Involvement of Phylogenetically Conserved Acidic Amino Acid Residues in Catalysis by an Oxidative DNA Damage Enzyme Formamidopyrimidine Glycosylase†

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ABSTRACT: Formamidopyrimidine glycosylase (Fpg) is an important bacterial base excision repair enzyme, which initiates removal of damaged purines such as the highly mutagenic 8-oxoguanine. Similar to other glycosylase/AP lyases, catalysis by Fpg is known to proceed by a nucleophilic attack by an amino group (the secondary amine of its N-terminal proline) on C1′ of the deoxyribose sugar at a damaged base, which results in the departure of the base from the DNA and removal of the sugar ring by β/δ-elimination. However, in contrast to other enzymes in this class, in which acidic amino acids have been shown to be essential for glycosyl and phosphodiester bond scission, the catalytically essential acidic residues have not been documented for Fpg. Multiple sequence alignments of conserved acidic residues in all known bacterial Fpg-like proteins revealed six conserved glutamic and aspartic acid residues. Site-directed mutagenesis was used to change glutamic and aspartic acid residues to glutamines and asparagines, respectively. While the Asp to Asn mutants had no effect on the incision activity on 8-oxoguanine-containing DNA, several of the substitutions at glutamates reduced Fpg activity on the 8-oxoguanosine DNA, with the E3Q and E174Q mutants being essentially devoid of activity. The AP lyase activity of all of the glutamic acid mutants was slightly reduced as compared to the wild-type enzyme. Sodium borohydride trapping of wild-type Fpg and its E3Q and E174Q mutants on 8-oxoguanosine or AP site containing DNA correlated with the relative activity of the mutants on either of these substrates.

DNA continuously undergoes oxidation damage from both exogenous and endogenous sources. These include X-rays, γ-rays, ultraviolet light, and products of metabolism. Replication of damaged DNA sometimes gives rise to mutations, which can contribute to disease and aging (for review, see refs *1* and *2*). One of the most mutagenic lesions caused by DNA oxidation is 8-oxoguanine, which, if not repaired, results in G → T transversions. For protection from such damage, cells primarily rely on the mechanism of DNA base excision repair (reviewed in ref *3*). In *Escherichia coli*, the enzyme formamidopyrimidine glycosylase (Fpg),[1] encoded by the *mutM* gene, initiates removal of 8-oxoguanine and a number of other lesions, e.g., formamidopyrimidines (*4*).

The 8-oxoguanine excising enzymes are classified as glycosylase/apurinic (AP) lyases, because they initiate repair of the damaged bases by hydrolysis of the glycosidic bond with concomitant scission of the DNA backbone by a lyase mechanism (*3*). Several of these enzymes have been characterized through biochemical and functional studies, while others have been identified through genomic sequencing. Collectively, these studies have revealed similar 8-oxoguanine repair enzymes that are closely related to *E. coli* Fpg in sequence, function, and substrate specificity (*4*). Recently, a number of Fpg-like enzymes that are active in removal of 8-oxoguanine lesions have been cloned and purified from eukaryotes. Although functionally similar to Fpg, these proteins are divergent in primary structure and in substrate preference (*3*).

Previously, a unified catalytic mechanism has been suggested for glycosylases/AP lyases, regardless of their origin or substrate specificity (*5*−*11*). These enzymes use either their N-terminal amino group or an ϵ-amino group of lysine to carry out a nucleophilic attack on C1′ of the damaged nucleotide sugar. This pathway proceeds through a transient covalent complex between the enzyme and DNA, releasing the damaged base by protonation of the damaged base or deoxyribose sugar O4′ oxygen, or alternatively, by the generation of the negative electrostatic environment in the enzyme active site around the C1′ atom. Subsequently, depending on the structure of the active site, a β- or β/δ-elimination reaction occurs, ultimately leading to cleavage of the damaged DNA and dissociation of the enzyme−DNA complex with the formation of the β- or β/δ-elimination product.

It is now known that Fpg, like T4 pyrimidine dimer glycosylase (pdg), also uses its N-terminus for an attack on the damaged nucleotide (*12*). In addition, two other basic

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[1] Abbreviations: AP, apurinic; PCR, polymerase chain reaction; DTT, dithiothreitol; Fpg, formamidopyrimidine glycosylase; PDG, pyrimidine dimer glycosylase; Na-HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid sodium salt.

