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Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: Sanitization of nucleotide pool

(spontaneous mutation/oxygen radicals/*mutT* homologue/mutator)

JIN-YAO MO*, HISAJI MAKI†, AND MUTSUO SEKIGUCHI‡§

Department of Biochemistry, Faculty of Medicine, Kyushu University 60, Fukuoka 812, Japan

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ABSTRACT **8-Oxoguanine nucleotide can pair with cytosine and adenine nucleotides at almost equal efficiencies. Once 8-oxodGTP is formed in the cellular nucleotide pool, this mutagenic nucleotide is incorporated into DNA and would cause transversion mutations. The MutT protein of *Escherichia coli* possesses enzyme activity to hydrolyze 8-oxodGTP to the corresponding nucleoside monophosphate and thus may be responsible for preventing the occurrence of such mutations. Here we show that the human cell has an enzyme specifically hydrolyzing 8-oxodGTP in a fashion similar to that seen with MutT protein. The human 8-oxodGTPase has been found in cell-free extracts from Jurkat cells and purified >400-fold. Analyses by gel filtration and gel electrophoresis revealed that the molecular mass of the native form of human 8-oxodGTPase is 18 kDa. Mg^{2+} ion is required for the enzyme action and the optimum pH for the reaction is pH 8.0. The enzyme hydrolyzes 8-oxodGTP to 8-oxodGMP with a K_m value of 12.5 μM. dGTP and dATP are also degraded to dGMP and dAMP, respectively, with K_m values 70 times greater than that for 8-oxodGTP. dTTP and dCTP are not hydrolyzed. These properties of the human 8-oxodGTPase are similar to those observed with the *E. coli* MutT protein, suggesting that the function of protecting the genetic information from the threat of endogenous oxygen radicals is widely distributed in organisms.**

Mutator mutants that show an increased frequency of spontaneous mutations have led to elucidation of the multiple pathways of spontaneous mutagenesis. Studies on *Escherichia coli* mutator genes and their products revealed that a major cause of spontaneous mutation is errors of DNA replication and that the cell possesses multistep mechanisms to correct such errors (1). In addition, certain types of spontaneous DNA damage would cause mutations (2) and the cell comes equipped with mechanisms to repair such damage. Among 15 known *E. coli* mutator genes, 12 are shown to be involved in correction of replicational errors and/or spontaneous DNA damage (1, 3–6). Recently we obtained evidence that the *mutT* mutator gene is involved in a hitherto unknown mechanism for reducing spontaneous mutation frequency (7).

A *mutT* mutator mutant shows a frequency of A·T → C·G transversion 100–10,000 times the level of the wild type (8). The MutT protein specifically prevented misincorporation of dGMP onto poly(dA)/oligo(dT)$_{20}$ template DNA *in vitro* (9). This antimutagenic effect of MutT protein appeared to be catalytic and was achieved through its action on dGTP but not on DNA or DNA polymerase (H.M. and M.S., unpublished results). We and others noted that the MutT protein has a weak nucleoside triphosphatase activity with a substrate preference to dGTP (9, 10). Subsequently we found that the nucleotide that is misincorporated opposite the dA residue of the template is not dGMP but rather its oxidized form, 8-oxodGMP. When 8-oxodGTP was added to an *in vitro* DNA replication system, 8-oxodGMP was incorporated opposite dA and dC residues of the template, with almost equal frequencies (7). It was also shown that the 8-oxodGMP in the template DNA could pair with incoming dCMP and dAMP, with varying frequencies depending on the DNA polymerases used (11). These results imply that the existence of 8-oxodGTP in the nucleotide pool would induce A·T → C·G transversion and that G·C → T·A transversion would also be induced if the 8-oxodGMP incorporated opposite the template dC residue leaves unrepaired. Consistent with these findings, the MutT protein hydrolyzes the potent mutagenic nucleotide, 8-oxodGTP, much more efficiently than dGTP (7). Based on these observations, we proposed that the major role of MutT protein is to prevent the A·T → C·G transversion caused by 8-oxodGTP, which is formed spontaneously in the nucleotide pool of the *E. coli* cell.

