Multiple Enzyme Activities of *Escherichia coli* MutT Protein for Sanitization of DNA and RNA Precursor Pools

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ABSTRACT: 8-OxoGua (8-oxo-7,8-dihydroguanine) is produced in nucleic acids as well as in nucleotide pools of cells, by reactive oxygen species normally formed during cellular metabolic processes. MutT protein of *Escherichia coli* specifically degrades 8-oxoGua-containing deoxyribo- and ribonucleoside triphosphates to corresponding nucleoside monophosphates, thereby preventing misincorporation of 8-oxoGua into DNA and RNA, which would cause mutation and phenotypic suppression, respectively. Here, we report that the MutT protein has additional activities for cleaning up the nucleotide pools to ensure accurate DNA replication and transcription. It hydrolyzes 8-oxo-dGDP to 8-oxo-dGMP with a K_m of 0.058 μM, a value considerably lower than that for its normal counterpart, dGDP (170 μM). Furthermore, the MutT possesses an activity to degrade 8-oxo-GDP to the related nucleoside monophosphate, with a K_m value 8000 times lower than that for GDP. These multiple enzyme activities of the MutT protein would facilitate the high fidelity of DNA and RNA syntheses.

There is a special class of mutations that increases the frequency of spontaneous mutation. These are collectively termed mutators and are useful tools for elucidating cellular mechanisms related to the high fidelity of DNA replication (*1*). Among many mutators found in *Escherichia coli*, *mutT* has drawn particular attention. MutT is the first mutator found in this organism (*2*) and specifically induces the transversion of adenine−thymine to cytosine−guanine (*3*). As a consequence of this unidirectional mutator activity, *mutT*$^-$ cells have increased GC content levels in their chromosomal DNA (*4*). Akiyama et al. (*5*) cloned the *mutT* gene and, on the basis of sequence analysis, identified the product as a protein with a molecular weight of 15 000. The MutT protein was purified to physical homogeneity and was shown to have the nucleoside triphosphatase activity preferentially hydrolyzing dGTP (*6*). Using an *in vitro* DNA synthesis system, Akiyama et al. (*7*) demonstrated that the MutT protein specifically prevented misincorporation of dGMP onto the poly(dA)-oligo(dT)$_{20}$ template primer. Subsequently, Maki and Sekiguchi (*8*) found that the nucleotide that is misincorporated opposite the adenine of the template is not dGMP but rather its oxidized form, 8-oxo-7,8-dihydro-2′-deoxyguanosine monophosphate (8-oxo-dGMP).[1] When 8-oxo-dGTP was added to an *in vitro* DNA replication system, 8-oxo-dGMP was incorporated opposite adenine and cytosine residues of the template with almost equal efficiencies.

Recently, mammalian counterparts of the MutT have been identified, which can be divided into two groups according to their substrate specificities. One group of proteins, including MTH1 and MTH2, degrades 8-oxo-dGTP to 8-oxo-dGMP, as is the case of MutT (*9*, *10*). Another group of proteins, such as NUDT5, has only a little activity toward 8-oxo-dGTP but efficiently hydrolyzes 8-oxo-dGDP to the related nucleoside monophosphate (*11*). Despite their different substrate specificities, all of these mammalian proteins have abilities to replace the MutT function; when each of the cDNAs for these proteins was expressed in *mutT*-defective *E. coli* mutant cells, the mutator phenotype was almost completely suppressed (*10*−*12*).

In *E. coli* cells, MutT protein, which has a potent 8-oxo-dGTPase activity, is almost solely responsible for reducing the mutagenic nucleotide level, on the basis of the finding that *mutT*-defective mutants show a 1000-fold higher frequency of spontaneous mutations, as compared with wild-type cells (*3*, *13*). It was shown, moreover, that the MutT has a potential to hydrolyze 8-oxo-GTP, an oxidized form of GTP, thereby preventing misincorporation of 8-oxoGua into RNA (*14*). Considering these facts, as well as the above-mentioned findings in mammalian cells, there arises a

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[1] Abbreviations: 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxo-dGTP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate; 8-oxo-dGDP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-diphosphate; 8-oxo-dGMP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-monophosphate; 8-oxo-GTP, 8-oxo-7,8-dihydroguanosine 5′-triphosphate; 8-oxo-GDP, 8-oxo-7,8-dihydroguanosine 5′-diphosphate; 8-oxo-GMP, 8-oxo-7,8-dihydroguanosine 5′-monophosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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question if the MutT acts on 8-oxo-dGDP and 8-oxo-GDP. The present study was performed to answer this question.

