Mutation of a Unique Aspartate Residue Abolishes the Catalytic Activity but Not Substrate Binding of the Mouse N-Methylpurine-DNA Glycosylase (MPG)*

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N-Methylpurine-DNA glycosylase (MPG) initiates base excision repair in DNA by removing a variety of alkylated purine adducts. Although Asp was identified as the active site residue in various DNA glycosylases based on the crystal structure, Glu-125 in human MPG (Glu-145 in mouse MPG) was recently proposed to be the catalytic residue. Mutational analysis for all Asp residues in a truncated, fully active MPG protein showed that only Asp-152 (Asp-132 in the human protein), which is located near the active site, is essential for catalytic activity. However, the substrate binding was not affected in the inactive Glu-152, Asn-152, and Ala-152 mutants. Furthermore, mutation of Asp-152 did not significantly affect the intrinsic tryptophan fluorescence of the enzyme and the far UV CD spectra, although a small change in the near UV CD spectra of the mutants suggests localized conformational change in the aromatic residues. We propose that in addition to Glu-145 in mouse MPG, which functions as the activator of a water molecule for nucleophilic attack, Asp-152 plays an essential role either by donating a proton to the substrate base and, thus, facilitating its release or by stabilizing the steric configuration of the active site pocket.

Cellular DNA is continuously exposed to endogenous or exogenous chemical or physical agents that induce DNA lesions. The DNA base damage threatens the genomic stability and cellular viability (1). Multiple DNA repair pathways exist in all organisms from bacteria to man to preserve the integrity of the genome (1).

Unrepaired, damaged bases could be mutagenic (2) and cause cytotoxicity by blocking DNA replication (3). The DNA base excision repair pathway is primarily responsible for repairing the small alkylation adducts and other base modifications. This repair process is initiated by DNA glycosylases for the recognition and removal of inappropriate bases by cleavage of the C1'-N-glycosyl bond (4). Simple DNA glycosylases, e.g. N-methylpurine-DNA glycosylase (MPG),1 remove abnormal and modified bases and generate apurinic/apyrimidinic (AP) sites. Mixed function DNA glycosylase/AP lyases, on the other hand, after base cleavage carry out a β (or βδ) elimination reaction on the free deoxyribose residue, causing a DNA strand break at the resulting AP sites, and generate 3'-phosphosugar (or 3'-phosphate) residue termini. AP endonucleases cleave DNA strands to the AP sites and generate 3'-OH and 5'-phosphodeoxyribonucleotides. Subsequent repair steps include removal of the 5'-phosphodeoxyribose by deoxyribosephosphate lyase, filling the resulting DNA gap with a DNA polymerase, and finally sealing of the repaired strand by DNA ligase (4–5).

Although MPGs from several mammalian sources with different molecular masses (25–68 kDa) were reported, only one MPG, homologous in the humans and rodents, has been extensively characterized after its cDNA was cloned by several laboratories including ours (6–7). This protein is similar to the inducible AlkA protein of Escherichia coli in regard to their broad substrate range. E. coli has a second MPG, the constitutive Tag protein, that is specific for 3-alkyladenine (8).

Mammalian MPGs, like many other DNA glycosylases, are monomeric in active form, do not have an absolute requirement for a cofactor for activity, and are active when expressed as recombinant proteins in E. coli (9). Human and mouse MPGs (hMPG and mMPG, respectively) share about 83% identity in their amino acid sequences. MPG is known to excise at least 17 structurally diverse damaged bases, such as alkylated bases (3-methyladenine, 7-methylguanine, and 3-methylguanine) and a broad range of other damaged bases from DNA including cycloethenoadducts, hypoxanthine, and various adducts of nitrogen mustards used in cancer chemotherapy (10–12). Because the alterations are located in both major and minor grooves of duplex DNA, the earlier hypothesis that the E. coli MPG (AlkA) recognizes alkyl adducts localized only in the minor groove of DNA may not be correct (13). Based on the x-ray crystallographic structure of human MPG complexed with a AP-site analog, it was recently proposed that MPG, while scanning the duplex DNA, completely or partially unstack nucleotides to find its substrate, a structurally diverse group of damaged bases that may or may not cause distortion of the DNA double helix. Once the enzyme recognizes its substrate, a unique tyrosine residue of the protein is inserted into the minor groove of the DNA, which then flips out the damaged nucleotide and intercalates into the vacated space. The damaged base then enters the active site pocket of the enzyme, where a bound water is poised for nucleophilic attack on the N-glycosyl bond (14–15). Although Glu-125 (Glu-145 in mMPG) was proposed to be the active site residue in hMPG, needed for activating the water molecule (15), an aspartic acid
residue has been identified as the active site in most DNA glycosylases and glycosylase/lyases including AlkA (16–23). To identify the potential role of aspartic acid residues in the catalytic activity of MPG, we decided to mutate those aspartic acid residues in mouse MPG one at a time that are conserved in all mammalian MPGs characterized so far. Our results show that only Asp-152 is essential for activity and that it is involved in catalysis but not in substrate binding.