It was of interest to see if such a mechanism to prevent mutations exists in higher organisms. Here we report the identification, purification, and characterization of a human enzyme that specifically hydrolyzes 8-oxodGTP to 8-oxodGMP.

MATERIALS AND METHODS

Materials. RPMI-1640 medium was obtained from GIBCO/BRL. Fetal bovine serum was from Whittaker Bioproducts. Protein inhibitors were from Sigma. α-^{32}P-labeled dNTPs (800 Ci/mmol, 10 mCi/ml, 1 Ci = 37 GBq) were from Amersham. Unlabeled dNTPs (FPLC-pure grade) were from Pharmacia LKB. Polyethylenimine-cellulose (PEI-cellulose) plates were from Merck. Silver stain kits and triethylammonium hydrogencarbonate solution (1.0 M, pH 7.0) were from Wako Pure Chemical (Osaka). The Spherisorb SAX column (5 μm, 4.6 × 250 mm) and DEAE-MemSep 1000 chromatography cartridge were from GL Science (Tokyo) and Millipore, respectively. Mono Q HR 5/5, Superdex 75 HR 10/30, HiTrap heparin, and HiTrap blue columns were products of Pharmacia. DEAE-Bio-Gel A and protein assay dye reagent were obtained from Bio-Rad. *E. coli* MutT protein was prepared as described (9).

Preparation of 8-OxodGTP. Radiolabeled and unlabeled 8-oxodGTP were prepared according to the method of Kasai and Nishimura (12), with minor modifications. A reaction mixture containing 100 mM sodium phosphate (pH 6.8), 6 mM dGTP, 30 mM ascorbic acid, and 100 mM H_2O_2 was incubated at 37°C for 2 hr in the dark. Fifty microliters of the

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Abbreviation: DTT, dithiothreitol.

*Present address: Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709.
†Present address: Division of Biophysics, Institute of Applied Microbiology, University of Tokyo, Tokyo 113, Japan.
‡Present address: Department of Biochemistry, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812, Japan.
§To whom reprint requests should be addressed.

reaction mixture was loaded onto a HPLC Spherisorb SAX column equilibrated with 150 mM potassium phosphate (pH 5.5) and chromatographic separation was carried out at 25°C with the same buffer at a flow rate 1 ml/min. 8-OxodGTP was monitored with an UV detector (254 nm) and eluted with a retention time about 1 min later that for dGTP. The fractions containing 8-oxodGTP were combined and applied to a DEAE-MemSep 1000 cartridge equilibrated with 50 mM triethylammonium hydrogencarbonate (pH 7.0). 8-oxodGTP was eluted with a linear gradient (50–500 mM) of triethylammonium hydrogencarbonate (pH 7.0) at a flow rate 1 ml/min. The fractions containing 8-oxodGTP were lyophilized, dissolved in 20 mM sodium phosphate (pH 6.8), and stored at −20°C. Purity and quantity of the preparation were examined in electrochemical measurements and UV spectrum analyses (12).

8-OxodGTPase Assay. 8-OxodGTPase activity was assayed by measuring the hydrolysis of 8-oxodGTP to 8-oxodGMP. The reaction mixture (12.5 μl) contained 20 mM Tris·HCl (pH 8.0), 4 mM $MgCl_2$, 40 mM NaCl, 20 μM 8-oxodGTP, 80 μg of bovine serum albumin per ml, 8 mM dithiothreitol (DTT), 10% glycerol, and the enzyme preparation to be examined. The reaction was run at 30°C for 20 min and was terminated by spotting an aliquot (2 μl) of the reaction mixture onto a PEI-cellulose plate. The product was separated from the substrate by PEI-cellulose TLC with 1 M LiCl for 1 hr and quantitated by autoradiographic analysis with a Fujix 2000 Bio-image analyzer (Fuji, Tokyo). One unit of 8-oxodGTPase was defined as the amount of enzyme that produced 1 pmol of 8-oxodGMP per min, under standard conditions.