EXPERIMENTAL PROCEDURES

Production of MutT Protein. cDNA for MutT was amplified from pHS:MutT (*15*) by PCR using two primers, 5′-CCAAGGATCCATGAAAAAGCTGCAAATTGCGGTAG G-3′ and 5′-GAAGGTCGACCTACAGACGTTTAGCTTC GCAATTACCG-3′. The PCR product was subcloned into the *Bam*HI/*Sal*I site of pQE-80L (QIAGEN) to produce MutT protein that carries the His tag at its N terminus. *E. coli* M15 strain (QIAGEN) was transformed with the plasmid, and the cells were cultured in 200 mL of LB broth containing 100 μg/mL ampicillin at 28 °C with shaking. When A_{600} reached 0.6−0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a final concentration of 1 mM and cells were harvested 2 h after addition of IPTG. The cells collected by centrifugation were lysed in 10 mL of phosphate buffer (50 mM sodium phosphate at pH 8.0 and 300 mM NaCl) containing 10 mM imidazole by sonication. The lysate was applied to Ni-NTA agarose (QIAGEN) following clarification by centrifugation. After the MutT protein was washed with phosphate buffer containing 100 mM imidazole, it was eluted in phosphate buffer containing 250 mM imidazole. The protein was concentrated by Microcon YM3 (Amicon) in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, and 10% glycerol.

Enzymatic Reactions. 8-Oxo-dGTP, 8-oxo-dGDP, 8-oxo-dGMP, 8-oxo-GTP, 8-oxo-GDP, and 8-oxo-GMP were prepared as described in Fujikawa et al. (*16*). Enzymatic reaction and detection of the products were carried out as mentioned in Ishibashi et al. (*11*). To determine the relative velocity for hydrolysis of nucleotides, time-course experiments were done with different concentrations of the substrates, 0.1−4 μM for 8-oxo-dGTP, 8-oxo-dGDP, and 8-oxo-GTP, 0.05−4 μM for 8-oxo-GDP, and 0.1−5 mM for other nucleotides. K_m and V_{max} values for hydrolysis of each nucleotide were obtained from Lineweaver−Burk plots of the data.

Analysis of Reaction Products. γ-^{32}P-Labeled 8-oxo-GTP was prepared by oxidation of γ-^{32}P-labeled GTP (MP Biomedicals, Inc.) and purified, as described in Fujikawa et al. (*16*). The enzymatic reaction was carried out as mentioned in Hayakawa et al. (*23*), and an aliquot of the reaction mixture was spotted onto a PEI-cellulose plate (MERCK) and developed in 2 M LiCl/0.2 M Na_2HPO_4 (1:1). Yeast pyrophosphatase was purchased from SIGMA, and the reaction was performed at 30 °C for 30 min. After development, the radioactivity was measured by an image analyzer.

RESULTS

Hydrolysis of 8-OxoGua Deoxyribonucleotides. MutT protein was produced as a His-tagged form in *E. coli* M15 cells and purified to near homogeneity. Examination on SDS−PAGE revealed the existence of a single protein band, corresponding to the MutT protein (Figure 1). On incubation of a purified preparation of MutT protein in the presence of Mg^{2+}, an efficient conversion of 8-oxo-dGTP to the related monophosphate was observed (Figure 2A). There was only little degradation of dGTP under the same conditions. This result is consistent with the previous finding (*8*).

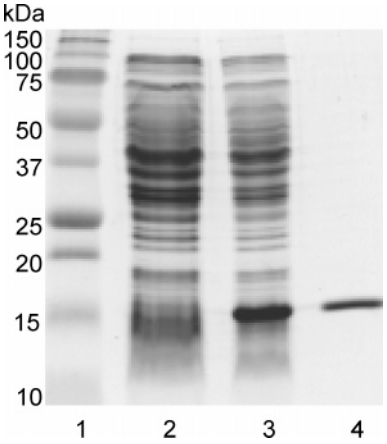

FIGURE 1: SDS−PAGE of MutT protein. Cell extracts were prepared from cultures of *E. coli* M15 (6.5×10^7 cells), and samples were subjected to 15% SDS−PAGE. Lane 1, molecular weight markers; lane 2, whole-cell extracts without IPTG induction; lane 3, whole-cell extracts with IPTG induction; lane 4, 0.5 μg of purified N-terminal His-tagged MutT protein.

