Purification and Biochemical Characterization of Recombinant *N*-Methylpurine-DNA Glycosylase of the Mouse†

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Received July 11, 1994; Revised Manuscript Received October 4, 1994⊗

ABSTRACT: The mouse *N*-methylpurine-DNA glycosylase (MPG), responsible for the removal of most *N*-alkyladducts in DNA, was purified to homogeneity as a recombinant nonfusion protein from *Escherichia coli*. Only 10−15% of the protein was present in the soluble form in *E. coli* cells. The *N*-terminal amino acid sequence of the purified protein which lacks 48 residues from the amino terminus of the wild type protein was identical to that predicted from the nucleotide sequence. The glycosylase hydrolyzes 3-methyladenine (m^3A), 7-methylguanine(m^7G), and 3-methylguanine (m^3G) from DNA, and the K_m and k_{cat} values were 130 nM and 0.8 min^{-1} for m^3A, and 860 nM and 0.2 min^{-1} for m^7G, respectively, when methylated calf thymus DNA was used as the substrate. A comparison of k_{cat}/K_m values for different bases indicates that the enzyme was more efficient in excising both m^3A and m^3G than m^7G from methylated DNA. The enzyme showed moderate binding affinities (K_A) for both methylated (5.8×10^7 M^{-1}) and nonmethylated DNAs (4.2×10^7 M^{-1}). The mouse protein has an extinction coefficient $E_{1\%}^{280nm}$ of 10.5 and a pI of 9.3. The enzyme activity was optimal in the presence of 100 mM NaCl, with a broad pH optimum of 8.5−9.5. The enzymatic release of both m^3A and m^7G was stimulated 50−75% by 0.5 mM $MgCl_2$ and 0.02 mM spermine but inhibited by higher concentrations of these agents. Product inhibition by 40−50% of the reaction occurred in the presence of 10 mM m^3A or m^7G. However, 1.0 mM m^3A stimulated release of m^7G. The enzyme was inhibited by 60% in the presence of 0.9 mg/mL DNA which, at the same time, protected it from thermal inactivation.

Simple, monofunctional alkylating agents induce alkyl adducts in DNA at various nucleophilic sites, including exocyclic and ring *N*-atoms, and *O*-atoms. The relative abundance of these adducts varies depending on the nature of the agent and on the alkyl group itself (Beranek, 1990; Singer & Grunberger, 1983). The major adducts formed by alkylating agents that react via the S_N2 mechanism are *N*-alkylpurines including 7-alkylguanine and 3-alkyladenine. In addition, methylating agents produce several minor methylation products including 3-methylguanine, O^6-methylguanine, 1-methyladenine, 7-methyladenine, O^2-methylthymine, 3-methylthymine, and O^4-methylthymine (Beranek *et al.*, 1980). Some of the methyl adducts are also produced by *in vivo* nonenzymatic reaction with *S*-adenosylmethionine as the endogenous methyl donor (Naslund *et al.*, 1983; Barrows & Magee, 1982).

N-alkyl adducts in DNA are repaired by a ubiquitous base excision repair pathway in which the lesions are first removed by the adduct-specific DNA glycosylases, followed by repair-resynthesis of the resulting abasic sites (Lindahl, 1982; Friedberg, 1985). *Escherichia coli* has two glycosylases for the removal of *N*-alkylpurines. The constitutive Tag protein, also called 3-methyladenine-DNA glycosylase I and the product of *tag* gene, is specific for 3-alkyladenine (Bjelland & Seeberg, 1987). The inducible AlkA protein (also called 3-methyladenine-DNA glycosylase II) is the product of the *alkA* gene, a component of the *ada* regulon, and has a broad substrate specificity. In addition to 3-alkyladenine, it removes 3-alkylguanine, 7-alkylguanine, and O^2-methylpyrimidines (Lindahl *et al.*, 1988; McCarthy *et al.*, 1984). Recently, the substrate specificity of this protein has been extended to N^2, 3-ethanoguanine, the product of reaction with 2-haloalkylnitrosourea (Habraken *et al.*, 1991), and N^2, 3-ethenoguanine (Matijasevic *et al.*, 1992), produced in DNA *in vivo* by vinyl chloride metabolites and related compounds (Laib *et al.*, 1986).

In contrast to the situation in *E. coli*, mammalian cells were shown to have only one type of *N*-methylpurine-DNA glycosylase (MPG)[1] which removes m^3A, m^7G, and m^3G from DNA (Helland *et al.*, 1987). Thus, this protein is similar to the AlkA protein. After the cloning and expression of the human and rodent MPG cDNAs (Chakravarti *et al.*, 1991; Samson *et al.*, 1991; O'Connor & Laval, 1991), the corresponding recombinant proteins were partially purified from *E. coli*, and the earlier observations on their substrate range were confirmed (Chakravarti *et al.*, 1991; Samson *et al.*, 1991). In all cases, m^3A was the preferred substrate of the mammalian MPGs as is also the case for the AlkA protein. Furthermore, the mammalian protein, like the AlkA protein, removes ethenoadducts of adenine, guanine, and cytosine from DNA (Dosanjh *et al.*, 1994b). Surprisingly, the human MPG releases 1,N^6-ethenoadenine (ϵA) from DNA at more than a 10-fold higher rate than does m^3A. Thus, the nature of MPG's primary substrate in the cell is uncertain (Dosanjh *et al.*, 1994a).

† This research was supported by U.S.P.H.S. Grant CA53791.

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1994.

[1] Abbreviations: MPG, *N*-methylpurine DNA-glycosylase; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; EDTA, ethylenediamine tetracetic acid; DMS, dimethyl sulfate; K_A, association constant; K_m, Michaelis constant; k_{cat}, catalytic constant.

