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Definitive Characterization of Human Thymine Glycol *N*-Glycosylase Activity†

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ABSTRACT: An *N*-glycosylase activity that released *cis*-[^{3}H]-5,6-dihydroxy-5,6-dihydrothymine (thymine glycol, TG) from chemically oxidized poly(dA-[^{3}H]dT) was unambiguously characterized both in extracts of HeLa cells and in purified *Escherichia coli* endonuclease III. This was accomplished by use of a microderivatization procedure that quantitatively converted *cis*-TG to 5-hydroxy-5-methylhydantoin (HMH). The reaction products were analyzed by high-pressure liquid chromatography before and after derivatization by using *cis*-[^{14}C]TG and [^{14}C]HMH, which had been independently synthesized, as reference compounds. This technique facilitated construction of a $v/[E]_t$ plot for the enzyme activity in HeLa cells, permitting estimation of its specific activity. The results obtained prove the existence of both human and bacterial *N*-glycosylase activities that effect removal of TG from DNA.

Thymine is the most susceptible of the DNA bases to modification by chemical oxidants (Iida & Hayatsu, 1971), oxygen-containing free radicals (Cadet & Teoule, 1978), and ionizing radiation (Scholes, 1976). The oxidative derivative that is most readily formed is the *cis*-5,6-dihydroxy-5,6-dihydrothymine (thymine glycol, TG)[1] moiety (Teoule et al., 1977). Thymine glycol has also been identified in DNA exposed to a variety of oxidative stresses (Hariharran & Cerutti, 1977; Frenkel et al., 1981a,b, 1986; Teebor et al., 1982a), where it has been shown to be a block to DNA replication in vitro (Ide et al., 1985; Rouet & Essigman, 1985). Thymine glycol is removed from DNA through the action of an *N*-glycosylase activity of *Escherichia coli* endonuclease III (Demple & Linn, 1980; Breimer & Lindahl, 1984). Its disappearance from the DNA of mammalian cells was first described in cell culture by Mattern et al. (1973). More recently, Cathcart et al. (1984) detected TG in both rodent and human urine and concluded that this was evidence of its ongoing formation in cellular DNA through endogenous oxidation and its removal by a repair enzyme. Yet, surprisingly, TG *N*-glycosylase activity has been difficult to characterize with certainty in mammalian cells. Breimer (1983) could not detect release of TG from an oxidized copolymer of dA and dT residues by an extract of calf thymus tissue. However, when the TG moieties were converted to urea residues, the enzymatic release of urea was easily detected. Only after partial purification of the urea *N*-glycosylase could Breimer detect TG release, and then it was at a rate less than 1% that of urea. Doetsch et al. (1986) described release of ^{3}H-labeled material from an oxidized poly(dA-[^{3}H]dT) substrate by calf thymus extracts. High-pressure liquid chromatographic (HPLC) analysis of the material showed the presence of three peaks, one of which eluted with the retention time of TG. The nature of the other two was not certain.

To definitively characterize this important enzyme activity, we devised a facile microderivatization scheme based on the work of Teoule et al. (1974) and modified by us for HPLC (Frenkel et al., 1982). This technique unambiguously and

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[1] Abbreviations: TG, thymine glycol (5,6-dihydroxy-5,6-dihydrothymine); dTG, thymidine glycol (5,6-dihydroxy-5,6-dihydrothymidine); HMH, 5-hydroxy-5-methylhydantoin; dT, thymidine; EDTA, disodium ethylenediaminetetraacetate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; BE buffer, 100 mM NaCl, 20 mM Tris-HCl, and 1 mM EDTA, pH 7.5; HPLC, high-pressure liquid chromatography; ODS, octadecylsilane; HAP, hydroxylapatite.

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quantitatively identified TG. We have been able to show that it is indeed TG that is enzymatically released by both *E. coli* endonuclease III and HeLa cell extracts. We have also constructed a $v/[E]_t$ plot for the human activity that permitted estimation of the actual specific activity of the TG *N*-glycosylase in human cells.