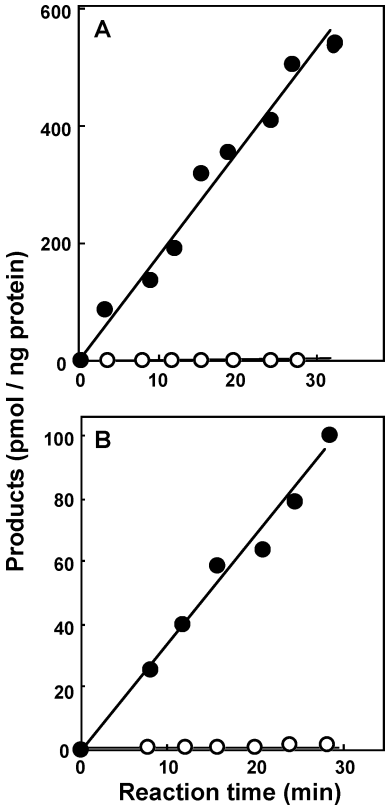

FIGURE 2: Specific hydrolysis of 8-oxo-dGTP and 8-oxo-dGDP by MutT protein. (A) Action of MutT on 8-oxo-dGTP and dGTP. A total of 10 pmol each of 8-oxo-dGTP (●) or dGTP (○) was incubated with 2.5 pg (for 8-oxo-dGTP) or 2 ng (for dGTP) of a purified preparation of MutT protein in 10 μL of reaction mixture containing 20 mM Tris-HCl at pH 8.0, 0.8 μg of bovine serum albumin, 8 mM $MgCl_2$, 40 mM NaCl, 5 mM DTT, and 2% glycerol. The reaction was carried out at 30 °C and terminated by adding SDS to 0.08%, and nucleoside monophosphates produced were determined by using HPLC. (B) Action of MutT on 8-oxo-dGDP and dGDP. A total of 10 pmol each of 8-oxo-dGDP (●) or dGDP (○) was treated with 20 pg (for 8-oxo-dGDP) or 2.5 ng (for dGDP) of MutT protein as described above.

We extended our studies to 8-oxoGua-containing deoxyribonucleoside diphosphate, which has not been examined before. 8-Oxo-dGDP was prepared as described in Fujikawa

Table 1: Action of *E. coli* MutT Protein on Various Deoxynucleotides

substrate	K_m (μM)	V_{max} [pmol min^{-1} (ng of protein)$^{-1}$]	V_{max}/K_m
8-oxo-dGDP	0.058	3.7	64
dGDP	170	5.9	0.035
dADP	1200	0.76	0.00063
dCDP	470	0.57	0.0012
dTDP	650	1.9	0.0029
8-oxo-dGTP	0.081	20	240
dGTP	1100	44	0.041
dATP	930	0.024	0.000 026
dCTP	1300	2.8	0.0021
dTTP	1700	16	0.0093

et al. (*16*) and purified to give a single peak in high-performance liquid chromatography (HPLC) analysis. When 8-oxo-dGDP was incubated with MutT protein under the same conditions as those for 8-oxo-dGTP hydrolysis, its rapid conversion to 8-oxo-dGMP was observed (Figure 2B). No apparent degradation of dGDP was observed in a similar treatment with MutT.

The kinetic parameters of the MutT enzyme (K_m and V_{max}) were measured for the hydrolysis of various nucleotides (Table 1). The K_m for the hydrolysis of 8-oxo-dGDP is approximately 3000 times lower than that for dGDP, which is the second best substrate among several deoxyribonucleoside diphosphates examined.

The kinetic parameters for hydrolysis of deoxyribonucleoside triphosphates were also determined, and the data are included in Table 1. The apparent K_m for the hydrolysis of 8-oxo-dGTP is 10 000 times lower than that for dGTP, whereas the V_{max} values for both nucleotides are almost the same. The MutT protein hydrolyzes other deoxyribonucleoside triphosphates with lower V_{max} and extremely high K_m values. From these results, we can conclude that both 8-oxo-dGDP and 8-oxo-dGTP are specific substrates for the MutT protein.