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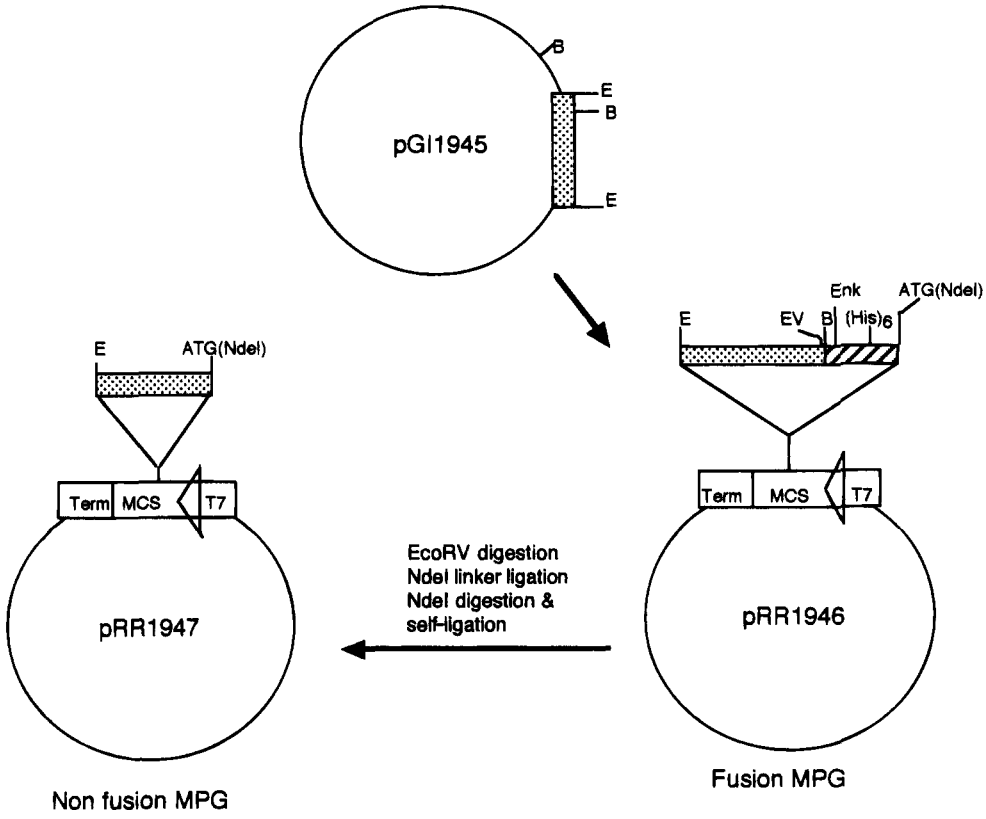

FIGURE 1: Construction of expression vectors for overproducing MPG. The *Bam*HI/*Eco*RI cDNA fragment was inserted in the multiple cloning site of pRSET A expression vector to create pRR1946. pRR1946 was further engineered to generate pRR1947, which encodes MPG polypeptide without the fusion sequence (striped region). Abbreviations: Term, termination cassette; MCS, multiple cloning site; T7, T7 promoter; $(His)_6$, polyhistidine-fusion; Enk, enterokinase cleavage site; B, *Bam*HI; EV, *Eco*RV; E, *Eco*RI.

Although only one kind of MPG has been identified in mammalian tissues and cell lines, the possibility of different MPG isozymes in different cell types of tissues cannot be ruled out. While several partially purified MPG preparations from mammalian sources were biochemically characterized [reviewed by Helland *et al.* (1987)], none of them has been purified to homogeneity because of their low abundance. While this paper was in preparation, O'Connor (1993) reported purification and preliminary characterization of the recombinant human MPG. However, we have recently shown with partially purified human and mouse MPGs that the mouse protein was more efficient than the human protein in releasing m^7G and 8-hydroxyguanine from DNA (Mitra *et al.*, 1993; Bessho *et al.*, 1993). In view of this significant difference in substrate specificity of the human and mouse MPGs, it is important to purify and characterize mouse MPG as well. It should be noted that nothing is known about the mechanism of catalysis by this enzyme. The fact that distinct substrates with alkyl residues present in both major and minor grooves of DNA duplex are recognized by MPG suggests involvement of distinct amino acid residues in base recognition and alternative conformations of the protein. This report represents the first step in a comprehensive investigation of the biochemical properties of the mammalian MPGs.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. E. coli MV1932 (Volkert et al., 1986) was used for the expression of MPG. The prokaryotic expression vectors pRSET (Invitrogen), pET11a (Novagen), and BL21(DE3) pLysS (Novagen) E. coli lysogen were used as host for the expression of mouse MPG polypeptide. The pUC plasmids (Viera & Messing, 1982) were used for subcloning and engineering the expression vector.

Construction of High Expression MPG Vector and Overproduction of Mouse MPG Protein. Low level of expression of the fusion MPG polypeptide was observed with pGI1945 (M. Tatsuka et al., submitted for publication). A 170 bp upstream sequence, including 44 bp of the untranslated sequence, was deleted from the plasmid by *Bam*HI digestion. After filling-in the single-stranded overhangs with the Klenow DNA polymerase I, the blunt ends were religated to generate pGI1946 (Figure 1). The 0.97 kb *Bam*H1/*Eco*R1 fragment from pGI1945 containing the complete coding sequence of mouse MPG was also ligated to *Bam*HI/*Eco*RI sites in both pET11a and pRSETA while maintaining the correct reading frame of the MPG coding sequence. The translation start sites (ATG) of the resulting expression plasmids, pSMPGHi and pRR1946, respectively, were used to encode fusion polypeptides. pSMPGHi yielded a fusion polypeptide with 1.43 kD a additional sequence, while pRR1946 gave rise to a fusion protein with 3 kD a additional sequence at the amino terminus of the mouse MPG protein. In subsequent studies, a new construct, pRR1947, encoding a nonfusion mouse MPG polypeptide, was generated by deleting the polyhistidine fusion as well as undesired multiple cloning sites from pRR1946. In order to achieve this, a *Nde*I linker (ending with ATG) was ligated at the 5′ blunt-ended *Eco*RV site and the resulting fragment was ligated directly to mouse MPG cDNA at the *Nde*I restriction site of pRSETA vector (Figure 1). The recombinant protein encoded by this cDNA sequence is predicted to have a 48 amino acid deletion

at the amino-terminal end of the protein (Engelward *et al.*, 1993; Tatsuka, M. et al., submitted for publication). However, it appears that the deleted region does not have a critical role in enzyme catalysis.

Both pRR1946 and pRR1947 were introduced into *E. coli* BL21(DE3)pLysS by transformation, and single colonies were grown at 37 °C in duplicate cultures of 5 mL of Luria broth containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. At A_{600} of 0.6, one set of cultures was transferred to 30 °C while the other set was left at 37 °C. After addition of IPTG (0.4 mM), aliquots from both 37 and 30 °C cultures were collected at 0 and 4 h. Cell pellets were resuspended in buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 5% glycerol), sonicated, and centrifuged at 12 000 rpm for 10 min. The supernatant was separated and the precipitate was dissolved in a sample loading buffer containing 1% SDS. Then the supernatant (10−15 μL) and the precipitate (3−5 μL) were analyzed separately by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) to test expression and solubility of the expressed recombinant proteins.