Experimental Procedures

Materials

[*methyl*-^{3}H]Thymidine 5′-triphosphate (dTTP) (80 Ci/mmol), [*methyl*-^{14}C]thymine (46.9 mCi/mmol), [*methyl*-^{14}C]thymidine (48.2 mCi/mmol), and [2-^{14}C]pyruvic acid were purchased from New England Nuclear. ϕX174 *Hin*dIII fragments were obtained through the courtesy of Opinder S. Bhanot. Poly(dA-dT) and DNA polymerase I (Klenow fragment) were obtained from Boehringer Mannheim, dTTP was obtained from U.S. Biochemical, and hydroxylapatite (HAP; DNA grade) and P-6 DG were obtained from Bio-Rad. Gelman Acro LC13 0.2-μm filters were used to filter samples prior to HPLC analyses. HPLC-grade acetone and acetonitrile were purchased from Fisher, growth medium was purchased from Gibco, and OsO_4 solution was purchased from Electron Microscopy Sciences. Pyridine (Mallinckrodt) was redistilled over CaH_2 and stored over molecular sieves under nitrogen in the dark. Acetic anhydride (Fisher) was redistilled and stored under nitrogen in the dark.

Methods

Synthesis of Marker Compounds cis-(±)-TG, cis-(±)-dTG, and 5-Hydroxy-5-methylhydantoin (HMH). The syntheses and purification of [^{14}C]-containing *cis*-TG and -dTG were accomplished as previously described (Frenkel et al., 1981a) and of HMH according to Murahashi et al. (1966) and Teebor et al. (1982b).

Preparation of Polydeoxyribonucleotide Substrate. (A) Synthesis of Poly(dA-[^{3}H]dT). Poly(dA-[^{3}H]dT) was synthesized on a poly(dA-dT) template (Fauvadon et al., 1985) of an average length of 1 kilobase, as determined by comparison with standard ϕX174 *Hin*dIII fragments on neutral agarose gels. The standard reaction mixture (150 μL) contained 850 μM (nucleotide equivalent) poly(dA-dT), 20 μM [*methyl*-^{3}H]dTTP (80 Ci/mmol), 230 μM dTTP (reducing the final specific activity of dTTP to approximately 8 Ci/mmol), 330 μM dATP, 5 units of DNA polymerase (Klenow fragment), 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7. This mixture was incubated for 2 h at room temperature. At that time, an additional 2 units of DNA polymerase was added in order to maximize yield, and the reaction continued for an additional 2 h. High molecular weight copolymer was precipitated by the addition of $^1/_{10}$ volume of 3 M sodium acetate and 2 volumes of absolute ethanol. After the sample was chilled for 60 min at −20 °C, it was centrifuged in an Eppendorf centrifuge for 10 min. The pellet was suspended in 70% ethanol, recentrifuged, and dissolved in BE buffer [100 mM NaCl, 20 mM Tris, 1 mM disodium ethylenediaminetetraacetate (EDTA), pH 7.5].

(B) Oxidation of Poly(dA-[^{3}H]dT). The *cis*-TG moiety was formed by oxidation of thymine residues in poly(dA-[^{3}H]dT) with OsO_4. Poly(dA-[^{3}H]dT) was denatured by heating for 2 min at 82 °C, OsO_4 (0.8%) solution was added, and the mixture was heated at the same temperature for 10 min, at which time the reaction was stopped by cooling to room temperature.

(C) Purification of Oxidized Poly(dA-[^{3}H]dT). The cooled sample was applied to a column of P-6 DG desalting gel (12 mL) equilibrated with dilute (1:100) BE buffer. The oxidized poly(dA-[^{3}H]dT) was eluted with the same buffer in the void volume, while OsO_4 was retained by the gel. The fractions containing ^{3}H were pooled and concentrated under low pressure to a volume of 50–100 μL.

(D) Reannealing. Unmodified poly(dA-dT) was added in an approximately 1:1 molar ratio (nucleotide equivalent) to the radiolabeled, oxidized poly(dA-[^{3}H]dT) dissolved in 0.2 M NaCl. The mixture was heated at 100 °C for 5 min and incubated at 37 °C overnight.