**EXPERIMENTAL PROCEDURES**

**Construction of Site-specific Mutants of MPG**—The mutants were generated using Stratagene’s Chameleon double-stranded site-directed mutagenesis kit. The primers used to mutate SacI site in the *amp* gene of the vector and different residues in the coding sequences are listed in Table I. The entire MPG amino acid-coding region was sequenced to ensure production of only the desired mutation.

**Expression and Assay of MPG Mutant Proteins**—Expression of MPG proteins in *E. coli* BL21(DE3) and assay of MPG activity in cell-free extract using methyl-1H-labeled methylated calf thymus DNA substrate were performed as described previously (10). One unit of MPG is defined as the activity needed to release 1 pmol of methylpurine/min at 37 °C under the assay conditions.

**Overexpression and Purification of MPG Proteins**—The wild type (NA1000C18) and mutant (Asn-152, Ala-152, and Glu-152) MPG proteins were expressed as a glutathione S-transferase fusion protein in *E. coli* MV1932 (alkA*tag*) and purified as described previously. The glutathione S-transferase domain was cleaved by digestion with thrombin and separated from MPG as described previously (20).

**N-Methylpurine DNA-Glycosylase Assay**—The wild type (5 ng) and three mutant enzymes (1 µg) were individually incubated with [3H]-labeled DNA (−10,000 cpm; the sequence is shown in Table IB) substrate for various times when necessary at 37 °C in an assay buffer (25 mM HEPES-KOH, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 150 mM NaCl, and 10% glycerol) in a total volume of 20 µl. The reaction was terminated by adding 20 µl of 2 M piperidine and heating at 90 °C for 30 min to cleave the labeled strand at the abasic site generated by MPG. The DNA was precipitated by ethanol in the presence of 0.3 M sodium acetate and 4 µg of tRNA, dried, dissolved in loading buffer containing 66% formamide and 0.33 M NaOH, and then resolved by electrophoresis in sequencing gel containing 8% polyacrylamide, 7 × urea.

**DNaI Footprinting Reactions**—DNaI footprinting was performed essentially as described by Leblanc and Moss (26). The binding reaction (50 µl) was performed with 4 fmol of 5'-32P-labeled oligonucleotide (−15,000 cpm; the sequence is the same as the substrate oligonucleotide), 5 ng of plasmid DNA, and varying amounts of the wild type NA1000C18 MPG or mutant proteins (Asn-152, Ala-152, and Glu-152) in the assay buffer. After incubating for 5 min at room temperature, the DNA was partially digested with 5 µl of 0.001 unit (Kunitz) of DNaI (Sigma) for 2 min. The reaction was stopped by the addition of reaction stop buffer (1% SDS, 200 mM NaCl, 20 mM EDTA, pH 8.0, and 40 µg/ml tRNA), and after extraction with phenol/chloroform, the DNA was precipitated in ethanol and dried, and the cleavage product was analyzed as before.

**Determination of Binding Affinities**—Two independent major DNase I-generated bands in the eA-containing strand protected by wild type and mutant MPGs were selected for quantitation of radioactivity in the PhosphorImager. The MPG concentration that reduced the radioactivity in the bands generated in the absence of added MPG to 50% was assumed to be the *K*~D~ values had less than 10% variability, independently determined from the two bands.

**Circular Dichroism Spectra Analysis**—The wild type and the mutant MPGs were dissolved in 20 mM sodium phosphate (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, and 150 mM NaCl and then analyzed for the secondary and tertiary structures by far UV and near UV CD spectra, respectively, using an AVIV 62 DS circular dichroism spectropolarimeter. The far UV and near UV CD spectra were obtained in fused quartz cuvettes with 0.1-cm and 1.0-cm path lengths, respectively, and the protein solutions had absorbance at 280 nm ranging from 0.6 to 0.7. Each spectrum was recorded with a 0.5-nm increment and 1-s interval. For each sample, three repetitive scans were obtained and averaged. The spectra were normalized to the same protein concentration.

