3′- and 5′-Strand Cleavage Reactions Catalyzed by the Fpg Protein from *Escherichia coli* Occur via Successive β- and δ-Elimination Mechanisms, Respectively†

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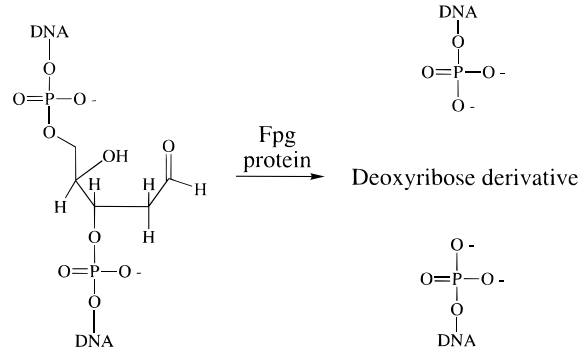
ABSTRACT: The Fpg protein from *Escherichia coli* is a multifunctional protein that excises damaged purine bases from DNA to generate aldehydic abasic sites and then catalyzes the successive cleavage of the phosphodiester bonds first on the 3′-side and then on the 5′-side of the abasic site to generate 5′- and 3′-phosphate ends, respectively, thereby excising the deoxyribose residue. The mechanisms of the 3′- and 5′-strand cleavage reactions have been studied by nuclear magnetic resonance spectroscopy (NMR) and gas chromatography−mass spectrometry (GC−MS). The 3′-strand cleavage reaction is a β-elimination reaction in which the 2′-hydrogen is abstracted and the 3′-phosphate is eliminated. The 5′-strand cleavage reaction is a δ-elimination reaction in which the 4′-hydrogen is abstracted and the 5′-phosphate is eliminated. Two types of experiments were performed to establish the occurrence of the sequential elimination reactions. First, when the reaction was performed in $H_2{}^{18}O$, ^{31}P NMR demonstrated that neither phosphate group contained ^{18}O. Second, the five-carbon product derived from the deoxyribose residue was stabilized by reduction with $NaBH_4$ and characterized by GC−MS. The mass spectrum of the reduced product was identical to that of authentic 4-oxo-2-pentenal, the tautomerized product of successive β- and δ-elimination reactions.

The Fpg protein from *Escherichia coli* [also known as fapy glycosylase (Boiteux *et al*., 1990), 8-oxoguanine glycosylase (Tchou *et al*., 1991), or MutM protein (Michaels *et al*., 1991)] is a DNA repair enzyme that is involved in the repair of DNA containing modified purine bases such as 2,6-diamino-4-oxo-5-(*N*-methylformamido)pyrimidine (methyl fapy) and 8-oxoguanine. The *fpg* gene encoding the Fpg protein has been cloned and encodes a protein of 269 amino acids (Boiteux *et al*., 1987). The enzyme has been purified to homogeneity (Boiteux *et al*., 1990) and is a globular monomer with a molecular weight of 30.2 kDa. This enzyme has at least three activities: (1) a DNA glycosylase activity in which various damaged bases are excised from DNA to produce an aldehydic abasic site; (2) an AP[1] nicking activity that cleaves both the 3′- and 5′-phosphodiester bonds at an AP site, leaving a gap in the DNA that has phosphate groups on both the 5′- and 3′-ends (Bailly *et al*., 1989; O'Connor & Laval, 1989) (Scheme 1); and (3) a deoxyribophosphodiesterase (dRpase) activity (Graves *et al*., 1992). Because of the multifunctional nature and broad substrate specificity of this enzyme, it has been referred to as Fpg protein instead of fapy glycosylase (Boiteux *et al*., 1990).

Scheme 1



The mechanisms by which the Fpg protein catalyzes these reactions have not been known. In this article we report the mechanisms of the AP nicking activities of the Fpg protein. Three mechanistic possibilities by which the Fpg protein can catalyze this reaction can be envisaged as outlined in Scheme 2: (1) the Fpg protein can hydrolyze both the 3′- and 5′-phosphodiester bonds (Scheme 2, path a); (2) the Fpg protein can abstract the 2′-proton to cleave the 3′-phosphodiester bond *via* a β-elimination reaction and hydrolyze the 5′-phosphodiester bond (Scheme 2, path b); and (3) the Fpg protein can cleave the 3′-phosphodiester bond *via* a β-elimination reaction as in path b but can cleave the 5′-phosphodiester bond *via* a δ-elimination reaction after abstracting the 4′-proton (Scheme 2, path c). The five-carbon aldehyde released *via* path a (hydrolysis, hydrolysis) will be 2-deoxyribose (I), *via* path b (β-elimination, hydrolysis) it will be 4,5-dihydroxy-2-pentenal (II), and *via* path c (β-elimination, δ-elimination) it will be 4-oxo-2-pentenal (III).

On the basis of indirect evidence using DNA which was 1′,2′-[^{3}H]-labeled and 5′-[^{32}P]-labeled at an abasic site, Bailly

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[1] Abbreviations: A_7, d(CGCACGC); AP, apurinic/apyrimidinic; D_7, d(GCGDGCG), where D is an aldehydic abasic site; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; EI-MS, electron impact mass spectrometry; GC−MS, gas chromatography−mass spectrometry; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TEAA, triethylammonium acetate; U_7, d(GCGUGCG).