(E) Separation of Double- from Single-Stranded Poly(dA-[^{3}H]dT). Batch-procedure HAP chromatography (Bernardi, 1965) was used to isolate reannealed, oxidized poly(dA-[^{3}H]dT). HAP equilibrated with 0.01 M potassium phosphate (0.5 mL) was centrifuged (IEC centrifuge with a 269 rotor) at 1500 rpm for 5 min. The mixture containing reannealed, oxidized poly(dA-[^{3}H]dT) was made 0.01 M with respect to potassium phosphate in a total volume of 0.5 mL. This mixture was added to an equal volume of HAP, gently vortexed, and allowed to stand at room temperature for 10 min before centrifuging (as above) at 1500 rpm for 5 min. A 10-μL aliquot of the supernatant was counted to determine ^{3}H content. The HAP was washed with 0.01 M potassium phosphate buffer (0.5 mL per wash) until less than 5% of the applied ^{3}H was present in the supernatant. This procedure was repeated with 0.1 and 0.3 M potassium phosphate to elute single- and double-stranded material, respectively.

(F) Identification and Quantitation of cis-dTG Content of Oxidized Poly(dA-[^{3}H]dT). These were accomplished by using a 5-μm semipreparative Ultrasphere ODS column (Altex; 1 × 25 cm) attached to an HPLC system (Beckman, Model 330) equipped with a gradient elution accessory (Beckman, Model 421). Oxidized poly(dA-[^{3}H]dT) was enzymatically digested to 2′-deoxyribonucleosides and analyzed together with the markers [^{14}C]dTG and [^{14}C]thymidine ([^{14}C]dT) (Frenkel et al., 1981b), with H_2O as eluent. The flow rate for all analyses was 2 mL/min. Fractions containing ^{3}H that cochromatographed with [^{14}C]dTG were collected, evaporated to dryness, and treated with acetic anhydride (600 μL) in dry pyridine (500 μL) overnight. The reaction was terminated by addition of an excess of distilled water. The sample was evaporated to dryness, extracted with a small volume of acetone, and chromatographed on an ODS column with acetonitrile–water (3:7) as eluent.

(G) Specific Radioactivity of Poly(dA-[^{3}H]dT). The specific radioactivities of poly(dA-[^{3}H]dT) and of the product, double-stranded [^{3}H]TG-containing poly(dA-[^{3}H]dT), were determined to be 2 and 0.4 Ci/mmol, respectively, assuming that 1 A_{260} unit = 50 μg/mL. The difference in specific activities between the starting material and the oxidized product was due to dilution of the oxidized radioactive polymer with unmodified, nonradioactive polymer during reannealing and the recovery of the oxidized copolymer as a double-stranded product from the HAP batch procedure.

Enzyme Preparations and Assay for TG N-Glycosylase Activity. (A) Preparation of HeLa Cell Sonicates. HeLa cells were grown at 37 °C on Corning tissue culture flasks (150 cm^2) in minimal essential medium (MEM; Joklik-modified) supplemented with 10% calf serum and L-glutamate. Cells were removed from the flasks with saline–EDTA buffer (150 mM NaCl, 100 mM Na_2EDTA, pH 8), collected by centrifugation, and washed 3 times with modified phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 6.8). The cells were suspended in hypotonic buffer (10 mM potassium phosphate, 1 mM EDTA,

pH 8) at a concentration of 2×10^7 cells/mL in a volume of approximately 1 mL and sonicated on ice for 30 s by using an Ultrasonics Model W-375 sonicator equipped with a microtip. The sonicate was centrifuged (Sorvall RC-2 with an SS-34 rotor) at 13 000 rpm for 10 min. The supernatant was removed, DTT was added to a final concentration of 0.5 mM, and the preparations were used immediately in enzymatic assays.

(*B*) *Preparation of Endonuclease III.* Purified endonuclease III (Breimer & Lindahl, 1984) was obtained through the courtesy of Lars Breimer.

(*C*) *Enzymatic Assays.* Oxidized poly(dA-[^{3}H]dT) was used as the substrate in assays to detect TG *N*-glycosylase activity in both HeLa cell sonicates and endonuclease III preparations. A typical reaction mixture (100 μL) consisted of oxidized poly(dA-[^{3}H]dT) containing 400 pmol (approximately 800 000 cpm) of thymine residues (of which 3–4% were oxidized to TG) in 0.3 M potassium phosphate buffer, 60 mM Tris-HCl, 1 mM EDTA, pH 7, and either HeLa cell sonicate or 4 μL of purified endonuclease III. After incubation at 37 °C (30 min with endonuclease III or 2 and 4 h with HeLa cell sonicate), 50 μg of bovine serum albumin (in the case of assays using endonuclease III) and 5 volumes of cold acetone (in both cases) were added to the reaction mixtures. The mixtures were chilled at −20 °C for 20 min and centrifuged (Sorvall RC-2 centrifuge with SS-34 rotor) for 10 min. The entire supernatant was evaporated under reduced pressure and dissolved in H_2O for HPLC analysis.