Cell-Free Extracts. Jurkat cells, a human T-cell leukemia cell line (stock of this laboratory), were grown under 5% CO_2 at 37°C in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum. When a density of 6×10^5 cells per ml was reached, the cells were harvested by centrifugation and washed twice with ice-cold phosphate-buffered saline. The cell pellets were frozen quickly and stored at −80°C until use. The preparation of cell-free extracts was essentially as described (13). Two liters of the cell culture was used for each preparation. All operations were carried out at 4°C. The cells were thawed on ice, suspended in 8 ml of hypotonic buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA/5 mM DTT), and left on ice for 20 min. After protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride and 0.5 μg/ml each of leupeptin, pepstatin, and chymostatin) were added to the suspension, the cells were homogenized in a Dounce homogenizer (B pestle). Eight milliliters of cold solution containing 50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM DTT, 25% sucrose, and 50% glycerol was then added, and the mixture was stirred. Two milliliters of neutralized saturated ammonium sulfate was slowly added, with gentle mixing. The lysate was stirred gently on ice for 30 min and centrifuged at 250,000 × *g* for 3 hr in a TLA 100.3 rotor (Beckman) at 2°C. The supernatant was recovered, and the protein was precipitated by adding solid ammonium sulfate (0.33 g/ml of supernatant). After dissolution of the ammonium sulfate, 1 M NaOH (10 μl/g of ammonium sulfate) was added and the mixture was left on ice for 30 min. The precipitate was collected by centrifugation at 15,000 × *g* for 20 min and resuspended in 1 ml of a buffer containing 25 mM Hepes-KOH (pH 7.9), 1 mM EDTA, 2 mM DTT, 17% glycerol, and the protease inhibitors. The suspension was dialyzed for 16 hr against two 500-ml changes of the same buffer (fraction I). The dialysate was centrifuged at 10,000 × *g* for 10 min, and the supernatant (≈5 ml) was quickly frozen in liquid nitrogen and stored at −80°C until use. The sample stored at −80°C retained full activity for at least 6 months. The cell-free extracts usually contained 4–5 mg of protein per ml.

Purification of Human 8-OxodGTPase. All operations were carried out at 4°C. The cell-free extracts were prepared from Jurkat cells as described above, and five 2-liter batches of the preparation were combined. The cell-free extracts (25 ml) were loaded onto a DEAE-Bio-Gel A column (bed volume, 50 ml) equilibrated with buffer A (25 mM Tris·HCl, pH 7.5/0.1 mM EDTA/2 mM DTT/15% glycerol), and the column was washed with 100 ml of the same buffer. Protein was eluted with 500 ml of buffer A containing a linear gradient (0–0.4 M) of NaCl at a flow rate of 0.5 ml/min. Fractions containing the 8-oxodGTPase activity were pooled (fraction II, 53 ml), dialyzed against buffer A, and applied to a HiTrap heparin column (bed volume, 5 ml) equilibrated with buffer A. All 8-oxodGTPase activity was recovered in a flow-through fraction (fraction III, 51 ml). Fraction III was loaded onto a HiTrap blue column (bed volume, 5 ml) equilibrated with buffer A containing 20 mM NaCl. The flow-through fractions containing the activity were pooled (fraction IV, 60 ml) and applied to a FPLC Mono Q HR 5/5 column (1 ml) equilibrated with buffer A containing 20 mM NaCl. The column was washed with 3 ml of the same buffer. Protein was eluted with 20 ml of buffer A containing a linear gradient (20–400 mM) of NaCl at a flow rate of 0.2 ml/min. The activity was recovered in a single peak at 120–150 mM NaCl in the linear gradient (fraction V, 0.8 ml).

Other Methods. $NaDodSO_4$/PAGE was done as described (14). Proteins were stained using a Wako silver stain kit. Protein concentrations were determined using the Bio-Rad protein assay dye reagent with bovine serum albumin as a standard.