Purification of Mouse MPG Polypeptides. BL21(DE3)-pLysS cells carrying pRR1947 were grown at 37 °C in a fermentor in 30 L of LB containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol and with the supplementation of oxygen. At A_{600} of about 1.5, IPTG (0.4 mM) was added and fermentation was continued for 3.5 h more until the O. D. at 600 nm plateaued off around 2.5. Cells were harvested (wet weight 272 g) and kept frozen at −80 °C until use. Cells (20 g) were thawed and suspended in 200 mL of buffer A containing 0.4 M NaCl and disrupted with sonication on ice. The lysate was centrifuged at 35 000*g* for 30 min. Polymin P (5% v/v, neutralized to pH 8.0) was added to the supernatant at 36 μg/A_{260} of the extract. The precipitated nucleic acids and some basic proteins were removed by centrifugation at 10000*g* for 20 min. The proteins in the supernatant were subjected to fractional precipitation with ammonium sulfate at 30−55% saturation, and the precipitate containing MPG was collected by centrifugation (10000*g*, 20 min). The precipitate was dissolved in 30−40 mL of buffer A containing 0.1 M NaCl and dialyzed against 2 L of the same buffer with one change at 4 °C. The dialysate was applied to a 60-mL column of the anion exchanger Q-Sepharose, and the flow-through was loaded directly onto a column of cation exchanger phosphocellulose P11 (Whatman, 25 mL). Both the columns were previously equilibrated with buffer A containing 0.1 M NaCl. The flow-through from the second column was recycled once through the combined columns which were subsequently washed with 400 mL of equilibration buffer. Subsequently, the phosphocellulose was washed with 100 mL of buffer A containing 0.2 M NaCl, before elution of MPG with buffer A containing 0.3 M NaCl. Fractions of 1.5 mL containing MPG were collected at a flow rate of 18 mL/h and then pooled and concentrated to 3.0 mL by aquacide II (Calbiochem). The concentrated sample was then loaded on a ACA 54 gel permeation column preequilibrated and then developed with buffer A containing 0.2 M NaCl. Fractions containing MPG activity were pooled and dialyzed against buffer A and then applied to a Mono S HR5/5 column (Pharmacia FPLC system) equilibrated with buffer A. After the column was washed with 7.5 mL of buffer A containing a linear gradient (0−0.3 M) of NaCl (flow rate, 1.5 mL/min), the gradient was held at 0.3 M NaCl for 5 min and then MPG eluted with a 20-mL linear gradient of 0.3−0.5 M NaCl in buffer A. Fractions containing the major peak with MPG activity were pooled and dialyzed against buffer A containing 0.1 M NaCl and 50% glycerol and then stored at −20 °C. In a typical preparation, approximately 13 mg of pure mouse MPG was recovered from 20 g (wet weight) of cells.

Isoelectric Focusing−Polyacrylamide Gel Electrophoresis. Isoelectric focusing was carried out in a nondenaturing vertical mini slab gel of 5% polyacrylamide containing ampholine (Pharmalyte pH 3.0−10.0, Pharmacia) (Robertson *et al.*, 1987). The pH gradient was calibrated by the use of isoelectric-point marker proteins (amyloglucosidase, pI 3.5; soybean trypsin inhibitor, pI 4.55; β-lactoglobulin A, pI 5.2; bovine carbonic anhydrase B, pI 5.85; human carbonic anhydrase B, pI 6.55; horse myoglobin, acidic band, pI 6.85; horse myoglobin, basic band, pI 7.35; lentil lectin, acidic band, pI 8.15; lentil lectin, middle band, pI 8.45; lentil lectin, basic band, pI 8.65; and trypsinogen, pI 9.3, Pharmacia) placed in the same gel. After electrophoresis (200 V for 1.5 h followed by another 1.5 h at 400 V), a section of the gel was cut into 0.5-cm slices, which were then incubated with 10 mM KCl overnight for measurement of the the pH in the eluate; the remaining gel was treated with 10% trichloroacetic acid for 10 min, followed by overnight incubation in 1% TCA and staining with Coomassie brilliant blue.

Polyacrylamide gel electrophoresis was performed in a 13% or 15% polyacrylamide gel containing 0.1% SDS according to Laemmli (1970). The gel was calibrated using marker proteins 14.3−200 kDa (Amersham), and the protein bands were stained as before.

N-Methylpurine-DNA-Glycosylase Assay. MPG was assayed as described (Chakravarti *et al.*, 1991) using [^{3}H]-methyl-labeled calf thymus DNA (370 cpm/μg; 109 fmol of m^7G and 20 fmol of m^3A in a total of 960 pmol of adenine and 640 pmol of guanine per μg of DNA) or [^{3}H]methyl-labeled DNA polymer poly(dG-dC) (18 800 cpm/μg; 7.7 pmol of m^7G and 100 fmol of m^3G in a total of 1600 pmol of guanine per μg of DNA) which were prepared by treating sonicated calf thymus DNA or the synthetic DNA polymer in 0.2 M cacodylate buffer, pH 7.2, with [^{3}H]dimethylsulfate, as described earlier (Washington *et al.*, 1989). The standard reaction mixture contained 70 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 5% glycerol, 100 mM NaCl, and 50 μg/mL bovine serum albumin in a total volume of 200 μL. The amounts of enzymes and substrates used are indicated in the figure legends. Incubation was carried out at 37 °C for 10 min, if not otherwise stated, and the reaction was terminated by addition of 200 μg of calf thymus DNA and precipitation with cold ethanol in the presence of 0.3 M sodium acetate. The released radiolabeled base adducts in the ethanol-soluble fraction were quantitated either directly by liquid scintillation counting or the ethanolic supernatant was evaporated to dryness, dissolved in 100 μL of water, and analyzed by high-pressure liquid chromatography on aminex A-6 column using ammonium borate buffer (pH 8.4) at 50 °C. One unit of MPG is defined as the activity needed to release 1 pmol of methylpurine per min, and the variation among independent experiments was no more than 10% under standard assay conditions.

Nitrocellulose Filter Binding Assay of Mouse MPG. The binding of mouse MPG to DNA was measured by the

procedure of Papoulas (1992) with minor modification. Two micrograms of plasmid DNA (3.6 Kb pSS601) (Shiota *et al.*, 1992) was linearized with *Hind*III and then labeled with Klenow DNA polymerase I in the presence of 1 mM each of dATP, dGTP, and dTTP, and 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmol) at room temperature for 15 min in a 25-μL reaction mixture.

The labeled DNA was purified by phenol/chloroform extraction followed by repeated ethanol precipitation. One microgram of [^{32}P] dCTP-labeled DNA was methylated with [^{3}H]dimethylsulfate; after methylation, unreacted dimethylsulfate was removed by alcohol precipitation as described earlier (Washington et al., 1989). Mouse MPG was diluted in DNA binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM DTT, 0.1 mM PMSF, and 20% glycerol). Ten nanograms methylated DNA (2000 cpm/ng; 8.1 fmol of m^3A/ng, 38.7 fmol of m^7G/ng in total amount of adenine and guanine) or nonmethylated DNA was incubated for 25 min at 30 °C with various amounts of enzyme (14−700 ng), or 100 μg of bovine serum albumin as a control in a 50-μL reaction mixture in the presence of DNA binding buffer. The reactions were stopped by transferring the reaction mixture onto a nitrocellulose membrane (Amersham) in a slot blot apparatus (Hybridot Manifold, BRL) under vacuum suction. The slots were washed twice with 0.5 mL of DNA binding buffer at a flow rate of ~1 mL/min. The individual sample blots were cut into pieces and transferred to scintillation vials containing 5 mL of Scintiverse II (Fisher) to count radioactivity in scintillation counter. The K_A value of the MPG was determined graphically according to Papoulas (1992).

