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Purification and characterization of human 3-methyladenine-DNA glycosylase

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ABSTRACT

A human cDNA coding sequence for a 3-methyladenine-DNA glycosylase was expressed in *Escherichia coli*. In addition to the full-length 3-methyladenine-DNA glycosylase coding sequence, two other sequences (resulting from differential RNA splicing and the truncated anpg cDNA) derived from that sequence were also expressed. All three proteins were purified to physical homogeneity and their N-terminal amino acid sequences are identical to those predicted by the nucleic acid sequences. The full-length protein has 293 amino acids coding for a protein with a molecular mass of 32 kDa. Polyclonal antibodies against one of the proteins react with the other two proteins, and a murine 3-methyladenine-DNA glycosylase, but not with several other *E.coli* DNA repair proteins. All three proteins excise 3-methyladenine, 7-methylguanine, and 3-methylguanine as well as ethylated bases from DNA. The activities of the proteins with respect to ionic strength (optimum 100 mM KCl), pH (optimum 7.6), and kinetics for 3-methyladenine and 7-methylguanine excision (average values: 3-methyladenine: K_m 9 nM and k_{cat} 10 min^{-1}, 7-methylguanine: K_m 29 nM and k_{cat} 0.38 min^{-1}) are comparable. In contrast to these results, however, the thermal stability of the full-length and splicing variant proteins at 50°C is less than that of the truncated protein.

INTRODUCTION

Chemical methylation of DNA is the consequence of exposure to environmental agents or cellular metabolism (1–3). Repair of cytotoxic lesions associated with DNA methylation in *E.coli* is performed mainly by the BER pathway. The first step in this ubiquitous pathway is the removal of a damaged or mispaired base by a DNA glycosylase to leave an abasic site (4,5). Subsequently, the abasic site is incised by an endonuclease or a lyase specific for abasic sites (6). Following incision at the abasic site, the 5'deoxyribose phosphate (7–9), the unsaturated 3'deoxyribose phosphate (10–13), or the phosphoryl group (10–13) is removed and repair is completed by DNA polymerase and DNA ligase.

The cytotoxicity of DNA methylating agents in *E.coli* is linked to the formation of 3-meAde bases (14,15). These modified bases block synthesis of DNA *in vitro*, which suggests that they are lethal lesions (16). *E.coli* mutant strains deficient in DNA glycosylases removing 3-meAde bases are hypersensitive to DNA methylating agents compared to the wild type strains (14,17–19). Experiments in mammalian cells suggest that 3-meAde bases may also be cytotoxic in eucaryotic cells if they are not repaired (20,21).

To study the biological role of mammalian 3-meAde-DNA glycosylases, we cloned cDNAs for murine and human DNA glycosylases removing that modified base (22,23). In addition to 3-meAde, crude extracts of *E.coli* which express these cDNAs also remove other alkylated bases (22–24).

In this report, I express the cDNA coding for full-length human 3-meAde-DNA glycosylase, purify the corresponding protein to physical homogeneity, and characterize some of its properties. In addition to the full-length protein, two coding sequences derived from this full-length sequence are also expressed and the corresponding proteins purified. Moreover, the isolation of polyclonal antibodies against 3-meAde-DNA glycosylase should facilitate future work in mammalian cells concerning the function of this protein.

MATERIALS AND METHODS

DNA, enzymes, and chemicals, DNA glycosylase assays

Calf thymus DNA (Type II) was obtained from the Sigma Chemical Co. (St. Louis, MO) and poly(dG-dC) was obtained from Boehringer-Mannheim. The pcDNAII plasmid was obtained from INVITROGEN and the plasmid pET3 and the BL21(DE3) strain from Drs. R. Devoret and J. Angulo. The pTO40 plasmid was constructed by inserting the BamH I/Hind III fragment containing the T4 RNA polymerase termination sequence from pET3 (25) into the pcDNAII plasmid digested with the same enzymes. Digestion of the pTO40 plasmid using Bgl II/Hind III and 'fill in' using Klenow fragment DNA polymerase, followed by ligation was used to generate the pTO50 plasmid. The

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pANPG10 plasmid with the truncated 3-meAde-DNA glycosylase cDNA was from laboratory stocks (23).

DNA restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim, and Taq polymerase was purchased from Promega or Bioprobe.

Radiolabelled reagents were obtained from the following sources: [^{3}H]-DMS (1 Ci/mmol) from New England Nuclear, γ-^{32}P-ATP (3000 Ci/mmol) from Amersham.

Methods for nucleic acid manipulation were performed according to Sambrook *et al.* (26).

Preparation of DNA substrates for [^{3}H]-DMS-DNA and [^{3}H]-DMS-poly(dG-dC) was as described using [^{3}H]-DMS (9,22). The specific activities of the [^{3}H]-labelled substrates were 550 cpm/pmole of [^{3}H]-methylated bases. The percentage of 3-meAde:(3-meAde + 7-meGua) in the [^{3}H]-DMS-DNA was 18.5% as assayed by HPLC separation of the purine bases following formic acid treatment of the substrate(75% formic acid, 80°C, 1 h). The [^{3}H]-DMS-poly(dG-dC) assayed using an identical method was 99.0% 7-meGua and 1% 3-meGua. The DNA substrates with abasic sites, uracil bases, or Fapy bases have been previously described (9).

The assay buffer for 3-meAde or 7-meGua-DNA glycosylase activity was 100 mM KCl, 50 mM Hepes-KOH pH 7.6, 1 mM Na_2EDTA, 5 mM β-mercaptoethanol and [^{3}H]-DMS-DNA. The standard assay is performed in 50 μL at 37°C for 5 min. using 6.4 pmoles of methylated bases in [^{3}H]-DMS-DNA. One unit of DNA glycosylase activity is defined as 1 pmol of methylated base released as ethanol soluble bases from [^{3}H]-DMS-DNA per min at 37°C. Further details are found in reference 22.

Determinations of Michaelis constants were performed as previously described using the [^{3}H]-DMS-DNA as substrate for 3-meAde excision and [^{3}H]-DMS-poly(dG-dC) for 7-meGua excision (9).