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residues have been implicated in Fpg catalysis: K57 and K155 (*13*, *14*). However, no active site acidic amino acid has been found so far, and at the time of the submission of this study, no Fpg crystal structure had been determined, further contributing to a lack of understanding about its catalytic activity. However, during the review, the crystal structure of Fpg from *Thermus thermophilus* HB8 was determined (*15*). In this study, we have probed the role of phylogenetically conserved glutamates and aspartates in the activities of Fpg protein and relate our data to this newly derived structure.

EXPERIMENTAL PROCEDURES

Targeted Mutagenesis of E. coli Fpg Protein. The entire Fpg ORF was amplified by PCR from pET-22Fpg plasmid. The PCR amplification introduced a unique *Xma*I site at the 3′ terminus of the ORF. This *Xma*I site and a unique *Nde*I site carried over from pET-22Fpg were used to subclone the ORF into the pTYB2 expression plasmid (New England Biolabs). The glutamic acid residues at positions 3, 6, 132, and 174 were changed into glutamine, and the aspartic acid residues at positions 107 and 161 into asparagine, using the Quick Change mutagenesis kit from Stratagene. The sequence of each primer and exact conditions for PCR and Quick Change mutagenesis reactions are available separately upon request from the authors.

The entire nucleotide sequences of wild-type Fpg and of the mutant Fpg genes were verified by dideoxy sequencing utilizing the UTMB NIEHS Center Molecular Biology Core (Thomas G. Wood, Director). pTYB2 plasmids encoding mutant Fpg proteins, as well as wild-type Fpg, were transformed into the CC104 (*mutM*$^-$) DE3 strain of *E. coli*. The CC104 (*mutM*$^-$) DE3 variant strain was created from CC104 (*mutM*$^-$) cells using the Novagen λDE3 lysogenization kit, as recommended by the supplier.

Expression and Purification of Fpg Proteins. Expression and isolation of wild-type and mutant Fpg proteins took advantage of New England Biolabs Impact T7 protein expression/purification system. CC104 (*mutM*$^-$) DE3 cultures, carrying expression plasmids for the Fpg proteins fused to a chitin binding domain (CBD) protein at their C-terminus, were each grown in 5 L of 2×YT medium to $OD_{600nm} = 0.5$. At this point, protein expression was induced in the cultures by addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG) to 0.3 mM final concentration and was carried out overnight at room temperature. Following expression, cells were centrifuged at 10000*g* for 15 min. The cell pellets were resuspended in 100 mL of 50 mM Na-HEPES, pH 7.5, 500 mM NaCl, 1 mM Na_2-EDTA, 0.5% Triton-X 100, 0.1 mM PMSF, 1 μM leupeptin, and 1 μM pepstatin. The cells were lysed by French press, and the debris was pelleted at 20000*g* for 10 min. Polymin P (Sigma) (10% w/v), pH = 7.6, was slowly added to the clarified lysates to a final 0.1% concentration and the solution stirred for 30 min. The precipitates from the polymin P fractionation were removed by centrifugation at 20000*g* for 10 min. $(NH_4)_2SO_4$ was slowly added to the supernatants to 60% of saturation. After 30 min of stirring, the precipitated proteins were centrifuged as above. The protein pellets were resuspended in small amounts of 50 mM Na-HEPES, pH 7.5, 1 mM Na_2-EDTA, and 0.1 mM PMSF to the final concentration of 0.3 M $(NH_4)_2SO_4$ (as judged by conductivity measurements). The proteins were loaded at a rate of 0.5 mL/min onto 15 mL chitin affinity columns (1.5 cm internal diameter), which had been preequilibrated with 50 mM Na-HEPES, pH 7.5, 1 M NaCl, 1 mM Na_2-EDTA, and 0.1 mM PMSF (chitin buffer). After the columns were washed with 20 column volumes of the chitin buffer, they were quickly flushed (15 min) with 3 column volumes of the chitin buffer containing 50 mM DTT. Following an overnight incubation of the proteins adsorbed on chitin with 50 mM DTT in the chitin buffer, wild-type Fpg and mutant proteins were eluted with 2 volumes of 25 mM Na-HEPES, pH 7.5, 1 M NaCl, and 1 mM Na_2-EDTA. The eluates were concentrated using Centriplus-30 concentration devices (Millipore). The concentrated target proteins were further purified on Superdex-75 gel filtration columns (dimensions 80 × 1.5 cm), preequilibrated in 100 mM Na-HEPES, pH 7.5, 200 mM NaCl, and 1 mM DTT. The fractions of pure Fpg proteins were pooled, and the proteins were again concentrated using Centricon-10 concentration devices (Millipore), diluted 2-fold with glycerol, and aliquoted for storage at −80 °C. All purification steps were performed at 4 °C. The purity of protein fractions and protein concentrations were assessed using the Bio-Rad protein assay kit (Bio-Rad), 12% SDS−polyacrylamide gels, and absorbance measurements at 280 and 260 nm.

