Partial Purification and Characterization of 3-Methyladenine–DNA Glycosylase from Human Placenta†

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ABSTRACT: A DNA glycosylase was isolated and purified over 1000-fold from human placentas by means of diethylaminoethylcellulose and double- and single-stranded DNA–Sepharose affinity chromatography. The procedure was rapid and yielded >15% of the initial enzyme activity. High-pressure liquid chromatographs of reaction products showed that 3-methyladenine was the predominant substrate in methylated native DNA. 7-Methylguanine and 3-methylguanine were also released by the partially purified enzyme, albeit at low rates; release was more evident when the substrate was methylated double-stranded poly(dG-dC). The enzyme preparation was essentially free of nuclease activity, retaining <0.001% of the initial cellular concentration of Mg^{2+}-requiring apurinic endonuclease activity. The glycosylase had a broad pH optimum between 7.2 and 7.7; it did not require metal ions but was stimulated by Na^+ or K^+ at 50 mM or by Mg^{2+} at 1 mM. Higher concentrations of these ions were inhibitory. Activity was unaffected by β-mercaptoethanol or dithiothreitol, but 1 mM *N*-ethylmaleimide or *p*-(hydroxymercuri)benzoate as well as 1 mM spermine or 10 mM sperimidine totally inhibited the enzyme. The apparent molecular weight of the glycosylase, determined by gel filtration, was 25 000, and the apparent K_m for 3-methyladenine in native methylated DNA was 3×10^{-8} M. The enzyme required double-stranded methylated DNA as a substrate and showed very low activity with denatured methylated DNA. It appeared that single-stranded regions in DNA inhibited 3-methyladenine–DNA glycosylase activity, but up to 1 mM concentrations of free methylated bases did not.

Methylation of DNA by agents such as methyl methanesulfonate, dimethyl sulfate, or methylnitrosourea results in the formation of two major products, 7-methylguanine and 3-methyladenine, and several minor methylated products, 3-methylguanine, O^6-methylguanine, 1-methyladenine, and 7-methyladenine (Strauss et al., 1975; Singer, 1979). In vivo studies show that 3-methyladenine, 7-methylguanine, and O^6-methylguanine are actively removed from alkylated DNA (Singer, 1979). A DNA glycosylase from *Escherichia coli* was first described by Kirtikar & Goldthwait (1974) and extensively purified by Riazuddin & Lindahl (1978); it cleaves the base–sugar bond specifically between 3-methyladenine and deoxyribose in methylated DNA to leave an apurinic site. Similar enzymes have been found in *Micrococcus luteus* (Laval, 1977), rodent cells (Cathcart & Goldthwait, 1981; Margison & Pegg, 1981; Male et al., 1981), and human lymphoblasts (Brent, 1979; Ishiwata & Oikawa, 1979). It is presumed that these enzymes initiate the repair of 3-methyladenine in vivo. The role of 3-methyladenine–DNA glycosylase as a repair enzyme is supported by the observation that *tag* mutants deficient in 3-methyladenine–DNA glycosylase (from *E. coli*) have an increased sensitivity to alkylating agents and a reduced ability to excise 3-methyladenine from methylated DNA (Karran et al., 1980).

7-Methylguanine from damaged DNA was thought to be removed by spontaneous chemical hydrolysis rather than by enzymatic cleavage. However, recent studies with extracts from *M. luteus* (Laval et al., 1981), *E. coli* (Thomas et al., 1982), rodent liver (Cathcart & Goldthwait, 1981; Margison & Pegg, 1981), and cultured human lymphoblasts (Singer & Brent, 1981) indicate that 7-methylguanine also is excised from methylated DNA by a DNA glycosylase activity. In the absence of enzyme preparations purified to homogeneity, it is not clear if 3-methyladenine and 7-methylguanine lesions are released by the same or different enzymes.

We describe the partial purification and characterization of a human placental DNA glycosylase activity that excises predominantly 3-methyladenine from alkylated double-stranded DNA and, to a lesser extent, 7-methylguanine and 3-methylguanine.

