MTH1 hydrolyzes oxidized purine nucleoside triphosphates such as 8-oxo-dGTP, 8-oxo-dATP, 2-hydroxy-dATP, and 2-hydroxy rATP to monophosphates, and thus avoids errors caused by their misincorporation during DNA replication or transcription, which may result in carcinogenesis or neurodegeneration. This substrate specificity for oxidized purine nucleoside triphosphates was investigated by mutation analyses based on the sequence comparison with the Escherichia coli homolog, MutT, which hydrolizes only 8-oxo-dGTP and 8-oxo-rGTP but not oxidized forms of dATP or ATP. Neither a replacement of the phosphohydrolase module of MTH1 with that of MutT nor deletions of the C-terminal region of MTH1, which is unique for MTH1, altered the substrate specificity of MTH1. In contrast, the substitution of residues at position Trp-117 and Asp-119 of MTH1, which showed apparent chemical shift perturbations with 8-oxo-dGDP in NMR analyses but are not conserved in MutT, affected the substrate specificity. Trp-117 is essential for MTH1 to recognize both 8-oxo-dGTP and 2-hydroxy-dATP, whereas Asp-119 is only essential for recognizing 2-hydroxy-dATP, thus suggesting that origins of the substrate-binding pockets for MTH1 and MutT are different.

Endogenous oxidation of nucleic acids, such as DNA, RNA, and their precursors by reactive oxygen species generated under normal metabolic conditions, appear to induce spontaneous mutagenesis and cell death, which have been implicated in aging and various diseases including cancer and neuronal degeneration (1). It has been established that 8-oxoguanine (8-oxoG), an oxidized form of guanine, is highly mutagenic and one of the main endogenous sources for spontaneous mutagenesis. During DNA replication, 8-oxo-deoxyguanosine triphosphate (8-oxo-dGTP) can be inserted into the nascent strand opposite adenine and cytosine in the template, with almost equal efficiency, thus resulting in a transition mutation (2, 3). Accumulation of 8-oxoG in double-stranded DNA is caused by direct oxidation of guanine in DNA or incorporation of precursor nucleotide, 8-oxo-dGTP, which is generated in nucleotide pools with oxidation of dGTP (4). A misincorporation of oxidized nucleotides also occurs during transcription, thus leading to an accumulation of abnormal proteins (5, 6).

To eliminate such deleterious oxidized nucleotides, organisms came equipped with elaborate mechanisms. In Escherichia coli the MutT protein hydrolizes 8-oxo-dGTP and 8-oxo-guanosine triphosphate (8-oxo-rGTP) to the monophosphate form, thus avoiding the misincorporation of mutagenic nucleotides into DNA and mRNA during replication and transcription (7, 8). The absence of the mutT gene increases the spontaneous occurrence of A:T to C:G transversion a thousandfold over the wild type level (7, 9), and further increases the occurrence of transcriptional errors (6). Enzymatic activities similar to that of MutT had been identified in both human and other mammalian cells (10–13). Based on the similarity in their amino acid sequences to that of MutT and their catalytic activities hydrolyzing 8-oxo-dGTP, the proteins were named MTH1 (MutT homolog-1) (14). Because the expression of the mammalian enzymes in mutT− E. coli cells suppresses the elevated level of spontaneous mutagenesis to an almost normal level, MTH1 appears to have the same antimutagenic capacity as E. coli MutT (10, 11, 15). In addition to the error avoiding mechanism against 8-oxo-dGTP, the base excision repair enzymes for 8-oxoG in DNA, such as OGG1/MutM and MYH/MutY, are conserved among various organisms from E. coli to mammals (16–18), thus indicating that the mutagenic potential of 8-oxoG is universal throughout living organisms.

Mice lacking the mth1 gene exhibit an increased occurrence of spontaneous carcinogenesis in lung, liver, and stomach (19), thus suggesting that the accumulation of 8-oxo-dGTP, namely a substrate for MTH1, triggers such malignant transformation in vivo. Furthermore, we previously reported that human MTH1 (hMTH1) appears to play a role in protecting neurons from oxidative stress (20, 21).