Assay of DNAse, AP-Endonuclease, and Exonuclease. The form I pSS601 plasmid (Shiota *et al.*, 1992), purified by equilibrium centrifugation in CSCl/ethidium bromide, was linearized with *Hind*III and then partially depurinated by treatment with 0.2% formic acid (Maniatis *et al.*, 1982). DNAse, AP-endonuclease, and exonuclease assays were performed by incubating 0.5 μg of supercoiled, depurinated, and linearized pSS601 plasmid DNA as substrates with 0.1 μg of mouse MPG protein or 0.1 μg of albumin, 9.3 ng of T4 endonuclease V, and 10 units of exonucleaseIII as controls at 37 °C for 15 min in 10 μL of reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, and 0.01% NP40). The reaction products were analyzed by electrophoresis on 1% agarose gel containing 0.5 μg/mL ethidium bromide according to Xanthoudakis *et al.* (1992).

Other Methods. Proteins were quantitated by the bicinchoninic acid procedure (Smith *et al.*, 1985) with bovine serum albumin as standard. Radioactivity in liquid samples was quantitated in a Beckman model LS6000SC liquid scintillation counter. Oligodeoxynucleotides were synthesized in an Applied Biosystems PCR-Mate. N-terminal sequencing of the protein was performed on an Applied Biosystem model 477A protein sequencer, while the amino acid composition was determined in a Hewlett Packard Aminoquant series II analyzer as described by Wang and Fried (1987).

RESULTS

Construction and Expression of Mouse MPG. Table 1 shows that the recombinant MPG was poorly expressed in both pUC and pET11a. We checked the solubility of expressed proteins after lysis of the plasmid-containing bacteria and subsequently analysis by SDS-PAGE (data not shown) and could not detect an MPG protein band based on the predicted size. We considered the possibility that the mouse MPG protein may be toxic to *E. coli*. We chose the pRSET expression vector, which expresses a fusion protein with an N-terminal polyhistidine tag to allow one-step purification by affinity chromatography nickel-chelating resin. The vector contains a T7 promoter driven by T7 RNA polymerase which is provided after infection with a recombinant M13 phage. The pRSET vector containing mouse MPG cDNA was transfected into *E. coli* BL21 (DE3) lysogen which provides T7 RNA polymerase, and also contains plyS in order to minimize the "leaky" transcription and hence toxicity. In both cases, although the MPG protein was

Table 1: Comparative Activity of MPG Encoded by Different MPG Expression Plasmids

E. coli	vehicle plasmid	expression plasmid	specific activity of MPG [fmol/(min·mg) of protein]
MV1932	pUC19	PGI1946	379
BL21 (DE3) plysS	pET11a	pSMPGHi	258
BL21 (DE3) plysS	pRSET	pRR1946	ND[a]
BL21 (DE3) plysS	pRSETΔ(his)$_6$	pRR1947	1150

[a] ND, not detectable.

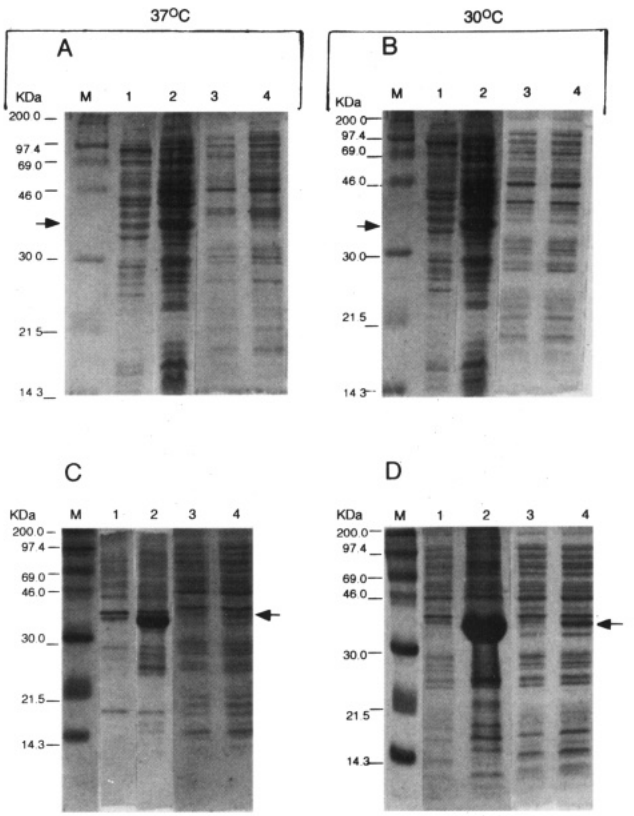

FIGURE 2: SDS−PAGE of recombinant MPG in *E. coli* extracts. BL21 (DE3) plysS *E. coli* carrying either pRR1946 (panels A and B) or pRR1947 (panels C and D) was grown at 37 °C. After addition of IPTG, cells were further grown at 37 °C (B & D) or at 30 °C (A and C). At 0 and 4 h, cells were harvested, and the cell-free extracts produced by sonication were fractionated by centrifugation into pellets and supernatants, which were then used for electrophoresis in 15% polyacrylamide. (Lanes 1 and 2) Pellet fractions at 0 and 4 h; (lanes 3 and 4) supernatant fractions at 0 and 4 h, respectively. The arrow indicates the IPTG-inducible band with the predicted size of MPG. Other details are given in Materials and Methods.

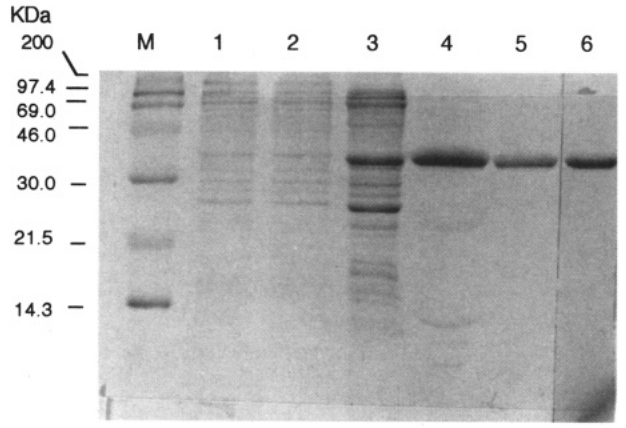

FIGURE 3: SDS−PAGE of purified MPG. Aliquots (10−15 μg) of protein of fractions I−VI (Table 1) were electrophoresed in 13% polyacrylamide, in lanes 1−6, respectively.

expressed at a high level, it was not detected in the soluble fraction either by analysis on SDS-PAGE, [Figure 2, lanes 3 and 4 in panel A (37 °C) and panel B (30 °C), respectively] or by MPG activity assay (Table 1). However, all of the expressed MPG protein was detected in the pellet of cell lysate [Figure 2, lanes 1 and 2 in panel A (37 °C) and panel B (30 °C), respectively]. Considering the possibility that the fusion sequence contributes to the protein aggregation, we deleted the coding sequence of the fusion polypeptides (Figure 1). We were then able to obtain ~10−15% of the MPG protein at 30 °C in soluble form as judged by SDS−PAGE (Figure 2, panel D, lane 4), and 3−4-fold higher glycosylase activity was also detected with this expression system as compared to others (Table 1). However, a larger fraction of soluble protein was observed when expressed at 30 °C (Figure 2, panel D, lane 4) as compared to 37 °C (Figure 2, panel C, lane 4).

