}Co γ-source at a dose of 80 Gy (dose rate 56 Gy/min). Another aliquot of the DNA solution was treated with 3 mM H_2O_2 in the presence of 25 μM $FeCl_3$ and 100 μM EDTA at 4°C for 30 min. $FeCl_3$ and EDTA were mixed before addition to the DNA solution. Subsequently, all DNA samples were dialyzed against 10 mM phosphate buffer (pH 7.4) for 18 h at 4°C.

Preparation of Fpg protein

The purification of homogenous Fpg protein from an overproducing strain of *E.coli* has been described elsewhere (24). The specific activity of enzyme preparations used for our studies was $> 2 \times 10^8$ U/mg.

Enzymatic assays

Aliquots of 100 μg of irradiated or H_2O_2/Fe(III)-EDTA-treated DNA samples were dried in a SpeedVac under vacuum. Samples were then dissolved in a 1 ml Eppendorf tube in the incubation mixture containing 50 mM phosphate buffer (pH 7.4), 100 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol and bovine serum albumin (0.1 mg/ml). Where indicated, 1, 2 or 5 μg of Fpg protein were added to each mixture. Some samples contained no Fpg protein, but the equivalent amount of buffer (25 mM HEPES, 200 mM NaCl, 1 mM EDTA and 50% glycerol). The total volume of the mixture was 240 μl. The enzyme was inactivated by heating at 100°C for 15 min. Three replicates of each mixture were incubated at 37°C in a water bath. Incubation time varied depending on the experiment.

For determination of excision as a function of the product concentration, 12.5, 20, 35, 50 and 75 μg of irradiated or H_2O_2/Fe(III)-EDTA-treated DNA samples were supplemented with 87.5, 80, 65, 50 and 25 μg of control DNA samples, respectively. An additional sample containing 100 μg of irradiated or H_2O_2/Fe(III)-EDTA-treated DNA samples was also used. Three replicates of these samples were incubated with 1 or 2 μg Fpg protein, or without Fpg protein at 37°C for 15 min as described above. For determination of excision as a function of incubation temperature, three replicates of 100 μg aliquots of irradiated or H_2O_2/Fe(III)-EDTA-treated DNA samples were incubated with 1 μg Fpg protein or without Fpg protein at 5, 15, 20, 25, 30 and 37°C for 15 min.

Following incubation, 540 μl of cold ethanol (–20°C) were added to each sample. Samples were kept at –20°C for 2 h and then centrifuged at 4°C for 30 min at 10000 r.p.m. DNA pellets and supernatant fractions were separated. The pellets were washed with 100 μl of a cold mixture (–20°C) of ethanol and water (80/20; v/v). DNA pellets were dried in a SpeedVac under vacuum, dissolved in 150 μl of 10 mM phosphate buffer (pH 7.4), and the concentration of DNA was determined by the absorbance at 260 nm (absorbance of 1 = 50 μg of DNA/ml). The recovery of DNA by precipitation with ethanol was ~100%.

Hydrolysis, derivatization and GC/IDMS

Aliquots of stable isotope-labeled analogues of modified DNA bases were added as internal standards to pellets with known amounts of DNA and to the supernatant fractions (19). Pellets were dried in a SpeedVac under vacuum and then hydrolyzed with 0.5 ml of 60% formic acid in evacuated and sealed tubes at 140°C for 30 min. The hydrolyzates were lyophilized in vials for 18 h. Supernatant fractions were freed of ethanol in a SpeedVac under vacuum for 30 min, and then lyophilized for 18 h. Supernatant fractions were not hydrolyzed. The derivatization of the lyophilized samples and the subsequent analysis by GC/IDMS with selected-ion monitoring were performed as described elsewhere (25).