Materials and Methods

Preparation of Radiolabeled Substrates. Procedures for the growth of PM2 bacteriophage, labeling with [^{3}H]thymidine, and subsequent isolation of the phage DNA have been described (Teebor & Brent, 1981). Labeled PM2 DNA was modified as follows. (1) Methylated DNA was prepared by reaction with 6×10^{-3} M methyl methanesulfonate at 37 °C for 20 min and was reisolated by Sephadex G-50 filtration (Brent, 1977). (2) Ultraviolet (UV)[1] irradiated DNA was prepared by exposure to 254-nm light (1500 J/m^2) from a germicidal lamp at room temperature. (3) Partially depurinated DNA was prepared by heating to 70 °C at pH 5.0 for 20 min.

^{3}H-Methylated substrates were prepared for determining specific base release. (1) Calf thymus DNA (4 mg, Sigma type I) was incubated with 5 mCi of [^{3}H]dimethyl sulfate (New England Nuclear, sp act. 4.5–4.7 Ci/mmol) in 2.0 mL

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[1] Abbreviations: UV, ultraviolet; AP, apurinic or apyrimidinic; HPLC, high-pressure liquid chromatography; DEAE, diethylaminoethyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; m^{3}A and m^{7}A, 3- and 7-methyladenine, respectively; m^{3}G and m^{7}G, 3- and 7-methylguanine, respectively; m^{6}G, O^6-methylguanine; Tris, tris(hydroxymethyl)aminomethane.

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of 0.2 M sodium cacodylate buffer (pH 7.9) with 1 mM EDTA, for 100 min at room temperature. The DNA was precipitated with 5.0 mL of absolute ethanol at −20 °C and washed 5 times with 10-mL aliquots of 80% ethanol; it was then redissolved in 2.0 mL of 0.01 M NaCl and dialyzed extensively against 0.01 M NaCl. The specific activity of the ^{3}H-methylated DNA was (2–12) × 10^4 dpm/μg. The labeled calf thymus DNA was denatured by alkaline treatment (pH 11.5) for 2 min at room temperature.

(2) Poly(dG-dC)·poly(dG-dC), 1.5 mg (P-L Biochemicals, Inc.), was incubated with 5.0 mCi of [^{3}H]dimethyl sulfate (New England Nuclear, sp act. 1.6 Ci/mmol) in 1.0 mL of 0.2 M sodium cacodylate buffer (pH 7.9) with 1 mM EDTA for 90 min at 37 °C. The polynucleotide was precipitated with 2.0 mL of absolute ethanol at −20 °C, washed 5 times with 5-mL aliquots of 80% ethanol, then redissolved in 1.0 mL of 0.01 M NaCl, and dialyzed extensively against 0.01 M NaCl. The ^{3}H-methylated poly(dG-dC)·poly(dG-dC) had a specific activity of 5.0 × 10^4 dpm/μg.

Preparation of Unlabeled Alkylated Calf Thymus DNA. Ten milliliters of calf thymus DNA (2 mg/mL) in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl, and 0.02% sodium azide (buffer A) was incubated with 6 × 10^{-3} M methyl methanesulfonate at 37 °C for 30 min. The reaction was terminated by cooling to 0 °C, and the DNA was precipitated by addition of 25 mL of absolute ethanol at −20 °C. The methylated DNA was washed twice with 20 mL of 80% ethanol and diluted in buffer A to a final concentration of 1 mg/mL. Methylated and unmethylated native calf thymus DNAs in buffer A were denatured by alkaline treatment (pH 11.5) for 2 min at room temperature.

Assays for Glycosylase Activity. Enzyme activity was measured by determining either the formation of apurinic sites in methylated PM2 DNA or the release of ^{3}H-methylated bases from methylated calf thymus DNA and poly(dG-dC)·poly(dG-dC).

(1) The formation of apurinic sites in PM2 DNA was measured by a nitrocellulose filter binding assay. Approximately 0.1 μg of methylated [^{3}H]thymidine labeled PM2 DNA was incubated at 37 °C for 10 min with active enzyme fraction that contained 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 1 mM β-mercaptoethanol (buffer B) in a final volume of 60 μL. The reaction was terminated by the addition of 25 μL of proteinase K (1 mg/mL) in 10 mM Tris-HCl buffer (pH 7.4) with 10 mM EDTA. After a 5-min incubation at 37 °C, 2.0 mL of 0.1 M sodium phosphate buffer (pH 11.8) with 0.6 M NaCl was added, and the mixture then stood at room temperature for 2 h. This treatment denatured the DNA and converted the alkali-labile apurinic sites, generated by glycosylase activity, to strand breaks. The solution was neutralized by 0.25 mL of 1.0 M disodium phosphate and 2.0 mL of 0.18 M sodium citrate buffer (pH 7.0) with 1.2 M NaCl and filtered through nitrocellulose membrane filters (Schleicher & Schuell, BA 85). The filters were washed with 0.09 M sodium citrate buffer (pH 7.0) that contained 0.9 M NaCl and then dried and assayed for radioactivity by liquid scintillation spectrometry. During renaturation, the nicked DNA remained single stranded and bound to nitrocellulose filters, while intact DNA molecules renatured and were not retained. This assay was used to measure glycosylase activity during purification and preliminary characterization. One unit of glycosylase activity was expressed as the activity that produced 1 pmol of alkali-labile sites in methylated DNA/min.