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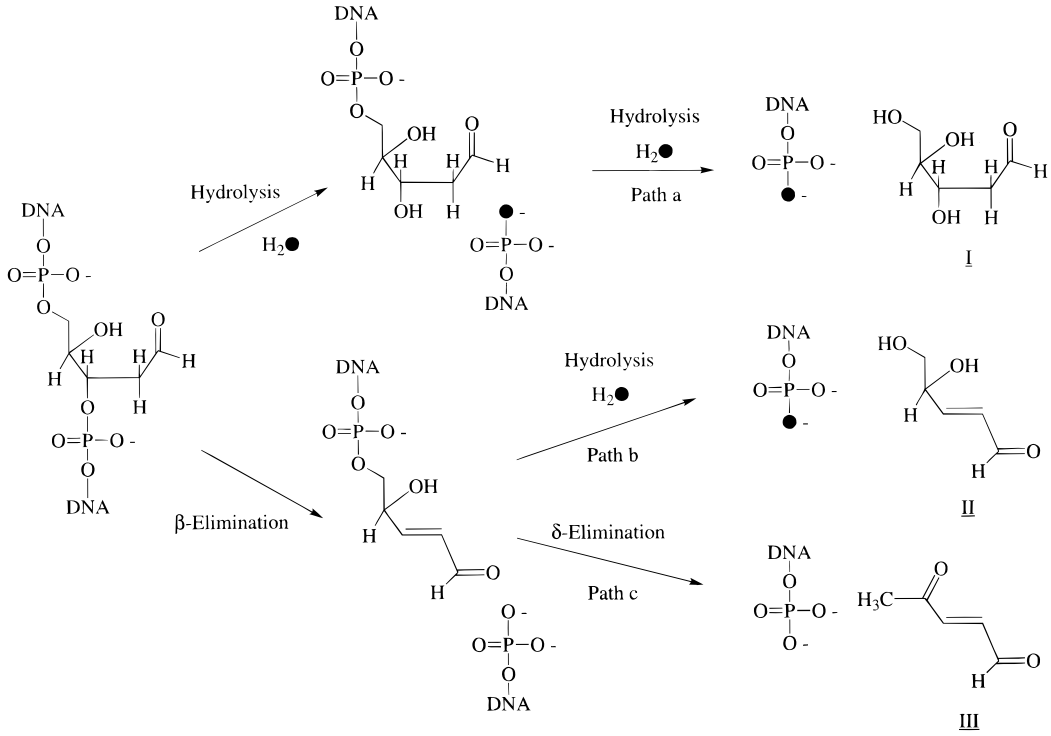
Scheme 2



et al. (1989a) proposed that the Fpg protein abstracts 2′- and 4′-hydrogens and cleaves the 3′- and 5′-phosphodiester bonds *via* successive β- and δ-elimination reactions (Scheme 2, path c). However, in the absence of direct experimental evidence, all three mechanistic possibilities must be considered. Although all three mechanistic possibilities lead to both 3′- and 5′-phosphate ends, the five-carbon deoxyribose derivative released (I, II, or III in Scheme 2) will be different.

We report the results of two independent sets of experiments that were performed to distinguish between these possible mechanisms. First, the Fpg reaction was performed in ^{18}O water, and the 3′- and 5′-phosphate ends of the Fpg reaction products were analyzed by 200 MHz ^{31}P NMR spectroscopy for the ^{18}O content. Second, the five-carbon deoxyribose derivative released by the reaction was identified by GC−MS and compared with the authentic expected products (I, II, or III). In both experiments, the results were consistent with successive β- and δ-elimination reactions (Scheme 2, path c).

MATERIALS AND METHODS

2-Deoxyribose was purchased from Aldrich, and $[^{18}O]H_2O$ was supplied by Isotec Inc. (Miamisburg, OH). Oligonucleotides were purchased from either Oligos Etc. Inc. (Wilsonville, OR) or the Protein Nucleic Acid Laboratory, University of Maryland, College Park. A Reacti-Vap evaporator, bis(trimethylsilyl)trifluoroacetamide with 1% chlorotrimethylsilane, and dry acetonitrile were purchased from Pierce. The PCR themocycler was purchased from Eppendorf. Mermaid kits were from Bio 101, Inc. (La Jolla, CA), and NAP-5 columns were from Pharmacia. All NMR and GC−MS analyses were performed at the University of Maryland, College Park. The N-terminal amino acid sequence analysis of Fpg protein was performed at the Protein Nucleic Acid Laboratory, University of Maryland, College Park.

Amplification of the fpg Gene from Chromosomal DNA by PCR. An *Eco*RI site at the beginning and a *Hin*dIII site at the end of the *fpg* gene were engineered using the primers d(GC**GAATTC**ATGCCTGAATTACC) and d(GC**AAGCTT**-TTACTTCTGGCAC), respectively. A 100 μL reaction mixture containing 1 μg of genomic DNA isolated from *E. coli* strain HB1100 (obtained from the *E. coli* Genetic Stock Center, Yale University) as template, 1 μM of each primer, 1 mM total dNTPs, and 1.5 mM $MgCl_2$ was incubated at 94 °C for 2 min, and then 2.5 units of *Taq* DNA polymerase was added. The reaction mixture was layered with 40 μL of silicone oil. The amplification of the *fpg* gene was carried out by PCR (using an Eppendorf thermocycler) using 30 cycles (2 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C).