Hydrolysis of 8-OxoGua Ribonucleotides. Taddey et al. (*14*) demonstrated that the MutT protein can degrade 8-oxo-GTP, the ribonucleotide counterpart of 8-oxo-dGTP. The result shown in Figure 3A confirms this finding. On incubation with a purified preparation of MutT, 8-oxo-GTP was rapidly converted to 8-oxo-dGMP, while no breakdown of GTP was observed.

We have then examined if 8-oxo-GDP is degraded to the related monophosphate under the same conditions. Here again, a specific conversion of 8-oxo-GDP to 8-oxo-GMP was observed (Figure 3B). The kinetic parameters for the hydrolysis of 8-oxo-GDP and GDP by a purified preparation of MutT enzyme are shown in Table 2. The K_m value for the hydrolysis of 8-oxo-GDP is approximately 8000 times lower than that for the degradation of GDP, whereas the maximal velocities observed with both substrates are much the same.

In Table 2, the kinetic parameters for hydrolysis of 8-oxo-GTP and GTP are also given. The K_m for the hydrolysis of 8-oxo-GTP is about 4000 times lower than that for GTP. These results clearly indicate that the MutT protein hydrolyzes two types of 8-oxoGua-containing ribonucleotides, 8-oxo-GDP and 8-oxo-GTP, at almost the same efficiencies.

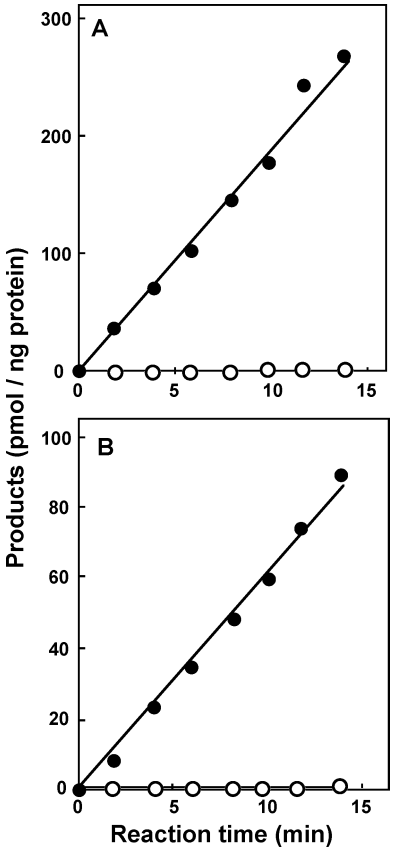

FIGURE 3: Specific hydrolysis of 8-oxo-GTP and 8-oxo-GDP by MutT protein. (A) Action of MutT on 8-oxo-GTP and GTP. A total of 10 pmol each of 8-oxo-GTP (●) or GTP (○) was treated with 5 pg (for 8-oxo-GTP) or 2 ng (for GTP) of a purified preparation of MutT protein in 10 μL of reaction mixture, under the conditions described in the caption of Figure 2. (B) Action of MutT on 8-oxo-GDP and GDP. A total of 5 pmol each of 8-oxo-GDP (●) or GDP (○) was treated with 20 pg (for 8-oxo-GDP) or 5 ng (for GDP) of MutT protein as described above.

Table 2: Action of *E. coli* MutT Protein on Various Ribonucleotides

substrate	K_m (μM)	V_{max} [pmol min^{-1} (ng of protein)$^{-1}$]	V_{max}/K_m
8-oxo-GDP	0.045	4.8	110
GDP	350	3.5	0.010
8-oxo-GTP	0.26	22	84
GTP	1000	24	0.023

Mode of Action of MutT. The fact that the MutT can hydrolyze 8-oxoGua-containing nucleoside diphosphates as well as triphosphates raises a question if nucleoside triphosphates are first converted to the related diphosphates and then degraded to the monophosphates. If this is the case, orthophosphate, rather than pyrophosphate, would be produced in the reaction. To examine this possibility, γ-^{32}P-labeled 8-oxoGua-containing ribonucleoside triphosphate was treated with a purified preparation of MutT protein and the products were analyzed on thin-layer chromatography. As shown in Figure 4, a spot for ^{32}P-labeled pyrophosphate appeared, which was further converted to orthophosphate by treatment with pyrophosphatase. It appears that the MutT protein has an ability to cleave the phosphoanhydride bond between the α and β phosphate of 8-oxoGua-containing nucleoside di- and triphosphates.