(2) The bases cleaved by DNA glycosylase activity were determined by measuring the release of ethanol-soluble products from ^{3}H-methylated polydeoxyribonucleotides. The standard reaction mixture of 2–10 μg of ^{3}H-methylated polydeoxyribonucleotide, buffer B, and the enzyme fraction, in a final volume of 60 μL, was incubated at 37 °C for various times as indicated under Results. The reaction was terminated by cooling to 0 °C and adding 5 μL of 2.0 M sodium acetate, 100 μL of denatured calf thymus DNA (2.0 mg/mL), and 350 μL of absolute ethanol at −20 °C. After the mixture stood for 1 h at −20 °C, the precipitate was removed by centrifugation, and the supernatant fraction was evaporated to dryness under a stream of nitrogen. The residue was redissolved in 100 μL of 10% methanol in 2.5 mM potassium phosphate buffer, pH 6.9, and a 90-μL aliquot was applied to a 25-cm reverse-phase column (Whatman Partisil 5-ODS). The column was eluted with 10% methanol in 2.5 mM potassium phosphate, pH 6.9, at a flow rate of 1.0 mL/min. The optical density was monitored continuously at 254 nm, and the radioactive content of fractions, collected at 0.5-min intervals, was determined by liquid scintillation spectrometry. One unit of glycosylase activity was expressed as the activity that released 1 pmol of 3-methyladenine/min. Authentic methylated bases were obtained from the following: 3-methyladenine, 7-methylguanine, and 3-methylguanine were purchased from Tridon-Fluka, New York, NY; 7-methyladenine was a gift from Dr. Ray Cox, VA Hospital, Memphis, TN; and O^6-methylguanine was a gift from Dr. David Ludlum, Albany Medical College, Albany, NY.

Assay for Endonuclease Activity. Endonuclease activity was measured in the presence of 0.5 mM $MgCl_2$ or 2 mM EDTA by the standard filter binding assay with either untreated, partially depurinated, UV-irradiated, or methylated PM2 DNA as a substrate. The 2-h room temperature incubation at pH 11.8 was omitted so that only endonuclease-induced strand breaks were detected and glycosylase-induced apurinic sites were not.

Enzyme Purification. Normal, full-term, human placentas were obtained immediately after delivery and were suspended in 0.9% saline at 4 °C. The placental tissue was processed within 1 h, and all subsequent steps were performed at 4 °C. The placenta was washed in saline to remove adhering blood clots; the membranes and connective tissues were removed, and the remaining tissue was blotted, weighed, and cut into small pieces. About 300 g of the tissue was suspended in 400 mL of 10 mM Tris-HCl buffer (pH 7.5), 2 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol, 0.02% sodium azide, 0.2 M NaCl, and 0.2 mM phenylmethanesulfonyl fluoride (buffer C). A second protease inhibitor, aprotinin (Sigma, 30 trypsin inhibitor units/L), was added to the extraction buffer. The tissue was homogenized for 3 min by intermittent blending in a Waring blender and was then centrifuged at 9000*g* for 15 min. The pellets were resuspended in 250 mL of buffer C, rehomogenized, and centrifuged. The supernatants from both centrifugations were pooled and centrifuged at 180000*g* in a Beckman Ti 50.2 rotor for 15 min. The supernatant constituted the crude extract (fraction I).

For ion-exchange DEAE-cellulose chromatography, the crude extract was loaded on a DEAE-cellulose column (Whatman DE-23, 25 × 9.2 cm diameter) previously equilibrated with buffer C and eluted with buffer C at a flow rate of about 100 mL/h. A protein peak, separated from the nucleic acids, was eluted. This material constituted fraction II.

