Mechanism of Action of Base Release by *Escherichia coli* Fpg Protein: Role of Lysine 155 in Catalysis†

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ABSTRACT: Fpg protein (formamidopyrimidine/8-oxoguanine DNA *N*-glycosylase) is a DNA repair enzyme that catalyzes the removal of oxidized purines, most notably the mutagenic 7-hydro-8-oxoguanine (8oxoGua) lesion, by an *N*-glycosylase action. Additionally, Fpg protein catalyzes β and δ elimination reactions subsequent to removal of the base lesions, as well as the analogous chemistry at abasic sites (AP sites). In this report, we show that of the two lysines that are conserved among the various putative prokaryotic Fpg proteins, a site specific alteration in one of them (lysine 155 changed to alanine) displays meaningful changes in substrate activities. However, lysine 155 is not required for the postulated covalent enzyme−substrate imine intermediate as demonstrated by trapping of the mutant protein−oligonucleotide complexes with cyanide or cyanoborohydride. The K155A mutant shows a decrease in activity with the 8oxoGua-substrate of ∼50-fold under both k_{cat}/K_m and k_{cat} conditions. This mutant also displays a similar reduction in activity with an oligonucleotide substrate possessing a single 2′-deoxy-8-oxonebularine site. In contrast, activity for a site specific 7-methylformamidopyrimidine-modified oligonucleotide is reduced ∼3−4-fold, a much more modest decrease in activity. Interestingly, there is a concomitant increase in AP lyase activity above wild-type for the K155A mutant (1.6-fold increase in k_{cat}, 32-fold increase in k_{cat}/K_m), demonstrating retention of functional β and δ lyase activities. Together these observations are readily accommodated by a model requiring a direct interaction of lysine 155 with the C_8 oxygen of 8-oxopurines. Thus, conservation of this amino acid residue during evolution appears to be essential for specific incision of the mutagenic 8oxoGua base lesion by Fpg protein.

DNA repair is a fundamental biological process that ensures the stability and integrity of the genome. Deficiencies in DNA repair have been implicated in aging and transformations to the cancerous state. DNA repair enzymes identified in prokaryotes and eukaryotes may exhibit either an astonishing degree of sequence homology (Demple & Harrison, 1994; Tainer et al., 1995) or an intriguing divergence in sequence and activity (Demple & Harrison, 1994; Eide et al., 1996; van der Kemp et al., 1996). The better-characterized prokaryotic enzymes can serve as general model systems for appreciating the similarities and differences among this group of important enzymes.

DNA repair enzymes are often classified according to the manner in which they promote removal of DNA damage. Included in the base excision repair category are enzymes that either catalyze removal of a modified base or additionally catalyze scission of the DNA phosphodiester backbone. Fpg protein[1] (formamidopyrimidine/8-oxoguanine DNA N-glycosylase) is a base excision repair enzyme that recognizes various modifications of purines, but also possesses an apyrimidinic/apurinic (AP) lyase activity that catalyzes successive β and δ elimination reactions (Bailly et al., 1989a; see Scheme 1). Action of this enzyme produces DNA strand scission 3′ and 5′ to the lesion site by the β and δ elimination steps, respectively (Bailly et al., 1989b). Consequently, the ultimate products arising from either a damaged base or an AP site include a monomeric five-carbon fragment derived from deoxyribose (Bhagwat & Gerlt, 1996) and a one-base gapped DNA terminated by 3′ and 5′ phosphates (Bailly et al., 1989b). Complete restoration of the damaged DNA is then accomplished by successive phosphatase-, DNA polymerase-, and DNA ligase-catalyzed reactions (Friedberg et al., 1995).

Fpg protein recognizes diverse but structurally related DNA base modifications including 7-hydro-8-oxoguanine (8oxoGua) (Tchou et al., 1991), formamidopyrimidines [FapyGua and FapyAde; ring opened forms of adenine and guanine; Chetsanga et al. (1981) and Breimer (1984)] and N_7-methylformamidopyrimidines [e.g., 7-MeFapyGua; Chetsanga et al. (1981)] by an *N*-glycosylase activity (see Chart 1 for structures). 8oxoGua in DNA has been shown to be mutagenic (Shibutani et al., 1991; Wood, et al. 1990), leading to the formation of G to T transversion mutations (Moriya, 1993); formamidopyrimidine residues in DNA are predominantly lethal lesions (Tudek et al., 1992). Fpg protein is the only *Escherichia coli* base excision repair enzyme that has been shown to recognize these damages.

The prokaryote *E. coli* has been, until recently, the primary organism for study of base excision repair enzymes. Endonuclease III and endonuclease VIII from *E. coli* are believed to be specific for pyrimidine damage products such as thymine glycol and dihydrothymine (Demple & Linn, 1980; Melamede et al., 1994). The activities of these enzymes provide a marked contrast to the recognition of

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[1] Abbreviations: Fpg protein, formamidopyrimidine/8-oxoguanine DNA *N*-glycosylase; 8oxoGua, 7-hydro-8-oxoguanine; AP site, abasic or apyrimidinic/apurinic site; 8oxodN, 2′-deoxy-8-oxonebularine; 7-MeFapyGua, 7-methylformamidopyrimidine or 2,6-diamino-4-hydroxy-5*N*-methylformamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; FapyAde, 4,6-diamino-5-formamidopyrimidine.

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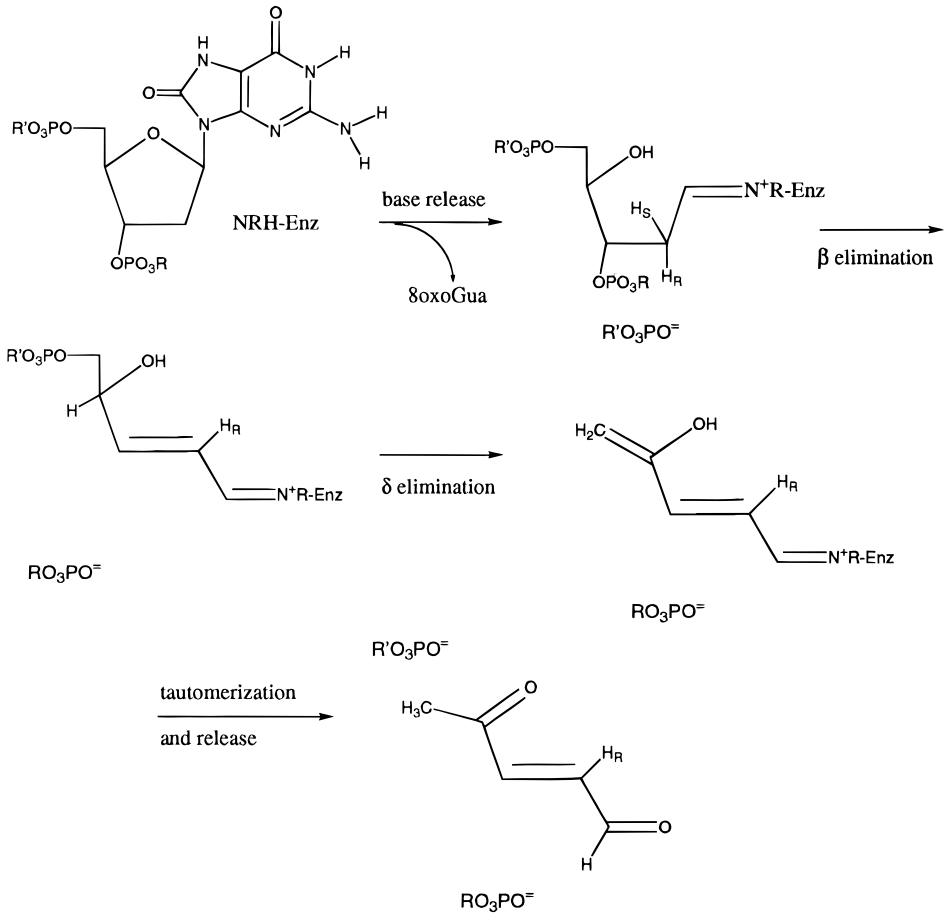
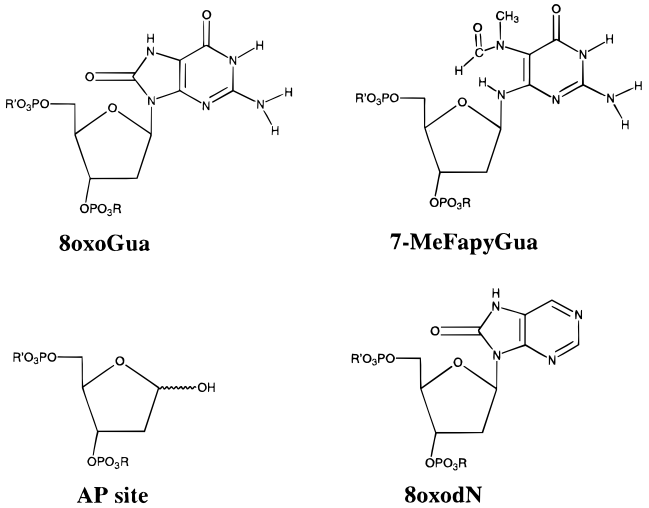
Scheme 1: Proposed Intermediates in Fpg Catalyzed Excision of 8oxoGua-Modified DNA[a]



[a] The reaction proceeds via a protein−substrate imine intermediate (Kow & Wallace, 1987). Free 8oxoGua and a modified deoxypentose fragment are liberated with formation of 3′ and 5′ phosphorylated DNA end groups. The proposed nucleophile is denoted as NHR-Enz to indicate that either a lysine ϵ-amino group or N-terminal secondary amine (α-amino group of proline) may be involved.