RESULTS

8-OxodGTPase Activity in Human Cell Extracts. The MutT protein of *E. coli* specifically hydrolyzes 8-oxodGTP to 8-oxodGMP (7). In search of similar activity in mammalian cells, we found that Jurkat cells (a human T-cell leukemia cell line) contained a high level of such activity. On incubation with the extract in the presence of Mg^{2+}, 8-oxodGTP was rapidly converted to the monophosphate. Fig. 1 *Left* shows a TLC analysis of the reaction product. The reaction is clearly time-dependent. It was noted that there is a concomitant formation of 8-oxodGDP in the reaction. When dGTP was incubated with the same extract, the formation of dGDP

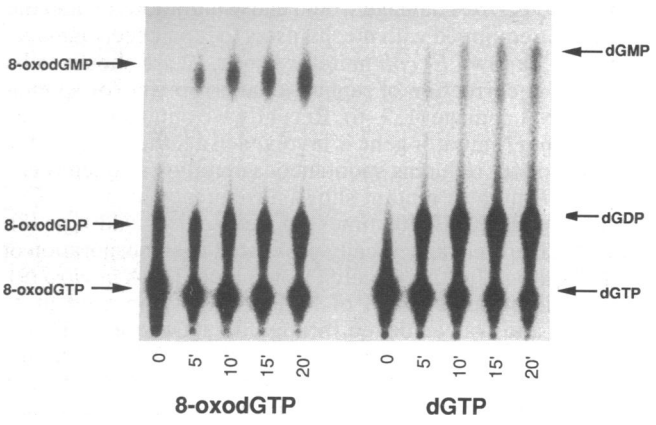

FIG. 1. Hydrolysis of 8-oxodGTP and dGTP by human cell-free extracts. The reaction mixtures (final volume, 25 μl) contained 40 μM 8-oxodGTP or dGTP. Reactions were initiated by the addition of 4-μl cell-free extracts and were carried out at 30°C. Aliquots (2 μl) of the reaction mixtures were withdrawn at the times indicated and spotted on a PEI-cellulose plate. An autoradiogram against the TLC plate was taken by a Fujix 2000 Bio-image analyzer.

was even more rapid (Fig. 1 *Right*). There was only little formation of dGMP from the dGTP. Thus the Jurkat cell extract probably contained two distinct activities; one to hydrolyze 8-oxodGTP to 8-oxodGMP and the other to hydrolyze dGTP preferentially to dGDP. It was not certain at this stage of investigation whether production of 8-oxodGDP is due to the former or the latter activity. To characterize the activity involved in cleavage of 8-oxodGTP, we initiated purification of the enzyme.

Purification of Human 8-OxodGTPase. Using TLC analysis, the activity related to the production of 8-oxodGMP was monitored and fractionated. After four steps of column chromatography, the human 8-oxodGTPase was purified ≈400-fold over the cell-free extract (Table 1). In each step of purification, the 8-oxodGTPase activity was recovered in a single-peak fraction and recovery of the activity was fairly high.

In an early step of the purification (DEAE-Bio-Gel A column chromatography), an activity that catalyzes the conversion of 8-oxodGTP to 8-oxodGDP was separated from the 8-oxodGTPase. The fraction containing the former activity had the capacity to degrade dGTP to dGDP and the rate of formation of dGDP was much greater than that for 8-oxodGDP. In addition, the K_m for hydrolysis of 8-oxodGTP by this fraction was much higher than the value observed with the 8-oxodGTPase. It seems likely that the degradation of 8-oxodGTP to 8-oxodGDP may be caused by a common dGTPase or by GTPase with a broad substrate specificity.

8-OxodGMP can be placed onto a poly(dA) template upon incubation of 8-oxodGTP with the α subunit of *E. coli* DNA polymerase III and this misincorporation is prevented by the *E. coli* MutT protein with a distinct 8-oxodGTPase activity (9). It thus seemed important to determine whether the human 8-oxodGTPase has such a potential. With the reconstituted system, we found that the activity to prevent 8-oxodGMP misincorporation was copurified with the 8-oxodGTPase and that the dGTPase fraction had no such activity. These results strongly suggest that human cells carry a single species of 8-oxodGTPase that functionally resembles the *E. coli* MutT protein.

Sizing Analyses of Human 8-OxodGTPase. To determine the size of native human 8-oxodGTPase, an aliquot of the purified 8-oxodGTPase (fraction V) was subjected to a gel-filtration column chromatography. As shown in Fig. 2, the 8-oxodGTPase activity was recovered in a single peak with a yield of 25%. From the elution volume for the human 8-oxodGTPase, the Stokes' radius was estimated to be 18.7 Å, a value corresponding to the molecular mass of 19 kDa, as a globular protein.