SDS-PAGE and protein sequencing

SDS-PAGE, transfer of proteins, and protein sequencing were performed as previously described (26,27). Protein molecular mass markers correspond to: α-lactalbumin (14.4 kD), trypsin inhibitor (20.1 kD), carbonic anhydrase (30 kD), ovalbumin (43 kD), albumin (67 kD), and phosphorylase b (94 kD). SDS-PAGE gels were stained using Coomassie Brilliant Blue dye.

PCR mutagenesis

The splicing variant and the full-length coding sequences from references 29 and 30 were constructed using PCR mutagenesis using the anpg cDNA from reference 23. Following each round of amplification, the corresponding fragments were gel purified before use in the next round of amplification. Each round of amplification started with approximately 10−20 ng of fragment containing the target sequence with 100 pmoles of primers and consisted of 5 cycles of polymerisation at a lower temperature and 10 cycles of amplification at a higher temperature to increase specific amplification (These two temperatures are indicated in parentheses in the list of oligonucleotides below). The time for denaturing, annealing, and elongation were 1 min., 2 min., and 2 min., respectively. The oligonucleotides used to construct the sequences are indicated (The bacterial ribosomal binding sites are underlined in the sequences of oligonucleotides *v*−*vii* .):
TGCCCCAGGGAGCGCTGCTTGGGACCGCCCACCACT-CCGGGCCCATACCGCAGCATCTATTTC (48°−60°C) *i*,
GAGCAGGGCAGCCACACAGCTCGTCCGACGCAGCCC-AGGCACCTGCAGAGCAGCCACACAGCTCGTCCGATGC-AGCCCAGGCACCTTGCCCCAGGGAGCG (50°−60°C) *ii*,
AGTTTTGCCGACGGATGGGGCAAAAGAAGCAGCG-ACCAGCTAGAGCAGGGCAGCCA (48°−60°C) *iii*,
GCTTTGCAGATGAAGAAACCAAAGCAGTTTTGCCGAC-GGA (46°C−60°C) *iv*,
CGGATCCGAAGGAGATATACATATGTCTAAAGACCGC-AGCATC (54°C) *v*,
CCGAATCCGAAGGAGATATACATATGGTCACCCCCGC-TTTGCAGATG (36°−70°C) *vi*,
CGCTCAGAGAAGGAGATATACATATGCCCGCGCGCAG-CGGGGCCCAGTTTTGCCGACGGATG (56°−68°C) *vii*,
GAAGATCTGCTCAGGCCTGTGTGTCCT *viii*.

Following the construction of the truncated coding sequence the DNA was inserted in the pUC18 plasmid in the BamHI site to form the pANPG20 plasmid. Restriction digestion mapping indicated that the 5'end of the coding sequence was oriented toward the XbaI site. The EcoRI/XbaI fragment from pANPG20 was placed in the EcoRI/XbaI site of pTO50 to generate pANPG40 producing the truncated 3-meAde-DNA glycosylase protein. Following insertion of the splicing variant sequence at the EcoRI/BamHI sites, an EcoRI/XbaI restriction, Klenow fragment DNA polymerase 'fill in', and ligation were performed to yield pANPG60. The full-length 3-meAde-DNA glycosylase coding sequence was inserted into the XbaI/BamHI site of the pTO50 plasmid to produce the pANPG70 plasmid. The sequences of cloned fragments were determined using the dideoxy chain termination method with Sequenase to verify the products of the PCR amplifications.

Similar procedures used to construct a plasmid expressing a murine 3-meAde-DNA glycosylase cDNA and the purification of that protein will be described elsewhere.

Protein purification

Truncated 3-meAde-DNA glycosylase. 10 g of frozen *E.coli* BL21(DE3) cells hosting the pANPG40 plasmid were resuspended in 5 volumes of 300 mM Tris−HCl 5 mM EDTA and a lysozyme solution added to a final concentration of 1 mg/ml lysozyme. The suspension was incubated at 0°C for 30 min. and then heated for 20 min. at 37°C. The suspension was then placed into an ethanol-dry ice bath (about −60°C) and allowed to freeze for a period of 15 min. This freeze-thaw cycle was repeated several times following which the suspension was centrifuged for 30 min at 30,000 rpm in a Beckman 42.1 rotor. The supernatant in this step was collected and designated the Crude Extract (53 ml). The Crude Extract was then subjected to PEI treatment to precipitate nucleic acids (53 ml). A 5% PEI stock solution (300 mM Tris−HCl pH 7.5, 5 mM EDTA) was added to the Crude Extract to a final concentration of 10% of the total volume (5.3 ml 5% PEI solution) over a period of 10 min. with stirring (31). The mixture was stirred for a period of 20 min. and the solution centrifuged to generate a supernatant of 56 ml identified as the PEI fraction. The PEI fraction was diluted to 300 ml using Buffer 1 and loaded onto a 15 ml Phosphoultrogel (1.5cm×15 cm) column equilibrated in Buffer 1 (70 mM Hepes-KOH pH 7.5, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 5% glycerol). The column was rinsed with Buffer 2 (50 mM NaCl, Buffer 1) and a 300 ml gradient between 50 and 600 mM NaCl in Buffer 1 was used to elute the protein. The active fractions were pooled and identified as the Phosophoultrogel fraction (60.5 ml). The proteins in the Phosphoultrogel fraction were precipitated using ammonium sulfate (0.5 g/ml), resuspended in less than 4 ml of Buffer 1, and loaded onto an AcA54 gel

exclusion column with a volume of 130 ml (1.5cm×1.2m) equilibrated in Buffer 3 (1 M NaCl, 0.8 M ammonium sulfate, Buffer 1 without glycerol). The active fractions were pooled and identified as the AcA54 fraction (11 ml). The AcA54 fraction was loaded onto a 5 ml Phenyl Superose HR5/5 FPLC column, rinsed with Buffer 3, and a 15 ml gradient from Buffer 3 to Buffer 1 performed. The active fractions were analyzed using SDS-PAGE, and fractions containing homogeneous truncated 3-meAde-DNA glycosylase were pooled.