Fpg Activity Assays. The sequences of the modified oligonucleotides used to create Fpg substrates were as follows: for the 30-mer, 5′ACCATGCCTGCACGAAog-TAAGCAATTCGTA3′, and for the 49-mer, 5′AGCTAC-CATGCCTGCACGAAuTAAGCAATTCGTAATCATGG-TCATAGCT3′, where the lower case "og" and "u" denote 8-oxoguanosine and uracil, respectively. The complementary oligonucleotides carried a C opposite og and u. All of these oligonucleotides were purchased from Midland Certified Reagent Co.

The activity of Fpg enzymes was assessed on double-stranded oligonucleotide substrates containing centrally located 8-oxoguanosine or an apurinic (AP) site opposite a cytosine in their respective complementary strands. The AP site containing double-stranded substrate DNA was prepared by treating 1 nM uracil containing 49-mer annealed to its complementary strand with 1 unit of uracil DNA glycosylase (Epicentre Technologies) in 25 mM Na-HEPES, pH 7.5, 50 mM NaCl, 2 mM Na_2-EDTA, 1 mM DTT, and 0.1 mg/mL BSA.

The 8-oxoguanosine 30-mer and the uracil 49-mer were labeled on the 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) and annealed to their complementary oligonucleotides. Fpg enzymes were mixed with the double-stranded 1 nM oligonucleotide substrates in 20 μL of 25 mM Na-HEPES, pH 7.5, 50 mM NaCl, 2 mM Na_2-EDTA, 1mM DTT, and 0.1 mg/mL BSA. Experiments involving trapping of the enzyme−DNA covalent reaction intermediates were performed in the presence of additional 100 mM $NaBH_4$ (or additional 100 mM NaCl in negative trapping controls). In these experiments, $NaBH_4$ from a freshly prepared stock solution (2 M) and the enzyme were added to DNA substrates simultaneously. The samples were incubated at 37 °C for 30 min. The reactions were stopped, and DNA products were piperidine treated or not and analyzed by denaturing gel electrophoresis as described (*16*). The images from the gels were obtained using the Phospho-

	1 10		101 120	121 134	135 154	155 177
E. coli	MPELPEVETS	...	KVLRYTDPRRFGAWLWTKEL	------EGHNVLTHLGPEPL	SDDFNGEYLHQKCAKKKTAI	KPWLMDNKLVVGVGNIYASESLF.
H. influenzae	MPELPEVETA	...	KLLRYNDPRRFGAWLWTENL	------DDFHLFLKLGPEPL	SDEFNAEYLFKKSRQKSTAL	KTFLMDNAVVVGVGNIYTNESLF.
D. radiodurans	MPELPEVETT	...	EKLYFNDPRRFGKVV----A	VAPGDYASMPTLAAMGPEPL	SDDFTEAEFV-ALAARCGPV	KPWLLSQKPVSGVGNIYADESLW.
T. thermophilus	MPELPEVETT	...	RTLYFHDPRRFGRLF----G	VRRGDYREIPLLLRLGPEPL	SEAFAFPGFFRGLKESARPL	KALLLDQRLAAGVGNIYADEALF.
B. subtilis	MPELPEVETV	...	TQLRYRDVRKFG----TMHL	FKPGEEAGELPLSQLGPEPD	AEEFTSAYLKDRLAKTNRAV	KTALLDQKTVVGLGNIYVDEALF.
S. mutans	MPELPEVETV	...	STLVYQDVRKFG----TFEL	LPKSQVEAYFVQKKIGPEPN	AKDFKLKPFEEGLAKSHKVI	KTLLLDQHLVAGLGNIYVDEVLW.
U. urealyticum	MPELPEVQTI	...	MQLRYNDTRQFGTF----HI	YEQQSFLDSKELKKIALDPL	DNNFSAQYLYEKLKKSNKAI	KTALLDQSVVSGIGNIYADEILF.
M. tuberculosis	MPELPEVEVV	...	TVLSFADQRTFGGWLLA-DL	VTVDGSVVPVPVAHLARDPL	DPRFDCDAVVKVLRRKHSEL	KRQLLDQRVVSGIGNIYADEALW.
M. genitalium	MPELPEVTTV	...	SQLNYHDTRQFGTF----HL	YEKLEQAA--QLNKLAFDPL	EAGFDYRKIFQKAQNSKRKV	KTFILDQTVISGIGNIYADEILF.