Identification of Enzymatically Released [^{3}H]TG. Analysis of Acetone-Soluble Products Released in Enzyme Assays. HPLC analysis of radioactive thymine derivatives released by incubation of oxidized poly(dA-[^{3}H]dT) with either purified endonuclease III or HeLa cell sonicate was performed on an ODS column with water as eluent and [^{14}C]TG as marker compound. The putative [^{3}H]TG isolated by HPLC analysis was converted to HMH by the method of Teoule et al. (1974) as modified for HPLC by Frenkel et al. (1982). Fractions in which ^{3}H-containing material had coeluted with [^{14}C]TG were evaporated under reduced pressure, and the residue was treated with 1 mL of 0.2 M sodium periodate at room temperature for 1 h. This treatment leads to the opening of the TG ring and formation of *N*′-formyl-*N*-pyruvylurea. The solution was evaporated to dryness, the residue extracted with methanol, and the solvent removed under reduced pressure. To effect cyclization to HMH, the residue was dissolved in 1 mL of H_2O and heated under a reflux condenser at 100 °C for 3 h. After removal of the solvent under reduced pressure, the residue was dissolved in H_2O and filtered through a 0.2-μm filter prior to HPLC analysis on an ODS column with water as eluent. The retention time of [^{14}C]HMH derived from authentic [^{14}C]TG was compared to that of authentic [^{14}C]HMH marker prepared by the method of Murahashi et al. (1966). Further proof of identity was obtained by acetylation of both [^{14}C]TG-derived and ^{14}C-containing authentic marker HMH and comparison of the HPLC profile of the ^{14}C-containing acetyl derivatives. Analyses of the putative HMH obtained from [^{3}H]TG released by HeLa cell sonicate or endonuclease III from the oxidized poly(dA-[^{3}H]dT) were carried out in the same manner; i.e., [^{3}H]HMH was acetylated together with [^{14}C]HMH and analyzed as above.

Results

Thymidine Glycol Content of Oxidized Poly(dA-[^{3}H]dT). Figure 1 shows the HPLC profile of an enzymatic hydrolysate of oxidized poly(dA-[^{3}H]dT) containing 2′-deoxyribonucleosides cochromatographed with [^{14}C]dTG and [^{14}C]dT

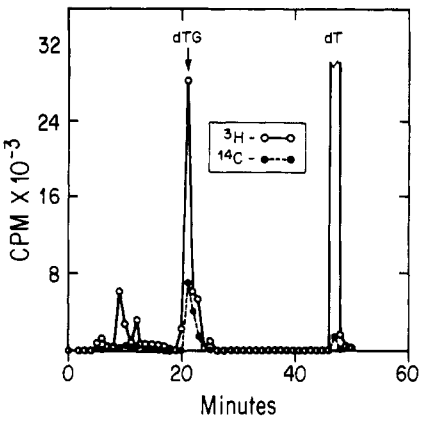

FIGURE 1: HPLC profile of enzymatic hydrolysate of oxidized poly(dA-[^{3}H]dT). The enzymatic hydrolysate of oxidized poly(dA-[^{3}H]dT) (o—o) was cochromatographed with [^{14}C]dTG and [^{14}C]dT (●---●) markers, with H_2O as eluent.

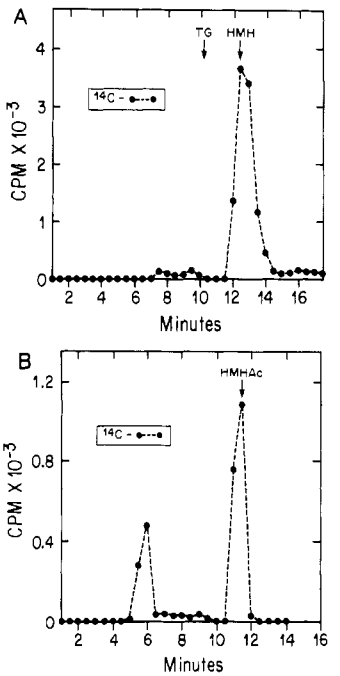

FIGURE 2: (A) HPLC profile of [^{14}C]HMH derived from authentic [^{14}C]TG. H_2O was used as eluent. (B) HPLC profile of the acetyl derivative of [^{14}C]HMH, chromatographed with acetonitrile–water (3:7) as eluent.