We also showed that hMTH1, but not E. coli MutT protein, has the ability to hydrolyze oxidized dATP and ATP, such as 2-hydroxy (OH)-dATP, 2-OH-rATP, or 8-oxo-dATP and 8-oxo-rATP, as well as 8-oxo-dGTP and 8-oxo-rGTP. However, oxidized pyrimidine nucleotides, such as 5-hydroxy-deoxycytidine triphosphate and 5-formyl-deoxyuridine triphosphate are not hydrolyzed by hMTH1 (22, 23). Based on the substrate specificity of hMTH1, we designated MTH1 to be an oxidized purine nucleoside triphosphatase (24). The broad spectrum of substrate specificity of MTH1 suggests that not only 8-oxo-dGTP but also oxidized forms of dATP contribute to the increased incidence of spontaneous carcinogenesis in mth1 knockout mice.

To understand the biological significance of oxidized purine...
nucleoside triphosphates in mammals, it is essential to unveil the molecular basis for substrate recognition by MTH1 and the mechanism by which each oxidized nucleotide causes cellular degeneration. In this study, we focused on three functional domains of MutT5.1, namely a phosphohydrolase module in MutT, and the molecular basis for substrate recognition by MTH1 and the nucleoside triphosphates in mammals, it is essential to unveil the molecular basis for its recognition of the two substrates. In this study, we focused on three functional domains of MutT5.1, namely a phosphohydrolase module in MutT, and the molecular basis for substrate recognition by MTH1 and the nucleoside triphosphates in mammals, it is essential to unveil the molecular basis for its recognition of the two substrates.

### EXPERIMENTAL PROCEDURES

#### Plasmids—Vectors harboring mutant hMTH1 cDNA were prepared by recombinant PCR (25). Expression vector for hMTH1 (P27A) was constructed, for example, with the following primers. The first PCR was performed with a template DNA of pET8c:hMTH1, which was previously reported as hMTH1 (26), with two sets of oligonucleotide primers, −1077 promoter and F27Ar and P27Af and SacI.

| Name | Sequence (5’-3’) |
|------|----------------|
| −1077 promoter | CCCCGGAAAATTAAAAAGC |
| +597T terminator | ATAGTTCCTCCTTTCAAGCA |
| F27Af | AACAGAGGCGGCCGCGCCGCGG |
| F27Ar | GCCGCCGCCGCCGCCCTTTTT |
| W117Af | TTTAGAGCACTGGGCGCGACAGCA |
| W117Ar | AGCTGTGCGCGCCGCACTTCTTT |
| W117Yf | TTCAACAGATGCTAGGCGACAGCTA |
| W117Yr | AGCTGTGCGCGCCGCACTTCTTT |
| D119Af | TTCAACAGATGCTAGGCGACAGCTA |
| D119Ar | AGCTGTGCGCGCCGCACTTCTTT |
| D119NY | ACATGTGCGCCGCAACAGGATCAGT |
| D119Nr | ACATGTGCGCCGCAACAGGATCAGT |
| SacI | TGCTGTGCTGATGCGGCAG |
| C5R | CGGAGTGTGTA |
| C4R | GACCGTGTCGTCGGG |
| CAGAAGACATGCATGTCCATGAG |
| SacI | IR |
| C25R, V156Ar, T155Ar, D154Ar, C12R, E152Ar, R151Ar, L150Ar, D156Ar, C16R | CTAGAAGACATGCATGTCCATGAG |
| C25R, V156Ar, T155Ar, D154Ar, C12R, E152Ar, R151Ar, L150Ar, D156Ar, C16R | CTAGAAGACATGCATGTCCATGAG |