For double-stranded DNA–Sepharose chromatography, 150–200 mL of fraction II was loaded on a column (11.5 × 2.7 cm diameter) of native calf thymus DNA covalently

Table I: Purification of DNA Glycosylase from Human Placental Tissue

purification step	fraction no.	volume (mL)	[protein] (mg/mL)	enzyme[a] activity (units × 10^{-3}/mL)	sp. act (units × 10^{-3}/mg)	purification factor	% activity recovered
crude extract	I	740	30.40	77.3	2.54	1.00	100.0
DEAE-23 column chromatography	II	740	17.80	67.6	3.80	1.50	87.5
double-stranded DNA-Sepharose column chromatography	III	176	0.133	116	865	343	35.7
single-stranded DNA-Sepharose column chromatography	IV	84	0.039	109	2795	1102	16.0

[a] Enzyme activity was determined by the nitrocellulose filter binding assay.

coupled to Sepharose 4B (Arndt-Jovin et al., 1975) preequilibrated with buffer C. The column was loaded at a flow rate of 50 mL/h, then washed with an additional 400 mL of buffer C, and eluted at 25 mL/h with a linear NaCl gradient (0.2–1.0 M) in 200 mL of buffer C. The bulk of glycosylase activity bound to the column and eluted as a peak between 0.25 and 0.45 M NaCl. This material was designated fraction III.

Fraction III was diluted with 10 mM Tris-HCl (pH 7.5), 2.0 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol, and 0.2 mM PMSF to lower the NaCl concentration to 0.15 M and loaded on a 5.5 × 2.1 cm diameter column that contained denatured calf thymus DNA covalently coupled to Sepharose 4B (Arndt-Jovin et al., 1975) preequilibrated with buffer D (10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol, 0.15 mM NaCl, and 0.2 mM PMSF). The column was washed with 250 mL of buffer D and eluted with a linear NaCl gradient (0.15–0.75 M) in 200 mL of buffer D at a flow rate of 25 mL/h. The peak glycosylase activity fractions eluting between 0.2 and 0.3 M NaCl were pooled, dialyzed against buffer D, and stored in liquid N_2 at −196 °C. This material, fraction IV, was used for subsequent characterization. For the HPLC assay, fraction IV was concentrated 5–10-fold by Amicon ultrafiltration with a YM 10 filter.

Molecular Weight Determination. The molecular weight of the enzyme was estimated by Sephadex G-100, Sephacryl S-200, and Spherogel-TSK (Altex SW-3000) gel filtration chromatography. Concentrated fraction IV (0.5 mL) was applied to a 55 × 1.2 cm diameter Sephadex G-100 column and eluted with buffer C at a flow rate of 3.0 mL/h. Fractions of 0.5 mL each were collected and assayed for glycosylase activity.

In another procedure, a 90 × 2.6 cm diameter Sephacryl S-200 column was loaded with 5.0 mL of concentrated fraction IV and then eluted with buffer C at 40 mL/h before 5.0-mL fractions were assayed for activity.

In the third method, HPLC fractionation of 0.5 mL of fraction IV was done on a 60 × 0.75 cm diameter Spherogel-TSK gel column with 50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 0.2 M KCl, and 5% glycerol buffer. Fractions of 0.5 mL each were eluted at a flow rate of 30 mL/h and collected before glycosylase activity was determined. All columns were calibrated with aldolase, ovalbumin, chymotrypsin, and ribonuclease.

Other Methods. Protein content was determined by the Lowry procedure (Lowry et al., 1951) after precipitation in 1.0 mL of 10% perchloric acid and 1% phosphotungstic acid. DNA was estimated from the absorbance at 260 nm or by the method of Burton (1956).

Results

Enzyme Purification. The results of a typical enzyme purification, normalized for 300 g of placental tissue, are summarized in Table I. The DEAE-cellulose column chromatography separated the major extracted protein from nucleic acids that potentially interfere with the enzyme assays. This step resulted in a high yield. The double-stranded DNA–Sepharose column chromatography (Figure 1) was the most effective purification step, resulting in several hundred fold purification with a 30–40% yield. The majority of protein, apurinic endonuclease, and nonspecific endonuclease washed through the column while the glycosylase activity bound and was eluted by a salt gradient. The residual apurinic endonuclease was essentially eliminated from the glycosylase activity by single-stranded DNA–Sepharose chromatography (Figure 2). The overall purification was over 1000-fold with 10–15% recovery of activity.