**Fluorescence Spectroscopy**—The structure of the wild type and mutant MPGs was also monitored by determining the emission spectra and the protein solutions had absorbance at 280 nm ranging from 0.6 to 0.7. Each spectrum was recorded with a 0.5-nm increment and 1-s interval. For each sample, three repetitive scans were obtained and averaged. The spectra were normalized to the same protein concentration.

**Other Methods**—Proteins were quantified by Bradford reagent (Bio-Rad) using bovine serum albumin as the standard. Oligonucleotides were synthesized in an Applied Biosystems model 394 DNA/RNA synthesizer. Adenine, thymine, guanine, and cytosine-β-cyanoethyl phosphoramidites were obtained from Applied Biosystems, and special ethenoadenine-β-cyanoethyl phosphoramidite was obtained from Glen Research.

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**TABLE I**

**Sequences of oligonucleotides used**

(A) To generate mutant MPGs:

- SacI site-abolishing primer, 5′-CTG GTG AGT ATT CAA CCA AGT-3′
- D112N, 5′-GCT GCG TGG TTA AAG AAT TCT GGT CC-3′
- D152N, 5′-GCA GCT TCA TCT TGG GCC CCC AAG TA-3′
- D152A, 5′-GCA GCT TCA TCT GCT GCG CCC AAG TA-3′
- D152E, 5′-GCA GCT TCT TCT TGG GCC CCC AAG TA-3′
- D231N, 5′-CAT TAC AGA GCT CAC GGT TCT TGA GGG-3′
- D231E, 5′-CTT GCT TAA AGC TCT TA-3′
- D255N, 5′-TGA GCC AGG TTG TGC TGG TC-3′
- D259N, 5′-CAC AGC ATC GTT TTG AGC C-3′
- D260N, 5′-CCA CAC AGT ATC ATG AG-3′
- D310N, 5′-CAG CCA CTC TGT TTA CCA CAC-3′

(B) As substrate:

- Ethenoadenine-containing oligonucleotide, 5′-TCG AGG ATC TCG AGC TCT AGT CAA CGA CGA TCG CTA ATG CTG CGG ATC CAA GC-3′

*The letters in italics are changed bases to generate site-specific mutants.*

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**FIG. 1.** SDS-polyacrylamide gel electrophoresis analysis of purified wild type and mutant MPG proteins. Lane M, protein molecular mass markers; lane 1, MPG wild type (NA1000C18), 2 µg; lane 2, mutant MPG, Asn-152, 2 µg; lane 3, mutant MPG, Ala-152, 2 µg; lane 4, mutant MPG, Glu-152, 2 µg.

**TABLE II**

**Specific activities of wild type and mutant MPGs in E. coli crude cell extracts**

Because the *E. coli* host contained endogenous MPG, the activity in BL21(DE3) cells harboring the empty vector was subtracted for each assay. The activities were determined in three or four experiments, and the mean values are listed. Wt, wild type.

| Protein type | Specific activity | units/mg |
|--------------|------------------|----------|
| Control      | 0.02             |          |
| Wt (NA1000C18) | 14              |          |
| Asn-112      | 13               |          |
| Asn-152      | 0.08             |          |
| Asn-231      | 7                |          |
| Asn-252      | 6                |          |
| Asn-255      | 7                |          |
| Asn-259      | 9                |          |
| Asn-360      | 10               |          |
| Asn-310      | 18               |          |
the positions of substrate and product DNAs, respectively.

The 50 bp piperidine to cleave DNA at abasic sites. The contains DNA substrate only. In all lanes samples were treated with piperidine to cleave DNA at abasic sites. The 50 bp and 26 bp indicate the positions of substrate and product DNAs, respectively.

RESULTS

We tested the role of aspartic residues in MPG in catalysis by targeted mutagenesis of eight such residues, at positions 112, 152, 231, 252, 255, 259, 260, and 310, to asparagine. All of these residues were conserved in three known mammalian MPGs. The levels of these mutant proteins in the soluble fraction of cell-free extract of E. coli BL21(DE3) carrying the corresponding expression plasmids were comparable with that of the wild type protein (N100C18). Only the Asn-152 mutant among the 8 Asn mutants showed substantial loss of enzymatic activity (Table II). The Asp-152 residue was then independently mutated to Ala and Glu, and all three mutant proteins (Asn-152, Ala-152, and Glu-152) were expressed as glutathione S-transferase fusion proteins in E. coli MV1932 (alkA-tag) and purified to apparent homogeneity (Fig. 1).