RESULTS

Three different DNA substrates were used in this work. Sixteen and 12 modified bases were identified and quantified by GC/IDMS in DNA samples damaged under anoxic (irradiation under N_2O) and oxic conditions [H_2O_2/Fe(III)-EDTA-treatment or irradiation under air], respectively. These modified bases were FapyGua, 8-OH-Gua, FapyAde, 8-OH-Ade, 2-hydroxyadenine, 5-hydroxy-5-methylhydantoin, 5-hydroxyhydantoin, 5-OH-Ura, 5-OH-Cyt, 5-(hydroxymethyl)uracil, thymine glycol, 5,6-dihydroxyuracil, 5,6-dihydrothymine, 5,6-dihydrouracil, 5-hydroxy-6-hydrothymine and 5-hydroxy-6-hydrouracil. Oxygen inhibits the formation of the latter four compounds (26,27). The levels of the modified bases in H_2O_2/Fe(III)-EDTA-treated DNA, in DNA irradiated under N_2O and in DNA irradiated under air were 4.9±0.2 (S.D.), 7.0±0.3 and 7.8±0.3 lesions per 10^3 DNA bases, respectively.

8-OH-Gua, FapyGua and FapyAde were excised from all three DNA substrates, in agreement with a previous report (15). Figure 1 illustrates the excision of these products from H_2O_2/Fe(III)-EDTA-treated DNA. The amounts recovered in the pellets of DNA samples incubated with the heat-inactivated enzyme were similar to those found after incubation without the enzyme (data not shown). These experiments show that the amounts of products in the supernatant fractions of DNA samples incubated with the active enzyme were similar to the amounts removed from the pellets of the same samples. None of the other lesions in the DNA substrates were excised significantly.

8-OH-Gua, FapyGua and FapyAde were released from DNA by Fpg protein in a dose-dependent manner. Excision increased

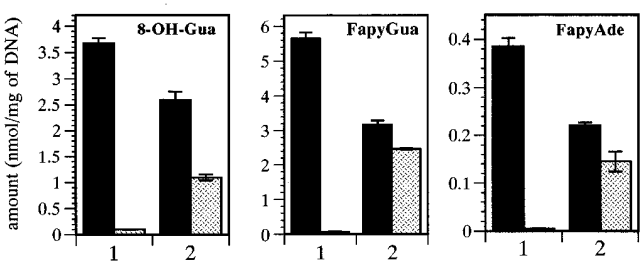

Figure 1. Excision of 8-OH-Gua, FapyGua and FapyAde by Fpg protein from DNA treated with H_2O_2/Fe(III)-EDTA. Dark columns, pellets; light columns, supernatant fractions. 1, Incubation at 37°C for 30 min without Fpg protein; 2, incubation at 37°C for 30 min with 2 µg of Fpg protein per 100 µg of DNA. Each column represents the mean (±standard deviation) of the values obtained from the analysis of three independently prepared samples. One nmol of a lesion/mg of DNA corresponds to ≈32 lesions/10^5 DNA bases.

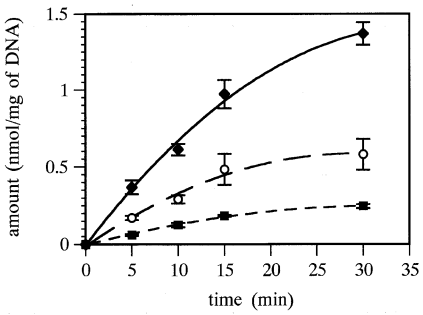

Figure 2. Excision of FapyGua (◆), 8-OH-Gua (○) and FapyAde (■) as a function of incubation time. DNA irradiated under N_2O was used. Enzyme amount was 1 µg per 100 µg of DNA. The amounts of products given on the Y-axis represent those found in supernatant fractions. Each data point represents the mean (±standard deviation) of the values obtained from the analysis of three independently prepared samples.

up to 5 µg of the enzyme and reached a plateau thereafter (data not shown). Figure 2 shows the time course of this reaction. Analysis of the data (28) revealed that excisions followed first-order kinetics.