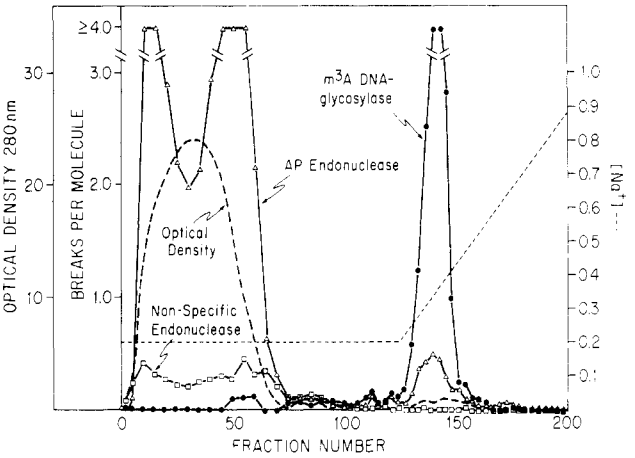

FIGURE 1: Double-stranded DNA–Sepharose chromatography of fraction II. DNA glycosylase activity (●) was determined with methyl methanesulfonate treated PM2 DNA by the nitrocellulose filter binding assay. Apurinic endonuclease activity (△) specific for partially depurinated PM2 DNA and nonspecific endonuclease activity (□) were assayed with 0.5 mM Mg^{2+}. The number of strand breaks per molecule in DNA incubated without enzyme was subtracted for each point. NaCl concentrations were determined by the refractive index.

Attempts to further purify the enzyme were unsuccessful. Gel filtration of fraction IV by Sephadex G-100, Sephacryl S-200, and Spherogel-TSK chromatography resulted in a sharp loss of enzyme activity. About 10–20% of the initial glycosylase activity loaded on these columns was recovered. Attempts to stabilize the enzyme with bovine serum albumin, glycerol, reducing agents, or various NaCl concentrations during subsequent purification were without effect. Other conventional methods of purification, such as phosphocellulose, hydroxylapatite, and Affi-gel blue column chromatography, resulted in total loss of activity.

Base Specificity. The identity of the bases cleaved from methylated DNA was determined by HPLC analysis of ethanol-soluble reaction products and compared to the retention times of the methylated base markers (Figure 3). The predominant base released from double-stranded methylated

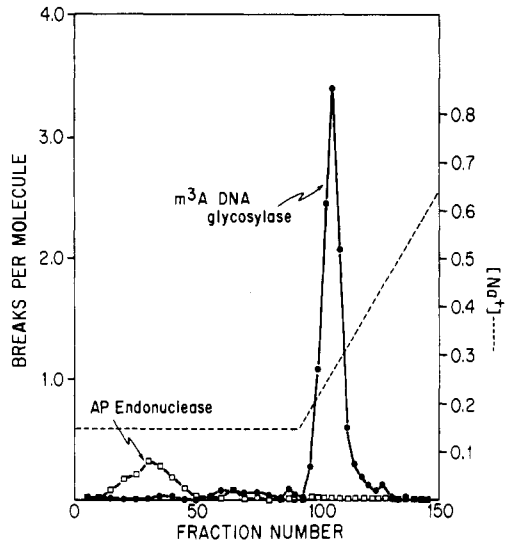

FIGURE 2: Single-stranded DNA–Sepharose chromatography of fraction III. DNA glycosylase activity (●) was measured by the filter binding assay described under Materials and Methods. Apurinic endonuclease activity (□) was assayed with partially depurinated DNA in the presence of 0.5 mM Mg^{2+}. The number of strand breaks per molecule in DNA incubated without enzyme was subtracted for each point. NaCl concentrations were determined by the refractive index.

Table II: DNA Glycosylase Release of Methylated Bases[a]

polydeoxyribonucleotide	incubation time (h)	pmol released m^3A	m^7G	m^3G
methylated calf thymus DNA	0.5	0.89[b]	0.06	ND[c]
	1.0	1.73	0.11	ND
	4.0	4.05	0.47	0.05
methylated poly(dG-dC)·poly(dG-dC)	4.0		2.76	0.33
denatured methylated calf thymus DNA	1.0	0.125	ND	ND

[a] Native or denatured ^3H-methylated polydeoxyribonucleotides were incubated with 8.0 μg of fraction IV at 37 °C for the indicated times. The ethanol-soluble reaction products were analyzed by HPLC as described under Materials and Methods. [b] Each value is corrected for depurination in controls incubated without enzyme. [c] ND = not detected.