Chart 1: DNA Modifications Used in This Study[a]



[a] 8oxoGua, 7-hydro-8-oxoguanine; 7-MeFapyGua, 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine; AP site, abasic or apyrimidinic/apurinic site; 8oxodN, 2′-deoxy-8-oxonebularine, or 2′-deoxy-7-hydro-8-oxopurine.

purine damage products by Fpg protein from the same organism. In this way, a strict dichotomy between the enzymes involved in excision of pyrimidine and purine damage products was believed to exist. However, the recent description of several base excision enzymes from *Saccharomyces cerevisiae* mandates a re-evaluation of the universality of this concept. While one partially purified enzyme activity appears to recognize formamidopyrimidines much more readily than 8oxoGua (de Oliveira et al., 1994), the recently cloned *ogg1* gene codes for a different enzyme that removes 8oxoGua residues in preference to formamidopyrimidines (van der Kemp et al., 1996). Perhaps even more strikingly, an endonuclease III-like protein from yeast (Eide et al., 1996) recognizes both thymine glycol and formamidopyrimidines, but is completely incapable of excising 8oxoGua residues from DNA.

In order to understand the substrate specificities of these base excision repair enzymes, one needs to appreciate the spatial arrangement of groups in the active site and the dynamics of the interaction of active site residues with the various substrates that are recognized. The three-dimensional structures for uracil glycosylase (Savva et al., 1995; Moi et al., 1995), 3-methyladenine glycosylase (Yamagata et al., 1996; Labahn et al., 1996), endonuclease III (Kuo et al., 1992; Thayer et al., 1995), and T4 endonuclease V (Morikawa et al., 1992; Vassylyev et al., 1995) have been solved using X-ray crystallography. In contrast, no three-dimensional structure of Fpg protein has been obtained. However, even when a physical structure is known, enzymological methods are also necessary to understand the dynamics of these reactions.

By using a combination of site-directed mutagenesis, enzyme kinetics, and protein chemistry, we have identified lysine 155 of *E. coli* Fpg protein as an important residue for

Table 1: Oligonucleotides Used in These Studies

8oxoGua-24mer[a]	5′ GAACTAGTG**OG**ATCCCCCGGGCTGC 3′
U-24mer	5′ GAACTAGTG**U**ATCCCCCGGGCTGC 3′
AP-24mer	5′ GAACTAGTG**AP**ATCCCCCGGGCTGC 3′
CComp-24mer	5′ GCAGCCCGGGGGATCCACTAGTTC 3′
8oxodN-23mer	5′ CTCTCCCTTC**ON**CTCCTTTCCTCT 3′
G-23mer	5′ CTCTCCCTTC**G**CTCCTTTCCTCT 3′
7-MeFapyGua-23mer	5′ CTCTCCCTTC**MG**CTCCTTTCCTCT 3′
CComp-23mer	5′ AGAGGAAAGGAGCGAAGGGAGAG 3′

[a] Abbreviations used for substituted deoxyoligonucleotides are as follows: OG, 8oxoguanine; U, uracil (2′-deoxyuridine); AP, abasic or apyrimidinic site; ON, 8-oxopurine (2′-deoxy-8-oxonebularine); MG, N_7-methylformamidopyrimidine.

specific recognition of the exocyclic oxygen at the C8 position of 8oxoGua. This model is derived from a comparison of the activities of wild-type Fpg protein and the K155A mutant using 8oxoGua-, 7-MeFapyGua-, 2′-deoxy-8-oxonebularine- (8oxodN), and AP-modified deoxyoligonucleotides. Evidence presented below demonstrates that lysine 155 does not have a role in covalent catalysis, but rather displays a noncovalent catalytic interaction with the O8 exocyclic oxygen that involves proton transfer or transient formation of an ion pair between the enzyme and substrate.

MATERIALS AND METHODS

Materials. Restriction endonucleases were from New England Biolabs (*Nco*I, *Nde*I, *Sma*I), Boehringer Mannheim (*Xho*I), and Gibco-BRL (*Pst*I). T4 DNA ligase, DNA polymerase I, and *E. coli* strain 5H11S were also Gibco-BRL products. T7 polymerase and terminal deoxynucleotidyltransferase were supplied by U.S. Biochemicals. Uracil DNA glycosylase (1 unit catalyzes the release of 1 nmol of uracil from DNA in 1 h at 37 °C) was a product of Epicentre Technologies or U.S. Biochemicals. *Pfu* DNA polymerase, exonuclease III, and *E. coli* strain XL1-B were obtained from Stratagene. Novagen supplied the vector pET22b, *E. coli* strains BL21(DE3) and BLR(DE3), phage λCE6, and the His-Bind resin. The nucleoside triphosphate dGTPαS (S_p isomer) was purchased from Amersham. The 8-oxo-dG-CE phosphoramidite was obtained from Glen Research and incorporated into the 10th position from the 5′ end of the 24mer using the Applied Biosystems Model 392 oligonucleotide synthesizer of the Department of Microbiology and Molecular Genetics at the University of Vermont. The complementary oligonucleotide was synthesized at the same facility. The 8oxodN-23mer was provided by Arthur P. Grollman of SUNY at Stony Brook (Tchou et al., 1994). Other oligonucleotides, including the 24mer possessing a single deoxyuridine residue (Table 1), were provided by Operon.

Construction of Wild-Type Fpg Expression Vector. The fpg coding region of pKKfapy2 (Michaels et al., 1991) was PCR-amplified using primers 5′-d(CCCCCCCCATATGCCTGAATTACCCGAAGTT)-3′ and 5′-d(CCCCCCCCTCGAGCTTCTGGCACTGCCGACAATA)-3′, which introduce *Nde*I and *Xho*I restriction sites near the ends of the PCR fragment. Restriction of pET22b and the PCR fragment with *Nde*I and *Xho*I followed by ligation with T4 ligase (16 °C for 17 h) and electroporation into XL1-B cells produced carbenicillin-resistant colonies containing the inserted DNA. The construct thus produced (pLRfpg16) codes for an Fpg protein modified to contain six C-terminal histidines.

Site-Directed Mutagenesis of fpg Gene. Preparation of vectors and site-directed mutagenesis was accomplished according to the procedures of Olsen et al. (1993) with slight modifications. The oligonucleotide used for site-directed mutagenesis contained two nucleotide changes to code for the lysine to alanine substitution and a one nucleotide change to produce a silent mutation and introduce a *Nco*I restriction site. The mutant primer was 5′-pd(CATCAGCCATGGTGCAATCGCCGTTTT)-3′ with the changes underlined. Single-stranded template was isolated following introduction of pLRfpg16 into *E. coli* strain 5H11S (Olsen et al., 1993). Primer and template were combined with T7 polymerase, T4 ligase, and dGTPαS, dATP, dCTP, and TTP to create one phosphorothioate-containing strand of the duplex plasmid. Preferential restriction of the unmodified strand by *Pst*I was followed by partial exonuclease III digestion of the unmodified strand and formation of duplex containing the oligonucleotide-directed substitutions using DNA polymerase I and T4 ligase. Plasmid DNA from individual carbenicillin-resistant XL1-B colonies was purified (Promega or Qiagen minipreps) and analyzed for the presence of the introduced restriction site. Of the colonies examined, 100% contained the mutation (five colonies were selected). Formation of the desired substitutions was verified by automated DNA sequencing of the region of interest (Applied Biosystems Sequencer Model 373A, Department of Microbiology and Molecular Genetics at the University of Vermont).

Expression in Host Cells. Mutant and wild-type constructs were introduced into strains BL21(DE3) and BLR(DE3) by electroporation. The BL21(DE3) strain may allow homologous recombination to occur between the chromosomal and plasmid DNA, potentially producing a mixed population of proteins, while the BLR(DE3) strain contains a recA deletion mutation that prevents this exchange. Expression was induced by IPTG as described below. Alternatively, induction was achieved via the introduction of λ phage CE6 into XL1-B cells, which prevents production of mutant enzyme prior to phage addition (protocol supplied by Novagen).

Purification of Fpg Proteins. The following methods were used to isolate Fpg protein from *E. coli* expression strain BLR(DE3). Cultures were grown to an absorbance of 0.6 at 600 nm, then induced with 1 mM IPTG and harvested after 3 h. Cells were resuspended in 50 mM Hepes-KOH, pH 7.6, 250 mM KCl, 0.1% Triton X-100, and 1 mg/mL lysozyme and then subjected to three freeze−thaw cycles (Boiteux et al., 1990). Following centrifugation at 39000*g* for 20 min, the supernatant was applied to a charged, equilibrated nickel affinity column (Novagen). Elution steps of 0.005, 0.06, and 1 M imidazole in 20 mM Tris-HCl, pH 7.9, 500 mM NaCl yielded Fpg protein of approximately 95% purity by silver staining of a 12% SDS−PAGE gel. The proteins were dialyzed in 50 mM Hepes, pH 7.6, 250 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, and 10% glycerol at 4 °C with four changes of buffer over 2 days. Separate nickel columns were reserved for the wild-type and mutant proteins to prevent cross-contamination. Purified wild-type Fpg protein possessing the C terminal histidines displayed a substrate profile and specific activity with polymeric DNA containing 7-MeFapyGua (54 nmol/min/mg of protein) that compares favorably with the analogous properties reported for native Fpg protein (specific activity of 43 nmol/min/mg of protein; Boiteux et al., 1990).

Preparation and Analysis of 7-MeFapyGua Oligonucleotide. The oligonucleotide containing 7-MeFapyGua was prepared essentially as described by Tchou et al. (1991). The G-23mer (Table 1, 20 μg) was reacted with 50 mM dimethyl sulfate in 200 mM sodium cacodylate/1 mM EDTA, pH 8.0, at room temperature for 30 min to effect alkylation at the N7 position of guanine. Following ethanol precipitation, the sample was resuspended in 7.6 M ammonium hydroxide and incubated at 37 °C for 1 h to produce the ring opened 7-MeFapyGua isomers. Ammonium hydroxide was removed *in vacuo* and the 7-MeFapyGua-23mer was resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl. The extent of formation of 7-MeFapyGua residues was ≈11% as determined by denaturing polyacrylamide gel electrophoresis of the 5′ end labeled oligonucleotide reaction products and HPLC analysis of free 7-MeFapyGua isomers produced by exhaustive digestion of the duplex oligonucleotide with wild-type Fpg protein (see below). Free 7-MeFapyGua was ≤1.9% of the total labeled strand (≤17% of modified DNA); this defines an upper limit of the extent of formation of AP sites in these preparations.