Determination of the DNA Sequence of the Cloned Fragment. The *Eco*RI/*Hin*dIII fragment containing the entire *fpg* gene was cloned into pKK223-3 to form an overexpression plasmid pFapy-2. The sequence of the *fpg* gene in pFapy-2 was determined by the Sanger dideoxy method using a Sequenase 2.0 kit (USB) and the following series of primers: a universal sequencing primer for pKK223-3 (Pharmacia), d(GCGAATTCATGCCTGAATTACC), d(G-CAAGCTTTTACTTCTGGCAC), d(ATTTAGGGATGTCT-GGC), d(GCGACGATTTCAATGGT), and d(TGATTAAA-GCGGTGTTG). The sequencing reactions were analyzed as described by Sambrook *et al*. (1989).

Purification of Fpg Protein. The purification procedure followed that described by Boiteux *et al*. (1990) with minor modifications. β-Mercaptoethanol and glycerol were omitted from the buffers used for the Phenyl-Superose column since β-mercaptoethanol could inhibit the δ-elimination reaction (M. Bhagwat and J. A. Gerlt, unpublished observations) or react with the five-carbon fragment derived from the abasic site and glycerol could interfere with product identification by NMR spectroscopy. The purity of the Fpg protein was determined by SDS−PAGE, and the Fpg protein was stored at 4 °C as eluted from the Phenyl-Superose column or as a suspension containing 500 mg of ammonium sulfate/mL.

Reaction of Fpg Protein with a Duplex Containing an Aldehydic Abasic Site. The reaction of Fpg protein with a duplex containing an aldehydic abasic site was analyzed by both polyacrylamide gel electrophoresis and HPLC.

Analysis by Polyacrylamide Gel Electrophoresis. d(GCGUGCG) (U_7, 10 nmol, 0.5 OD) was 5′-end labeled using [γ-^{32}P]ATP and purified from a 20% polyacrylamide gel using the Mermaid kit (Bio 101 Inc.). After purification, the labeled U_7 was dissolved in 250 μL of water. An oligonucleotide containing an aldehydic abasic site (D_7, where D represents the centrally located aldehydic abasic site) was generated from U_7 by reaction of 2.5 μL of the solution containing the end-labeled oligonucleotide with 50−100 ng of uracil−DNA glycosylase in 50 mM HEPES-KOH, pH 7.6, 100 mM NaCl, and 1 mM EDTA at 37 °C for 80 min in a final volume of 10 μL. The complementary strand d(CGCACGC) (A_7, 100 pmol) was added to prepare a double-stranded duplex containing an aldehydic abasic site. Reactions in the presence of varying amounts of Fpg were performed at 37 °C for 2 h or at 17 °C for 2 h. The reactions were analyzed using a 20% denaturing polyacrylamide gel after adding one-sixth of the volume of the gel loading buffer.

Analysis by HPLC. U_7 (1 OD, 10 μL) was incubated overnight with 1−1.5 μg of uracil−DNA glycosylase in 50 mM HEPES-KOH, pH 7.6, containing 100 mM NaCl and 1 mM EDTA at room temperature. An aliquot of the reaction was analyzed by HPLC for the complete excision of uracil using a C_8 column (Rainin, 300 Å particle size). The mobile phase consisted of 100 mM triethylammonium acetate (TEAA), pH 7.0, from pump A and acetonitrile from pump B at a total flow rate of 1 mL/min. The column was equilibrated with 5% acetonitrile−95% 100 mM TEAA, pH 7.0, prior to loading the sample. The oligonucleotides were eluted by holding the mobile phase at 5% acetonitrile−95% 100 mM TEAA, pH 7.0, for 5 min and then using a gradient of 5% acetonitrile−95% 100 mM TEAA, pH 7.0, to 15% acetonitrile−85% 100 mM TEAA, pH 7.0, in 50 min. Typically, uracil eluted at 4 min, the aldehydic abasic site containing oligonucleotide (D_7) at ∼34.5 min, and unreacted U_7 at ∼36 min. A_7 (1 OD) was added either directly to the reaction mixture or after removal of uracil using an NAP-5 column. The reaction with Fpg protein (4−8 μg) was performed at 17 °C for 20 min. An aliquot was analyzed by HPLC. The reaction products d(GCGp) and d(pGCG) coeluted as a broad peak at ∼22 min.

The presence of d(GCGp) was confirmed using the 3′-phosphatase activity of T_4 polynucleotide kinase. The reaction conditions used for the 3′-phosphatase activity of polynucleotide kinase were as follows. The Fpg reaction was diluted with a equal volume of 0.2 M imidazole hydrochloride, pH 5.0, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 unit of T_4 polynucleotide kinase. The mixture was incubated at 37 °C for 30 min. An aliquot of the reaction mix, when analyzed by HPLC, showed a decrease in the peak at 22 min and an appearance of a new peak at ∼25 min. Authentic d(GCG) (from Oligos Etc. Inc.) also eluted at ∼25 min.

The presence of d(pGCG) was confirmed using the 5′-phosphatase activity of alkaline phosphatase. An aliquot of the polynucleotide kinase reaction was treated with 1 unit of alkaline phosphatase in 100 mM Tris-HCl, pH 8.5, containing 10 mM $MgCl_2$ and 0.07 mM $ZnCl_2$. The mixture was incubated at 37 °C for 30 min. An aliquot of this reaction, when analyzed by HPLC, showed a further decrease in the area under the peak at 22 min and an increase in the d(GCG) peak at ∼25 min.