DNA by fraction IV was 3-methyladenine. The activity was proportional to protein level up to 10 μg/reaction or to a reaction time up to 2 h. Under these conditions, the release of 7-methylguanine was also detected, albeit at relatively low levels (Table II). However, if the reaction was allowed to proceed beyond completion for release of 3-methyladenine, the enzymatic cleavage of 7-methylguanine as well as 3-methylguanine could be more readily detected (Table II). The initial rate of 7-methylguanine release was about 6% of that for 3-methyladenine, whereas the initial rate of 3-methylguanine release was about 12% of that for 7-methylguanine. The enzymatic cleavage of the methylated guanine derivatives was more clearly shown when ^3H-methylated poly(dG-dC)·poly(dG-dC) was used as a substrate. As shown in Table II, the rate of release of 3-methylguanine was again about 12% of the rate of release of 7-methylguanine. The release of both methylated guanine derivatives was proportional to protein levels up to 12 μg/reaction.

Substrate Specificity. Methylated double-strand DNA was the preferred substrate for the enzyme. The rate of 3-methyladenine release from denatured methylated DNA was <10% of that from methylated native DNA (Table II), suggesting that the double-stranded structure was important for

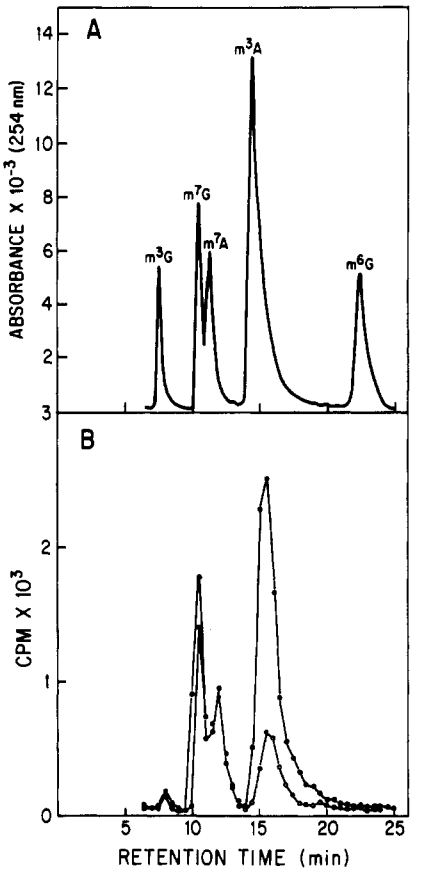

FIGURE 3: HPLC separation of methylated bases. (A) Absorbance profile of authentic methylated bases separated by HPLC as described under Materials and Methods. (B) Bases released from methylated DNA by fraction IV. Radioactivity profile of ethanol-soluble supernatants of 10 μg of ^3H-methylated calf thymus DNA incubated with buffer (○) or 8.0 μg of enzyme fraction IV (●) at 37 °C for 1.0 h, as determined by HPLC analysis.

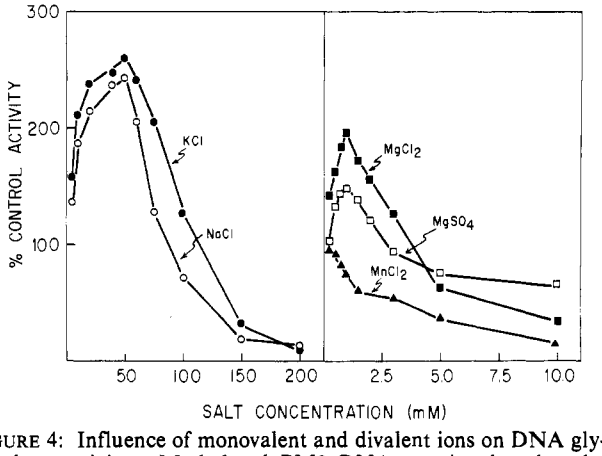

FIGURE 4: Influence of monovalent and divalent ions on DNA glycosylase activity. Methylated PM2 DNA was incubated under standard conditions with 0.05 μg of fraction IV and glycosylase activity determined by the filter binding assay. Each value represents the average number of strand breaks per molecule of PM2 DNA corrected for the nonenzymatic background.

enzyme activity. Under standard assay conditions with 2 mM EDTA or with Mg^{2+}, the enzyme preparation did not contain detectable endonuclease activity for untreated, UV-irradiated, or methylated DNA. Trace amounts of apurinic endonuclease activity were found in fraction III; however, <0.001% of the total placental apurinic endonuclease was detected in fraction IV.