marker compounds. The major product formed by oxidation of the thymine moiety in the polymer coeluted with [^{14}C]dTG, which had a retention time of 22 min. This product represented 15% of the total ^{3}H content of the oxidized poly(dA-[^{3}H]dT). The ^{3}H-containing, putative dTG isolated by HPLC was acetylated, and the acetoxy-dTG derivatives were analyzed by HPLC using acetonitrile–water (3:7) as eluent. The acetylation procedure yields the tri- (5′,3′,6) and tetra- (5′,3′,5,6) acetoxy derivatives of the (+) and (−) diastereoisomers of dTG (Teebor et al., 1987). This procedure was used to corroborate the identity of the ^{3}H-containing peak coeluting with [^{14}C]dTG marker as [^{3}H]dTG and to calculate the amount of dTG present in the oxidized polymer.

Identification of HMH as the Product of the Derivatization of TG. Figure 2A shows the HPLC elution profile of [^{14}C]HMH obtained by oxidative ring opening of authentic [^{14}C]TG followed by recyclization. The retention time of HMH is 12 min, as opposed to 9.5 min for TG. This retention time is identical with that of authentic [^{14}C]HMH synthesized as previously described (Teebor et al., 1982b). Further proof

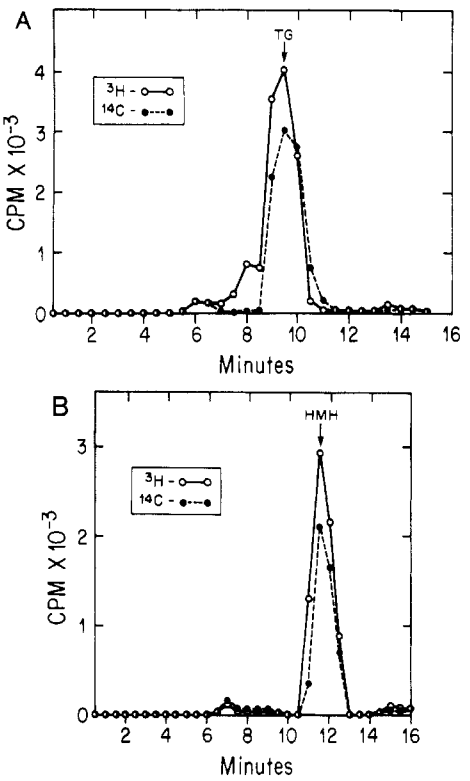

FIGURE 3: (A) HPLC profile of acetone-soluble products (O—O) released from oxidized poly(dA-[^{3}H]dT) by endonuclease III after 30 min of incubation. Products were cochromatographed with [^{14}C]TG (●---●), with H_2O as eluent. (B) HPLC profile of [^{3}H]HMH derived from [^{3}H]TG released from oxidized poly(dA-[^{3}H]dT) by endonuclease III. The products of the conversion reaction (O—O) were cochromatographed with authentic [^{14}C]HMH (●---●), with H_2O as eluent.

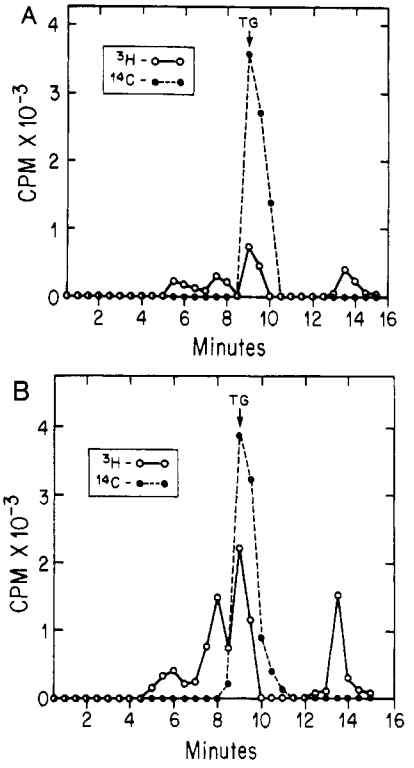

FIGURE 4: (A) HPLC profile of acetone-soluble products (O—O) released from oxidized poly(dA-[^{3}H]dT) by HeLa cell sonicate after 2 h and cochromatographed with [^{14}C]TG marker (●---●), with H_2O as eluent. (B) HPLC profile of acetone-soluble products (O—O) released from oxidized poly(dA-[^{3}H]dT) by HeLa cell sonicate after 4 h and cochromatographed with [^{14}C]TG marker (●---●), with H_2O as eluent.