FIGURE 1: Comparison of partial amino acid sequences of putative Fpg proteins from various prokaryotic organisms. The predicted sequences were from the SwissProt database. The alignment was performed using the DNAsis program, version 2.6 (Hitachi Software Engineering). Conserved glutamates and aspartates are highlighted in black.

rimager system (Molecular Dynamics) and analyzed with Molecular Dynamics ImageQuant software.

RESULTS

Mutagenesis, Expression, and Purification. To identify potential acidic amino acid residues (glutamic and aspartic acid) in Fpg that could be involved in glycosidic and phosphodiester bond catalysis, a search was conducted for strictly conserved (invariant) Glu and Asp residues from an alignment of all known prokaryotic Fpg-like protein sequences. A portion of such an alignment, which compares enzymes from several evolutionarily diverse bacteria, is presented in Figure 1. A total of six acidic amino acid residues were found conserved in the alignment: glutamic acids at positions 3, 6, 132, and 174 and aspartic acids at positions 107 and 160.[2]

The 30 kDa wild-type *E. coli* Fpg and its mutants each carrying a single amino acid substitution at one of the indicated positions, were cloned, expressed, and purified. The purity of the proteins following a final Superdex 75 sizing column was determined to be greater than 95% (data not shown).

Activity on 8-Oxoguanine Containing Substrate. Overexpressed recombinant Fpg proteins were assayed for dual glycosylase/AP lyase activity on a DNA substrate containing 8-oxoguanine opposite a cytosine, in which the damaged base was centrally located in the deoxyoligonucleotide duplex. Figure 2a depicts the relative ability of each of the mutant enzymes to cleave the 8-oxoguanine containing oligonucleotide in the duplex substrate. While aspartate mutants (D107N and D160N) had as much, if not more, activity as the wild-type Fpg, the glutamate mutant enzymes were found to be compromised in their ability to cleave DNA containing 8-oxoguanine. Although the activity of two mutants, E6Q and E132Q, was diminished more than 3- and 20-fold, respectively, the most dramatic loss of activity occurred with E3Q and E174Q mutants. Separate experiments were designed to determine activity of these mutants relative to the wild-type enzyme, and these data revealed over 500- and 100-fold decreases in 8-oxoguanine specific nicking for E3Q and E174Q, respectively, as determined by ImageQuant analyses (data not shown). Since E3Q and E174Q mutants had essentially lost all of their glycosylase activity, these seemed likely candidates for an essential role in catalysis and, thus, became the focus of the remainder of this study.

The assays described above tested for the ability of the wild-type and the mutant forms of Fpg to catalyze both glycosylase and β/δ-elimination. However, it was possible that these mutants could have retained glycosylase activity without the concomitant phosphodiester bond scission activity, and thus, no change in electrophoretic mobility would have been detected. This possibility was addressed by heating the Fpg-reacted DNAs with piperidine (*17*). Incubation of all reaction products in the presence of piperidine resulted in no additional cleavage (data not shown). Therefore, it was concluded that the catalytic proficiency of the glutamate mutants in processing of 8-oxoguanosine was reduced at the glycosidic bond scission step.