Large-Scale Reaction of Fpg Protein with D_7−A_7 in [^{18}O]-H_2O and Sample Preparation for ^{31}P NMR Spectroscopy. The reaction of uracil−DNA glycosylase with 24 OD of U_7 to create an abasic site (D) was performed as in the previous section. An aliquot was checked by HPLC for complete excision of uracil. Uracil was removed from the reaction mix using a NAP-5 column, and the effluent containing D_7 was lyophilized. The complementary strand containing A opposite D (A_7, 25 OD) was also lyophilized. The D_7 and A_7 were resuspended together in 30 μL of water, 20 μL of buffer (1 M HEPES-KOH, pH 7.6, containing 2 M NaCl and 20 mM EDTA), and 400 μL of [^{18}O]H_2O. Fpg protein (275 μg in 275 μL of buffer containing [^{16}O]H_2O) was added, and the reaction proceeded at 17 °C for 30 min. An aliquot was checked by HPLC to determine the extent of both the 3′- and the 5′-strand cleavage reactions. The reaction mixture was passed through a column of Chelex-100 (0.5 mL), which was then washed with 1.5 mL of water. The eluents were combined and lyophilized. The residue was resuspended in 500 μL of D_2O and 20 μL of 500 mM EGTA. The pH was adjusted to 8.1, and the solution was passed through a second column of Chelex-100. Proton-decoupled and proton-coupled ^{31}P NMR spectra were recorded at 200 MHz using a Bruker AMX-500 NMR spectrometer equipped with a ^{31}P probe operating at 300 K. Phosphoric acid (85%) was used as the external standard.

Synthesis of Authentic Deoxyribose-Derived Products. 2-Deoxypentitol was synthesized from 2-deoxyribose by the procedure of Rabow *et al*. (1990). 4,5-Dihydroxy-2-pentenal was synthesized by the method of Esterbaur *et al*. (1975). 4-Oxo-2-pentenal was synthesized from 2-methylfuran by ring opening with bromine (Ohno *et al*., 1990).

Reaction of Fpg Protein with D_7−A_7 for Analysis by GC−MS. U_7 (5 OD) was treated with uracil−DNA glycosylase as described previously for the analysis of the reaction of Fpg protein with U_7 by HPLC. An aliquot was checked by HPLC for completion of the uracil−DNA glycosylase reaction. Uracil was removed from the reaction by using a NAP-5 column. After lyophilization, the residue was resuspended in 100 μL of reaction buffer (same as for uracil−DNA glycosylase reaction) containing 5 OD of A_7. Fpg protein (20−40 μg) was added, and the reaction proceeded at 17 °C for 20 min. A negative control reaction was also performed simultaneously in which the Fpg protein was substituted with the buffer in which the Fpg protein was stored. At the end of 20 min, each reaction mixture was diluted to 800 μL and reduced with 200 μL of 1 M $NaBH_4$ at 4 °C for 1−2 h. The reaction was passed through a column of AG 501-X8 (Bio-Rad) in a Pasteur pipet. The column was washed with 2−3 mL of water. The eluents were pooled and lyophilized in a round-bottom flask. The residue was dissolved in 1−1.2 mL of methanol and transferred to a reaction vessel (Pierce). Methanol was removed by rotary evaporation or using a Reacti-Vap evaporator (Pierce). The remaining residue from the round-bottom flask was further transferred to the reaction vessel by washing the round-bottom flask twice with methanol (400−800 μL). After concentration, the residue in the reaction vessel was further dried by dissolving in 100−200 μL of ethanol and concentrating *in vacuo* or using a Reacti-

Vap evaporator. The reaction vessel was then stored over P_2O_5 under vacuum for at least 12 h to dry completely. *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide containing 1% chlorotrimethylsilane (either 7.5 or 15 μL) and an equal volume of dry acetonitrile (all reagents from Pierce) were added, and the contents were dissolved by shaking at room temperature for 30 s. Trimethylsilylation was performed under N_2 at 55 °C for 1 h. The samples were analyzed by 70 eV EI-MS on a Hewlett-Packard 5890GC/5988 MS with a 12 m DB-1 column (J & W Scientific). The samples were loaded at 50 °C and eluted with a temperature gradient of 15 °C/min.

RESULTS AND DISCUSSION

The *fpg* gene from *E. coli* (strain HB1100; Boiteux *et al*., 1987) was amplified using PCR so that we could obtain large amounts of the Fpg protein free of glycerol and β-mercaptoethanol that could interfere with the identification of the reaction products. The *Eco*RI/*Hin*dIII fragment containing the entire *fpg* gene so produced was cloned into pKK223-3 to form the overexpression plasmid pFapy-2. This plasmid places expression of the Fpg protein under the control of the strongly inducible *tac* promoter. The sequence of the cloned *Eco*RI/*Hin*dIII fragment was determined by the Sanger dideoxynucleotide sequencing method. The sequence was in agreement with the published sequence of the *fpg* gene from *E. coli* with the exception of the 266th codon (Boiteux *et al*., 1987); this codon was CAA instead of CAG, which represents a silent mutation (both codons encode Gln).