Proteins recovered from the gel-filtration column were analyzed by NaDodSO$_4$/PAGE and visualized by silver staining (Fig. 3). A single band corresponding to an 18-kDa protein was found in lanes for fractions containing 8-oxodGTPase. The intensity of the band increased in parallel with the 8-oxodGTPase activity. Thus, it seems probable that the human 8-oxodGTPase, in a native form, is composed of a single polypeptide with a molecular mass of ≈18 kDa. Since the protein concentration of the peak fractions was too low

Table 1. Purification of human 8-oxodGTPase

Fraction	Protein, mg	Total activity, units × 10^5	Specific activity, units × 10^4/mg	Yield, %
I Cell-free extract	54.0	2.20	0.41	100
II DEAE-Bio-Gel A	7.74	2.82	3.64	128
III HiTrap heparin	0.77	2.60	34.0	118
IV HiTrap blue	0.59	2.57	43.7	117
V Mono Q	0.07	1.20	174	54.5

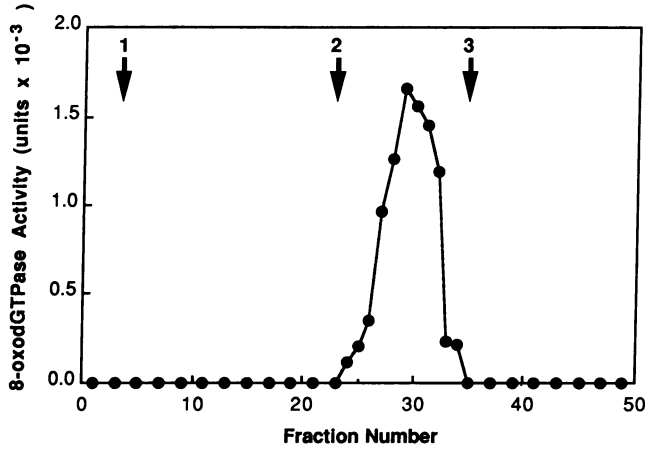

FIG. 2. Elution profile of human 8-oxodGTPase in gel-filtration chromatography. One-hundred ninety microliters of fraction V (3.7 × 10^4 units) was filtered through a Superdex 75 HR 10/30 column equilibrated with a buffer containing 25 mM Tris·HCl (pH 7.5), 2 mM DTT, 0.1 mM EDTA, 100 mM NaCl, and 15% glycerol at a flow rate of 0.2 ml/min. The 8-oxodGTPase activity in each fraction was determined. Molecular mass standards are ovalbumin (arrow 1; 43 kDa, 30.5 Å), chymotrypsinogen A (arrow 2; 25 kDa, 20.9 Å), and ribonuclease A (arrow 3; 13.7 kDa, 16.4 Å).

for an accurate determination, we could not estimate the specific activity of the 8-oxodGTPase. From the staining pattern of the gels, purity of the 18-kDa protein in the fraction V seemed to be 5–10%.

Characterization of Human 8-OxodGTPase. Using purified preparations, several properties of 8-oxodGTPase were examined. The pH optimum for the activity was at pH 8.0. Mg^{2+} ion was essential for the activity and the maximal activity was obtained with 2–6 mM $MgCl_2$. The addition of 40 mM NaCl to the assay stimulated the activity 1.6-fold; however, further increased concentrations of NaCl inhibited the activity and the reaction with 160 mM NaCl showed only 20% of the maximum activity.