An important intermediate step in processing of 8-oxoguanosine and the AP site by Fpg is formation of a transient covalent bond between the N-terminal proline and C1′ of the damaged nucleotide (*12*, *18*). This covalent enzyme−DNA complex, when reduced with agents such as $NaBH_4$, is stabilized, thus facilitating its detection. The relative abilities of wild-type, E3Q, and E174 enzymes to be trapped on 8-oxoguanine containing oligonucleotide were compared (Figure 2b). In the absence of $NaBH_4$ (lanes 1−7), the wild-type and the mutant enzymes did not trap 8-oxoguanine oligonucleotides. Also as expected, E3Q and E174Q mutants had barely detectable nicking activity on this substrate as compared to the wild-type enzyme. When $NaBH_4$ was added (lanes 8−14), the 8-oxoguanine DNA was covalently trapped and migrated much slower, near the origin of the gel. The E3Q and E174Q mutants' ability to trap the substrate was miniscule and correlated with the relative nicking activities of these mutants in the absence of the reducing agent (Figure 2a and lanes 1−7 of Figure 2b). The presence of $NaBH_4$ in the trapping reactions has been previously shown to inhibit Fpg nicking activity (*17*). The decrease in the amount of nicking by wild-type Fpg after treatment with the reducing agent (as can be seen in Figure 2b) was consistent with such an inhibition.

Activity on AP Site Containing Substrate. The effect of the glutamate mutations at positions 3 and 174 on AP lyase activity of Fpg is illustrated in Figure 3a. In contrast to their 8-oxoguanine glycosylase/AP lyase activities, E3Q and E174Q mutants retained substantial AP lyase activity. E3Q was approximately 2-fold less active, and E174Q was about 4-fold less active than the wild-type enzyme on an AP site substrate. The AP activity of other glutamate mutants−E6Q and E132Q−was comparable to that of the wild-type enzyme and E174Q mutant, respectively (data not shown).

Glycosylases/AP lyases are able to covalently trap AP containing DNA in the presence of a reducing agent.

[2] Our study had been initiated before the *D. radiodurans* Fpg-like amino acid sequence was deposited into the sequence databases, and therefore, *E. coli* Asp 160 was presumed ubiquitously conserved.