Analysis of Cleavage Activity by Denaturing Gels. Activities of the mutant and wild-type enzymes were analyzed with 23mer and 24mer oligonucleotide duplex substrates containing a unique damage site at the 11th or 10th position from the 5′ end, respectively (Table 1). Oligonucleotides (5−10 pmol) were labeled using [γ-^{32}P]ATP (2-fold excess at 6000 Ci/mmol) and T4 polynucleotide kinase (3−6 units) at 37 °C for 30 min. Labeled oligonucleotide was separated from ATP using gel filtration (G-50) or reversed-phase (NENSORB, Dupont) columns after 5 min heat inactivation of the enzyme at 65 °C. The oligonucleotide strand containing the modification (8oxoGua, uracil, 7-MeFapyGua or 8oxodN; see Table 1) was annealed with a 1.5-fold molar excess of the complementary strand containing cytosine opposite the modified base. The U-24mer was converted to an AP oligonucleotide by treatment with 1 unit of uracil DNA glycosylase per pmol of oligonucleotide in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl at 37 °C for 15 min. The production of essentially quantitative yields of AP-24mer was established by conversion to the expected degradation products by exhaustive heat treatment (95 °C for 90 min), endonuclease III digestion, or endonuclease VIII digestion. The reactions of Fpg proteins with duplex substrates were conducted in 10 mM Tris-HCl, pH 7.5, and 50 mM KCl at 37 °C for 5 or 10 min and quenched with an equal volume of formamide loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, and 10 mM EDTA in 98% formamide). Kinetic data were typically collected using an oligonucleotide concentration range of 2−100 nM at 0, 30, 60, and 90 s. Product and substrate were separated on a 12.5% denaturing polyacrylamide gel. Dried gels were analyzed by autoradiography and band intensities were quantified with a Fujix BAS 1000 phosphorImager system. The percent of product formed was calculated as the ratio of the product to total measured intensity of the substrate band and the product band(s). Absolute amounts were calculated by assuming that the total of the substrate and product bands was equal to the amount used in the reaction. Lanes containing oligonucleotide without enzyme were used for background correction.

Reversible Trapping of Enzyme−Substrate Intermediates with NaCN. The wild-type or mutant Fpg protein was added to a NaCN dilution series on ice. Duplex 8oxoGua-24mer or AP-24mer (20 nM final concentration) was added in 10 mM Tris-HCl, 50 mM KCl, and the entire mixture was incubated at 37 °C for 10 min. The final NaCN concentrations were 6.25, 12.5, 25, 50, and 100 mM, and parallel control reactions contained matching NaCl concentrations (Dodson et al., 1993). Reactions were quenched with an equal volume of formamide buffer and analyzed by denaturing polyacrylamide gel electrophoresis and phosphorImager measurements as described above.

Irreversible Trapping of Enzyme−Substrate Intermediates with $NaBH_3CN$. Fpg−oligonucleotide covalent complexes were formed using the method of Tchou and Grollman (1995), with minor modifications. The wild-type or K155A mutant protein was combined with $NaBH_3CN$ to which was added the duplex 8oxoGua-24mer or AP-24mer in 100 mM potassium phosphate, pH 6.8, and 60 mM NaCl. Control reactions contained enzyme and $NaBH_3CN$ or enzyme and duplex 24mer without the third component. Final concentrations were 28.6 mM $NaBH_3CN$, 1.65 μM Fpg mutant or wild-type protein, and 3.3 μM duplex 24mer, the concentrations reported by Tchou and Grollman (1995). Following 2.5 h of incubation at 16 °C, glycerol/DTT sample loading buffer (final concentrations: 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) was added and samples briefly heated prior to loading on a 12% SDS−polyacrylamide gel. Bands were visualized by silver staining (Poehling & Neuhoff, 1981), and relative mobility was determined in comparison to protein molecular mass standards. For other preparations, either the strand containing the AP-modification or the complementary strand (cytosine opposite the damage site) was 5′ or 3′ end labeled. Labeling at the 5′ end was performed as described above. Labeling at the 3′ end was accomplished using 2 pmol of oligonucleotide, 4 pmol of [α-^{32}P]cordycepin triphosphate (5000 Ci/mmol), and 4 units of terminal deoxynucleotidyltransferase at 37 °C for 30 min. The reaction was terminated by addition of EDTA to 45 mM. Separation of labeled oligonucleotide and unincorporated cordycepin triphosphate proceeded as described above for 5′ labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase. A single-stranded oligonucleotide containing one of the four possible 5′ or 3′ radiolabeled species was added to unlabeled AP-24mer/C duplexes. Heat denaturation at 75 °C for 5 min followed by slow reannealing to room temperature permitted the formation of specifically radiolabeled duplexes. Trapping experiments were performed as described above with wild-type and K155A enzyme in the presence of one of the oligonucleotide labels and analyzed by SDS−polyacrylamide gels as described above. Gels were dried and subjected to autoradiography.

Measurement of 8oxoGua Release by HPLC. Release of the 8oxoGua free base was measured using a C18 reversed-phase HPLC column (Rainin Microsorb-MV, 5 μm, 4.6 mm × 25 cm) with electrochemical detection (Bioanalytical Systems, Inc. model BAS 200A, isocratic elution in 20 mM potassium citrate, pH 5.5, flow rate of 1 mL/min, electrical potential at +600 mV). Reactions were conducted with mutant or wild-type Fpg protein using 100 nM 8oxoGua-24mer in 10 mM Tris-HCl, pH 7.5, and 50 mM KCl at 37 °C for 30 min. The free base 8oxoGua eluted with a retention time of 7.4 min, and identification of the product

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                 48              63
E. coli          PVLSVQRRAK YLLLEL
H. influenzae    KIVDLTRRAK YLIIHT
N. meningitidis  QVLSCGRRAK YLIVRF
B. firmus        TIRSIRRRGK FLLFEF
L. lactis        TIQGISRRGK YLIFEI
S. mutans        TFESIGRRGK YLLLNL
M. tuberculosis  RINGTDRRGK YLWLTL
M. pneumoniae    SFVDIKRRGK YIIFCL
M. genitalium    FFTDIKRKGK YIIFLL
                 50              65

                 140                                     179
E. coli          GEYLHQKCAK KKTAIKPWLM DNKLVVGVGN IYASESLFAA
H. influenzae    AEYLFKKSRQ KSTALKTFLM DNAVVVGVGN IYTNESLFIC
N. meningitidis  ADYLYVRLKA QKRAVKLALM DNAVVVGVGN IYANESLFRA
B. firmus        SELLMNAFQK TNRKIKVALL DQKTVVGLGN IYVDEALFRA
L. lactis        EKLFREKLRK STKKIKPYLL EQTLVAGLGN IYVDEVLWLA
S. mutans        LKPFEEGLAK SHKVIKTLLL DQHLVAGLGN IYVDEVLWAA
M. tuberculosis  CDAVVKVLRR KHSELKRQLL DQRVVSGIGN IYADEALWRA
M. pneumoniae    HQAIFNKGHK SNKKIKTFIL DQTNISGIGN IYADEILFAS
M. genitalium    YRKIFQKAQN SKRKVKTFIL DQTVISGIGN IYADEILFAS
                 146                                     185
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FIGURE 1: Comparison of primary amino acid sequences of Fpg proteins (partial). Predicted sequences of putative Fpg proteins were aligned using the Pile Up program of the Wisconsin Sequence Analysis Package [species, accession no.: *E. coli*, X06036; *Haemophilus influenzae*, U32776; *Neisseria meningitidis* (group B), U21808; *Bacillus firmus*, X53930; *L. lactis*, X74298; *Streptococcus mutans*, D26071; *Mycobacterium tuberculosis*, Z74697; *Mycoplasma pneumoniae*, AE000045; *Mycoplasma genitalium*, Robison et al. (1996)]. Conserved lysines are indicated in boldface; in addition, conserved lysine residues of *E. coli* Fpg protein are underscored.

peak was confirmed by coinjection of authentic 8oxoGua standard.

Measurement of 7-MeFapyGua Release by HPLC. Release of the 7-MeFapyGua isomers was measured using isocratic elution (10 mM ammonium phosphate, pH 5.2, with 2% methanol, flow rate of 0.8 mL/min) from a C18 reversed-phase column (BAS Phase-II ODS, 3 μm, 100 mm $\times$ 3.2 mm) with UV detection at 260 nm. Two peaks corresponding to 7-MeFapyGua isomeric forms (Boiteux et al., 1984) eluted at 2.2 and 2.9 min and were identified in comparison to standards produced by treatment of 7-methylguanosine with 0.25 N KOH for 1 h at room temperature followed by heating at 90 °C for 10 min in $\approx$0.2 N HCl (Hemminki, 1989).

Circular Dichroism Measurements. CD spectra of wild-type and K155A proteins were collected using an Aviv Model 62 DS circular dichroism spectrometer. The ellipticity of the protein at a concentration of 72 μg/mL in 50 mM sodium phosphate, pH 7.5, 50 mM NaCl, and 5% glycerol was determined at 20 °C using a 2 mm path-length cuvette. Measurements were taken from 250 to 200 nm, with four scans of 5 points/nm. Buffer background collected in the same manner was subtracted from each sample.