of the identity of TG-derived HMH came from comparison of the HPLC profile of the acetyl derivative synthesized from authentic [^{14}C]HMH with that of [^{14}C]TG-derived HMH. Both had the same retention time, 11 min, when chromatographed on the ODS column with acetonitrile–water (3:7) as eluent (see Figure 2B). Thus, derivatization and two different chromatographic analyses showed that [^{14}C]TG was converted to HMH.

Enzyme Assays. (A) Endonuclease III. Figure 3A shows the HPLC profile of the products released from the oxidized poly(dA-[^{3}H]dT) after incubation with purified endonuclease III for 30 min. Of the ^{3}H-containing material present in the supernatant (18 000 cpm) obtained by precipitation of the reaction mixture, the majority (11 500 cpm) coeluted with [^{14}C]TG marker. The fractions containing both ^{3}H and ^{14}C cpm were pooled and used for the subsequent conversion to HMH.

Figure 3B shows the HPLC profile of the products obtained by conversion of the putative [^{3}H]TG, which had been released by endonuclease III, to [^{3}H]HMH. Approximately 60% of both the ^{3}H- and ^{14}C-containing material that eluted between 9 and 10.5 min (Figure 3A) was recovered as HMH (Figure 3B, 10.5–13 min). These results prove that the ^{3}H-containing, acetone-soluble material released upon incubation with endonuclease III that coeluted with [^{14}C]TG marker was indeed [^{3}H]TG.

There was no release of ^{3}H-containing base or nucleoside products from the oxidized substrate when heat-denatured endonuclease III was used (data not shown).

(B) HeLa Cell Sonicate. Figure 4A shows the HPLC profile of the products released from oxidized poly(dA-[^{3}H]dT) after 2 h of incubation with HeLa cell sonicate. Approximately 1500 ^{3}H cpm coeluted with [^{14}C]TG marker. HPLC analysis of the products released after 4 h of incubation revealed an increase in the amount of ^{3}H-containing material (4300 cpm) that coeluted with [^{14}C]TG (Figure 4B). Heat-denatured HeLa cell sonicate was incubated with copolymer for 4 h in the control assay, and there were no ^{3}H-containing base or nucleoside products released from the oxidized substrate under those conditions (data not shown).

The ^{3}H-containing fractions were pooled and converted to HMH. The recoveries of both ^{3}H- and ^{14}C-containing material from the conversion reaction were about 50%. Thus, derivatization confirmed that the release of [^{3}H]TG increased with time of incubation with TG *N*-glycosylase activity present in HeLa cells.

The need for derivatization was emphasized by the presence of two additional peaks of ^{3}H-containing material eluting at 8 and 13.5 min, respectively, as shown by the HPLC analysis of the products released by HeLa cell sonicate from poly(dA-[^{3}H]dT) (Figure 4). Comparison of the relative magnitudes of the peaks in the HPLC profile of acetone-soluble products released at 2 vs. 4 h indicates that these products were also released in a time-dependent fashion. Endonuclease III activity released smaller amounts of both products relative to TG than did the enzymatic activity (activities) present in HeLa cell sonicate (Figures 3A and 4A). These peaks were not present in the HPLC profile of the supernatant of oxidized copolymer incubated for 4 h with heat-denatured HeLa cell sonicate or endonuclease III. (Only the ^{3}H peak eluting at 5–6 min was present in that control.) Incubation of authentic [^{14}C]TG marker for 4 h under the conditions of the enzymatic assay did not yield any other ^{14}C-containing peaks. Therefore, those eluting at 8 and 13.5 min (Figure 4) do not represent products formed by either hydrolysis of TG in solution or

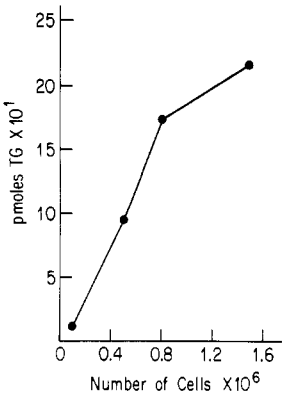

FIGURE 5: v vs. $[E]_t$ plot. Amount of [^{3}H]TG released by HeLa cell sonicate after 4 h.

isomerization of *cis*-TG to *trans*-TG. These results indicate that, like TG, these two thymine derivatives were also enzymatically released.