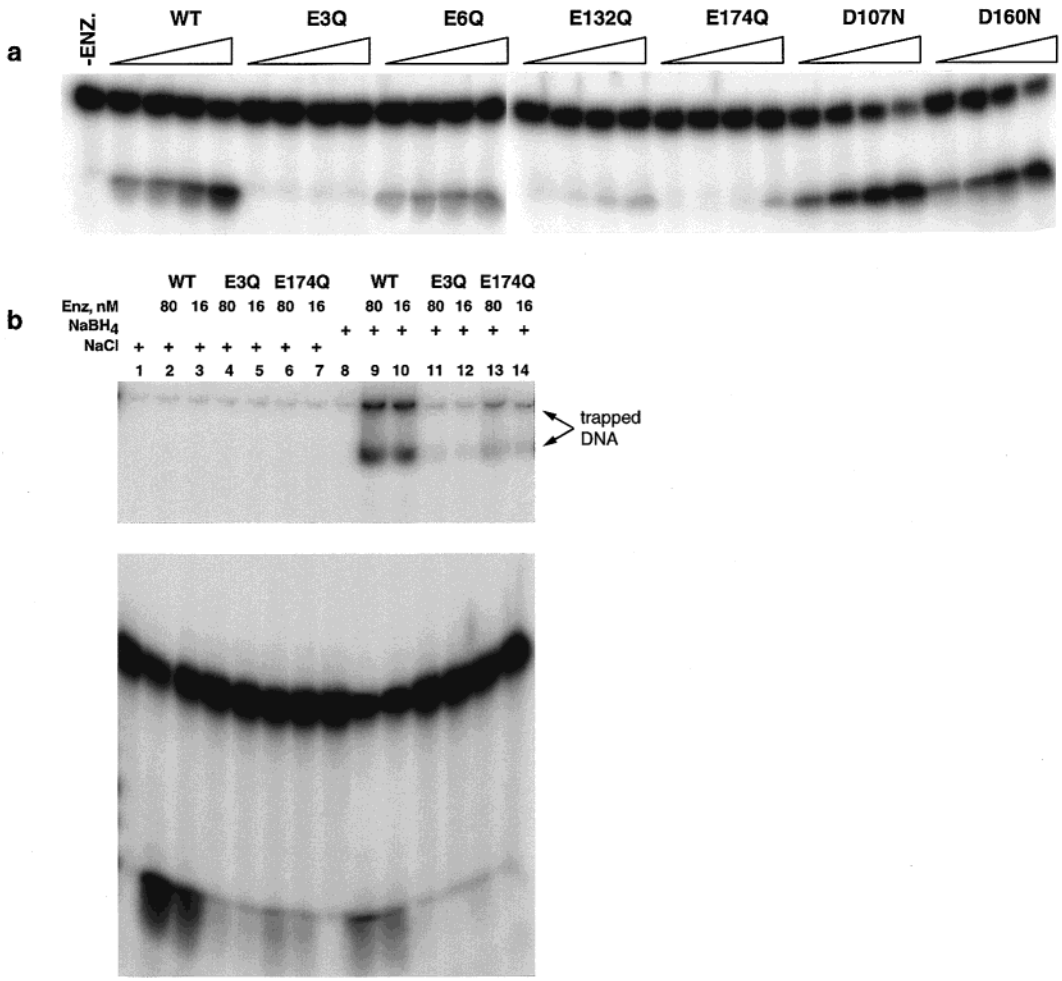

FIGURE 2: Activity of Fpg and its mutants on the 8-oxoguanine containing DNA substrate. (a) Glycosylase/AP lyase activity. The upper band in each lane represents the substrate 8-oxoguanine DNA 30-mer; the lower band is the product of the cleaved 30-mer. Lane 1: no enzyme. Four enzyme concentrations were used in the assays for each of the Fpg variants: 1, 2, 4, and 8 nM (left to right). (b) Trapping of the covalent reaction intermediate. 8-Oxoguanine containing 30-mer DNA duplexes were assayed with no enzyme or with wild type, E3Q, or E174Q Fpg at the indicated concentrations in the presence of either 100 mM NaCl or 100 mM $NaBH_4$.

However, the enzyme and the reducing agent must be added to the AP substrate simultaneously in order to avoid loss of the substrate due to either a rapid lyase reaction (if the enzyme is added first) or reduction of the deoxyribose, rendering it untrappable (if the reducing agent is added first). Fpg trapping with cyanide and cyanoborohydride has been previously demonstrated (*13*). We examined the ability of E3Q and E174Q to covalently trap the AP site 49-mer. Figure 3b demonstrates that the AP DNA is trapped by the wild-type Fpg (lanes 9 and 10) and by the mutants in the presence of $NaBH_4$; albeit E3Q and E174Q (lanes 13 and 14) exhibit less trapping. The trapping by the mutants relative to the wild type was consistent with the relative AP site activities of these variants, as seen in Figure 3a.

DISCUSSION

The current model of action of glycosylases/AP lyases postulates that the coordinated activities of either a primary or secondary amine and an acidic amino acid residue initiate catalysis by formation of a transient covalent complex between the amine and the C1′ atom of the damaged nucleoside. This nucleophilic attack is accompanied by either glycosyl bond hydrolysis with subsequent deoxyribose sugar ring opening or by the sugar ring opening with subsequent glycosyl bond hydrolysis. The departure of the base and opening of the sugar result in formation of the positively charged DNA−enzyme Schiff base intermediate. The abstraction of the 2′-*pro-S* hydrogen from this DNA−enzyme covalent intermediate triggers β- or β/δ-elimination reactions in the sugar moiety, resulting in DNA backbone cleavage (*3*). There are several well-established examples, where the active site amine and acidic residues have been mapped. The α-NH_2 group of the N-terminal Thr 2 and Glu 23 of T4-pdg have been implicated in both the glycosylase and lyase steps of the catalytic mechanism (*10*, *19*). Lys 120 (ϵ-amine) and Glu 112 are the residues critical for catalysis by endonuclease III (*20*). Mutational analysis of the yeast 8-oxoguanine repair enzyme, yOgg1, demonstrated that Asp 260 and the ϵ-amino group of Lys 241 were necessary for the efficient trapping of the enzyme on its substrate (*21*).