General Methods. Protein concentrations were determined using the method of Bradford (1976; Bio-Rad protein dye) with bovine serum albumin as the standard. Kinetic data were analyzed using the Macintosh program k·cat (Biometallics, Inc.).

RESULTS

Rationale for Mutagenesis. We employed interspecies amino acid sequence comparisons as our primary tool for selecting substitution mutations of *E. coli* Fpg protein (Figure 1). Although the *E. coli* Fpg protein was the first member of the group to be purified and studied biochemically (Chetsanga et al., 1981), other potential Fpg sequences were identified via DNA sequencing as gene products with such high homology to the *E. coli* protein that it is very improbable that such similarity would occur by chance. In addition, corroborating functional information has been described for the *Lactococcus lactis* Fpg gene product (Duwat et al., 1995). Among the nine putative Fpg genes, two lysines are absolutely conserved, at positions 57 and 155 (*E. coli* numbering). We were interested in the possible involvement of one of these lysines in covalent catalysis, although there is ample enzymological precedent for acid/base catalysis, ion pair interactions, and structural roles for the side chain of this amino acid residue. Lysine 57 appears to be required for the maintenance of secondary structure (Rabow & Kow, 1996), but the K155A mutant exhibits a CD spectrum that is indistinguishable from wild-type (data not shown), suggesting a role in catalysis.

N-Glycosylase Activities of Crude Supernatants. The initial activity analysis was conducted on crude supernatants (see Materials and Methods) derived from induced *E. coli* strains BL21(DE3), BLR(DE3), and λCE6-infected XL1-B harboring the mutant or wild-type plasmid, with the 8oxo-Gua-24mer as the substrate. The K155A protein exhibits a significant reduction in activity compared to wild-type. There is no detectable activity with cells harboring the parental pET22b vector, and detectable cleavage of the λ infected XL1-B cells is only observed at supernatant concentrations 10-fold greater than the highest concentration used with the transformed cells (data not shown). Coomassie blue staining of the total protein separated on a 12% SDS−polyacrylamide gel reveals similar high levels of induction of wild-type and K155A Fpg proteins (data not shown). Since the levels of expression of enzyme are high and background levels are low, the activity assays in crude supernatant allow for meaningful comparisons when levels of expression of mutant and wild-type are comparable. At a 500-fold dilution, crude supernatants from λ infected XL1-B cells exhibited 89% cleavage of the 8oxoGua-24mer at 20 nM substrate concentration in 10 min at 37 °C when expressing the wild-type protein and 5% cleavage activity with the K155A mutant. The values obtained at 5000-fold dilutions of crude supernatant were 36 and 1% cleavage for wild-type and K155A proteins, respectively. Quantitatively similar results were obtained following transformation of the BL21(DE3) or BLR(DE3) *E. coli* strains. Because of its ease of use and the ability to scale up the size of the culture, Fpg variants were induced in the recA$^-$ BLR(DE3) cells for protein purification.

Activities of Purified Fpg Variants. The mutant and wild-type Fpg proteins were processed using a nickel affinity column [His-Bind resin (Novagen)] as the essential method for rapid purification. Analysis of cleavage products of the 8oxoGua- and AP-24mers is shown in Figure 2, panels A and B. 8oxoGua *N*-glycosylase activity is diminished for the K155A site specific mutant. In striking contrast, the AP lyase activity is actually enhanced in the K155A mutant compared to the wild-type. For the 8oxoGua-24mer, the ratio of specific activities of wild-type versus K155A mutant is 51:1. The corresponding ratio with the AP-24mer is 1:2.1. There is clear evidence for multiple rounds of turnover under all conditions with the exception of the 8oxoGua *N*-glycosylase activity for the K155A mutant, where moles of product formed never exceeded the molar quantity of K155A mutant protein added.

In addition to DNA strand scission (Figure 2), release of the monomeric product 8oxoGua would be expected when

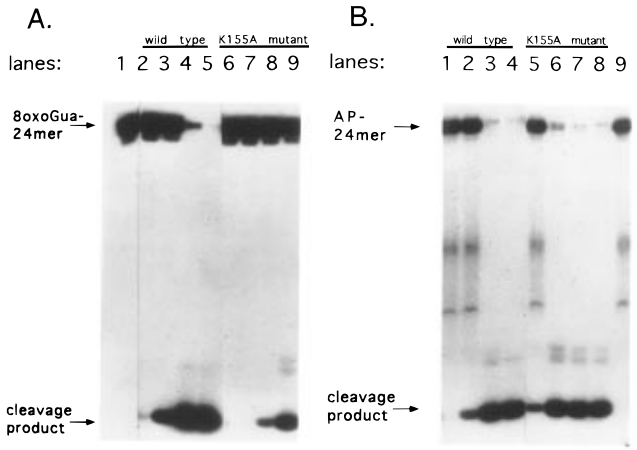


FIGURE 2: Activity of affinity-purified Fpg protein variants. Strand scission was measured with (A) a 5′ ^{32}P-labeled 8oxoGua-24mer or (B) a 5′ ^{32}P-labeled AP-24mer (20 nM of labeled strand) in 10 mM Tris-HCl, pH 7.5, 50 mM KCl and incubated at 37 °C for 5 min. (A) Lane 1, no enzyme; lanes 2 and 6, 1:50000 dilution; lanes 3 and 7, 1:5000 dilution; lanes 4 and 8, 1:500 dilution; lanes 5 and 9, 1:50 final dilution of enzyme stock. (B) Lanes 1 and 5, 1:50000 dilution; lanes 2 and 6, 1:5000 dilution; lanes 3 and 7, 1:500 dilution; lanes 4 and 8, 1:50 final dilution of enzyme stock; lane 9, no enzyme. Stock concentrations were 0.158 and 0.198 mg/mL for wild-type and K155A protein, respectively. Substrate 8oxoGua-24mer (A) or AP-24mer (B) and cleavage product are indicated by arrows at left.

the 8oxoGua-24mer is used as a substrate. In order to affirm that base release also occurs, we employed HPLC using a C18 reversed-phase column with electrochemical detection. Figure 3A shows release of the free base 8oxoGua following treatment of the 8oxoGua-24mer with excess K155A mutant. Coinjection of authentic standard produces enhancement of the product peak (Figure 3B) while standard alone also displays the same retention time (data not shown). Comparisons of the peak heights and areas indicate that wild-type and K155A mutants release 95−99% of the 8oxoGua following analysis of 2 or 5 pmol of 8oxoGua-24mer incubated with excess enzyme. Thus, the mutant enzyme is fully competent to produce the free base that is characteristic of the Fpg *N*-glycosylase reaction.

Evidence for Sodium Cyanide Trapping of Fpg Variants. The hypothesis that one of the conserved lysines is involved in formation of an imine covalent intermediate leads to the prediction that chemical trapping should be possible for the native enzyme but not one possessing a mutated lysine (Scheme 2). The methods of sodium cyanide and sodium cyanoborohydride complex formation are well-established biochemical techniques that have demonstrated applicability to DNA repair glycosylases/lyases (Tchou & Grollman, 1995; Dodson et al., 1993). Figure 4, panels A and C, illustrates the result of combining 8oxoGua-24mer with either wild-type or K155A mutant in the presence of successive doublings of the sodium cyanide or sodium chloride concentration from 6.25 to 100 mM. Sodium chloride serves as a control for ionic strength, which is known to influence the Fpg enzymatic reactions (Boiteux et al., 1990). The wild-type strand scission reaction is insensitive to chloride ion at the enzyme concentration used, while the K155A mutant shows diminished activity with increasing sodium chloride (Figure 4, panels A and C). Incubation and electrophoresis in the presence of sodium cyanide yields radioactive material from above the position of the 8oxoGua-24mer substrate

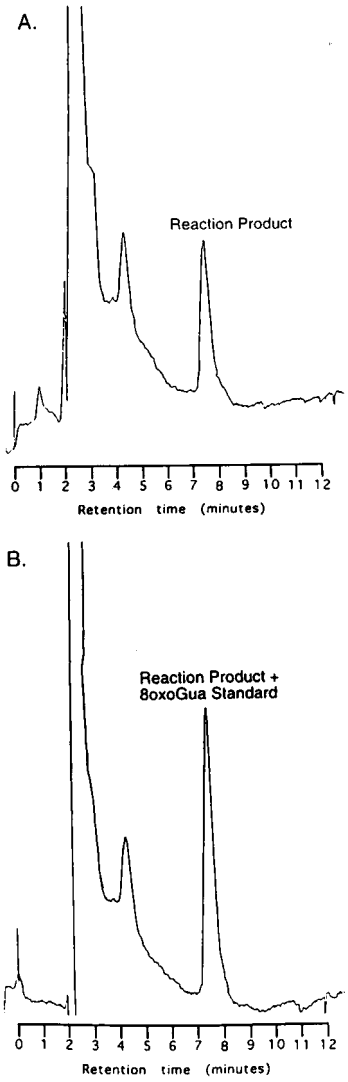
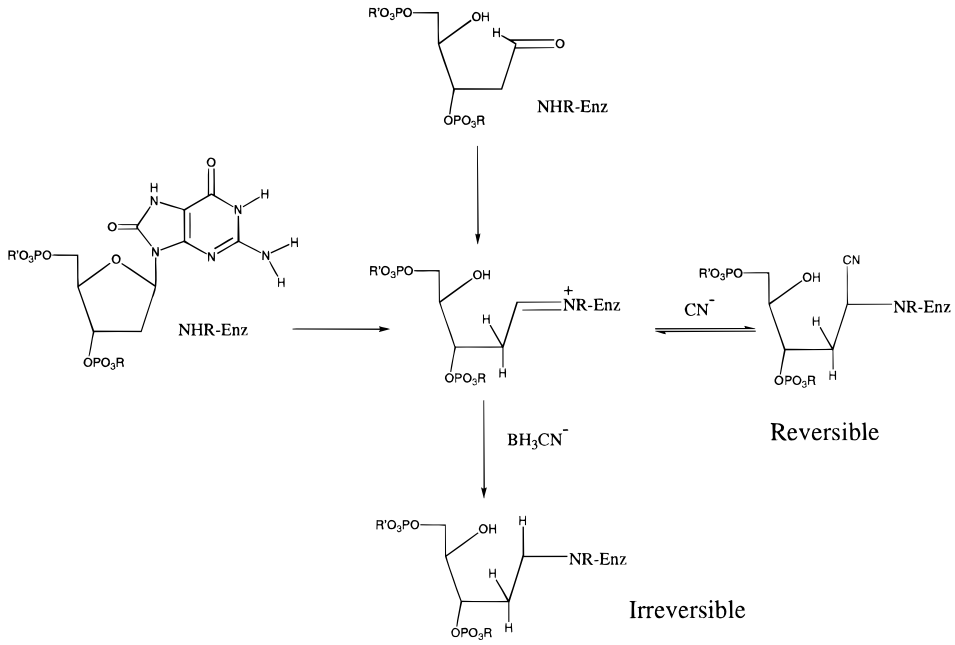