MPG Activity of Wild Type and Mutant Proteins—With eA-containing duplex oligonucleotide substrate, no eA-cleaving activity was observed for any of three mutants, even when incubated for an extended time and with 200-fold excess protein (Fig. 2). Also with hypoxanthine-containing duplex oligonucleotide, we could not detect any cleavage activity for any of the three mutants (data not shown). Although some activity was observed in crude E. coli extract containing the Asn-152 mutant protein (Table II) using a less sensitive assay, we conclude that the purified enzyme lacks detectable MPG activity. Thus Asp-152 appears to play an essential role in the catalytic function of the enzyme.

Footprinting Analysis of MPG-bound Substrates—Although the three proteins mutated at the Asp-152 site underwent some structural reorganization (as described later) and completely lost catalytic activity, they showed identical behavior as the wild type enzyme in protecting the substrate DNA from DNase I digestion as indicated in the footprinting analysis (Fig. 3). Furthermore, all mutants had similar affinity for the substrate DNA as the wild type enzyme (Table III). These results further support our conclusion that Asp-152 is essential for catalytic activity but not for substrate binding.

CD and Fluorescence Spectral Analysis of Mutant Proteins—The far UV circular dichroism spectra of Asn-152, Ala-152, and Glu-152 mutant proteins are closely similar to that of wild type enzyme (Asp-152), indicating similar secondary structure or backbone conformation of the mutant and wild type protein (Fig. 4A). The near UV CD spectrum, which generally depends on the atomic environment and closeness of packing of the aromatic residues including their accessibility to solvent (27), shows that one or more of the five tyrosine residues of MPG are in an asymmetric environment, causing a decrease in positive ellipticity of the Glu-152 and Asn-152 mutants and generating a marked negative ellipticity of Ala-152 mutant compared with that of the wild type protein (Fig. 4B). These changes in aromatic CD reflect changes in conformation that are limited strictly to the immediate neighborhood of the relatively few aromatic residues. On the other hand the emission spectra of
tryptophan (total of three residues) fluorescence of these mutants are superimposable with that of the wild type protein (Fig. 4C). Therefore, it appears that a small local structural perturbation observed due to mutation of the Asp-152 residue is not around the tryptophan residues but more likely the tyrosine residues.

**DISCUSSION**

Aspartic acid was shown to be the catalytic residue in the cases of most DNA glycosylases and DNA glycosylase/lyases, including *E. coli* alkA, the MPG ortholog, uracil-DNA glycosylase, MutY, *E. coli* endonuclease III, hNTH1, Fpg, human 8-oxoguanine-DNA glycosylase-1, yeast 8-oxoguanine-DNA glycosylase-1, and yeast 8-oxoguanine-DNA glycosylase-2 (16–23). These observations prompted us to carry out a systematic investigation to test whether an Asp residue plays a critical role in MPG activity as well. We generated site-directed mutants one at a time of those aspartate residues that are conserved in mammalian MPGs. Among eight such aspartate residues, mutations of only Asp-152 caused complete loss of catalytic activity. While our experiments were in progress, the tertiary structure of hMPG was elucidated by x-ray crystallography, upon which basis it was proposed that Glu-125 in hMPG (Glu-145 in mMPG) is the active site residue (15). This residue presumably activates a bound water molecule by abstracting a proton, which then attacks the C1-9 atom of the deoxynucleoside substrate (15). Thus, as expected, Ala-125 or Gln-125 mutants of hMPG had no detectable catalytic activity; however, both retained high affinity for an inhibitor, pyrrolidine-containing DNA.2 It was proposed earlier that the net positive charge in the N-alkylpurine substrates such as 7-methylguanine facilitates in cleavage of the glycosyl bond (14). The results reported here indicate that, in addition to Glu-125, Asp-132 in hMPG (Glu-145 in mMPG) is the active site residue (15). This residue presumably activates a bound water molecule by abstracting a proton, which then attacks the C1 atom of the deoxynucleoside substrate (15). 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It is interesting to note that among all aspartates, only Asp-152 of mMPG is conserved in all putative glycosylases cloned from the eukaryotic sources (e.g. human, rat, and *Arabidopsis thaliana*) as well as among from several bacteria (e.g. *Bacillus subtilis*, *Borrelia burgdorferi*, and *Mycobacterium tuberculosis*; Fig. 5). Furthermore, among all Asp residues Asp-132 is the only one located near the DNA binding site, whereas the other Asp residues are widely scattered on the surface of hMPG (15). In the case of several DNA glycosylases, a conserved residue(s) other than the active site has(have) been shown to be involved in lesion recognition and catalysis. For example, although the N-terminal proline (Pro-2) in *E. coli* Fpg was shown to be involved in catalysis by producing an imino (Schiff base) intermediate with its substrate (28), Lys-57 and Lys-155, conserved in Fpg proteins of several bacteria, were implicated specifically in recognition and catalysis of 8-oxoguanine in DNA (29–30).