Biochemical and computer modeling studies from several laboratories have proposed several possible roles for acidic amino acid residues in the active site. According to the current model, removal of a damaged base by all glycosylases/AP lyases is aided by the protonation of this leaving base. Also consistent with this mechanism would be protonations of O4′ or O1′ of the deoxyribose sugar ring. Generation of positive charge at these two atoms facilitates

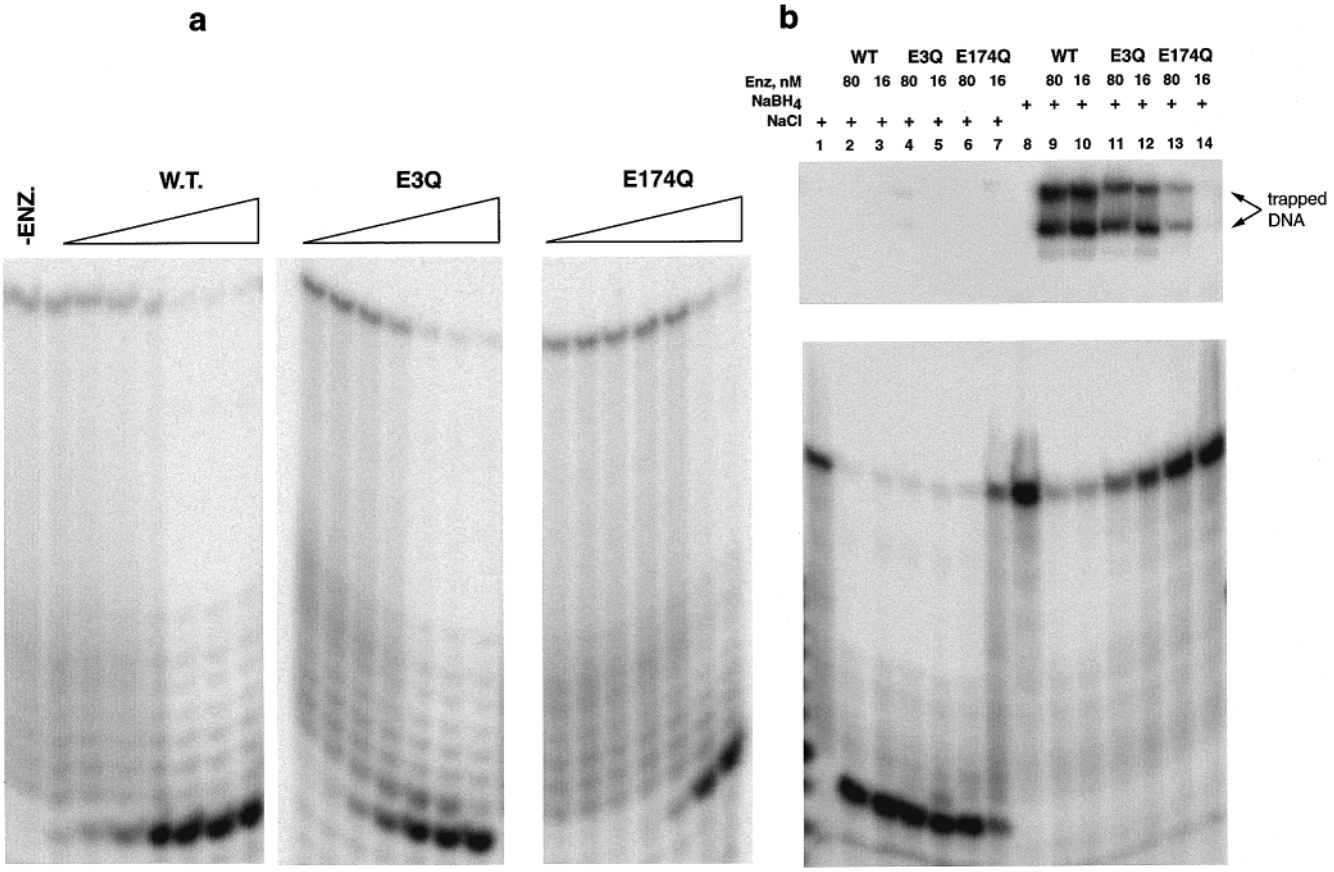

FIGURE 3: Activity of Fpg and its mutants on the AP site containing substrate. (a) Concentration-dependent AP lyase activity of the Fpg mutants. The upper band in each lane is the substrate AP 49-mer; the lower band is the cleavage product. Lane 1: no enzyme. Enzyme concentrations used in the assays were 0.12, 0.25, 0.5, 1, 2, 4, and 8 nM (left to right). (b) Trapping of the covalent reaction intermediate. The AP containing 49-mer duplex was assayed with no enzyme and with wild type, E3Q, or E174Q Fpg at the indicated concentrations in the presence of either 100 mM NaCl or 100 mM $NaBH_4$.

the sugar ring opening and the attack on C1′ by the enzyme (*3*). An amino acid carrying a side-chain carboxyl group could supply the necessary proton, provided it is in proximity to the acceptor atom, and its pK_a at that point is high enough and close enough to the pK_a of the acceptor to make it an efficient general acid (proton donor). Also, if the side chain of Glu in the active site becomes negatively charged (ionized), it may aid the base departure by stabilizing the positively charged enzyme−DNA covalent intermediate or act as a general base in proton abstraction from C2′ in the β-elimination step. Another possible role for the involvement of the acidic residues in catalysis could be that, by being juxtaposed to the attacking nucleophile, they could decrease the pK_a of the amino group of the nucleophile, rendering it more reactive.

This investigation of essential acidic residues in Fpg did not find any catalytic role for aspartic acid residues 107 and 160. Interestingly, shortly after the project had begun, a new Fpg homologue sequence (from *Deinococcus radiodurans*) was deposited into databases, which lacked an acidic residue at the position corresponding to amino acid 107 in alignment with *E. coli* Fpg (see Figure 1 and footnote 2). The discovery of a lack of conservation of Asp 107 is consistent with the absence of any detectable involvement of this residue in catalysis. After completion of this study, the crystal structure of Fpg from *T. thermophilus* was solved (*15*), and the *E. coli* D107 would be predicted to be at the start of a loop between β8 and β9, and D160 would be predicted to reside at the end of α-helix D, both of which are far from the active site pocket.