Purification of Mouse MPG Polypeptide. Purification of the homogeneous protein was achieved in five steps (Figure 3); Table 2 shows the result of a typical purification procedure. Actually chromatography of purified MPG on MonoS column separated two polypeptides one of which (peak I) corresponded to the predicted *N*-terminal sequence (MDHEVGQMP...), while the other, having a 14 amino acid deletion, was eluted in peak II and III (Figure 4). In any case, these two species had the same specific activity for m^3A release from DNA (data not shown). Although the specific activity of MPG did not increase significantly by FPLC on Mono S column in the last step, the chromatography resulted in a homogeneous preparation. The protein has a calculated molecular mass of 31.4 kDa, but it migrated as a single band of 34.4 kDa. Isoelectric focusing of the purified protein shows it to be highly basic with a pI of 9.3,

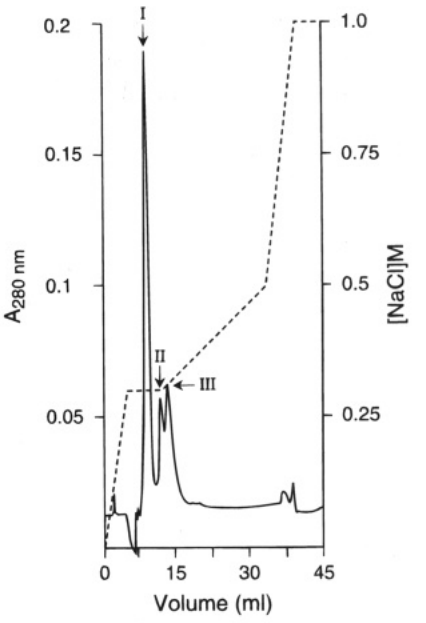

FIGURE 4: Elution profile of MPG on MonoS cation exchange by FPLC. The broken line (- - -) denotes NaCl concentration.

similar to the value calculated from its amino acid composition (Table 3).

Enzymatic Properties of Mouse MPG. The purified MPG released methylated bases in a dose-dependent manner (Figure 5A). Figure 5B shows that the amounts of both m^3A and m^7G released from DNA increased with time up to 20 min. After 20 min, addition of fresh enzyme extended the reaction up to 60 min. The rate of release of m^3A was faster than that of m^7G. The Lineweaver−Burk plots of initial velocity versus m^3A concentration (Figure 5C) and m^7G concentration (Figure 5D) at a fixed concentration of 2.2 μg/μL calf thymus DNA were used to determine various kinetic constants, namely, K_m, k_{cat}, and k_{cat}/K_m by using the software ENZFITTER version 1.05 (EGA) created by Robin J. Leatherbarrow (c) (1987) and published by Elsevier-Biosoft (Table 4). The (k_{cat}/K_m) for m^3A was 26-fold higher than that for m^7G when methylated calf thymus DNA was used as substrate. However, the absolute value depended on the nature of substrate DNA because (k_{cat}/K_m) for m^7G was 4-fold higher with methylated poly(dG·dC) (at a fixed concentration of 0.16 μg/μL) than that obtained with methylated calf thymus DNA as the substrate. We could not detect release of m^3G from methylated calf thymus DNA in which the base adduct is present in very low abundance. The (k_{cat}/K_m) for m^3G was determined with methylated poly(dG·dC) and was 9-fold higher than that for m^7G in the same substrate. This indicates the preference of the enzyme for m^3G over m^7G.

Table 2: Purification of Recombinant Mouse MPG

step	volume (mL)	total protein (mg)	total MPG activity (units)	specific activity (units/mg)	purification fold	recovery (%)
(I) crude extract	200	600	30400	50		100
(II) Polymin P supernatant	200	546	30200	55	1.1	99
(III) ammonium sulfate supernatant	100	316	29600	93	1.8	97
(IV) Q-Sepharose and phosphocellulose chromatography	97.5	17.5	8350	476	9.5	27.4
(V) ACA 54 chromatography	22	13.2	7600	575	11.4	25.0
(VI) Mono S chromatography	26.4	13.1	7590	579	11.6	24.9