Full-length and splicing variant 3-meAde-DNA glycosylases. The initial steps in the purification of the full-length and splicing variant 3-meAde-DNA glycosylases were identical to those described for the truncated protein until after the AcA54 gel exclusion chromatography. The AcA54 fractions were loaded onto a 5 ml Phenyl Sepharose column and rinsed with Buffer 3. A 70 ml gradient from Buffer 3 to Buffer 1 was used to elute proteins, and the fractions with 3-meAde-DNA glycosylase activity were pooled (Phenyl Sepharose fractions). The Phenyl Sepharose fractions were diluted to reduce the ionic strength and loaded onto a MonoS FPLC HR5/5 column. The column was rinsed with Buffer 1 and gradients from 0 to 150 mM KCl (5 ml) and 150 to 800 mM KCl (30 ml) were used to develop the column. The fractions containing 3-meAde-DNA glycosylase activity were analyzed using SDS-PAGE and fractions containing homogeneous, full-length 3-meAde-DNA glycosylase or the splicing variant 3-meAde-DNA glycosylase were pooled.

Polyclonal anti-3-meAde-DNA glycosylase antibodies and Western blots. 100 μg splicing variant 3-meAde-DNA glycosylase was suspended in complete Freund's Adjuvant and injected in a female New Zeland wite rabbit (age about 60−70 d., weight 2 kg) prior to which a blood sample was withdrawn. Blood samples (5 ml) were removed each week, the serum cleared, and assayed for antigenic reaction to the 3-meAde-DNA glycosylase using a dot blot technique. In this technique, 1 μg and 0.1 μg of the splicing mutant protein were spotted on a Hybond-C nitrocellulose filter with an identical quantity of bovine serum albumin as control, and treated as in the Western blotting protocol (26). 5 weeks after the first injection a booster injection was performed using incomplete Freund's adjuvant. The Western blots shown in this report were performed using serum isolated 7 weeks following the first inoculation.

Affinity column purified polyclonal anti-3-meAde-DNA glycosylase antibodies were obtained using two Sepharose CL4B (Pharmacia) columns. The first column had the purified truncated 3-meAde-DNA glycosylase (26 kDa) coupled to the resin and the second column had a crude extract of total proteins from BL21 hosting pTO50 coupled to the resin. These columns were a gift from Dr. K. Kleibl (Villejuif, this laboratory). The methods used in purifying the antibodies are described in reference 32.

For Western blots, proteins were transferred to Hybond C nitrocellulose filters or ECL membranes (Amersham), and reacted with antibodies following blocking of nonspecific sites using 5% w/v non-fat dried milk (26). Bound antibodies were detected using an anti-Rabbit IgG(H+L) antibody (at 1/30000 dilution) conjugated with peroxidase (Pasteur Institute, Paris), and revealed using ECL detection (Amersham). In this detection system, the peroxidase catalysed oxidation of luminol generates a chemiluminescent species which is detectable on X-ray film.

HPLC. Products were analysed using HPLC on a C18 μBondapak column (Waters) developed isocratically at 1.5 ml/min with a mobile phase of 10 mM $NH_4H_2PO_4$, pH 4.5 in 2% methanol or in 20 mM $NH_4H_2PO_4$, pH 4.5 in 5% methanol (21). Radioactivity was quantitated using scintillation spectroscopy. The retention times for the bases in the first buffer indicated were as follows: 11 min for 3-meGua and 20 min for 7-meGua, whereas in the second buffer, the retention times were: 7 min 3-meAde and 13 min 7-meGua.

5′ labeling of a 205 bp fragment. To perform chemical and enzymatic reactions a 205 bp fragment from the anpg cDNA (23) was 5′ end labelled using PCR BioTaq polymerase. 0.5 μg of one of the two primers used in the PCR reaction (GACCTGCC-ACAGGATGAAG *ix*) was ^{32}P (15μCi, 3000 Ci/mmol)labelled at the 5′ end using T4 polynucleotide kinase. Oligonucleotide *viii* was used for the amplification at the 3′end. The first five cycles of amplification were performed with an annealing temperature of 62°C with an elongation time of 0.5 min. Following five cycles of amplification, a second series of amplifications was performed for 20 cycles with an annealing temperature of 68°C and an elongation time of 0.5 min. The temperatures of denaturation and elongation in both cases were 94°C and 74°C, respectively. The DNA was then phenol−chloroform extracted and separated using 15% PAGE.

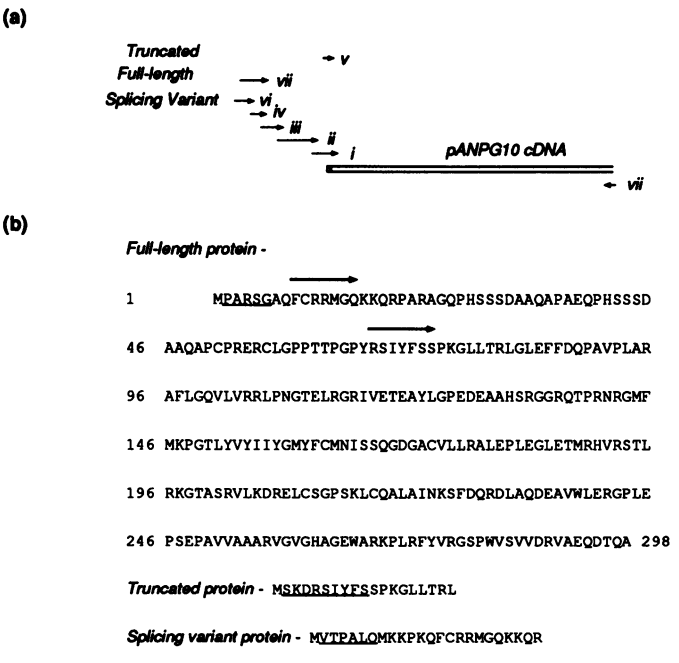

Figure 1. Construction of the cDNA coding sequences and protein sequences of the 3-meAde-DNA glycosylases purified in this study. **(a)** Scheme showing the construction of the full-length, truncated, and splicing variant cDNA coding sequences using PCR mutagenesis. The oligonucleotide sequences and the methods are described in the Materials and Methods. The starting sequence, the anpg cDNA, is indicated by the double line. The small box indicates part of the anpg sequence which was associated with the cDNA cloning. The oligonucleotides containing the bacterial ribosomal binding sites are indicated as *Full-length*, *Truncated*, and *Splicing Variant*. The pANPG70 plasmid has DNA coding for the 293 amino acid, 32 kDa full-length 3-meAde-DNA glycosylase. The pANPG40 plasmid has DNA coding for the 230 amino acid, 26 kDa truncated 3-meAde-DNA glycosylase. The pANPG60 plasmid has DNA coding for the 298 amino acid, 32 kDa splicing variant 3-meAde-DNA glycosylase. **(b)** Amino acid sequences of the proteins isolated in this study. The underlined amino acids were sequenced. The beginning of the truncated and the splicing variant protein sequences are indicated by arrows.