Substrate specificity of the enzyme was examined with α-^{32}P-labeled dNTPs (Fig. 4). Forty micromolar normal dNTP and 40 μM 8-oxodGTP were added to the standard reaction mixture. Nineteen units of the human 8-oxo-

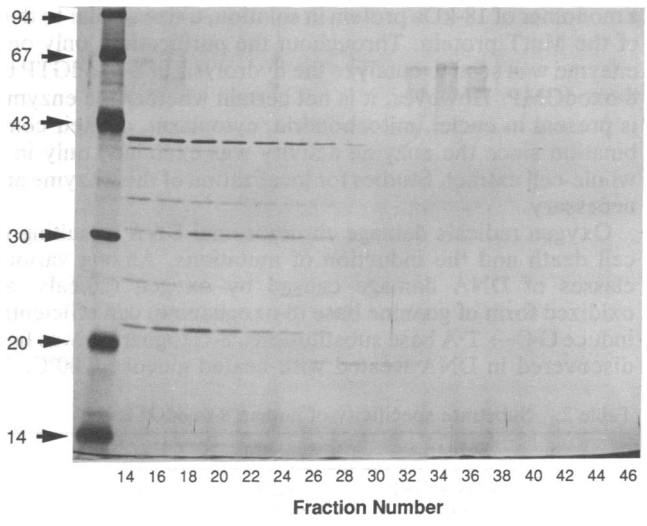

FIG. 3. NaDodSO$_4$/PAGE analyses. Proteins in each fraction recovered from the gel-filtration column were run in 12% NaDodSO$_4$/PAGE and visualized with silver staining. Molecular mass standards are phosphorylase B (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

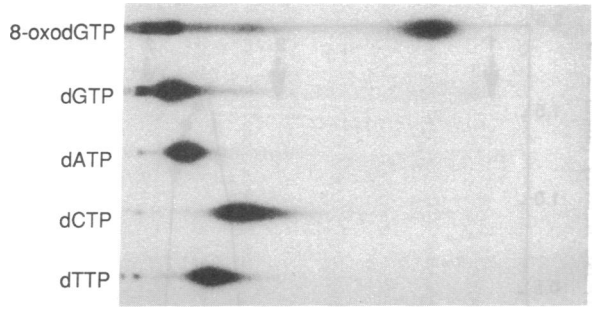

FIG. 4. Substrate specificity of human 8-oxodGTPase. Each nucleotide was present at 40 μM. Other assay conditions were as described in the text. Nineteen units of purified human 8-OH-dGTPase was used for each reaction. Two microliters of the reaction mixtures was analyzed by PEI-cellulose TLC.

dGTPase almost completely hydrolyzed the 8-oxodGTP to 8-oxodGMP, in 20 min at 30°C. Although dGTP and dATP were also hydrolyzed to the corresponding nucleoside monophosphate, the amounts of products were ≈5% of that of 8-oxodGMP. Neither dTTP nor dCTP was hydrolyzed by the enzyme. We also examined kinetic parameters for hydrolysis of 8-oxodGTP or dGTP by the purified enzyme (Table 2). The apparent K_m for hydrolysis of 8-oxodGTP was 70 times lower than that for the degradation of dGTP, whereas the maximal velocities observed with both substrates were much the same. Based on these observations, we concluded that 8-oxo-dGTP is a specific substrate for the human 8-oxodGTPase.

DISCUSSION

We have shown here that the human cell has an enzyme similar to the MutT protein of *E. coli*. The human enzyme specifically hydrolyzes 8-oxodGTP to 8-oxodGMP with a relatively low K_m value, comparable to one determined with MutT protein (7). Therefore, like the MutT protein, this enzyme can maintain very low the level of 8-oxodGTP in the nucleotide pool of the human cell. We found that the human enzyme inhibits the misincorporation of 8-oxodGMP opposite dA residues of template DNA. This implies that the human 8-oxodGTPase has the same antimutagenic capacity as the MutT protein. The human 8-oxodGTPase seems to be a monomer of 18-kDa protein in solution, a size similar to that of the MutT protein. Throughout the purification, only one enzyme was seen to catalyze the hydrolysis of 8-oxodGTP to 8-oxodGMP. However, it is not certain whether the enzyme is present in nuclei, mitochondria, cytoplasm, or their combination since the enzyme activity was examined only in a whole-cell extract. Studies for localization of this enzyme are necessary.