The Fpg protein was overexpressed and purified to homogeneity. To test the AP nicking activity of the Fpg protein, an aldehydic abasic site (D) was created by the action of uracil−DNA glycosylase on the heptamer d(GCGUGCG) (U_7). The reaction of the Fpg protein with the D_7-A_7 duplex was analyzed by both polyacrylamide gel electrophoresis and HPLC to confirm cleavage on both the 3′- and 5′-sides of the abasic site. As described in the previous section, two products were obtained from the abasic site-containing strand: d(GCGp) resulting from 5′-strand cleavage and d(pGCG) resulting from 3′-strand cleavage.

Reaction of Fpg Protein with D_7-A_7 in 55% [^{18}O]H_2O. The reaction of the Fpg protein with the duplex formed from D_7 and A_7 was performed in ∼55% enriched [^{18}O]H_2O. If the reaction proceeds by hydrolysis (Scheme 2, path a) on the 3′-side of an abasic site, then the 5′-phosphate of the d(pGCG) product will contain ^{18}O. Alternatively, if the reaction proceeds by a β-elimination reaction (Scheme 2, path b or c), then the 5′-phosphate of d(pGCG) will not contain ^{18}O. Similarly, if the reaction proceeds by a hydrolysis mechanism on the 5′-side of an abasic site (Scheme 2, path a or b), then the 3′-phosphate of the d(GCGp) product will contain ^{18}O. If the reaction proceeds by a δ-elimination reaction (Scheme 2, path c), then the 3′-phosphate will not contain ^{18}O.

Thus, if a strand cleavage reaction proceeds by a hydrolytic mechanism, the terminal phosphate in the corresponding product will contain ^{18}O. One ^{18}O oxygen bonded directly to a ^{31}P nucleus in a phosphoryl group causes an upfield shift of about 0.020 ppm (Gerlt, 1984). Since the ^{18}O content of the solvent for the Fpg reaction mixture was ∼55%, the ^{31}P NMR resonance of the terminal phosphate will exist as two signals of approximately equal intensity if the strand cleavage reaction occurs by a hydrolysis mechanism; the separation of the signals is expected to be 4 Hz at 200 MHz. In contrast, if a strand cleavage reaction proceeds by an elimination mechanism, a single resonance corresponding to a phosphate monoester containing only ^{16}O will be observed. The presence or the absence of ^{18}O in the 3′- and the 5′-phosphate of the reaction product was analyzed by 200 MHz ^{31}P NMR.

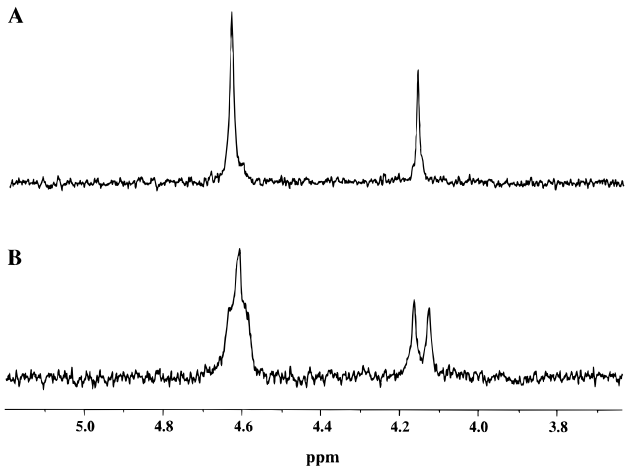


FIGURE 1: A, 200 MHz proton-decoupled ^{31}P NMR spectrum of the products from the Fpg-catalyzed reaction with the D_7-A_7 duplex in about 55% [^{18}O]H_2O. B, 200 MHz proton-coupled ^{31}P NMR spectrum of the Fpg reaction products. From the coupling patterns observed in spectrum B, the resonance at 4.15 ppm corresponds to the 3′-phosphate of the d(GCGp) and the resonance at 4.62 ppm corresponds to the 5′-phosphate of the d(pGCG) product.

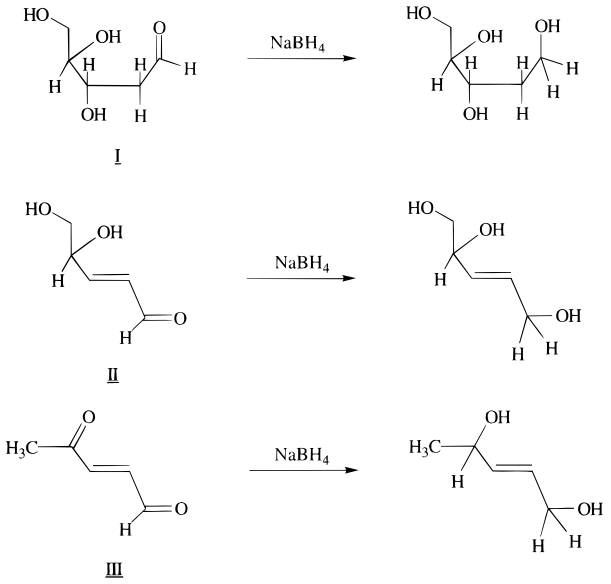
The proton-decoupled ^{31}P NMR spectrum of the Fpg reaction products obtained from D_7-A_7 in ∼55% [^{18}O]H_2O is shown in Figure 1A. As deduced by examination of a proton-coupled spectrum (shown in Figure 1B), the resonance at 4.15 ppm can be associated with the 3′-phosphate of d(GCGp) (coupled to one proton), and the resonance at 4.62 ppm can be associated with the 5′-phosphate of d(pGCG) (coupled to two protons). The line widths of the peaks are about 1.5 and 2.2 Hz, respectively, demonstrating that an isotope induced shift of 4 Hz would have been observed if ^{18}O had been incorporated into either phosphate monoester. Neither of the ^{31}P NMR resonances for the Fpg reaction products, d(GCGp) or d(pGCG), showed the presence of two signals separated by 4 Hz which would be diagnostic for the incorporation of solvent-derived oxygen into the reaction products.