General Properties. As shown in Figure 4, the glycosylase activity was stimulated by several mono- and divalent ions.

Table III: Inhibitory Effects of Deoxyribonucleic Acids on 3-Methyladenine-DNA Glycosylase Activity[a]

addition	enzyme activity (units $\times 10^{-3}$)	% control
none	3.57	100.0
native DNA	3.05	85.4
denatured DNA	1.51	42.2
native methylated DNA	1.18	33.0
denatured methylated DNA	0.72	20.1

[a] ^{3}H-Methylated calf thymus DNA (10 μg) was incubated with an equal concentration of the indicated nonradioactive calf thymus DNA and fraction IV for 2.0 h at 37 °C. The ethanol-soluble reaction products were analyzed by HPLC as described under Materials and Methods.

Na^+ or K^+ at 50 mM more than doubled the activity, whereas 0.8 mM Mg^{2+} stimulated maximally. The addition of 0.2–1 mM Mn^{2+} did not result in stimulation. The salt effects were inhibitory at higher concentrations. The glycosylase activity was not affected by up to 10 mM EDTA or EGTA, so that metal ions are apparently not required for activity. For maximum glycosylase activity, 50 mM concentrations of NaCl or KCl were maintained in the standard reaction mixtures.

The enzyme activity was stable at 4 °C for several days and for >6 months at −196 °C. The glycosylase activity had a broad pH optimum between pH 7.2 and 7.7 with 50 mM Tris-HCl or 55 mM Hepes–KOH. About 50% of the activity remained at pH 6.0 and 8.0, whereas <25% was observed at pH 8.5. The apparent molecular weight of the DNA glycosylase, estimated by Sephadex G-100, Sephacryl S-200, and Spherogel-TSK gel filtration, was 25 000. Less than 10–20% of the initial activity loaded on these columns was detected in the collected fractions. The apparent K_m of the enzyme for 3-methyladenine was 3×10^{-8} M; this value, determined by HPLC analysis, was obtained by varying the concentration of methylated calf thymus DNA with a known 3-methyladenine content.

Inhibitors. Addition of various unlabeled calf thymus DNAs at a concentration equal to that of the ^{3}H-methylated substrate inhibited 3-methyladenine–DNA glycosylase activity (Table III). The enzyme activity was inhibited 15% and 67% in the presence of nonmethylated and methylated native DNAs, respectively. Denaturation of the calf thymus DNAs before their addition to the reaction mixture further increased the inhibition.

Glycosylase activity was totally inhibited by 1 mM *N*-ethylmaleimide or by *p*-(hydroxymercuri)benzoate, whereas reducing agents such as 1 mM β-mercaptoethanol or dithiothreitol were without effect. Thus, essential sulfhydryl groups were likely present. Addition of 1 mM spermine or 10 mM spermidine also totally inhibited glycosylase activity. By contrast, free 3-methyladenine at 1 μM–1 mM did not inhibit the 3-methyladenine–DNA glycosylase activity. Other methylated purines such as 7-methylguanine, 3-methylguanine, O^6-methylguanine, 1-methyladenine, or 7-methyladenine at up to 1 mM had no effect on 3-methyladenine release.

Discussion

A 3-methyladenine–DNA glycosylase from human placentas has been purified over 1000-fold with >15% yield. Attempts to purify the enzyme further were unsuccessful. Cathcart & Goldthwait (1981) reported similar difficulty with rat liver 3-methyladenine–DNA glycosylase and attributed it to instability of the enzyme. The separation of a necessary cofactor or subunit from the enzyme during purification is an alternate possibility. Loss of activity during purification of 3-methyladenine–DNA glycosylase from human lymphoblasts was correlated with the loss of an apparent stimulatory factor (Gallagher & Brent, 1981), and we have preliminary evidence of a similar phenomenon during purification of the placental enzyme (P. E. Gallagher and T. P. Brent, unpublished experiments).