The velocity of the *N*-glycosylase-mediated release of TG was assayed at the 4-h time point by using various amounts of HeLa cell sonicate (Figure 5). After 4 h of incubation with oxidized poly(dA-[^{3}H]dT), ^{3}H-containing material having 240, 1900, and 3480 cpm was released by HeLa cell extracts from 1×10^5, 5×10^5, and 8×10^5 cells, respectively. The specific activity of the enzyme was calculated from the values that determined the linear portion of the v vs. $[E]_t$ plot and was expressed as femtomoles of TG released per minute. The specific activity of TG *N*-glycosylase in HeLa cell sonicate was determined to be 22 fmol/(min·mg of protein).

Discussion

The unambiguous identification of TG *N*-glycosylase activity was accomplished by using an oxidized substrate of high specific radioactivity that facilitated the detection of enzymatically released [^{3}H]TG. It was necessary to reanneal the oxidized poly(dA-[^{3}H]dT) to unmodified poly(dA-dT) to form a double-stranded heteroduplex polydeoxyribonucleotide, for virtually no activity was detected if pure single-stranded oxidized polymer was used as substrate. The reason for this is not certain. It may be that the heavily modified (15%) poly(dA-[^{3}H]dT) was so distorted that the enzyme did not recognize it as a helical DNA-like substrate. The requirements for a double-stranded substrate by both endonuclease III and the mammalian urea *N*-glycosylase activity have been well documented (Breimer, 1983; Breimer & Lindahl, 1984).

The reannealing procedure unavoidably diluted the oxidized heteroduplexes with unmodified poly(dA-dT) by a factor of about 5 (see Methods, section G). It previously was shown (Caradonna & Cheng, 1980) that addition of a 5-fold excess of unmodified DNA to uracil-containing PBS2 DNA resulted in a 40% inhibition of human uracil *N*-glycosylase activity. This degree of substrate dilution is similar to ours. Therefore, the specific activity of the TG *N*-glycosylase activity in HeLa cells may be closer to 40 fmol/(min·mg of protein). This value is comparable to that of another mammalian *N*-glycosylase directed against a different oxidatively modified thymine residue, 5-(hydroxymethyl)uracil. The specific activity of that enzyme was estimated to be 200 fmol/(min·mg of protein) in mouse cells (Hollstein et al., 1984).

Results of our HPLC analyses of the products released by both *E. coli* endonuclease III and HeLa cell sonicate are quite similar to those of Doetsch et al. (1986), who also observed three peaks of ^{3}H-containing material released from OsO_4-oxidized poly(dA-[^{3}H]dT), one of which had the retention time of authentic *cis*-TG. They suggested the other peaks were "isomers" of TG. We feel this interpretation may not be correct. *cis*-TG prepared by the chemical oxidation of thymine is a mixture of (±) enantiomers that are, by definition, structurally identical and not easily separable by nonchiral chromatographic matrices such as ODS, particularly when retention times are so short. They may be in equilibrium in aqueous solution with their corresponding *trans*-(±) diastereomers, which are also an enantiomeric pair and thus elute as a single peak from nonchiral columns. Therefore, at least one of the three peaks cannot be a TG isomer. Furthermore, we incubated marker *cis*-(±)-[^{14}C]TG under conditions of the enzyme assay and saw no second peak after 4 h. This corroborates observations of Latarjet et al. (1961), Barszcz et al. (1963), Iida and Hayatsu (1970, 1971), Cadet et al. (1975), and Frenkel et al. (1981a), all of whom found the cis form to be favored in aqueous solution and could not detect trans until after vigorous heating and then only to a level of 20% that of cis. We suggest that the other two peaks are as yet unidentified thymine products resulting from OsO_4 oxidation that are different from TG. They were released by both the HeLa sonicate and purified endonuclease III, albeit to differing degrees. Whether they were enzymatically released by multiple proteins of mammalian cells or by a protean *N*-glycosylase/AP endonuclease having activity similar to that of *E. coli* endonuclease III is not yet known with certainty. Doetsch et al. (1986) reported that their purified calf thymus preparation released photochemically modified cytosine residues in addition to TG, as did endonuclease III. Breimer's (1983) partially purified calf thymus preparation released TG at only 1% the rate of urea. It should be noted that the specific activity of TG *N*-glycosylase of HeLa cells we obtained is about 1% that of the specific activity of urea *N*-glycosylase reported to be present in crude calf thymus extracts. This finding supports the contention of Doetsch et al. (1986) that mammalian cells do indeed contain a protean *N*-glycosylase/AP endonuclease activity analogous to endonuclease III. The complete substrate range of this activity will be known only through the use of defined substrates and precise chemical characterization of the released products. This approach will aid our understanding of the relative contribution of those modified pyrimidine residues to cell lethality, mutagenicity, and carcinogenicity.