A unified catalytic mechanism for DNA glycosylases was proposed, which postulates that protonation of the substrate base is necessary for making it a better leaving group in efficient N-glycosylic bond cleavage. In such a case, catalysis involves a proton donor as well as a proton acceptor residue in the enzyme molecule (31). Thus, in the cases of *E. coli* Mut Y and uracil-DNA glycosylase of herpes simplex virus type I, Glu-37 and His-210, respectively, were proposed to be involved in protonation of the substrate bases, whereas Asp-138 and Asp-
88, respectively, in these enzymes act as activators of water molecules, which attack the N-glycosyl bonds (17–18).

X-ray crystallographic structure of hMPG bound to pyrrolidine-containing oligonucleotide allowed identification of Glu-125 as being responsible for activation of water based on its location relative to the pyrrolidine residue (15). In this structure, Asp-132 (corresponding to Asp-152 in mMPG) is localized on the protein surface facing away from the DNA binding site (15) and, thus, may not be involved in DNA binding. The fact that all mutations of the Asp-152 residue in mMPG did not affect the substrate DNA binding is consistent with this prediction. In considering the possible causes for the loss of activity of mutants at residue 152, we should point out that a trivial reason for such inactivation is a drastic structural change of the mutant proteins. However, spectroscopic studies and DNA footprinting analysis indicate that conservative substitution of Asp-152 with Asn or Glu or even nonconservative substitution with Ala did not cause a global change in the tertiary structure of the protein. No significant change was observed in the far UV CD spectra and the intrinsic tryptophan fluorescence as a result of mutation. Furthermore, DNA footprinting pattern was indistinguishable among the wild type and mutant proteins. At the same time, a localized change in the environment and closeness of packing of aromatic residues, most likely tyrosine residues, was produced in the Asn-152 and Glu-152 mutants as indicated by a decrease in positive ellipticity in the near UV region. Such tyrosine residues are localized near the binding site of DNA. Thus the loss of activity of at least Asn-152 and Glu-152 mutants are likely to be due to a local effect. This conclusion is further supported by the lack of detectable environmental perturbation surrounding the Trp residues, which are located away from Asp-152. If the role of Asp-152 is to donate a proton to the substrate base, then the complete loss of activity of the Glu-152 mutant was unexpected because Glu, in principle, is capable of donating a proton. It is likely that perturbation of the local conformation in the neighborhood of aromatic residues could have prevented the optimum geometry for such proton transfer from Glu-152.

Because Asp-132 (Asp-152 in mMPG) in the crystal structure of hMPG complexed with pyrrolidine-containing DNA is located 10.52 Å away from the C1′ of pyrrolidine, it is unlikely that in such a configuration Asp-152 could be a proton donor to the base. However, we should point out that pyrrolidine is not a substrate of the enzyme. It is thus possible that the binding of a true substrate, e.g. eA to the enzyme, could cause movement of the loop containing the Asp-152 residue (Fig. 6), to an optimum distance from the base to allow the proton transfer. Alternatively, the proton transfer may involve a bridging H2O molecule. At the same time, we are not able to exclude the possibility of an indirect role of Asp-152 in which its substitution mutation destabilized the architecture of the catalytic pocket. Thus the local hydrogen-bonding network with the neighboring residues, particularly the critical Tyr residues at positions 147, 179, and 182 of mMPG in the hydrophobic active site pocket, may be disrupted in the absence of Asp-152. This disruption may lead to loss of hydrogen bonding between the OH group of Tyr-147 and Glu-145 (Glu-125 in hMPG), which holds the side chain of Glu-145 in position for activating the water molecule. These alternatives could be directly tested by elucidation of the structure of active or Asp-152 mutant MPG bound to the substrate base and/or to a substrate oligonucleotide.

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