Oxygen radicals damage chromosomal DNA, resulting in cell death and the induction of mutations. Among various classes of DNA damage caused by oxygen radicals, an oxidized form of guanine base (8-oxoguanine) can efficiently induce G·C → T·A base substitutions. 8-Oxoguanine was first discovered in DNA treated with heated glucose (200°C, 20 min) (15). X-ray irradiation also generates 8-oxoguanine in DNA (12). Shibutani *et al.* (11) showed that dCMP and dAMP are incorporated opposite 8-oxodG residues of template DNA in a DNA synthesis system *in vitro*. Thus, 8-oxodG formed in DNA is a potent mutagenic lesion that induces G·C → T·A transversion. This is consistent with the observation that a significant portion of mutations induced by chemical oxidants is G·C → T·A transversion (16). This modified base is also present in DNA from cells untreated with such agents, albeit the level being low, approximately two molecules of 8-oxodG per 10^5 dG residues in *E. coli* chromosome DNA (T. Tajiri, H.M. and M.S., unpublished result). It seems, therefore, that the level of active oxygen species produced by cellular metabolic intermediates may be sufficient to oxidize the guanine base of the DNA, even in normally growing cells.

Table 2. Substrate specificity of human 8-oxodGTPase

Substrate	K_m, μM	V_{max}
8-OxodGTP	12.5	1.0
dGTP	870	1.3

The relative velocity for production of nucleoside monophosphate from nucleoside triphosphate was determined in time course experiments with substrate concentrations ranging from 3 μM to 40 μM for 8-oxodGTP and 100 μM to 4 mM for dGTP. K_m and V_{max} were obtained from Lineweaver–Burk plots of the data. V_{max} was expressed as the ratio to that for 8-oxodGTP.

8-OxodGTP is a potent mutagenic substrate for DNA synthesis (7). As the oxidation of guanine proceeds *in vitro* more rapidly in forms of free nucleotide than in paired polynucleotides (ref. 12; H.M. and M.S., unpublished result), it is conceivable that the oxidative damage to the base occurs more frequently in the nucleotide pool of the cell than in its chromosomal DNA. In contrast with the consequence of 8-oxoguanine arising in DNA, 8-oxodGTP can induce A·T → C·G as well as G·C → T·A transversion (17). However, in normally growing cells, frequencies of these two types of transversions are kept at low levels (18, 19). From studies on *E. coli* mutator mutants, this organism possesses special mechanisms to prevent mutations caused by oxidation of the guanine base, in both DNA and free nucleotide forms. The oxidized DNA can be repaired by MutM (Fpg) protein that has an activity to remove the 8-oxoguanine base from the damaged DNA (6). A mutant defective in the MutM protein showed a 10-fold higher frequency of G·C → T·A mutation, as compared with the wild-type level (20). On the other hand, 8-oxodGTP can be eliminated from the nucleotide pool by MutT protein that hydrolyzes the mutagenic nucleotide to 8-oxodGMP (7). *mutT*-defective mutants increase sharply the occurrence of A·T → C·G transversion but not G·C → T·A (8). In the *mutT* mutant, 8-oxodGMP misincorporated opposite dC residues of template may possibly be removed by the MutM protein, before the next round of DNA replication. Since the frequency of G·C → T·A mutation was still low when a *mutM* mutation was introduced into the *mutT* mutant strain (T. Tajiri, H.M. and M.S., unpublished results), it seems probable that more than one mechanism is operative in preventing G·C → T·A transversion in *E. coli* cells. Another mutator gene (*mutY*), the mutations of which induce specifically the G·C → T·A transversion (21–23), might have a role in such mechanisms.

A certain portion of spontaneous mutagenesis in higher organisms may be caused by endogenous oxygen radicals. It has been proposed that the steady-state level of oxidative damage to DNA may be an important determinant of longevity (2). A significant amount of 8-oxoguanine is formed in the chromosome DNA of mammalian cells and most of the damaged nucleotides are excized from the DNA and excreted into the urine (2, 24). An enzyme activity for cleaving DNA at the position of 8-oxoguanine was detected in mammalian tissues (25). In the present study, we found that 8-oxo-dGTPase that sanitizes the nucleotide pool is also present in mammalian cells. The wide distribution of these enzymes implies that protection of the genetic material from the threat of endogenous oxygen radicals is of utmost importance for living systems.

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