Excision of 8-OH-Gua, FapyGua and FapyAde was determined simultaneously as a function of concentration. The product amounts in the supernatant fractions were in good agreement with those removed from pellets and were used for determination of kinetic constants. Lineweaver–Burk plots representing initial velocity versus product concentration were used to determine kinetic constants (28). Figure 3 illustrates two such analyses, which reveal a distinct dependence of FapyAde excision on the DNA substrate. The kinetic constants and corresponding standard deviations were obtained by a linear least squares analysis of the Lineweaver–Burk plots. Statistical analysis of the data was performed by the Student *t*-test. Kinetic constants and the results of the statistical analysis are given in Table 1. Values were obtained from measurements at six different concentrations of each lesion. Concentration ranges used for these measurements were: FapyAde, 0.05–0.16 µM; FapyGua, 0.53–2.35 µM; 8-OH-Gua, 0.39–1.54 µM in H_2O_2/Fe-EDTA-treated DNA; FapyAde, 0.08–0.63 µM; FapyGua, 0.47–3.38 µM; 8-OH-Gua, 0.27–1.47 µM in DNA irradiated under N_2O; FapyAde, 0.07–0.33 µM; FapyGua, 0.35–1.7 µM; 8-OH-Gua, 0.75–3.4 µM in DNA irradiated under air.

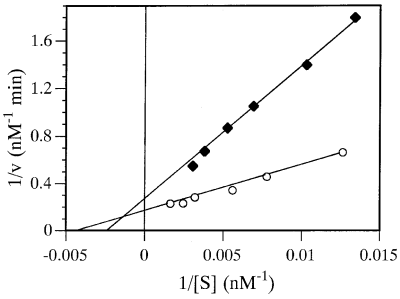

Figure 3. Lineweaver–Burk plot for excision of FapyAde from DNA irradiated under air (◆), and from DNA irradiated under N_2O (○). Enzyme amount was 1 µg per 100 µg of DNA. Incubation time was 15 min. [S], concentration of FapyAde; v, initial velocity. The amounts of products found in supernatant fractions were used for initial velocity.

Table 1. Catalytic constants (k_{cat}) for excision of FapyAde, FapyGua and 8-OH-Gua by Fpg protein

DNA substrate	k_{cat} (min^{-1})			
	FapyAde	FapyGua	8-OH-Gua	
	(1)	(2)	(3)	
1 µg Fpg protein/100 µg DNA:				
H_2O_2/Fe-EDTA	0.054±0.015^{b,c,d}	0.446±0.050^{a,b,d}	0.127±0.014^{b}	(1)
irradiation/N_2O	0.048±0.003^{b,c,d}	0.267±0.030^{b,d}	0.111±0.012	(2)
irradiation/air	0.027±0.003^{c,d}	0.067±0.013	0.082±0.003	(3)
2 µg Fpg protein/100 µg DNA:				
H_2O_2/Fe-EDTA	0.106±0.010^{a,b,c,d}	0.625±0.060^{a,b,d}	0.178±0.021^{b}	(1)
irradiation/N_2O	0.044±0.005^{b,c,d}	0.230±0.037^{b}	0.160±0.016	(2)
irradiation/air	0.026±0.009^{c,d}	0.075±0.018	0.112±0.011	(3)

[a]Statistically different from the value in line 2 ($P < 0.05$).
[b]Statistically different from the value in line 3 ($P < 0.05$).
[c]Statistically different from the value in column 2 ($P < 0.05$).
[d]Statistically different from the value in column 3 ($P < 0.05$).

Table 2. Michaelis constants (K_M) for excision of FapyAde, FapyGua and 8-OH-Gua by Fpg protein

DNA substrate	K_M (nM)			
	FapyAde	FapyGua	8-OH-Gua	
	(1)	(2)	(3)	
1 µg Fpg protein/100 µg DNA:				
H_2O_2/Fe-EDTA	412±79^{a,c,d}	2761±330^{a,b,d}	1004±303	(1)
irradiation/N_2O	275±14^{b,c,d}	1037±139	861±72	(2)
irradiation/air	424±39^{c,d}	1250±194	914±37	(3)
2 µg Fpg protein/100 µg DNA:				
H_2O_2/Fe-EDTA	1632±320^{a,b,c}	7063±955^{a,b,d}	2405±291^{a}	(1)
irradiation/N_2O	389±78^{c,d}	1384±182	1406±108	(2)
irradiation/air	509±122^{c,d}	1490±278	2029±602	(3)

[a]Statistically different from the value in line 2 ($P < 0.05$).
[b]Statistically different from the value in line 3 ($P < 0.05$).
[c]Statistically different from the value in column 2 ($P < 0.05$).
[d]Statistically different from the value in column 3 ($P < 0.05$).