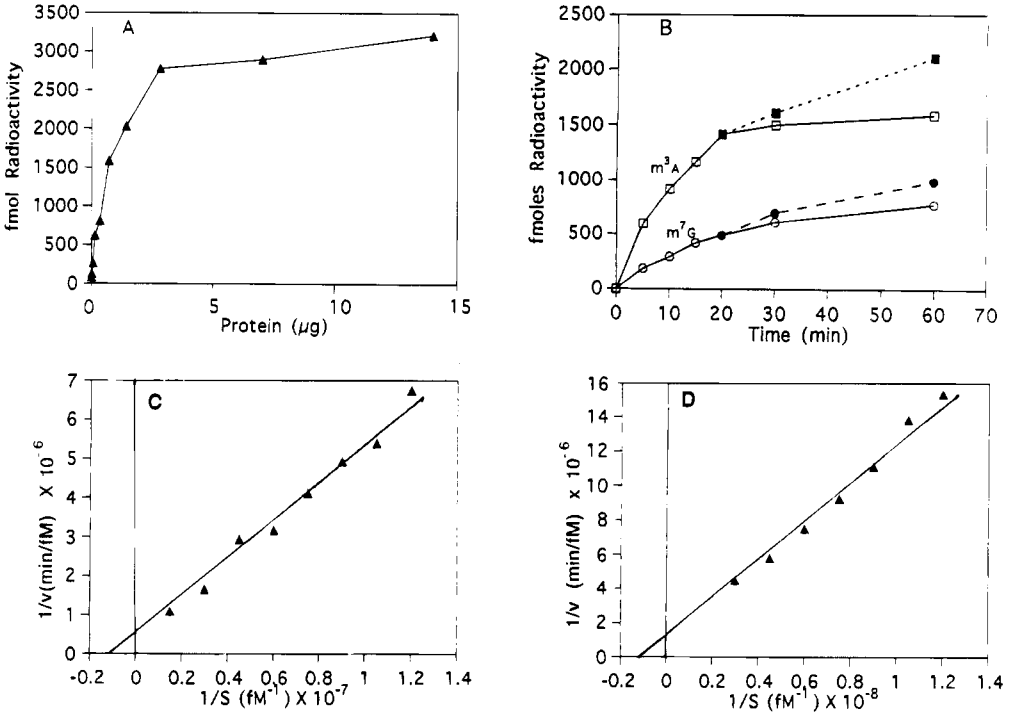

FIGURE 5: Kinetics of the MPG reaction. (A) Effects of MPG concentration on the release of m^3A and m^7G from DNA. Various amounts of the protein were incubated in 0.2 mL of standard assay buffer for 10 min at 37 °C with 24 μg of methylated DNA (containing 4.7 pmol of m^7G and 860 fmol of m^3A, 41.3 nmol of adenine and 27.5 nmol of guanine). (B) Kinetics of release of m^3A and m^7G. The enzyme (0.98 μg) was incubated with 170 μg of methylated DNA containing 46 pmol methylated purines in 1.5 mL at 37 °C. At various times, released m^3A (■) and m^7G (●) bases were quantitated in 0.2-mL aliquots. At 20 min, additional enzyme (0.14 μg) was added to a separate reaction mixture for further analysis of the rate of release of the methylated bases. (C) Lineweaver–Burk plot of MPG reaction for m^3A release. The enzyme (0.14 μg) was incubated with a constant amount (2.2 μg/μL) of DNA (adjusted by the addition of unmodified DNA) containing increasing concentration of m^3A (0.2–70 fmol/μL) and m^7G (1.2–420 fmol/μL). Other details are described in Materials and Methods. (D) Lineweaver–Burk plot for the excision of m^7G by MPG. The details are as in panel C.

Table 3: Physicochemical and Biochemical Properties of Recombinant Mouse MPG

molecular mass	
observed SDS–PAGE	34 400
predicted	31 350
isoelectric point (pI)	
IEF	9.3
predicted from amino acid sequence	9.2
extinction coefficient ($E^{280nm}_{1\%}$)	
calculated	9.9
experimental	10.5
optimum pH	8.5–9.5
optimum NaCl concentration	100 mM
effect of spermine	stimulation, 40–50% at 0.02 mM inhibition, ~50% at 1.0 mM
effect of $MgCl_2$	stimulation, 75% m^3A; 35% m^7G release at 0.5 mM inhibition, 80% m^3A and m^7 release at 50 mM

The binding of the MPG with untreated and DMS-treated plasmid DNA was studied by a nitrocellulose filter binding assay. The mouse MPG has moderate affinity for DNA binding, but the K_A, in the range of $4–6 \times 10^7$ M^{-1}, was not significantly different between methylated and non-methylated DNA (Table 4).

Effect of Polyamines. Polyamines were shown to affect the activity of a variety of DNA-modifying enzymes (Kleppe *et al.*, 1981) or to have direct effect on AP sites in DNA (Male *et al.*, 1982). Forty to fifty percent stimulation of both m^3A and m^7G release by the MPG was observed in the presence of 0.02 mM spermine. However, ~50% inhibition was observed in 1.0 mM spermine (Table 3), an effect which may be due to polyamine-induced aggregation of DNA (Osland & Kleppe, 1977).

Interaction of Mouse MPG with Nucleic Acids. Calf thymus DNA at 500 and 900 μg/mL inhibited the release of m^3A and m^7G by 32% and 60%, respectively. At the same time, the DNA protected MPG from heat inactivation. The enzyme lost ~80% of its activities for both m^3A and m^7G after incubation when the substrate DNA at a noninhibitory concentration (0.12 μg/μL) was present for 20 min at 37 °C.

Table 4: Kinetic Constants of Recombinant Mouse MPG for the Binding to Methylated DNA and Excision of m^3A, m^7G, and m^3G from Methylated DNA[a]

DNA	base hydrolyzed	methylated K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} nM^{-1}) ($\times 10^{-4}$)	K_A (M^{-1}) ($\times 10^7$)
calf thymus DNA	m^3A	130	0.8	61.0	
	m^7G	860	0.2	2.3	
poly(dG)·poly(dC)	m^7G	500	0.46	9.2	
	m^3G	1.4	0.013	93.0	
non-methylated plasmid DNA					4.3
methylated plasmid DNA					5.8

[a] Experimental details are described in materials and methods and the legend to Figure 4C.

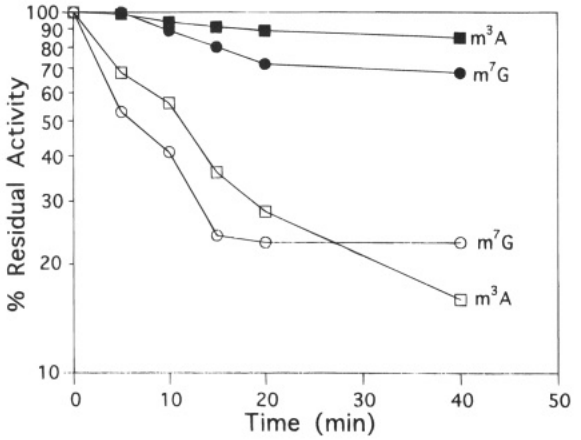

FIGURE 6: Thermal stability of MPG. The kinetics of inactivation of MPG in the presence (solid symbols) or absence (open symbols) of duplex DNA (0.4 μg/μL) was measured by incubating the enzyme at 37 °C and then assaying for its residual activity.

However, 25% and 10% loss of activity for m^7G and m^3A, respectively occurred when DNA concentration was raised to 2 μg/μL by adding nonmethylated calf thymus DNA (Figure 6). The differential inactivation of the enzyme for m^7G and m^3A suggests that MPG may have distinct recognition mechanisms for the two bases.

Effect of Salts on MPG Activity. The MPG was maximally active for release of both m^3A and m^7G in 100 mM NaCl (Table 3). Fifty and twenty five percent of this activity was observed in the presence of 10 and 200 mM NaCl, respectively (data not shown). Human MPG was also reported to show maximal activity at 100 mM KCl, and the activity of human MPG declined rapidly above 200 mM KCl (O'Connor, 1993).

Although the mouse MPG does not require Mg^{2+} for activity, its activity in releasing both m^3A and m^7G was stimulated by 75% and 35%, respectively, in 0.5 mM $MgCl_2$. However, 50 mM $MgCl_2$ inhibited 80% of the activity (Table 3).

Product Inhibition of MPG Activity. High concentration of m^7G (10 mM) inhibited release of both m^3A and m^7G from methylated calf thymus DNA by 50−60%, while 0.6 mM m^7G caused a slight stimulation (20%) (Figure 7B). In contrast, 1 mM m^3A stimulated release of m^7G and m^3A by 67% and 30%, respectively. Again, higher concentration (11 mM) of the base inhibited release of both m^3A and m^7G by 50% at (Figure 7A). tRNA-guanine ribosyltransferase is inhibited by m^7G and pterine or tetrahydrobiopterin, but not by adenine or adenine derivatives, presumably due to structural similarity of pterins to guanine (Farkas *et al.*, 1984). Pteridines are present in significant amounts in mammalian cells (Abou-Donia & Viveros, 1981). MPG was also inhibited by pterine but at a high concentration. Ten millimolar pterine inhibited the release of both m^3A and m^7G by 40% (Figure 7C). However, other bases, such as adenine, deoxyadenine, 7-methyldeoxyguanosine, O^2-methyl thymine, or O^2-methylcytosine, in millimolar concentration, did not show any effect on MPG activity (data not shown).