The band corresponding to the fragment was imaged using Ethidium Bromide staining and excised from the gel. The labelled fragment was then eluted from the gel matrix, filtered, extracted using phenol and chloroform, and the DNA was precipitated (26). Following a second precipitation, the DNA was resuspended in 10 mM Tris−HCl, pH 7.5, 1 mM EDTA buffer.
The sequence generated was as follows: GACCTGGCACAGG-ATGAAGCTGTATGGCTGGAGCGTGGTCCCCTGGAGCC-CAGTGAGCCGGCTGTAGTGGCAGCAGCCCGGGTGGG-CGTCGGCCATGCAGGGGAGTGGGCCCGGAAACCCCT-CCGCTTCTATGTCCGGGGCAGCCCCTGGGTCAGTGTG-GTCGACAGAGTGGCTGAGCAGGACACACAGGCCTG-AGCAGATCTTG.
The underlined triplet is discussed in the Results section.

Modification of the ^{32}P-labelled fragment with DMS and DES. The labelled fragment (250000 cpm counted by Cherenkov radiation) from the previous section in 200 mM sodium cacodylate at pH 7 was reacted with either DMS (5 mM) or DES (100 mM) for 30 min at 25°C. The reaction was stopped by the addition of 11 µl of β-mercaptoethanol, and the DNA was ethanol precipitated, the pellets rinsed, dried, and resuspended in 25 µl of water. The modified fragments were then subjected either to chemical or enzymatic reactions to reveal the modified bands. Chemical cleavage was performed using 1 M piperidine treatment at 90°C for 30 min. Enzymatic removal of damaged bases was performed using 9 ng of the 32 kDa splicing variant 3-meAde-DNA glycosylase. The 3-meAde-DNA glycosylase sensitive sites were revealed by the addition of 18 ng of *E.coli* Fpg protein, which incises DNA at abasic sites leaving the same ends in DNA as chemical sequencing and is active in the same buffer as the human 3-meAde-DNA glycosylase (9). Following removal of the piperidine, or the phenol extraction and ethanol precipitation of the enzymatically treated samples, the DNA was analysed on a 7M urea, 10% PAGE sequencing gel.

RESULTS

Construction and expression of a cDNA sequence encoding human 3-meAde-DNA glycosylase

Figure 1a shows the scheme for the construction of the coding sequence of the full-length human 3-meAde-DNA glycosylase (30) using the truncated cDNA isolated in reference 23 as a template for PCR mutagenesis. This coding sequence was inserted into the pTO50 vector under the control of a promoter specific for the bacteriophage T7 RNA polymerase (25). In addition to the full-length sequence, two other coding sequences for a truncated 3-meAde-DNA glycosylase and a splicing variant protein were also inserted into the pTO50 vector. The splicing variant 3-meAde-DNA glycosylase differs in the splicing of the RNA between the first and second exons (30). The corresponding protein has 13 amino acids associated with the first exon, whereas the full-length protein has 8 amino acids associated with the first exon. Figure 1b shows the amino acid sequences of the proteins which were studied in this report.

Following the insertion of the cDNAs into the expression vectors, the activities releasing methylated bases present in crude extracts of *E.coli* cells hosting the pANPG plasmids were assayed as a function of time following induction with IPTG. The maximal 3-meAde-DNA glycosylase activities in these crude extracts of BL21 cells hosting the plasmids expressing the cDNAs were observed 4−5 h. post-induction (data not shown). The 3-meAde-DNA glycosylase activity in the crude lysates of *E.coli* BL21 harboring the pANPG plasmids compared to the activity in crude lysates of the same strain with only the pTO50 vector indicated that over 95% of the activities in the crude lysates hosting the pANPG plasmids was associated with the plasmid coded 3-meAde-DNA glycosylases.

Table I. Purification of 3-meAde-DNA glycosylase proteins following expression of the cDNAs in *E. coli* BL21 cells. 26 g of cells hosting the pANPG70 plasmid, 10 g hosting the pANPG40 plasmid, and 20 g hosting the pANPG60 plasmid were used in each of the purifications. The first line in each of the purification steps indicates the purification of full-length protein (FL), the second line the truncated protein (T), and the third line the splicing variant protein (SV). Cells were harvested following 5 h. induction with IPTG. MonoS FPLC chromatography of the truncated 3-meAde-DNA glycosylase protein did not increase the specific activity (purity) of the protein.

Step	Protein	Volume (mL)	Total Proteins (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Purification Factor (-fold)
Crude extract	FL	117	1875	102000	55	100	1
	T	53	244	85000	296	100	1
	SV	92	775	577000	745	100	1
PEI	FL	126	1765	95000	53	93	1
	T	56	240	72000	300	84	1
	SV	100	720	545000	758	94	1
Phosphoultrogel	FL	116	50	30000	600	29	11
	T	60.5	16	27000	1070	32	32
	SV	72	48	526000	10900	91	14
AcA54	FL	19	6.7	23000	3500	23	64
	T	11	1.9	16000	8700	19	29
	SV	20	13	230000	18000	40	24
Phenyl Sepharose	FL	10	1	7000	7000	7	127
Phenyl Superose	T	2	0.38	12000	32000	14	108
Phenyl Sepharose	SV	50	9	181000	20000	32	27
Mono S	FL	4	0.20	5800	29000	6	527
	T	-	-	-	-	-	-
	SV	2.5	1.6	54000	33000	9	44

Purification of the 3-meAde-DNA glycosylases

The steps used in the purification of the three 3-meAde-DNA glycosylase proteins from *E.coli* harboring the recombinant plasmids are summarized in Table I. The full-length human protein was purified over 500-fold to yield a single band migrating in SDS-PAGE with a molecular mass of 39 kDa. Although this molecular mass is considerably different from that of the predicted 32 kDa, the N-terminal amino acid sequence of the protein is identical to that predicted by the nucleic acid sequence, indicating that the full-length human 3-meAde-DNA glycosylase has 293 amino acids (Figure 1d).