The human placental 3-methyladenine–DNA glycosylase described here resembles the DNA glycosylases in general (Duncan, 1981). All glycosylases reported thus far have a relatively low molecular weight and specifically cleave the base–sugar bond of an abnormal or damaged base, leaving an apurinic or apyrimidinic site. Properties of the placental 3-methyladenine–DNA glycosylase, such as the broad pH optima, essential sulfhydryl groups, and absence of metal ion requirements, are similar to those of other partially purified 3-methyladenine–DNA glycosylase activities studied (Duncan, 1981). Product inhibition of placental 3-methyladenine–DNA glycosylase did not occur. In this respect, the placental enzyme resembled the 3-methyladenine–DNA glycosylase II of *E. coli* (Thomas et al., 1982) but differed from the *E. coli* glycosylase I (Riazuddin & Lindahl, 1978; Thomas et al., 1982) as well as mouse L cells (Male et al., 1981).

Enzymatic release of 7-methylguanine in vitro was observed with the 1000-fold-purified placental enzyme fraction, which thus resembled enzyme activities from bacteria (Laval et al., 1981; Thomas et al., 1982), rodent liver nuclei (Cathcart & Goldthwait, 1981; Margison & Pegg, 1981), and human lymphoblasts (Singer & Brent, 1981). The levels of 7-methylguanine released by extracts of placenta, *E. coli* (Thomas et al., 1982), and rodent liver (Cathcart & Goldthwait, 1981; Margison & Pegg, 1981) were very low compared to that of 3-methyladenine. In contrast to the results reported here, the rat liver enzyme did not release 7-methylguanine from poly(dG-dC). Because none of these preparations were homogeneous, it is not yet clear whether 7-methylguanine excision activity is an integral part of the 3-methyladenine–DNA glycosylase or a result of a separate contaminating enzyme. *E. coli* has at least two species of 3-methyladenine–DNA glycosylase activities: one is highly specific for 3-methyladenine excision, and the other has a broader specificity (Thomas et al., 1982). Further purification and characterization are needed to determine if similar multiple enzymes exist in human placenta cells.

Unlike the uracil–DNA glycosylase that acts preferentially on single-stranded DNA (Duncan, 1981), 3-methyladenine–DNA glycosylase activity requires the double-stranded structure. The small amount of glycosylase activity observed with denatured methylated DNA probably was due to double-stranded regions remaining after denaturation. It appears the single-stranded regions in methylated or nonmethylated DNA inhibit placental 3-methyladenine–DNA glycosylase. Addition of unlabeled denatured DNA to an equal amount of labeled methylated DNA in the reaction mixture inhibited enzyme activity 58%; this inhibition was more than 3 times that observed if unlabeled native DNA was added. The even greater inhibition caused by the addition of denatured methylated DNA was likely due to the combined effect of inhibition by single-stranded regions and isotope dilution caused by residual double-stranded methylated regions remaining after denaturation. These results are consistent with the observation that double-stranded methylated DNA is the preferred substrate. However, it is surprising that the enzyme more effectively binds to methylated denatured DNA, a poor substrate, then to methylated native DNA. It also suggests that

excision repair of 3-methyladenine cannot occur in or near single-stranded regions such as a replicating fork, thereby preventing DNA fragmentation or random base pairing.

In *tag* mutants of *E. coli*, deficient in 3-methyladenine–DNA glycosylase, 3-methyladenine in DNA is cytotoxic and mutagenic (Karran et al., 1980). The presumed function of 3-methyladenine–DNA glycosylase is the excision repair of lesions introduced by methylating agents. However, it is not clear to what extent exogenous methylating species derived from the environment react with DNA. It may be that the enzyme has evolved to repair illicit DNA methylation produced endogenously (Lindahl, 1982). The ubiquitous nature of 3-methyladenine–DNA glycosylase supports this idea. Barrows & Shank (1981) observed aberrant methylation of DNA in rodent livers treated with hepatotoxins and showed that *S*-adenosylmethionine acts as the methyl donor. Other recent evidence indicates that *S*-adenosylmethionine can donate its methyl group to DNA by a nonenzymatic process to yield 7-methylguanine and 3-methyladenine (Lindahl, 1982). Thus, 3-methyladenine–DNA glycosylase may have evolved to protect against chronic low level DNA methylation by the intracellular methyl donor pool. If so, major deficiencies of the enzyme could be lethal, and minor variations in enzymatic activity might affect the frequency of somatic cell mutation and the incidence of "spontaneous" carcinogenesis.

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