In a control experiment, the ability to observe an ^{18}O upfield shift for a ^{31}P NMR resonance was confirmed. Adenosine 5′-monophosphate was treated with calf intestinal phosphatase in ∼40% enriched [^{18}O]H_2O, and a 200 MHz ^{31}P NMR spectrum of the reaction product showed the expected upfield-shifted resonances (data not shown). As a result, the absence of an upfield-shifted signal for the resonances of the 3′- and the 5′-phosphate monoesters in the Fpg protein-derived reaction products can be interpreted as evidence against hydrolytic mechanisms for the cleavage of the respective phosphodiester bonds (Scheme 2, paths a and b). The most reasonable mechanistic alternative is successive β- and δ-elimination reactions (Scheme 2, path c).

In order to obtain positive evidence for successive β- and δ-elimination reactions, we identified the deoxyribose derivative released during the reaction.

Trapping and Identification of the Deoxyribose Derivative Released by the Fpg Protein. In order to identify the five-

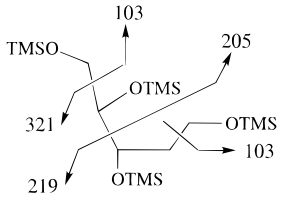
carbon fragment derived from the deoxyribose moiety of the aldehydic abasic site, authentic samples of 2-deoxyribose (I), 4,5-dihydroxy-2-pentenal (II), and 4-oxo-2-pentenal (III) were stabilized by reduction with NaBH4.



I II III

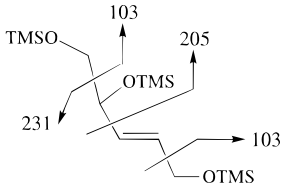
Examination of ^{1}H NMR spectra of 4-oxo-2-pentenal at different times revealed that 4-oxo-2-pentenal (III) is stable only for an hour under the reaction conditions before it starts to decompose. Therefore, the Fpg protein-catalyzed reaction with the D_7-A_7 duplex was performed for 20 min, and the five-carbon product was stabilized by reduction with $NaBH_4$ to the corresponding alcohol. The mass spectra of the alcohols obtained from authentic samples of I−III and of the Fpg reaction product were recorded after trimethylsilylation.

Figure 2 shows the mass spectrum of reduced and trimethylsilylated 2-deoxyribose (I; elution time, 10.05 min); the total mass is 424, and the expected fragmentation pattern is shown below.



The observed *m*/*e* ratios included 321, 219, 147, 103, and 73.

Figure 3 shows the mass spectrum of reduced and trimethylsilylated 4,5-dihydroxy-2-pentenal (II; elution time, 8.81 min); the total mass is 334, and the expected fragmentation pattern is as follows:



The observed *m*/*e* ratios included 231, 147, 103, and 73. A small molecular ion at *m*/*e* 334 can be discerned.

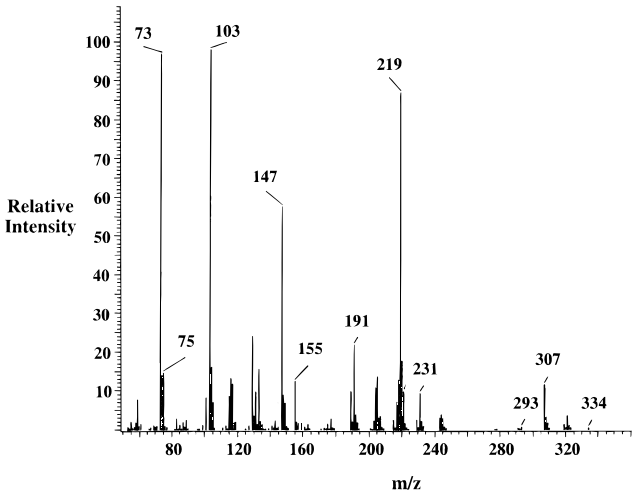


FIGURE 2: Mass spectrum of reduced and trimethylsilylated 2-deoxyribose (I).

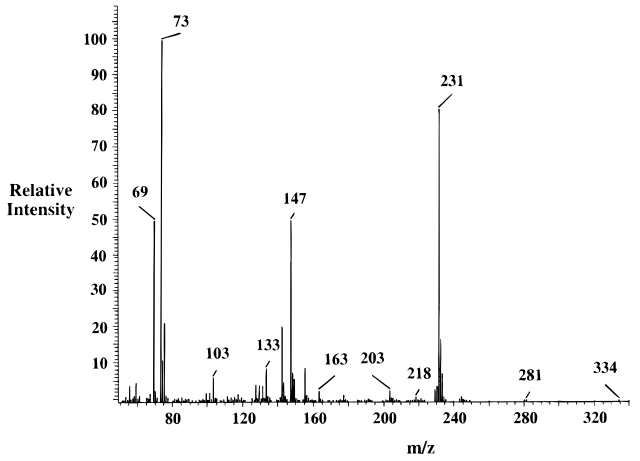
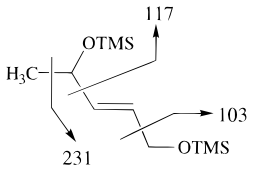


FIGURE 3: Mass spectrum of reduced and trimethylsilylated 4,5-dihydroxy-2-pentenal (II).