FIGURE 3: Release of 8oxoGua free base by K155A mutant. (A) Chromatogram from C18 reversed-phase HPLC analysis (electrochemical detection, +600 mV) of products from reaction of 2 pmol of 8oxoGua-24mer with 1.6 μg of K155A Fpg mutant at 37 °C for 30 min. (B) Standard addition of 2 pmol of authentic 8oxoGua free base to reaction mixture of panel A prior to injection onto column. Elution was accomplished with 20 mM potassium citrate buffer, pH 5.5, at a flow rate of 1 mL/min.

band to the product band below it for both K155A mutant and wild-type enzymes, although the relative intensity is greater for wild-type (Figure 4A). The K155A mutant displays a complete inhibition of strand scission activity at the two highest sodium cyanide concentrations. Additionally, there is evidence of radioactive material that barely penetrates the gel for both wild-type and K155A mutant Fpg protein incubated with sodium cyanide and 8oxoGua-24mer (not illustrated). These autoradiograms provide evidence for the formation of a reversible enzyme−substrate complex that partially decomposes during electrophoresis. A semiquantitative comparison of the effect of sodium cyanide on product formation is obtained by calculating the ratio of product band to the total of product and substrate using the 8oxoGua-24mer (Figure 4C) and AP-24mer (Figure 4D). With the 8oxoGua-24mer substrate, the cyanide-dependent inhibition of K155A cleavage activity is greater than that of the control at the same sodium chloride concentration (Figure 4C). Both wild-type and the K155A mutant display similar inhibition tendencies when each is incubated with sodium

Scheme 2: Schematic Representation of Reversible or Irreversible Trapping of 8oxoGua- or AP-24mer and Wild-Type or K155A Mutant Enzyme by NaCN or $NaBH_3CN$[a]



[a] The central enzyme−substrate imine intermediate is the presumptive species trapped by either reagent.

cyanide and the AP-24mer (Figure 4, panels B and D). With the AP-24mer substrate, both mutant and wild-type enzyme display a cyanide concentration-dependent decrease in product formation with concomitant streaking of radioactive material (Figure 4B), similar to that described above. At the higher cyanide concentrations, the wild-type enzyme is less cyanide sensitive than the K155A mutant using either substrate. This may be a consequence of the lower activity of the K155A mutant with the 8oxoGua-24mer and the potentially greater accessibility of the active site of the mutant enzyme to small ions. In sum, these observations are consistent with the interpretation that cyanide forms a transient covalent complex with both the mutant and wild-type Fpg enzyme−substrate imine intermediates as illustrated in Scheme 2. Lysine 155 is not required for cyanide trapping of the enzyme−substrate complex and therefore not essential for covalent catalysis.

Evidence of Sodium Cyanoborohydride Modification of Fpg Variants. The recent report by Tchou and Grollman (1995) includes conditions for irreversible trapping of an Fpg protein−substrate complex with sodium cyanoborohydride. We have adapted these conditions to probe for the existence of enzyme−substrate complexes in the presence of Fpg mutant or wild-type, 8oxoGua-24mer or AP-24mer, and sodium cyanoborohydride. Figure 5, panels A and B, represents essentially similar experiments except for the choice of oligonucleotide (Figure 5A corresponds to the 8oxoGua-24mer and Figure 5B corresponds to the AP-24mer). For either panel, the three lanes following the protein molecular mass markers contain wild-type Fpg protein and the next three lanes contain K155A protein. The first of the three lanes for each variant was incubated at 16 °C for 2.5 h in the presence of 28.6 mM sodium cyanoborohydride without oligonucleotide, the second lane contains protein and 8oxoGua-24mer (Figure 5A) or AP-24mer (Figure 5B), and the third lane for either variant contains all three components. Two major lower mobility bands are observed with both variants (apparent molecular mass of 39 and 44 kDa[2]). The extent of formation of these two bands is greater for the wild-type enzyme than for the K155A mutant when the 8oxoGua-24mer is trapped (Figure 5A). When the AP-24mer is used as a substrate, both low-mobility species are apparent for both wild-type and K155A mutant Fpg protein in lanes combining oligonucleotide, enzyme, and sodium cyanoborohydride (Figure 5B). The band of low intensity below the doublet but above the monomeric Fpg protein (apparent molecular weight of 35 kDa) may represent cleaved substrate complexed with protein (either covalent or noncovalent). It is also possible to adjust the relative amounts of damage-containing and complementary strand so that the lower mobility band (molecular mass of 44 kDa) of the doublet becomes the predominant or sole low-mobility band observed (data not shown).

A series of control experiments demonstrate that an active enzyme and an intact AP site are required for specific formation of the doublet species. Incubation of heat denatured wild-type Fpg protein (90 °C for 30 min) with the AP-24mer and $NaBH_3CN$ results in a very low level of trapping; in addition, trapping of at least four sets of products of increasing apparent molecular weights were observed, suggesting indiscriminate complex formation (data not shown). Incubation of the AP-24mer with 100 mM $NaBH_4$ for 1 h prior to enzyme addition produces a reduced AP site (deoxypentitol site) and prevents formation of the characteristic doublet upon Fpg addition. No complex is formed between Fpg protein and the U-24mer in the presence of $NaBH_3CN$ (data not shown).

To further elucidate the nature of the low-mobility bands observed by silver staining, either the damage-containing AP-24mer strand or the complementary strand was 5′- or 3′-end labeled (see Materials and Methods). The autoradiogram

[2] Since the gels have been calibrated using polypeptide markers, the apparent molecular mass of the Fpg protein−oligonucleotide complexes would not necessarily equal the total of the molecular mass of the separate protein and DNA components.