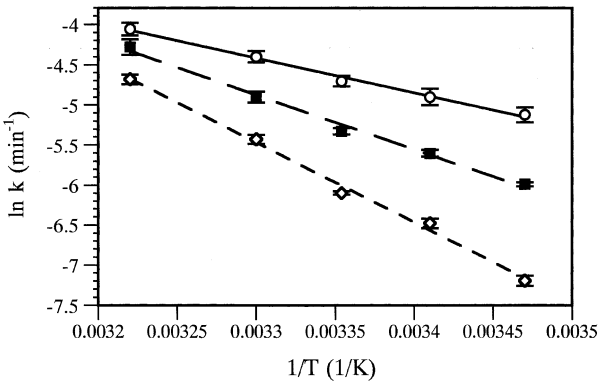

Figure 4. Dependence of first-order rate constants (k) for excision of 8-OH-Gua (◇), FapyGua (■) and FapyAde (○) on temperature in the range from 15°C to 37°C. DNA treated with H_2O_2/Fe(III)-EDTA was used. Enzyme amount was 1 µg per 100 µg of DNA. Incubation time was 15 min. The amounts of products found in supernatant fractions were used for the calculation of rate constants. Each data point represents the mean (±standard deviation) of the values obtained from the analysis of three independently prepared samples.

Table 3. Specificity constants (k_{cat}/K_M) for excision of FapyAde, FapyGua and 8-OH-Gua by Fpg protein

DNA substrate	$k_{cat}/K_M \cdot 10^3$ (min$^{-1} \cdot$ nM^{-1})			
	FapyAde	FapyGua	8-OH-Gua	
	(1)	(2)	(3)	
1 µg Fpg protein/100 µg DNA:				
H_2O_2/Fe-EDTA	0.132±0.035[b]	0.162±0.018[a,b]	0.127±0.014[b]	(1)
irradiation/N_2O	0.176±0.012[b]	0.257±0.029[b,d]	0.130±0.013[b]	(2)
irradiation/air	0.063±0.007[d]	0.053±0.011[d]	0.090±0.003	(3)
2 µg Fpg protein/100 µg DNA:				
H_2O_2/Fe-EDTA	0.065±0.006[a]	0.090±0.009[a,b]	0.074±0.012[a,b]	(1)
irradiation/N_2O	0.112±0.012[b]	0.165±0.026[b]	0.114±0.012[b]	(2)
irradiation/air	0.052±0.0017	0.050±0.010	0.055±0.005	(3)

[a]Statistically different from the value in line 2 ($P < 0.05$).
[b]Statistically different from the value in line 3 ($P < 0.05$).
[c]Statistically different from the value in column 2 ($P < 0.05$).
[d]Statistically different from the value in column 3 ($P < 0.05$).

Excision of 8-OH-Gua, FapyGua and FapyAde was determined as a function of temperature between 5 and 37°C. At 5°C, no significant excisions were observed. According to the Arrhenius equation (28), the logarithm of the first-order rate constants (ln k) was plotted against the reciprocal of the absolute temperature (1/T). Figure 4 presents Arrhenius plots for excision from H_2O_2/Fe(III)-EDTA-treated DNA. Activation energies (E_a) and corresponding standard deviations, obtained by the linear least squares analysis of the plots, are given in Table 4, along with the results of the statistical analysis of the data. Values were obtained from measurements at five different temperatures.

Table 4. Activation energies (E_a) for excision of 8-OH-Gua, FapyGua and FapyAde by Fpg protein

DNA substrate	$k_{cat}/K_M \cdot 10^3$ (min$^{-1} \cdot$ nM^{-1})			
	8-OH-Gua	FapyGua	FapyAde	
	(1)	(2)	(3)	
H_2O_2/Fe-EDTA	83.9±3.5[b,c,d]	59.6±3.2[a,b,d]	36.1±1.7[a]	(1)
irradiation/N_2O	90.8±4.3[b,d]	83.3±3.4[b]	74.3±1.9[b]	(2)
irradiation/air	66.1±0.6	48.8±1.4	31.0±3.	(3)

[a]Statistically different from the value in line 2 ($P < 0.05$).
[b]Statistically different from the value in line 3 ($P < 0.05$).
[c]Statistically different from the value in column 2 ($P < 0.05$).
[d]Statistically different from the value in column 3 ($P < 0.05$).