Figure 4 shows the mass spectrum of reduced and trimethylsilylated 4-oxo-2-pentenal (III; elution time, 6.20 min); the total mass is 246, and the expected fragmentation pattern is as follows:

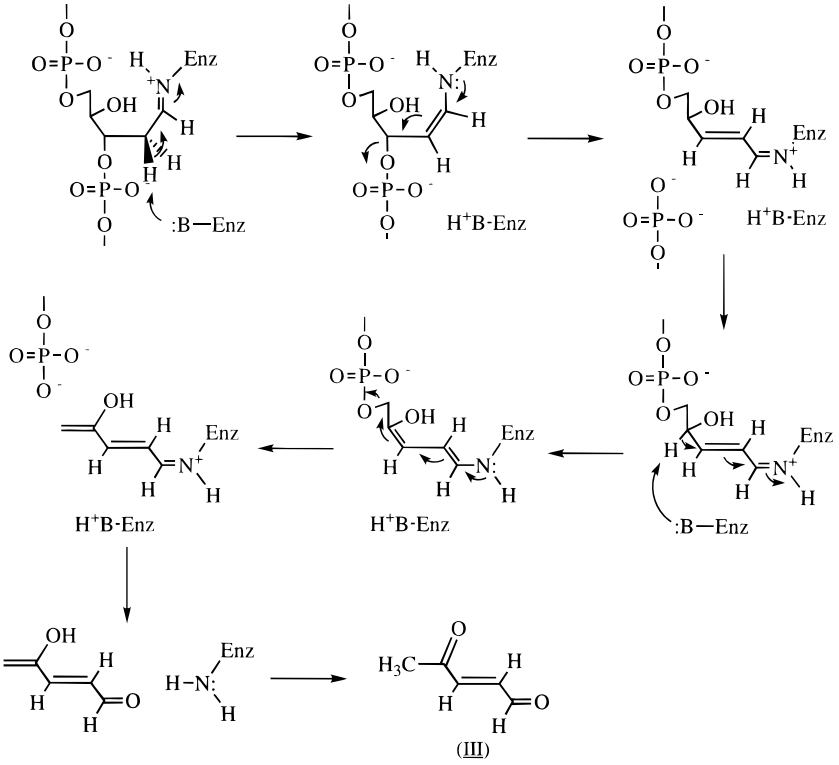


The observed *m*/*e* ratios included 231, 147, 143, 117, and 73. A small molecular ion at *m*/*e* 246 can be discerned.

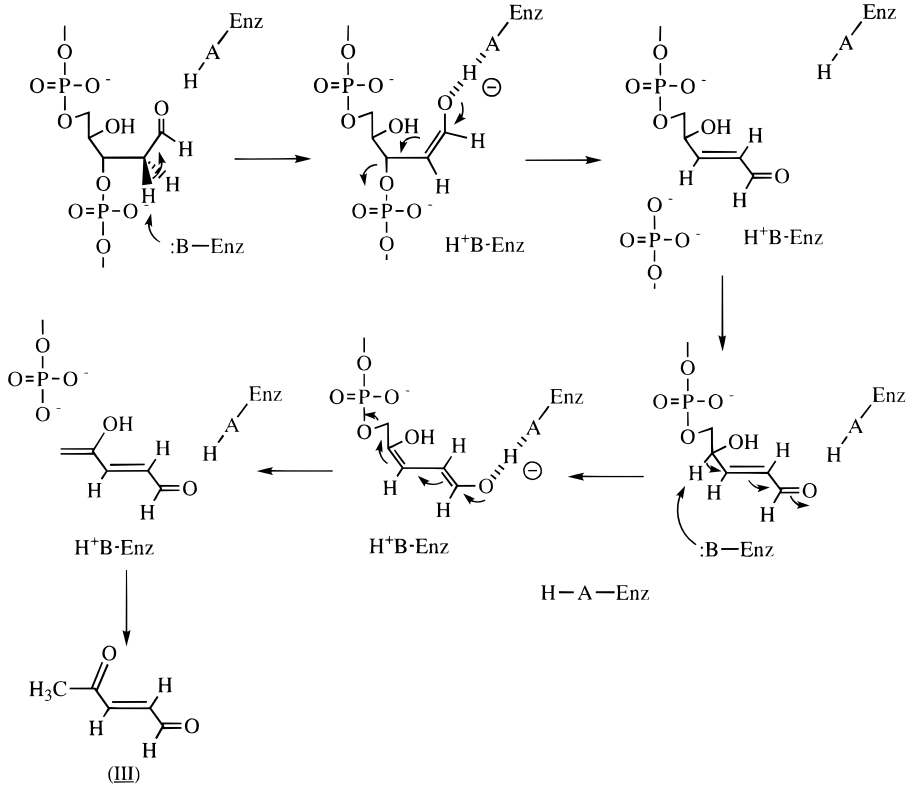
Figure 5 shows the mass spectrum of the reduced and trimethylsilylated Fpg protein-derived reaction product (elution time, 6.22 min). The mass spectrum of the Fpg protein-derived product contains *m*/*e* ratios of 231, 147, 143, 117, and 73, matching the spectrum of reduced 4-oxo-2-pentenal (III; Figure 4), which is the product expected from successive β- and δ-elimination reactions. This spectrum is unequivocal evidence for successive β- and δ-elimination mechanisms for the 3′- and 5′-strand cleavage reactions, respectively, catalyzed by the Fpg protein.