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Effect of Single Amino Acid Changes in the Region of the Adenylylation Site of T4 RNA Ligase

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ABSTRACT: Preparation and analysis of a series of mutants of bacteriophage T4 RNA ligase that carry single amino acid changes at or near the site of covalent reaction with ATP (adenylylation) are described. The mutant proteins were constructed by site-directed mutagenesis of the gene for T4 RNA ligase (g63) cloned in M13 vectors, transfer of the mutant genes into a λpL-containing expression plasmid, and subsequent expression in *Escherichia coli*. The results give further evidence that Lys-99 is the adenylylation site and that the residue is also important to step 3 in the RNA ligase mechanism (ligation between acceptor and adenylylated donor). Mutations at Glu-100 or Asp-101 have no effect on adenylylation, but Asp-101 is shown to be crucial to both step 2 (transfer of adenylyl to donor) and step 3.

Bacteriophage T4 RNA ligase (EC 6.5.1.3) is a useful enzyme that catalyzes a variety of inter- and intramolecular nucleic acid joining reactions (Uhlenbeck & Gumport, 1982). Of all currently known ligases, it is the only one that catalyzes the formation of a 3′-5′-phosphodiester bond between one nucleic acid strand containing a 5′-terminal phosphate and another containing a 3′-terminal hydroxyl group without needing a template. The enzyme has therefore been of particular value in the synthesis of defined-sequence oligoribonucleotides and in the 3′ labeling of RNA using nucleoside 3′,5′-bisphosphates (Uhlenbeck & Gumport, 1982).

The enzyme mechanism for joining reactions catalyzed by RNA ligase involves three reversible steps. First, the enzyme reacts with ATP to form a covalently adenylylated enzyme intermediate (E-pA) and pyrophosphate. In the second step, the adenylyl moiety is transferred to the 5′-terminal phosphate of a donor molecule [pN(pN)$_n$] to form an adenylylated donor. Finally, a 3′-5′-phosphodiester bond is formed by reaction of the 3′-hydroxyl group of an acceptor molecule [N(pN)$_m$pN] with the adenylylated donor releasing AMP.

$$E + ATP \rightleftarrows E\text{-}pA + PP_i \quad (1)$$

$$E\text{-}pA + pN(pN)_n \rightleftarrows [A\text{-}5'pp5'N(pN)_n]\cdot E \quad (2)$$

$$N(pN)_mpN + [A\text{-}5'pp5'N(pN)_n]\cdot E \rightleftarrows N(pN)_mpNpN(pN)_n + AMP + E \quad (3)$$

Adenylylated enzyme intermediates appear to be important to the mechanism of many ligase enzymes. A covalent phosphoramidate bond between the adenylyl moiety and enzyme has been shown to be formed in the case of T4 DNA ligase (Gumport & Lehman, 1971), T4 RNA ligase (Juodka et al., 1980; Juodka & Markuckas, 1985), and wheat germ RNA ligase (Pick et al., 1986). Similar adenylylated intermediates have been postulated for a yeast tRNA ligase (Phizicky et al., 1986) and an RNA ligase (Perkins et al., 1985) and a cyclase (Filipowicz et al., 1985) from HeLa cells. In the cases of T4 DNA ligase and T4 RNA ligase, a lysine residue has been implicated as the site of adenylylation. By

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