Other Properties of MPG. No DNA endo- or exonuclease activity was detected in the purified MPG after 0.1 μg of protein was incubated with supercoiled, linear, and depurinated plasmid DNAs at 37 °C for 15 min (Figure 8).

The enzyme showed maximum activity at pH 8.5−9.5 (Table 3). EDTA at upto 10 mM had no effect on its activity,

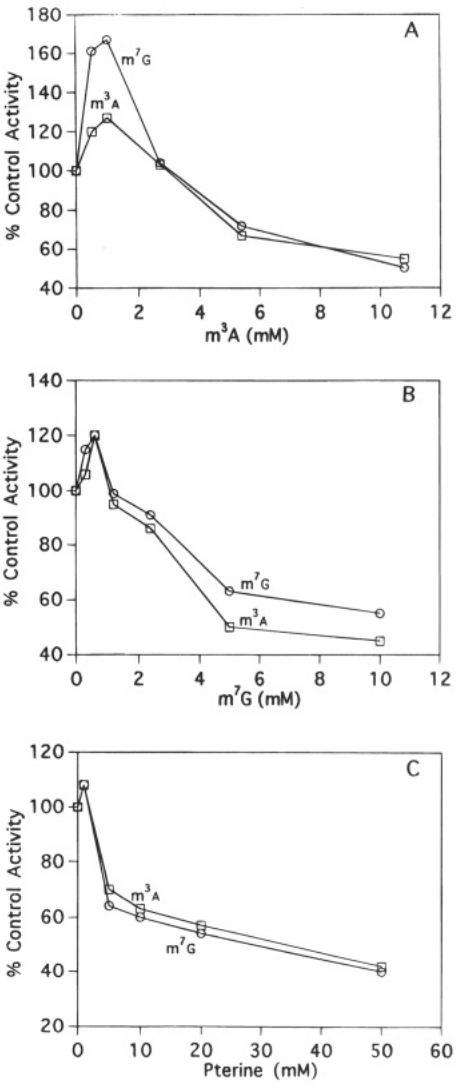

FIGURE 7: Effects of m^3A, m^7G, and pterine on MPG reaction. The release of m^3A and m^7G was measured in the presence of m^3A (A), m^7G (B), and pterine (C). The experimental details are as given in the legend to Figure 4A.

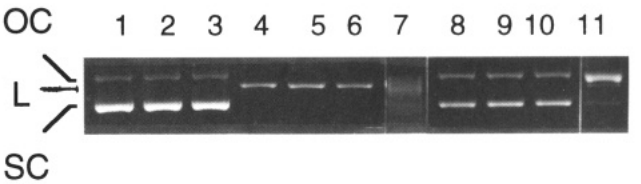

FIGURE 8: Absence of endonuclease, exonuclease, and AP-endonuclease in purified MPG. Details of experimental details are described in Materials and Methods. Supercoiled plasmid DNA was treated with either bovine serum albumin (lane 2), purified MPG (lane 3), or buffer alone (lane 1). Linearized plasmid DNA was treated with either albumin (lane 5), purified MPG (lane 6), exonuclease III (lane 7), or buffer alone (lane 4). Acid-depurinated plasmid DNA was incubated with either albumin (lane 9), purified MPG (lane 10), T4 endonuclease V (lane 11), or buffer alone (lane 8). Supercoiled DNA (SC), open circular DNA (OC), and linear DNA, (L).

although at 50 mM inhibited it by 50%. Glycerol 10−20% stimulated the activity by 30%. The purified MPG preparations can be stored in a buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 M NaCl, and 50% glycerol at −20 °C for several months without degradation or loss of activity. Two cycles of freezing and thawing or keeping on ice for 2−3 days in the presence of 5% glycerol

led to a loss of 20% and 5% activity, respectively.

The extinction coefficient of the MPG protein was calculated from its absorbance at 280 nm in the presence of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 200 mM NaCl. An aliquot of protein was hydrolyzed for amino acid analysis. The experimental value of the extinction coefficient ($E_{1\%}^{280nm}$) of 10.5 was close to the calculated value (Table 3).

DISCUSSION

Although MPG (also called 3-methyladenine-DNA glycosylase) had been purified from several mammalian sources and some of its properties have been reported, none of the endogenous proteins was isolated in a homogeneous form. Few earlier studies showed that partially purified enzymes from human sources released m^7G and m^3G in addition to m^3A from DNA (Gallagher & Brent, 1982, 1984; Singer & Brent, 1981).

Preliminary studies of the kinetics and substrate specificity of partially purified MPGs from calf thymus were published earlier (Male *et al.*, 1987). Recently, the recombinant human MPG was purified to homogeneity, and some of its kinetic properties were investigated (O'Connor, 1993). However, our studies showed a significant difference between the human and mouse glycosylases in their substrate preferences, particularly in regards to 7-methylguanine in DNA (Mitra *et al.*, 1993). This observation underscores the importance of a detailed study of both mouse and human proteins for subsequent structure−function analysis. Furthermore, the mouse MPG has been more thoroughly characterized than the human protein. Although the lack of endonuclease and AP-endonuclease activities, in partially purified endogenous human MPG, was observed earlier (Gallagher & Brent, 1982), we have shown by a sensitive gel electrophoresis method that the homogenous mouse MPG is devoid of AP-endonuclease and DNA endo- and exonuclease activities. Thus, the mammalian MPG is similar to *E. coli* MPGs in this regard (Sakumi & Sekiguchi, 1990). That recombinant mammalian MPGs can rescue MPG-negative *E. coli* mutants indicates that such proteins can be isolated in active and homogeneous form from *E. coli* for biochemical and physicochemical studies. We expressed a shorter-than-full length mouse MPG polypeptide because of the relative ease of its overproduction in soluble and active form. However, we believe that deletion of 48 amino acid residues from the amino terminus of the protein does not affect its catalytic activity for the following reasons. First, the ratios of activity for the release of m^3A and m^7G by the partially purified full length and deleted mouse enzymes were the same (R. Roy, unpublished results). Second, O'Connor (1993) did not find a significant difference in the kinetic properties of the wild-type human MPG and a mutant enzyme with deletion of 68 amino acid residues from the amino terminus. In any case, we encountered significant difficulties during overexpression of the mouse MPG in *E. coli* because of its aggregation. We had more difficulty when the mouse protein was expressed in the form of amino-terminal fusion polypeptides. These suggest that the amino terminus region of the protein contributes significantly to its aggregation, when overexpressed in *E. coli*.