Mechanistic Implications. That the Fpg protein catalyzes both β- and δ-elimination reactions can be best understood by considering the relative pK_as of the 2′- and 4′-protons

Scheme 3



Scheme 4



that are abstracted by an active site general basic functional group. In the open chain form, the 2′-protons of the aldehydic abasic site can be expected to have pK_as of ~18 (Gerlt *et al.*, 1991). While the pK_a of the 2′-proton is undoubtedly significantly greater than that of the conjugate acid of the active site general base, the enolic intermediate generated by proton abstraction can be kinetically competent if it is stabilized in the active site (Gerlt & Gassman, 1993a,b; Cleland & Kreevoy, 1994). Although sufficient structural and mechanistic data are not yet available for the Fpg protein to describe conclusively the mechanism of stabilization of the intermediate, it could occur either by (1) formation of a Schiff base between the aldehyde functional group of the abasic site and an active site amino group (Scheme 3; either that of an active site lysine or perhaps the N-terminal α-amino group of the polypeptide chain) or (2) preferential

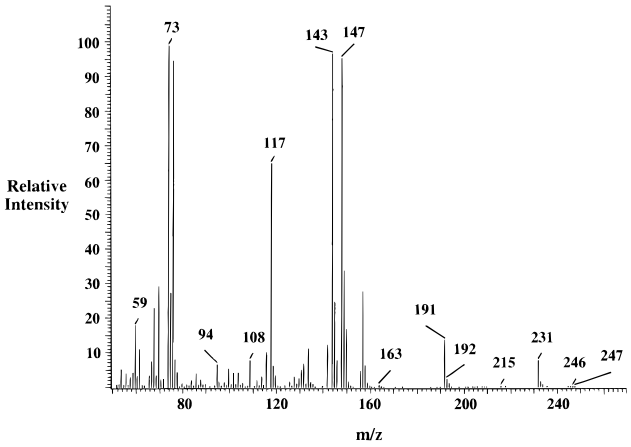


FIGURE 4: Mass spectrum of reduced and trimethylsilylated 4-oxo-2-pentenal (III).

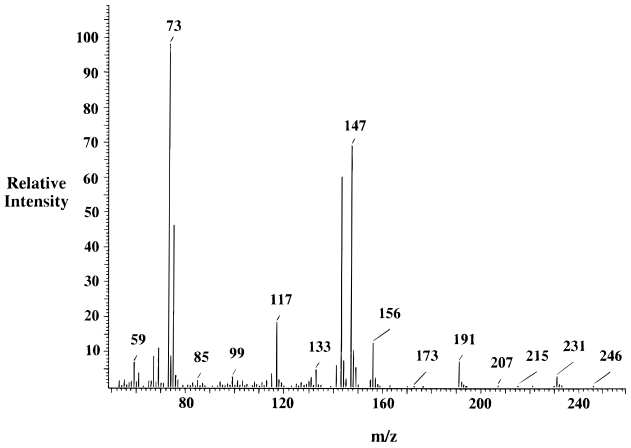


FIGURE 5: Mass spectrum of the reduced and trimethylsilylated Fpg protein reaction product.

stabilization of an enolic intermediate by hydrogen-bonding interactions with an active site electrophilic group (Scheme 4). In the case of endonuclease V from bacteriophage T_4, the available data suggest that the N-terminal α-amino group of the polypeptide chain does, in fact, form a Schiff base with the aldehydic abasic site (Schrock & Lloyd, 1991; Dodson *et al.*, 1993); in the case of endonuclease III, the available structural data suggest that a lysine in the active site forms a Schiff base with the substrate (Kuo *et al.*, 1992).

In the aldehydic abasic site, the pK_a of the 4′-proton can be expected to have a pK_a of ∼50; however, once the β-elimination reaction occurs, the 4′-proton is vinylogous to the aldehyde functional group and, therefore, significantly more acidic. Thus, we predict that the δ-elimination reaction will necessarily occur after the β-elimination reaction. Consistent with this prediction is the observation that in the presence of 10 mM β-mercaptoethanol the 5′-cleavage reaction is inhibited (M. Bhagwat and J. A. Gerlt, unpublished observations). Either this exogenous nucleophile can enter the active site and intercept the reaction after the β-elimination reaction has occurred or the product of the β-elimination reaction can dissociate from the active site of the enzyme prior to the δ-elimination reaction. In either case, the δ-elimination reaction is prevented by addition of the β-mercaptoethanol to the α,β-unsaturated aldehyde that is the product of the β-elimination reaction since the pK_a of the 4′-proton of the product of the addition reaction will be increased such that it cannot be abstracted by the active site general basic catalyst.

Although a "δ-elimination reaction" has been reported for the reactions catalyzed by endonuclease V from bacteriophage T_4 (Latham & Lloyd, 1995), the experiments reported in this manuscript provide the first documented examples of successive β- and δ-elimination reactions that are catalyzed by an AP lyase.

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