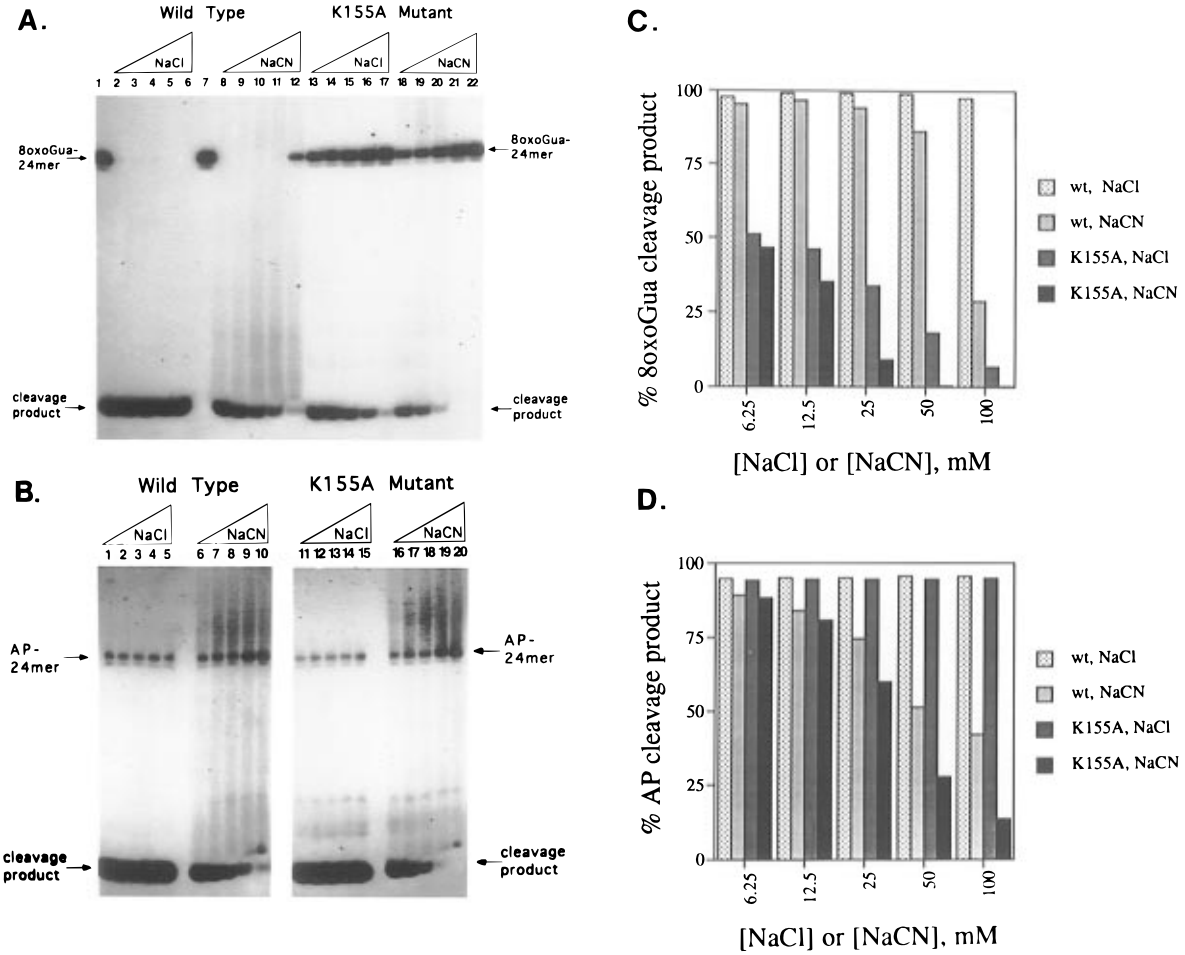


FIGURE 4: Reversible trapping of 8oxoGua- and AP-24mer with wild-type or K155A Fpg protein by NaCN. (A) Autoradiogram of 12.5% denaturing polyacrylamide gel illustrating cleavage of 8oxoGua-24mer in the presence of increasing concentrations of NaCl or NaCN. Lanes 2−6 and 8−12 contain wild-type protein. Lanes 13−22 contain K155A mutant protein. NaCl or NaCN concentration increases by a factor of 2 from left to right: lane 1, 100 mM NaCl, no enzyme; lanes 2 and 13, 6.25 mM NaCl; lanes 3 and 14, 12.5 mM NaCl; lanes 4 and 15, 25 mM NaCl; lanes 5 and 16, 50 mM NaCl; lanes 6 and 17, 100 mM NaCl; lane 7, 100 mM NaCN, no enzyme; lanes 8 and 18, 6.25 mM NaCN; lanes 9 and 19, 12.5 mM NaCN; lanes 10 and 20, 25 mM NaCN; lanes 11 and 21, 50 mM NaCN; lanes 12 and 22, 100 mM NaCN. Substrate and product bands are indicated by arrows. (B) Autoradiogram of 12.5% denaturing polyacrylamide gel illustrating cleavage of AP-24mer in the presence of increasing concentrations of NaCl or NaCN. Lanes 1−10 contain wild-type protein. Lanes 11−20 contain K155A mutant protein. NaCl or NaCN concentration increases by a factor of 2 from left to right: lanes 1 and 11, 6.25 mM NaCl; lanes 2 and 12, 12.5 mM NaCl; lanes 3 and 13, 25 mM NaCl; lanes 4 and 14, 50 mM NaCl; lanes 5 and 15, 100 mM NaCl; lanes 6 and 16, 6.25 mM NaCN; lanes 7 and 17, 12.5 mM NaCN; lanes 8 and 18, 25 mM NaCN; lanes 9 and 19, 50 mM NaCN; lanes 10 and 20, 100 mM NaCN. Substrate and product bands are indicated by arrows. Enzyme concentrations were 26 and 78 μg/mL for wild-type and K155A mutant protein, respectively, for both 8oxoGua-24mer and AP-24mer trapping experiments. (C) Bar graph of percentage of 8oxoGua-24mer cleavage product at indicated [NaCl] or [NaCN] for wild-type and K155A mutant Fpg protein. (D) Bar graph of percentage of AP-24mer cleavage product at indicated [NaCl] or [NaCN] for wild-type and K155A mutant Fpg protein.

of Figure 5C shows that 5′- or 3′-labeled AP-24mer or cytosine-Comp-24mer strand comigrates with the upper band of the silver-stained doublet (molecular mass ≈ 44 kDa) for either Fpg variant. The AP-containing strand also appears to be a part of the lower band of the doublet (molecular mass ≈ 39 kDa), as revealed by both 5′ phosphorylation and 3′ extension with cordycepin triphosphate. This labeling pattern is consistent with a covalent protein−double-stranded oligonucleotide complex for the upper band and a covalent protein−single stranded oligonucleotide complex containing only the AP-strand for the lower band. In addition, overexposure of the autoradiogram showed the presence of label from the 5′ end of the AP-containing strand comigrating with the faint silver-stained band with a mobility between that of Fpg protein alone and the doublet complex (molecular mass ≈ 35 kDa). This suggests that a minor component of sodium cyanoborohydride trapping of all Fpg variants contains the protein plus the first nine nucleotides from the 5′ end, presumably formed subsequent to the 3′ β-lyase step (Scheme 1). The results of these $NaBH_3CN$ trapping experiments demonstrate that specific enzyme−oligonucleotide complex formation occurs with both the K155A mutant and wild-type Fpg protein. These experiments provide further evidence that lysine 155 of Fpg protein in not essential for covalent catalysis.

Kinetic Analysis of Oligonucleotide Substrates. In order to gain further insight into the role of lysine 155 in the reaction of Fpg with its substrates, we completed steady state kinetic analyses of the reaction of various base modified substrates and an AP-containing substrate. The four substrates utilized provide a comprehensive analysis of the interaction of the various portions of the substrate molecule with the wild-type and mutant enzyme. The K155A mutant had demonstrated, in studies described above, a 50-fold reduction in activity with an 8oxoGua-24mer substrate at 20 nM (Figure 2A) and a slight enhancement of activity

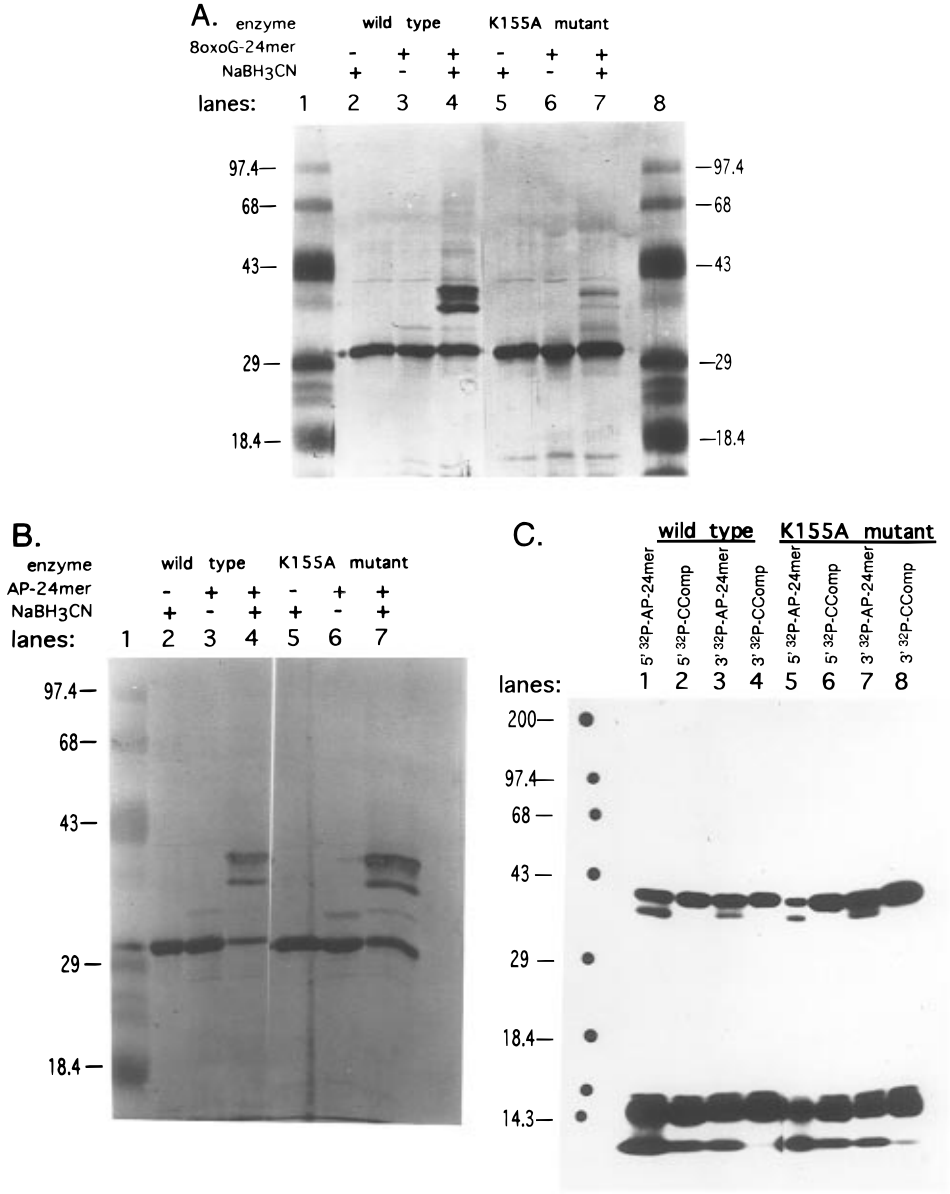


FIGURE 5: Irreversible trapping of 8oxoGua- and AP-24mer and wild-type or K155A Fpg protein by $NaBH_3CN$. (A) Twelve percent Laemmeli SDS−polyacrylamide gel developed by silver staining. Lanes 1 and 8, molecular mass markers; lanes 2−4, wild-type protein; lanes 5−7, K155A mutant protein; lanes 2 and 5, protein and $NaBH_3CN$; lanes 3 and 6, protein and 8oxoGua-24mer; lanes 4 and 7, protein, $NaBH_3CN$ and 8oxoGua-24mer. (B) Twelve percent Laemmeli SDS−polyacrylamide gel developed by silver staining. Lane 1, molecular mass markers; lanes 2−4, wild-type protein; lanes 5−7, K155A mutant protein; lanes 2 and 5, protein and $NaBH_3CN$; lanes 3 and 6, protein and AP-24mer; lanes 4 and 7, protein, $NaBH_3CN$ and AP-24mer. (C) Autoradiogram of 12% Laemmeli SDS−polyacrylamide gel of AP-24mer with wild-type (lanes 1−4) or K155A (lanes 5−8) Fpg protein. Lanes 1 and 5, 5′ ^{32}P end labeled AP-24mer strand; lanes 2 and 6, 5′ ^{32}P end labeled CComp-24mer strand; lanes 3 and 7, 3′ ^{32}P end labeled AP-24mer strand; lanes 4 and 8, 3′ ^{32}P end labeled CComp-24mer strand. Monomeric Fpg protein migrates as 31 kDa polypeptide. Molecular mass markers (Gibco-BRL high-range prestained protein molecular mass markers) are as follows: lysozyme (14.3 kDa), β-lactoglobin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorylase b (97.4 kDa), myosin (H-chain) (200 kDa).

compared to wild-type with an AP-24mer at the same substrate concentration (Figure 2B). In order to explore the interactions of K155A with the base portion of the oligonucleotide molecule, substrates containing either 8oxodN or 7-MeFapyGua were examined as examples of loss of functional groups at the pyrimidine ring or rupturing of the imidazole ring of 8oxoGua, respectively (see Chart 1 for structures).