DISCUSSION

We have studied the kinetics of excision of purine lesions by *E.coli* Fpg protein from three differently prepared DNA substrates. Each DNA substrate contained a variety of pyrimidine- and purine-derived lesions. In this respect, this work differs from structure-function studies that use a single chemically-defined lesion embedded in an oligonucleotide substrate at a defined position (14,17,29). Previously, the excision of 8-OH-Gua, FapyGua and FapyAde by *E.coli* Fpg protein from oxidatively damaged DNA has been demonstrated using GC/MS; however, the kinetics of excision of these lesions has not been reported (15). The present paper is the first report on the simultaneous measurement of the kinetics of excision of 8-OH-Gua, FapyGua and FapyAde by *E.coli* Fpg protein from oxidatively damaged DNA. The salient feature of this work is the evidence that these purine lesions were excised by Fpg protein with similar specificity from various oxidatively damaged DNA substrates and there was a significant influence of the nature of DNA substrate on the efficiency of excision. The results suggest that, in addition to 8-OH-Gua which is thought to be the main physiological substrate of Fpg protein (14,17,29), FapyGua and FapyAde may be important physiological substrates of this enzyme. It should be pointed out that calf thymus DNA was used as a model for the present study. The experimental conditions used may not be necessarily similar to those found *in vivo*.

Values for k_{cat} (maximum velocity/[enzyme]) and K_M were generally higher for excision of 8-OH-Gua, FapyGua and FapyAde from H_2O_2/Fe(III)-EDTA-treated DNA than from other substrates. Kinetic constants varied significantly. For example, k_{cat} for excision of FapyGua from H_2O_2/Fe(III)-EDTA-treated DNA was twice and seven times as high as k_{cat} for excision from DNA substrates irradiated under N_2O and under air, respectively. Values for k_{cat}/K_M were greater for excision from DNA irradiated under N_2O than from other substrates, indicating the preferred excision of lesions from this DNA substrate. The preference for FapyGua excision was pronounced as k_{cat}/K_M was almost twice as high as the comparable value for H_2O_2/Fe(III)-EDTA-treated DNA and three to five times higher than for DNA irradiated under air. The k_{cat}/K_M ratios for excision from H_2O_2/Fe(III)-EDTA-treated DNA were greater than those for excision from DNA irradiated under air. Taken together, these results indicate a strong dependence of excision of purine lesions by Fpg protein on the nature of the DNA substrate.

A comparison of kinetic constants for the same DNA substrate reveals that k_{cat}/K_M ratios for excision of 8-OH-Gua, FapyGua and FapyAde from H_2O_2/Fe(III)-EDTA-treated DNA were similar, indicating comparable preference of the enzyme for these lesions. The k_{cat}/K_M ratio for excision of FapyGua from DNA irradiated under N_2O was significantly greater than that for excision of 8-OH-Gua and similar to that for excision of FapyAde. FapyGua and FapyAde showed similar k_{cat}/K_M ratios for DNA irradiated under air. In this case, k_{cat}/K_M for excision of 8-OH-Gua was significantly greater than for excision of FapyGua and FapyAde. When the concentration of the enzyme was doubled, no significant differences between the k_{cat}/K_M ratios for excision of 8-OH-Gua, FapyGua and FapyAde were observed for any of the DNA substrates. These results suggest a comparable preference of the enzyme for all three lesions.