The purification of the truncated and splicing variant 3-meAde-DNA glycosylases also yield photographically homogeneous species migrating as single bands in SDS-PAGE as indicated in Figures 2b and 2c. The major difference in the purification of the splicing variant and full-length human 3-meAde-DNA glycosylases compared to the purification of the truncated protein is the use of a MonoS FPLC column in the final step. The sequences of the N-terminal amino acids (Figure 1d) indicate that these two proteins are also identical to those predicted by their nucleic acid sequences. Therefore, the truncated 3-meAde-DNA glycosylase has 230 amino acids with a molecular mass of 26 kDa and the splicing variant 3-meAde-DNA glycosylase has 298 amino acids with a molecular mass of 32 kDa. The specific activities of all three proteins are virtually identical (Table I).

Polyclonal antibodies against the human splicing variant 3-meAde-DNA glycosylase recognize other mammalian 3-meAde-DNA glycosylases

The purification of the splicing variant 3-meAde-DNA glycosylase in quantity has allowed the production of polyclonal

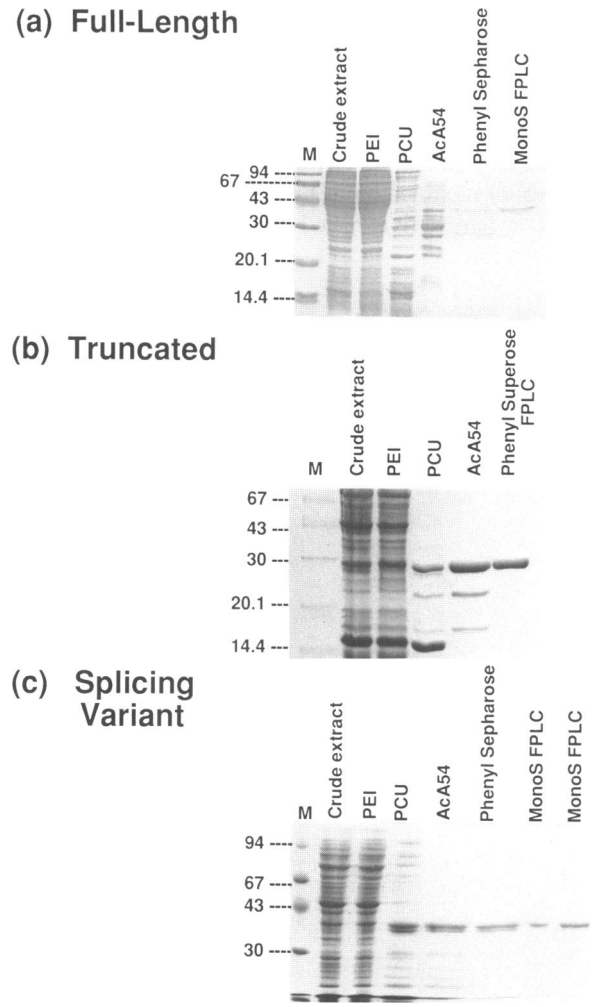

Figure 2. SDS-PAGE analysis of the fractions for the purification of the truncated, splicing variant, and full-length 3-meAde-DNA glycosylases. Molecular mass markers (M) are indicated. **(a)** Purification steps for the full-length 3-meAde-DNA glycosylase. The crude extracts and the PEI fraction lanes have 30 μg of total protein, the PCU (Phosphoultrogel) fraction has 15 μg of total protein, the AcA54 fraction 10 mg of total protein, the Phenyl Sepharose fraction 2 μg, and the MonoS FPLC fraction 0.5 μg of full-length 3-meAde-DNA glycosylase. **(b)** Purification steps for the truncated 26 kDa 3-meAde-DNA glycosylase. The quantities of protein loaded for the crude lysates, the PEI, the PCU, and the AcA54 fractions were identical to those in part (a). The FPLC Phenyl Superose fractions contained 3 μg truncated 26 kDa 3-meAde-DNA glycosylase **(c)** Purification steps for the splicing variant 3-meAde-DNA glycosylase. The quantities of protein loaded for all fractions is identical to those loaded in part (a). For the Phenyl sepharose column fractions, 2 μg of protein was loaded. The MonoS FPLC lanes contained 0.5 and 1 μg of protein.

antibodies against the protein in rabbits. Figure 3a shows a Western blot of the Crude Extract fractions from the purification of the 3-meAde-DNA glycosylases isolated in this report. This Figure demonstrates that all three 3-meAde-DNA glycosylase proteins are detected using these polyclonal antibodies.

Figure 3b shows that the detection of the 3-meAde-DNA glycosylase protein is possible at 50 ng and that the polyclonal antibodies are not specific for the N-terminal sequence of the splicing variant 3-meAde-DNA glycosylase, since they also recognize the 26 kDa protein. These polyclonal antibodies although useful in Western blot detection assays, do not inhibit the DNA glycosylase activities of the 26 and 32 kD proteins.