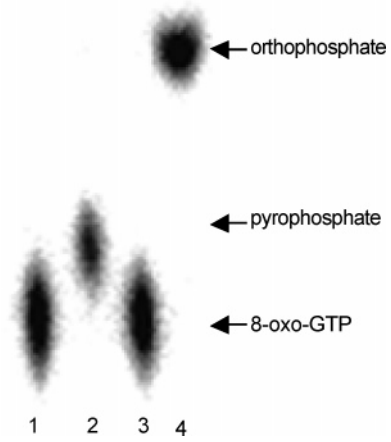

FIGURE 4: Mode of nucleoside triphosphate cleavage. A total of 5 μL of reaction mixture containing γ-^{32}P-labeled 8-oxo-GTP (50 pmol) was incubated with or without enzyme(s) at 30 °C for 30 min and then applied to thin-layer chromatography. Lane 1, no enzyme; lane 2, treated with 8 ng of purified MutT protein; lane 3, treated with 0.05 unit of yeast inorganic pyrophosphatase; lane 4, treated with both 8 ng of MutT protein and 0.05 unit of pyrophosphatase.

DISCUSSION

Oxidation of guanine proceeds in the cellular nucleotide pool, and 8-oxo-dGTP, the oxidized form of dGTP, is the mutagenic substrate for DNA synthesis. It can be incorporated opposite adenine or cytosine residues of template DNA, with the result being A:T to C:G and G:C to T:A transversions (*8*, *17*). However, in normally growing cells, the frequency of these types of mutations remains low, owing to the action of enzymes degrading such mutagenic substrates (*9*, *18*). The MutT protein of *E. coli* hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP, thereby preventing misincorporation of 8-oxoGua into DNA (*8*).

Recently, the mammalian counterparts of MutT were identified. These include MTH1 (NUDT1), MTH2 (NUDT15), and NUDT5, all of which carry the 23-residue MutT-related sequence (Nudix box) (*9*−*11*, *19*−*21*). Despite their structural similarity, MTH1/MTH2 and NUDT5 have opposite preferences for substrates; NUDT5 cleaves 8-oxo-dGDP but not 8-oxo-dGTP, whereas MTH1 and MTH2 preferentially degrade 8-oxo-dGTP. Nevertheless, all of these proteins can replace the MutT function; expression of cDNA for these proteins significantly suppressed the high frequency of spontaneous mutation in *E. coli mutT*$^-$ cells.

Considering these facts, it seemed important to know whether the MutT protein of *E. coli* carries an enzyme activity to degrade 8-oxo-dGDP, in addition to its well-characterized 8-oxo-dGTPase activity. The present study was designed to answer this question, and it was revealed that the MutT indeed carries such an activity. The K_m of the MutT enzyme for the hydrolysis of 8-oxo-dGDP is 3000 times lower than that for dGDP, which is the second best substrate among various deoxyribonucleoside diphosphates examined. It is noted that this K_m value for 8-oxo-dGDP (0.058 μM) is comparable to that for 8-oxo-dGTP (0.081 μM).

Another notable feature of the MutT protein is its ability toward 8-oxoGua-containing ribonucleotides. It has been shown that the MutT hydrolyzes 8-oxo-GTP, which is otherwise incorporated in RNA opposite template adenine (*14*). This finding has been substantiated in the present study by comparing the enzymic parameters for normal and oxidized forms of guanosine triphosphate. The K_m values of the MutT protein for 8-oxo-GTP and GTP are 0.26 and 1000, respectively, with the former being 4000 times lower than the latter. Of interest is the finding that this preference of the MutT for oxidized nucleotides can be extended to ribonucleoside diphosphate. The K_m for the hydrolysis of 8-oxo-GDP is much lower than that for 8-oxo-GTP. On the basis of these findings, we can conclude that the MutT protein has an ability to degrade all four forms of 8-oxoGua-containing precursors for both DNA and RNA syntheses.