We tested various protocols of denaturation and renaturation to reactivate the insoluble MPG present in inclusion bodies in *E. coli*, which results presumably from improper folding of the protein. However, we were uniformly unsuccessful. Finally, we found induction with IPTG at low temperature (30 °C) rather than at 37 °C increased the soluble fraction of MPG in *E. coli* extract. It is possible that lower temperature promotes correct folding of the protein as was also observed with the tail spike protein of phage P22 (Mitraki *et al.*, 1991). We finally succeeded in purifying the MPG with 48 amino acid deletion at the amino terminus to homogeneity from the soluble fraction of *E. coli* extract. In a preliminary study, the molecular weight of the homogeneous protein was calculated from equilibrium sedimentation to be about 45 000, higher than the calculated value of 31350 (S. Rajendran, R. Roy, S. Mitra, and J. Lee, unpublished experiment). This raises the possibility that the protein may be present in a monomer−dimer equilibrium, which is currently being investigated. It is interesting to note that Male *et al.* (1981) observed proteins of 27 and 68 kDa molecular masses with MPG activity in a partially purified enzyme preparation from mouse L cells. It is, thus, tempting to speculate that the 68-kDa species was a dimer of the monomeric MPG polypeptide with a predicted molecular mass of 36 kDa.

The availability of the homogeneous MPG allowed us to compare its amino-terminal sequence with that predicted from the nucleotide sequence of the cDNA. The monoS column provided us the opportunity to separate the MPG protein with expected N-terminal sequence from its deletion mutant which had a 14 amino acid deletion from the N-terminus. This deletion presumably resulted from the action of *E. coli* protease(s).

It is interesting that DNA protects the glycosylase from heat inactivation while inhibiting its activity at the same time. A similar phenomenon was observed earlier with O^6-methylguanine-DNA methyltransferase (Bhattacharya *et al.*, 1990). We have also shown that DNA inhibits MPG activity, presumably by its nonspecific binding. Male *et al.* (1987) and O'Connor (1993) found that the release of m^7G by calf thymus MPG or human MPG did not increase with increasing the amount of methylated calf thymus DNA as substrate and thus could not use that substrate for determining kinetic parameters for m^7G. It is not clear whether their studies took into account the nonspecific inhibition of reaction by DNA whose concentration was raised in order to increase the concentration of m^7G in the reaction mixture. Thus, it is important to ensure that while the concentration of the actual substrate (*N*-methylpurine residues) is varied, the concentration of DNA remains unchanged. We achieved this by adding appropriate amounts of unmodified DNA to the reaction mixtures. This observation also implies that the K_m and k_{cat} values determined in this study are apparent and for a particular concentration of DNA and thus are useful only for comparative purposes. For example, the K_m (m^3A) values for both the calf thymus and human MPGs were reported to be 9 nM, but the K_m (m^7G) of calf thymus MPG was 15-fold lower than that of the human glycosylase (Male *et al.*, 1987; O'Connor, 1993). On the other hand, the K_m values of mouse MPG for m^3A and m^7G are 15−30-fold higher than those of calf thymus and human MPGs. The k_{cat} value for m^3A was reported to be 10-fold higher for the human MPG (O'Connor, 1993) than for the mouse MPG described here, while the k_{cat} value for m^7G appeared to be comparable for the human and mouse MPGs. However, in view of the

potential problems in such measurement, as discussed above, it is not clear whether such comparisons are meaningful.

The preferred substrate among all methylated base adducts for both human and mouse MPGs is m^3A, although m^7G is generally a more abundant DNA adduct (Beranek, 1990). There may be a teleological reason for this, i.e., that it may be more important to remove m^3A, a toxic lesion, than m^7G, which appears to be benign (Boiteux *et al.*, 1984). Nonetheless, our results strongly suggest that, in addition to its spontaneous hydrolysis, m^7G is enzymatically removed by MPG *in vivo*. In any case, the K_m and k_{cat} values for m^3A and m^7G in the same DNA suggest that the preference of m^3A over m^7G by mouse MPG occurs both at the level of affinity for the substrate and at the catalytic step. Because we did not observe a significant difference in the affinity of methylated versus nonmethylated DNA for the protein, it appears that the free energy change in the binding of MPG to the alkylated base adducts is small compared to that of nonspecific binding of MPG to DNA. Further experiments will be necessary to investigate the mechanism by which MPG recognizes its substrate in the presence of a vast excess of DNA.

The mouse MPG, like other DNA glycosylases, does not have an absolute requirement for a cofactor. However, our studies, as well as previous reports with partially purified proteins from mammalian cells, showed a small but significant activation of the protein by low concentration of Mg^{2+}. The biochemical mechanism of this activation is not clear. Similarly, the product inhibition studies showed that both m^3A and m^7G are inhibitory, although at a rather high concentration. Structurally related pterine also inhibited the MPG activity. It is noteworthy that tetrahydrobiopterine, similar to pterin, is present in mammalian cells (Abou-Donia & Viveros, 1981). It significantly inhibited the incorporation of queuine into tRNA by tRNA-guanine ribosyltransferase which is also inhibited by m^7G (Farkas *et al.*, 1984). It is interesting that a low concentration of m^3A stimulated the release of m^7G more than that of m^3A. This observation suggests that the binding sites of the two adducts during catalysis are different. This possibility is supported by the differential thermal inactivation and Mg^{2+} effects for the two substrates.

It is surprising that the mouse MPG, like the *E. coli* AlkA protein, has an unusually broad substrate specificity, and that its substrate range includes 8-hydroxyguanine and $1,N^6$-ethenoadenine (Bessho *et al.*, 1993; Dosanjh *et al.*, 1994a). Because the protein recognizes only modified DNA, the modification should be a component of its recognition element. Contrary to an earlier suggestion that MPG may recognize only lesions in the minor groove in DNA (McCarthy *et al.*, 1984), it is evident that the presence of the methyl group either in the major or the minor groove is recognized by the protein. Our preliminary experiments indicate that the mouse MPG contains distinct domains (R. Roy, *et al.*, unpublished observation). We are currently examining the role of these domains in the recognition of specific base lesions.

ACKNOWLEDGMENT

We thank Dr. S. J. Kennel and Dr. K. B. Jacobson (Biology Division, Oakridge National Laboratory) for providing us with mouse lung cDNA library and for suggesting the experiment with pterine respectively. We also thank Dr. R. S. Lloyd (Sealy Center for Molecular Science, UTMB) for providing the purified T4 endonuclease V. We thank Drs. G. Ibeanu and S. Shiota for construction of plasmids pGI1946 and pSMPGHi. We thank Mr. Stefan Serabyn for protein microsequencing and amino acid composition analysis. Finally, we thank Dr. David Konkel for critically reading the manuscript and Ms. Wanda Smith for excellent secretarial help.

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