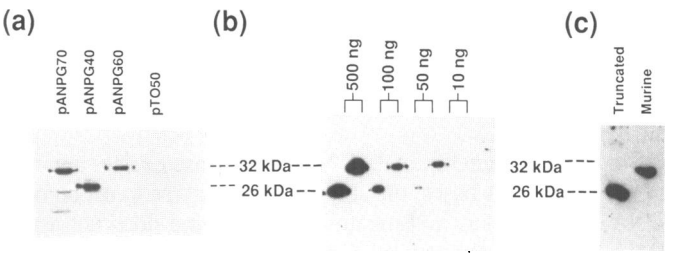

Figure 3. Western blots of 3-meAde-DNA glycosylases using anti-human splicing variant 3-meAde-DNA glycosylase antibodies. **(a)** Western blots using crude extracts of *E.coli* BL21 hosting plasmids coding for the truncated (pANPG40), splicing variant (pANPG60), and the human 3-meAde-DNA glycosylase (pANPG70). The extracts from *E. coli* BL21 hosting the vector are indicated as pTO50. Each lane contains 0.6 μg of total protein, except the lane with the crude extract of the E. coli hosting pANPG70 which has 6 μg of total protein. **(b)** Detection limit for the splicing variant and truncated 3-meAde-DNA glycosylases. The quantity of protein loaded for the 15% SDS-PAGE separation and the position of the two proteins are indicated. **(c)** Detection of murine and truncated human 3-meAde-DNA glycosylases by anti-human splicing variant 3-meAde-DNA glycosylase antibodies. 50 ng of each protein was transferred and detected as in the Figure 2b. The purified murine 3-meAde-DNA glycosylase has a molecular mass of 32 kDa and the truncated 3-meAde-DNA glycosylase protein a molecular mass of 26 kDa.

Figure 3c shows the detection of the murine 3-meAde-DNA glycosylase at 50 ng is also possible. Since the detection levels using the polyclonal anti-human 3-meAde-DNA glycosylase antibodies for the human and murine proteins are similar, these antibodies should prove useful in the study of other mammalian 3-meAde-DNA glycosylases.

Western blots were also performed with a variety of purified *E.coli* DNA glycosylases. The anti-human 3-meAde-DNA glycosylase antibodies do not cross react with the purified 3-meAde-DNA glycosylase proteins of *E.coli* (Tag and AlkA proteins) (data not shown). In addition to the Tag and AlkA proteins, no cross reactivity of the antibodies was noted with the Fpg protein, Nth protein, or Uracil-DNA glycosylase.

Purified human 3-meAde-DNA glycosylases also release 7-meGua, and 3-meGua from [^{3}H]-DMS-DNA and [^{3}H]-DMS-poly(dG-dC)

The capacities of the three forms of 3-meAde-DNA glycosylase isolated in this study to release several modified bases were examined using HPLC chromatography as a function of several parameters. Figure 4a shows that release of 3-meAde and 7-meGua bases as a function of enzyme concentration for these proteins is almost identical. For these enzymes, the 3-meAde is excised much faster than the 7-meGua. Figure 4b shows that the release of 3-meAde and 7-meGua bases as a function of time at a fixed enzyme concentration for the three proteins is also similar.

Figure 4c shows the Lineweaver–Burk plots for the excision of 3-meAde and Table II summarizes the kinetic constants for the three proteins. The kinetic constants obtained are the same for all 3 proteins. The ratio k_{cat}/K_m suggests that the reaction of the all three proteins is much more specific for the release of 3-meAde than for the release of 7-meGua. Therefore, although the 26 kDa protein is missing more than 20% of the N-terminal amino acids compared to the 32 kDa proteins, the DNA glycosylase function is not significantly altered.

In addition to the release of 3-meAde and 7-meGua, the release of a minor base from a [^{3}H]-DMS-poly(dG-dC) substrate, 3-meGua, was detected by HPLC (data not shown). This base represented 1% of the total bases modified and is removed by all 3 proteins.

Since the human 3-meAde-DNA glycosylase excises a variety of modified DNA bases, the ability of this enzyme to act on other DNA damages was investigated. This enzyme does not act on DNA containing abasic sites, Fapy bases, or uracil residues (data not shown).

3-meAde-DNA glycosylase releases modified bases from ethylated DNA

E.coli strains deficient in the repair of 3-meAde are also sensitive to DNA ethylating agents such as DES (23). The sensitivity of these deficient strains is reduced, however, if they harbor a plasmid expressing the truncated 3-meAde-DNA glycosylase cDNA (23).

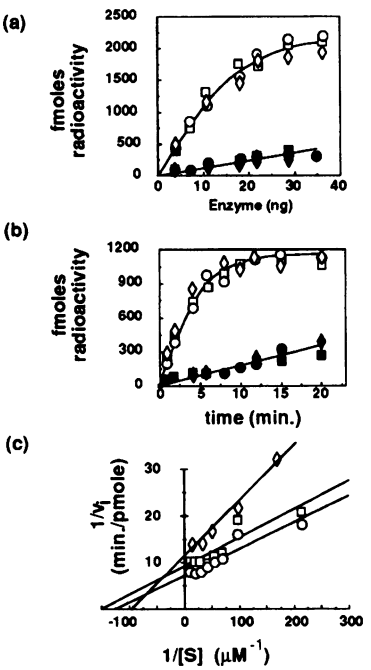

Figure 4. Excision of 3-meAde and 7-meGua from [^{3}H]-DMS-DNA by the 3-meAde-DNA glycosylases. Symbols: 3-meAde excised by the truncated 3-meAde-DNA glycosylase (□), the splicing variant 3-meAde-DNA glycosylase (○), and the full-length 3-meAde-DNA glycosylase (◇), 7-meGua excised by the truncated 3-meAde-DNA glycosylase (■), the splicing variant 3-meAde-DNA glycosylase (●), and the full-length 3-meAde-DNA glycosylase (◆). **(a)** Excision of 3-meAde and 7-meGua from [^{3}H]-DMS-DNA as a function of protein concentration. The proteins were incubated for 5 min. in 100 μl of assay buffer with 15.5 pmoles of [^{3}H]-DMS-DNA (2.75 pmoles 3-meAde bases). **(b)** Excision of 3-meAde and 7-meGua from [^{3}H]-DMS-DNA as a function of time at constant 3-meAde-DNA glycosylase concentration. 15.5 pmoles of [^{3}H]-DNA-DMS (2.75 pmoles 3-meAde bases) was incubated in the standard assay buffer in 100 μl total volume with 7.2 ng of 3-meAde-DNA glycosylase. **(c)** Lineweaver–Burk plots for the excision of 3-meAde by the three proteins. The plots were generated using identical protein concentrations from 100 μl total volume using 0.1 nM, 0.14 nM, and 0.11 nM enzyme concentrations for the full-length, truncated, and splicing variant proteins, respectively. The assays for the determination of the kinetic constants of 7-meGua excision were performed using 2.1 nM enzyme concentrations. Since the 7-meGua is the major modified base in the [^{3}H]-[CH_3]-poly(dG-dC), the products were not subjected to HPLC to separate the modified bases.