Our study also demonstrated that catalysis by Fpg employs all evolutionally conserved glutamic acids. While none of the glutamates proved to be essential for pure AP lyase activity, as demonstrated by general AP site nicking, two of the glutamates, E3 and E174, were found necessary for efficient combined glycosylase/AP lyase activity on 8-oxoguanine containing DNA. Interestingly, the crystal structure would predict that *E. coli* E3 and E174 would be on opposite sides of an active site cleft, assuming that the computer modeling of the positioning of substrate DNA is correct. This helps to substantiate our interpretation that these are critical residues. E3 would be predicted to protonate N7 of the 8-oxoguanine. Although E6 is predicted to be in α-helix A with E6 potentially stabilizing a H_2O molecule with Pro2, our data do not conclude that this is an essential function. Additionally, E132 is predicted to be in an extended loop, distal to the active site.

The significant reduction in 8-oxoguanine specific catalysis, in combination with the relatively mild effect on AP-specific nicking, reveals that E3 and E174 are critical for interaction with the damaged base and/or for the chemistry of the glycosylase step and have little involvement in the lyase step. However, our data do not rule out the possibility that either E3 or E174 participates in the catalysis and that mutation at only one site is insufficient to eliminate this reaction. Creation of double mutants could potentially address

this possibility. Thus, our data are not sufficient to describe indispensable roles for these glutamates in sugar ring opening, or C2 hydrogen abstraction, which are integral parts of the lyase reaction.

The involvement of two Fpg conserved lysine residues, K57 and K155, in the catalysis has been investigated (*13*, *22*). The findings revealed contributions of the basic residues to the enzyme's glycosylase activity and, similar to our results, documented no role for these lysines in the lyase step. The authors suggested that K57 or K155 might be directly interacting with O8 of 8-oxoguanine via an ion pair or acting as proton donors.

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