The results of steady state kinetic analyses of 8oxoGua-, AP-, and 7-MeFapyGua-containing oligonucleotides are summarized in Table 2. The kinetic parameters with the 8oxoGua-24mer substrate indicate that the rate of reaction with the K155A mutant is consistently 50-fold lower than the wild-type enzyme over the entire substrate concentration range. In contrast, for the 7-MeFapyGua-23mer, the relative k_{cat}/K_m and k_{cat} values for the wild-type are 4-fold and 3-fold greater than the values for the K155A mutant, respectively. Thus, replacement of lysine with alanine at position 155 produces a much larger reduction in activity with the 8oxoGua-24mer substrate than the 7-MeFapyGua-23mer. For the AP-24mer, the kinetic parameters of the mutant exceed those for the wild-type. The relative k_{cat}/K_m and k_{cat} values are 32 and 1.6 times greater for the K155A mutant. It is also interesting to note that the K_m values for the glycosylase

Table 2: Kinetic Parameters of Mutant and Wild-Type Fpg Protein

protein	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} nM^{-1})
8oxoGua Modification[a]			
wild-type	6.9 ± 3.7	1.11 ± 0.18	0.16 ± 0.09
K155A mutant	7.5 ± 1.5	0.023 ± 0.001	0.003 ± 0.0006
7-MeFapyGua Modification[b]			
wild-type	23.1 ± 0.35	0.23 ± 0.002	0.0101 ± 0.00017
K155A mutant	28.7 ± 3.7	0.0720 ± 0.0035	0.0025 ± 0.0003
AP-Substrate[a]			
wild-type	19 ± 5.7	3.41 ± 0.34	0.182 ± 0.056
K155A mutant	0.91 ± 0.13[c]	5.30 ± 0.12	5.83 ± 0.84

[a] Substrate concentration range 2−100 nM. [b] 7-MeFapyGua from partial conversion of a unique G of 23mer (11% modified sites); substrate concentrations range 4.4−220 nM. [c] K_m of 0.25 ± 0.03 nM when lower concentration range used (0.2−10 nM substrate concentration).

substrates (8oxoGua-24mer and 7-MeFapyGua-23mer) are insensitive to the change at position 155, while the K_m value of the AP-24mer displays considerable sensitivity.

To probe for possible interactions of lysine 155 with the functional groups of the pyrimidine ring of 8oxoGua, we studied the interaction of the mutant and wild-type enzyme with a 23mer containing a unique 8oxodN site. As the wild-type enzyme has very low activity with this substrate and the K155A mutant is even less active (see below), it was not feasible to perform a complete kinetic analysis with the 8oxodN-23mer. Instead, time dependence and enzyme dependence were evaluated at several substrate concentrations. The dependence of cleavage of 4 and 20 nM 8oxodN on wild-type and K155A mutant enzyme concentrations is shown in Figure 6A, while the graph in Figure 6B illustrates the time-dependent cleavage of 3 and 30 nM 8oxodN for both enzyme variants. Using the K155A mutant, the cleavage of the 8oxodN oligonucleotide is greatly diminished at all concentrations studied. The relative activity of the mutant ranges from 1.3 to 7.3% of the value of wild-type (mean: 4.0 ± 2.0%, using five substrate concentrations between 3 and 30 nM). Therefore, the substrate containing a unique 8oxopurine (8oxodN) site displays an activity profile that is very similar to that observed for the 8oxoGua substrate. These results support the assertion that lysine 155 does not interact with the functionality of the pyrimidine ring of 8oxoGua.

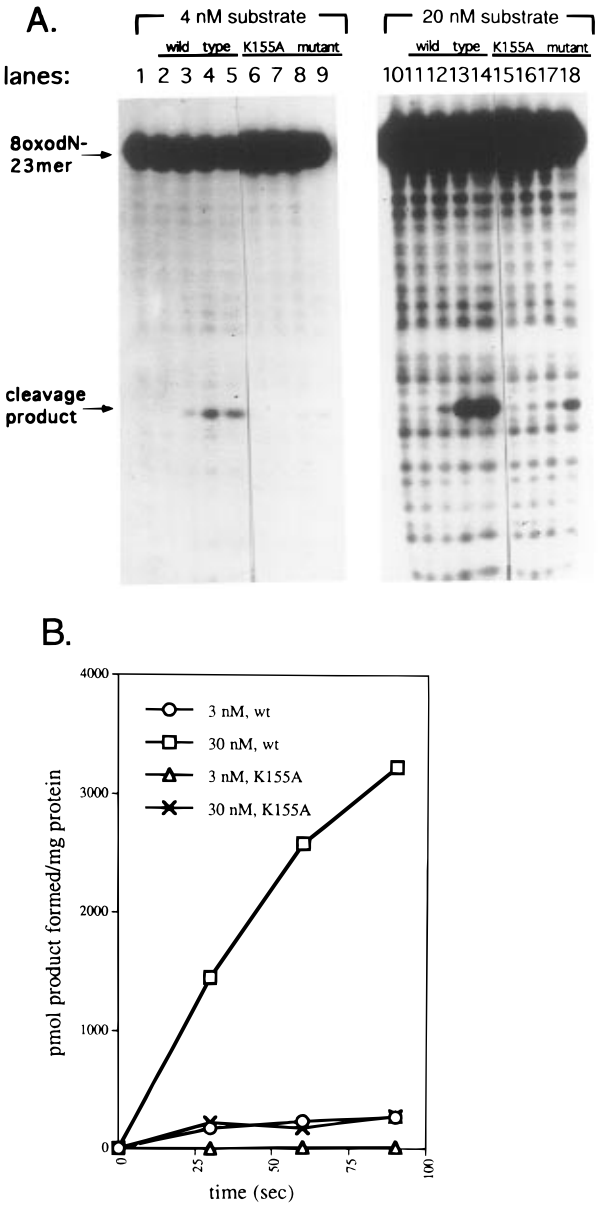


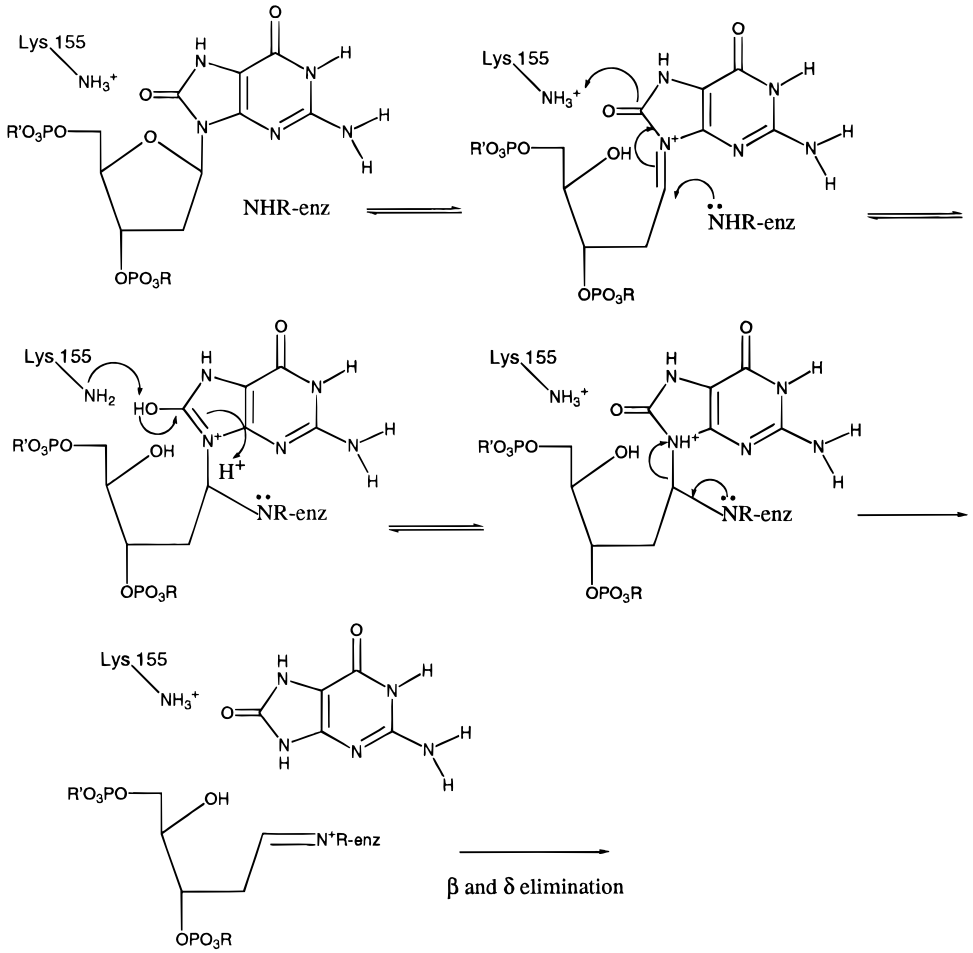
FIGURE 6: Activity of purified Fpg protein variants with 8oxodN-23mer substrate. (A) The dependence of strand scission on enzyme concentration was measured using a 5′ ^{32}P-labeled 8oxodN-23mer at 4 nM (lanes 1−9) or 20 nM (lanes 10−18). The autoradiogram of a 12.5% denaturing polyacrylamide gel for the reaction of 8oxodN duplex in 10 mM Tris-HCl, pH 7.5, 50 mM KCl and incubated at 37 °C for 5 min with wild-type (lanes 2−5 and 11−14) or K155A mutant (lanes 6−9 and 15−18) Fpg protein is shown. Lane 1 and 10, no enzyme; lanes 2, 6, 11, and 15, 1:5000 dilution; lane 3, 7, 12, and 16, 1:500 dilution; lanes 4, 8, 13, and 17, 1:50 dilution; lanes 5, 9, 14, and 18, 1:5 final dilution of enzyme stock. Stock concentrations were 0.132 and 0.39 mg/mL for wild-type and K155A protein, respectively. The autoradiogram was overexposed to permit visualization of the product band. The intensities of the minor bands remain unchanged as protein concentration is increased. (B) Graph of time dependence of cleavage of duplex 8oxodN-23mer by wild-type and K155A mutant Fpg enzymes (0.5 μM) at 3 and 30 nM substrate from 0−90 s (single determinations at each time point).