To demonstrate that methylated and ethylated bases are released by this protein, a 5′- ^{32}P end-labelled fragment of 205 bp was modified using either DMS or DES. The modified fragments were incubated in the presence of the splicing variant 3-meAde-DNA glycosylase to release methylated and ethylated bases which leaves an abasic site in the DNA. The abasic sites generated by the removal of modified bases by the splicing variant 3-meAde-DNA glycosylase were revealed using the Fpg protein of *E.coli*

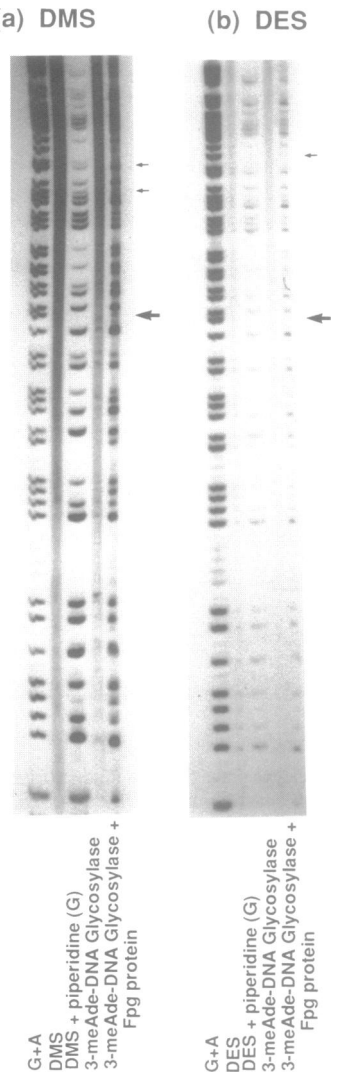

Figure 5. Removal of methylated and ethylated bases from a DNA fragment by the human splicing variant 3-meAde-DNA glycosylase. The Ade which is not repaired is indicated by an arrow. Other bases which show reduced repair are indicated by smaller arrows. **(a)** Repair of methylated bases by the human 3-meAde-DNA glycosylase. **G+A**—Formic acid depurination of the labeled DNA fragment followed by piperidine treatment revealing Ade and Gua residues. **DMS**—Methylation by DMS without further treatment, **DMS+piperidine (G)**—Methylation by DMS followed by 1 M piperidine treatment at 90°C revealing Gua. **3-meAde-DNA glyscosylase**—Methylation by DMS followed by treatment with splicing variant human 3-meAde-DNA glycosylase (20 ng). **3-meAde-DNA glycosylase+Fpg protein**—Methylation by DMS followed by treatment with splicing variant human 3-meAde-DNA glycosylase and then Fpg protein (10 ng) of *E.coli*. Treatment with Fpg protein alone did not reveal any of the modified sites (data not shown). **(b)** Repair of ethylated bases by the human 3-meAde-DNA glycosylase. The lanes are identical to those found in part (a), except that the DNA was alkylated using DES.

which has an associated lyase activity incising DNA at abasic sites.

Figure 5a shows that the splicing variant 3-meAde-DNA glycosylase removes methylated Ade and Gua residues from the DNA fragment. Figure 5b shows that ethylated Ade and Gua in DNA are also removed by the human splicing variant 3-meAde-DNA glycosylase. Similar results were obtained using the other proteins isolated in this study (data not shown). Therefore, the human 3-meAde-DNA glycosylase is most probably responsible for the repair of ethylated Ade and Gua bases associated with cytotoxicity.

At the enzyme concentrations used in these experiments, virtually all the modified bases are excised from the DNA. Thus, the formic acid depurination (G+A) lanes are identical to the 3-meAde-DNA glycosylase + Fpg protein treatments, except for one base. This unrepaired Ade is indicated by an arrow in Figure 5a and 5b is in the triplet TAG (see MATERIALS AND METHODS). It is not possible to distinguish, however, if the Ade is modified, since piperidine treatment of methylated and ethylated purines reveals only modified Gua bases. A larger sequence sampling and further studies are required to establish if there is a difference in repair or modification of this base. In addition to this base several other bases which are not repaired efficiently are indicated in Figures 5a and 5b.

Table II. Kinetic constants for the full-length and truncated Anpg proteins for the excision of 3-meAde from DNA-DMS and 7-meGua from [^{3}H]-DMS-poly(dG-dC). Details are found in the legend to Figure 4c.

Protein	Base Excised	K_M (nM)	kcat (1/min)	kcat/ K_M (1/(min-nM))
Full-length	3-meAde	11	9.9	0.90
Truncated	3-meAde	8	11	1.4
Splicing variant	3-meAde	7	10	1.4
Full-length	7-meGua	32	0.40	0.013
Truncated	7-meGua	25	0.35	0.014
Splicing variant	7-meGua	30	0.40	0.013

The 26 kD truncated 3-meAde-DNA glycosylase is more stable than the 32 kD proteins

The purification of the 3-meAde-DNA glycosylases permitted the comparison of some physical properties of these enzymes. Figure 6a shows that there is no significant difference in the ionic strength dependence of the 3-meAde excision for the full-length, splicing variant, and truncated proteins. The maximal 3-meAde-DNA glycosylase activity with respect to KCl concentration is observed between 100 mM for all three proteins. The 26 kDa protein is about 30% more active than the 32 kDa proteins, however, at KCl concentrations below 10 mM. The activity of all three proteins declines quite rapidly at KCl concentrations above 200 mM. Figure 6b shows that the optimal 3-meAde-DNA glycosylase activity for the proteins with respect to pH is at approximately pH 7.6 for all proteins. In addition, the DNA glycosylase activities of all three proteins are EDTA resistant between 0.1 and 10 mM EDTA.