The measurement of base excision as a function of temperature revealed a profound dependence of this reaction on temperature. 8-OH-Gua had the highest activation energy (E_a) in the case of H_2O_2/Fe(III)-EDTA-treated DNA and DNA irradiated under air. In both cases, E_a of FapyGua was greater than that of FapyAde. With DNA irradiated under N_2O, E_a of 8-OH-Gua was similar to that of FapyGua, but greater than that of FapyAde. FapyAde had the lowest E_a in all cases. Significant differences between DNA substrates were noted. Values of E_a for excision from DNA irradiated under N_2O were higher than those for excision from other substrates. Values of E_a were lowest for DNA irradiated under air. Taken together, the results indicate a significant dependence of activation energies on the nature of the DNA substrate and a significant difference between activation energies for each DNA substrate tested.

Differences in the mechanisms by which H_2O_2/Fe(III)-EDTA and ionizing radiation cause damage to DNA may affect the excision rates of modified purines. Oxygen profoundly modifies radiation damage in DNA (30), and alters the distribution of lesions (3,26,27). Ionizing radiation may generate multiply damaged sites consisting of base damages and strand breaks within less than 20 base pairs (31). Closely spaced lesions on opposite strands of DNA may present difficulties for DNA repair enzymes (32,33). There may be differences between end groups due to strand breaks in DNA. The ratio of base damage to strand breaks among DNA substrates and the nucleotide sequence context of damage may vary. In fact, there is evidence that the excision of 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine by Fpg protein from methylated, alkali-treated DNA shows some sequence specificity (34). Some or all of these factors may significantly influence the kinetics of excision of modified bases from different DNA substrates. The methodology used in this work is not conducive to a precise molecular explanation of the effect of different DNA substrates on excision rates.

Excision of synthetically prepared 5-OH-Ura and 5-OH-Cyt by Fpg protein from defined oligonucleotides containing a single lesion has been observed (18). In our study, these oxidized pyrimidines were not excised from any of three DNA substrates tested, all of which contained substantial amounts of these products. Generally, enzymatic excision of single oxidized bases from oligodeoxynucleotide substrates parallels their excision from oxidized DNA. The reason for the apparent discrepancy is unclear.

The action of Fpg protein on defined oligodeoxynuclotides containing 8-OH-Gua and structurally related lesions has been systematically investigated; binding parameters and specificity constants were related to the three dimensional structure of duplex DNA containing either 8-OH-Gua:Cyt or 8-OH-Gua:Ade (17,35,36). The presence of the 8-oxo function in the major groove of DNA correlates with the apparent binding affinity of Fpg protein for its several substrates. Both 8-oxo and 6-oxo functions are present in the major groove in bases that were efficiently excised from duplex DNA. A catalytic mechanism involving attack at C1′ by a nucleophilic residue in Fpg protein, accompanied by O-protonation of the 6-oxo group has been postulated to explain these observations (17,37). Similar mechanisms have been proposed for Fpg protein and other DNA *N*-glycosylases by Lloyd *et al.* (38,39). The kinetic data presented here for excision of 8-OH-Gua and FapyGua support these mechanisms and account for the lack of excision that was noted when Fpg protein acts on damaged DNA containing 8-OH-Ade, 5-OH-Ura or 5-OH-Cyt. However, they fail to explain satisfactorily the efficient excision of FapyAde. The latter may exist in two rotameric forms as was shown for another formamidopyrimidine (40), and the conformational flexibility inherent in ring-opened structures may be important if modified bases are flipped out of the duplex prior to excision by the cognate DNA glycosylase (41). Comparative studies of the chemical and biological properties of 8-OH-Ade and FapyAde will be required to elucidate the interaction of these lesions with Fpg protein.

In conclusion, we have shown that Fpg protein excised 8-OH-Gua, FapyGua and FapyAde efficiently from three heterogenous DNA substrates. The rates of excision of these lesions among DNA substrates varied, even though each substrate contained similar levels of oxidative damage. Differences in the mechanisms of DNA damage may account for these observations. The simultaneous measurement of the kinetic constants revealed that the enzyme had similar specificity for 8-OH-Gua, FapyGua and FapyAde. These data suggest that, in addition to 8-OH-Gua, FapyGua and FapyAde may be important physiological substrates for this enzyme. The results also emphasize the utility of the GC/IDMS technique as an assay of high specificity and sensitivity to measure the kinetics of excision of oxidatively damaged bases from DNA by DNA repair enzymes.

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