DISCUSSION

Substitution of phylogenetically conserved lysine 155 of *E. coli* Fpg protein with alanine has revealed some details of the interaction of the protein with various substrates and substrate-analogs. Defined oligonucleotides containing a single 8oxoGua- or 8oxodN-modified nucleotide (Chart 1) display virtually the same reduction in activity with the K155A mutant compared to wild-type (25−50-fold). This is true despite the large difference in activity of 8oxoGua and 8oxodN oligonucleotides with wild-type protein (Tchou et al., 1994; see discussion below). In contrast, the decrease in activity with a 7-MeFapyGua-oligonucleotide is much more modest; the activity is reduced approximately 3−4-fold. It is also interesting that the magnitude of these effects is almost independent of substrate concentration and the K_m values are virtually unaffected by the K155A substitution. In contrast to the activity with base-substituted oligonucleotides, the activity with an oligonucleotide possessing a unique AP site exhibits enhanced activity at low substrate concentration (k_{cat}/K_m conditions) but only slightly elevated activity under k_{cat} conditions.

The data are readily accommodated by a model requiring a direct interaction between lysine 155 and the carbonyl oxygen at the C8 position of 8-oxopurines (Scheme 3). The first step is postulated to be substrate imine formation at the

Scheme 3: Postulated Role for Lysine 155 of Fpg Protein[a]

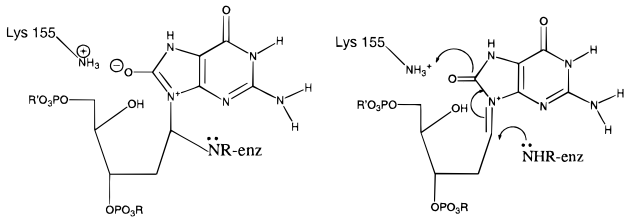


[a] Lysine 155 interacts with the O8 oxygen of 8oxoGua and functions as a general acid catalyst. Alternatively, the reaction may proceed via formation of an ion pair between the lysine 155 ammonium ion and the O8 oxyanion (see text).

enzyme active site. The movement of the modified DNA base relative to enzyme that would be required for this ring-opening step may place the C8 oxygen of 8oxoGua in close proximity to the ϵ-ammonium group of lysine 155. Attack of the proposed nucleophile (a different lysine ϵ-amino group or the N-terminal proline secondary amino group; Tchou & Grollman, 1995) would then be accompanied by a shifting of the electron density, culminating in proton transfer from lysine 155 to the oxyanion of 8oxoGua. The lysine ϵ-amino group could then abstract the proton and further electronic rearrangement would transiently protonate the N9 position of 8oxoGua. Collapse of the tetrahedral intermediate at C1′ would yield the free base 8oxoGua and the proposed enzyme−substrate imine intermediate. The complete base release sequence would require only one other acid/base catalyst in addition to lysine 155, and all steps would be reversible prior to release of 8oxoGua.

The initiating step of this model is the formation of a substrate−imine that would precede formation of the covalent enzyme−substrate imine intermediate, as originally proposed by Kow and Wallace (1987). Support for this mechanistic feature has been obtained from studies of alkoxyamine-modified DNA (Purmal et al., 1996). The alternative model for formation of the covalent enzyme−substrate imine intermediate is a direct displacement model with significant S_N2 character (Dodson et al., 1994). The postulated role for lysine 155 would be identical if a direct displacement elimination were postulated, with lysine 155 of Fpg protein playing a role that is very similar to what has been proposed for glutamic acid 23 of T4 endonuclease V (Doi et al., 1992; Vassylyev et al., 1995).

While the data presented in this communication are completely consistent with a direct interaction between lysine 155 and the C8 oxygen of 8oxoGua, acid/base catalysis is not the only role for lysine 155 that is compatible with the known information. Alternatively, the ammonium group of lysine 155 and the oxyanion of 8oxoGua could form a transient ion pair. The two possibilities are shown below:



Both of these models have ample precedent in the enzymological literature. Evidence for lysine involvement in covalent and acid/base catalysis at pH values below the pK_a for the ϵ-amino group of the free amino acid lysine (p$K \approx$ 10.5) is well-documented for acetoacetate decarboxylase (Schmidt & Westheimer, 1971; Highbarger et al., 1996), mandelate racemase, and muconate lactonizing enzyme

(Babbitt et al., 1995; Landro et al., 1994). Formation of an oxyanion stabilized by the interaction with lysine ϵ-ammonium residues has been described for ribulose bisphosphate carboxylase (Schneider et al., 1992; Harpel et al., 1995) and a second lysine of mandelate racemase (Landro et al., 1994).

A study by Tchou et al. (1994) examined the substrate specificity of native Fpg protein using a variety of substrate analogs. These investigators examined the interactions of the exocyclic functionality at the pyrimidine (six-membered) ring of 8oxopurines with Fpg protein. The relative k_{cat}/K_m and k_{cat} values were 388- and 41-fold higher, respectively, for oligonucleotides possessing a unique 8oxoGua nucleotide compared to an 8oxodN nucleotide within the same sequence when cytosine is the base opposite the modification. These results and others described by Tchou et al. (1994) suggest that interactions with the N1 position and exocyclic O6 positions are of predominant importance.

In the studies reported here, we have altered the protein component using site-directed mutagenesis and examined the effect on substrate specificity. The interactions of Fpg protein are reduced almost 2 orders of magnitude with oligonucleotides possessing a unique 8oxoGua or 8oxodN nucleotide when alanine replaces lysine at position 155 of the enzyme. The loss of activity with the K155A variant is strikingly similar for these two-base modifications and suggests that lysine 155 does not interact with the functional groups and hydrogen bond donor/acceptors of the six-membered pyrimidine ring of 8oxopurines (see Chart 1 for structures). In contrast, the 7-MeFapyGua-modified oligonucleotide retains 25−31% activity with the K155A mutant. This suggests that the binding interaction that would be possible between the formamido oxygen of 7-MeFapyGua and lysine 155 of Fpg protein is a relatively minor contributor to the rate acceleration provided by interaction of enzyme and substrate. This, then, strengthens the assertion that the interaction of lysine with the 8oxo group of the 8oxopurines requires steps along the reaction coordinate, such as those detailed in Scheme 3. The robust reaction catalyzed by the K155A protein with AP sites provides support for a specific interaction between lysine 155 and the modified DNA bases, and further suggests that β and δ elimination steps (Bailly et al., 1989a, b; Bhagwat & Gerlt, 1996) are unaffected by the amino acid substitution. Taken together, the evidence is compelling that the five-membered imidazole ring is involved in the interaction with lysine 155, and the chemical identity of lysine with its reactive ϵ-amino/ammonium group favors a specific interaction with the C8 oxygen anion.

No physical description of the three-dimensional structure of Fpg protein is yet available, but several proteins involved in DNA repair have been examined by X-ray crystallography. Endonuclease III forms a hydrophilic pocket that can recognize oxidized pyrimidine DNA modifications (Thayer et al., 1995). In contrast, the active site cleft of 3-methyladenine DNA glycosylase II (Alk A) is rich in electron donating aromatic residues (Yamagata et al., 1996; Labahn et al., 1996). On the basis of the biochemical studies reported here and by Tchou et al. (1994), we would anticipate that hydrophilic residues line either side of the specificity pocket of Fpg protein, including lysine 155, which is poised to interact with the carbonyl oxygen of C8 of 8oxopurines.

Many prokaryotic homologs of *E. coli* Fpg protein have been discovered by DNA sequencing (Figure 1), although homologous eukaryotic sequences have yet to be described. Nevertheless, this group of prokaryotic organisms spans a vast range (Olsen & Woese, 1993), including Gram-positive and -negative eubacteria, and bacterial mycoplasma that possess very small genomes. For all these organisms, the position corresponding to *E. coli* lysine 155 is completely conserved. In contrast, the *E. coli* repair enzyme endonuclease VIII that recognizes pyrimidine damage (Melamede et al., 1994) shares significant regions of homology with Fpg protein in both the N-terminal and C-terminal regions (Jiang and Wallace, accession no. U38616). Importantly, the central portion of the endonuclease VIII and Fpg polypeptides differ significantly, and there is no lysine in endonuclease VIII that corresponds to lysine 155 of Fpg protein.

If 8oxoGua is the primary damage recognized by Fpg protein *in vivo*, a mutation in the fpg gene coding for lysine at position 155 would greatly diminish removal of 8oxoGua. The enzyme would remain an effective AP lyase; however, given the rapid rate of formation of 8oxoGua sites in oxidizing environments, G to T transversion mutations would increase in frequency (Michaels et al., 1991) with deleterious consequences for the organism.

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