Although the activities associated with ionic strength and protein charge do not reveal significant differences between the proteins, the thermal stabilities of these proteins are different. Figure 6c shows that the truncated protein is more stable than the full-length and splicing variant 3-meAde-DNA glycosylases by at least a factor of 2.5. Therefore, the sequences at the N-terminal end of the proteins render the 32 kDa proteins as produced in *E.coli* less stable than the truncated protein. Alternatively, although less likely, the 3 additional amino acids found in the truncated protein may convey increased stability on the truncated protein compared to the other two proteins.

DISCUSSION

The isolation and characterisation of the full-length human 3-meAde-DNA glycosylase protein provides a basis for the study of the biochemical properties and the role of this protein *in vivo*.

The full-length and splicing variant 3-meAde-DNA glycosylases have a direct repeat in the amino acid sequence (full-length: 25−36 and 39−50, splicing variant: 30−41 and 44−55)

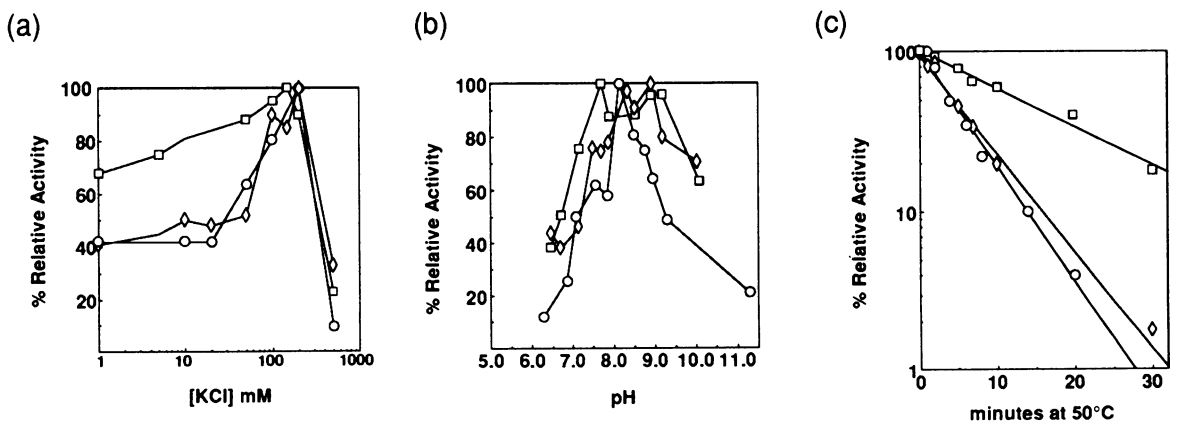

Figure 6. Properties of the purified 3-meAde-DNA glycosylases. All assays were performed using 2 ng of protein. **(a)** Dependence of 3-meAde-DNA glycosylase activity on KCl concentration. **(b)** Dependence of 3-meAde-DNA glycosylase activity on pH. **(c)** Thermal stability of the 3-meAde-DNA glycosylases. The truncated (□), splicing variant (○), or full-length human (◇). The 3-meAde-DNA glycosylases were pre-incubated at 50°C as a function of time, and the standard assays were performed using 2 ng of enzyme. The relative activity is defined as the ratio of the activity without incubation at 50°C to the activity observed following incubation at a given time.

(Figure 1c). This direct repeat sequence is interesting in that it is not observed in the rat 3-meAde-DNA glycosylase (22) which is evolutionarily close to human. This sequence is apparently not important in the DNA glycosylase function, since the 26 kD protein is fully functional with respect to DNA glycosylase activity.

The specific activity of the physically homogeneous 3-meAde-DNA glycosylase, is at least 500−1000 fold more active than previously isolated mammalian proteins (33−36). The number of protein molecules can be estimated by the activity observed in cells (33−35) and the specific activity of the 3-meAde-DNA glycosylase. This type of calculation suggests that there are between 1000−2000 3-meAde-DNA glycosylase molecules per cell. Therefore, the 3-meAde-DNA glycosylase is in relatively low abundance in human cells.

The 3-meAde-DNA glycosylases isolated in this report excise modified bases associated with both methylation and ethylation of purines in DNA. Such repair of ethylated bases was suggested for the partially purified placental 3-meAde-DNA glycosylase (34). The properties of the human 3-meAde-DNA glycosylase suggest that this protein has many analogous substrates to the bacterial AlkA protein and the calf thymus 3-meAde-DNA glycosylase (37−40). Although the excision of 7-meGua was not observed in an initial report (29), this study shows that the purified 3-meAde-DNA glycosylase releases both 7-meGua and 3-meGua in addition to 3-meAde as reported for crude extracts of *E.coli* hosting cDNAs in expression vectors (23,24). In addition to these modified bases, crude extracts hosting plasmids expressing the truncated cDNA coding sequence and partially purified human placental 3-meAde-DNA glycosylase remove ethenoAde lesions from DNA (41). Such a wide substrate specificity suggests that the protein may more generally be referred to as an alkylpurine-DNA glycosylase. Further studies on the substrate specificity are required, however, to help elucidate the *in vivo* roles of this enzyme in BER and other cellular processes. The purification of this protein to homogeneity contributes to this goal.

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ABBREVIATIONS

AlkA protein: 3-methyladenine-DNA glycosylase II; anpg: human alkyl-N-purine-DNA glycosylase; apdg: murine alkyl-N-purine-DNA glycosylase; BER: Base Excision Repair; DES: diethylsulfate; DMS: dimethylsulfate; [^{3}H]-DMS-DNA: calf thymus DNA modified by treatment with [^{3}H]-labelled dimethylsulfate; [^{3}H]-DMS-poly(dG-dC): poly(dG-dC) DNA modified by treatment with [^{3}H]-labelled dimethylsulfate; ECL: Enhanced Chemiluminescence; Fapy: 2,6-diamino-4-hydroxy−5-N-methylformamidopyrimidine; Fpg protein: formamidopyrimidine-DNA glycosylase, 8-oxoguanine-DNA glycosylase; IPTG: isopropyl-β-D-thiogalactopyranoside; Nth protein: endonuclease III; PEI: polyethyleneimine; Tag protein: 3-methyladenine-DNA glycosylase I; K_m and k_{cat} values are apparent values.

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