Figure 5 illustrates a possible mechanism for exclusion of 8-oxoGua-containing nucleotides from the DNA and RNA precursor pools of *E. coli* cells. Enzymic conversion of ribonucleotides to deoxyribonucleotides occurs at the level of nucleoside diphosphate, and ribonucleotide reductase, the enzyme responsible, has relatively broad substrate specificity (*22*). Nevertheless, this enzyme does not catalyze conversion of 8-oxo-GDP to 8-oxo-dGDP (*23*), and it seems that 8-oxoGua-containing deoxyribonucleotides are derived from the normal counterparts in the deoxyribonucleotide pool. 8-Oxo-dGTP and 8-oxo-dGDP, thus formed, are intercon-

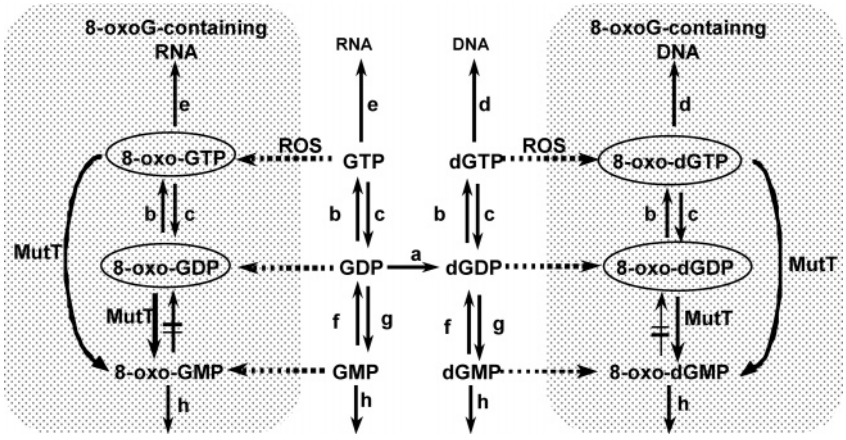

FIGURE 5: Model for exclusion of 8-oxoGua-containing nucleotides from the DNA and RNA precursor pools in *E. coli* cells. 8-OxoGua-containing nucleotides are produced by the action of reactive oxygen species (ROS), as shown by dotted lines, and converted by cellular enzymes. MutT protein degrades both 8-oxo-dGTP and 8-oxo-dGDP to 8-oxo-dGMP, a form unutilizable for DNA synthesis. It also hydrolyzes 8-oxo-GTP and 8-oxo-GDP to 8-oxo-GMP, a form unutilizable for RNA synthesis. a, ribonucleotide reductase; b, nucleoside diphosphate kinase; c, nucleoside triphosphatase; d, DNA polymerase; e, RNA polymerase; f, guanylate kinase; g, nucleoside diphosphatase; h, nucleotidase.

vertible by the actions of nucleoside diphosphate kinase and nucleoside triphosphatase, which do not discriminate 8-oxo-Gua-containing nucleotides from normal ones (*24*). The significance of hydrolysis of 8-oxo-dGDP, which is not the direct substrate for DNA polymerase, can be explained in this model. This situation may be applied to the ribonucleotide counterparts.

Once 8-oxo-dGTP and 8-oxo-GTP are formed, these can be utilized for DNA and RNA syntheses, respectively (*8*, *14*). As shown in the present study, the MutT protein hydrolyzes four types of 8-oxoGua-containing nucleoside diphosphates and triphosphates to the related monophosphates, thereby preventing misincorporation of 8-oxoGua into DNA and RNA. 8-Oxo-GMP and 8-oxo-dGMP, produced by the action of MutT protein, cannot be rephosphorylated, because guanylate kinase, which phosphorylates both GMP and dGMP to corresponding nucleoside diphosphates, is totally inactive for 8-oxoGua-containing nucleotides (*23*, *24*). From the action of a kind of nucleotidase, 8-oxoGua-containing nucleoside monophosphates are further degraded to nucleosides, forming readily excretable to the cell exterior (R. Ito, unpublished data).

In mammalian cells, there are at least three enzymes with different degrees of preference for 8-oxo-dGDP and 8-oxo-dGTP (*9*−*11*, *19*). It is of interest to see if some of these enzymes act on ribonucleotide counterparts, 8-oxo-GDP and 8-oxo-GTP, or whether mammalian cells possess an enzyme(s) specific for 8-oxoGua-containing ribonucleotides. Studies are in progress in this laboratory to answer these questions.

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