#### Recognition of Oxidized Purine Nucleotides by MTH1

Recruitment of hMTH1 and MutT in E. coli strain BL21T, preparation of crude extracts, and purification of recombinant proteins were performed as previously described (26). MutT activities were measured as previously described (22, 28). Briefly, each reaction was carried out in reaction mixtures (10–100 μl), which contained 20 mM Tris-HCl, pH 8.0, 4 mM MgCl₂, 0.4 M NaCl, 80 mM bovine serum albumin, 8 mM diethylenetriamine, and 10% glycerol, and up to 20 μM substrates (8-oxo-dGTP and 2-OH-dATP) with or without positive inhibitors (up to 500 μM). After incubation at 30 °C for 2 min, crude extracts (2–1,000 ng of total protein or 1 μM purified hMTH1 were added and the reaction at 30 °C for 2 to 6 min was terminated by an ice-cold EDTA solution. During the interval established, the reaction rate was constant, and thus the velocity of each reaction represents the initial reaction rate. The entire reaction mixture was injected into a TSK-Gel DEAE-2SW column (Tosoha, Tokyo, Japan) equilibrated with 75 mM sodium phosphate, pH 7.0, and 20% acetonitrile, and the amounts of nucleoside triphosphates and monophosphates were then quantified as previously reported (23). Lineeweaver-Burk plots were drawn from the initial velocity of nucleoside triphosphatase for each mutant hMTH1 protein. The k₅ and V₉₅ values were derived from intercepts of regression lines using least square fittings to the plots.

#### RESULTS

**Phosphohydrolase Module in hMTH1 Is Not Involved in the Differential Recognition of Oxidized Purine Nucleotides**—We previously reported that a chimeric protein hMTH1-Ec, in which its phosphohydrolase module was replaced with that of MutT, has the ability to hydrolyze 8-oxo-dGTP, and thus concluded that phosphohydrolase modules of MutT and hMTH1 proteins are equivalent in structure and function. Furthermore, the saturation mutagenesis of the module in hMTH1 revealed that 14 of the 23 residues constituting the module are essential for its catalytic function (28, 32). Among the 14 essential residues, 9 residues are conserved in MutT, whereas the others are not (Fig. 1, A and B). Considering the fact that the phosphohydrolase module in MutT is involved in substrate binding together with Mg²⁺ ion (35), it is likely that the unique 5 residues for hMTH1 may determine its broad spectrum of substrate specificity.

To address this question, we prepared crude extracts from BL21T (mutT) cells expressing MutT, hMTH1, or hMTH1-Ec, and measured the activities hydrolyzing 2-OH-dATP and 8-oxo-dGTP in each extract (Fig. 1C). 8-Oxo-dGTP was efficiently hydrolyzed to 8-oxo-dGMP in the presence of MutT, whereas 2-OH-dATP was never hydrolyzed in the same reaction condition. In the presence of hMTH1-Ec as well as hMTH1, on the other hand, 2-OH-dATP was more efficiently hydrolyzed to the monophosphate than was 8-oxo-dGTP. Neither 2-OH-
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in gray and MutT. The conserved residues among MTH1 and MutT are compared in the primary structures between mammalian MTH1 and prokaryotic MutT homologs is an additional sequence in C termini of mammalian MTH1 (Fig. 1A). Because 8 of 27 amino acid residues of the unique C-terminal region of hMTH1 are identical to those in C-terminal region of human MYH (hMYH) protein, a DNA glycosylase that excises 2-OH-A in DNA as well as adenine opposite 8-oxog in DNA, it has been suggested that the C-terminal region of hMTH1 is involved in the recognition of 2-OH-dATP (34). To explore its functional significance, we manipulated hMTH1 cDNA to introduce various deletions into the C-terminal region (Fig. 2A). Western blotting analyses of crude extracts prepared from BL21T cells expressing these mutant hMTH1 revealed that relative amounts of mutant proteins recovered in the insoluble fraction significantly increased as the number of residues deleted increased (Fig. 2B). Based on a quantitative Western analysis of the mutant hMTH1 remaining in the soluble fraction (Fig. 2C), we determined the specific activities of 2-OH-dATPase and 8-oxo-dGTPase for each mutant protein (Table II). The specific activities of wild type hMTH1 determined in crude extracts, 302.2 × 10^2 units of 2-OH-dATPase/μg of protein and 308.6 × 10^2 units of 8-oxo-dGTPase/μg of protein, were very close to the values determined with purified hMTH1, 336.2 × 10^2 units/μg and 296.6 × 10^2 units/μg, respectively. Neither 2-OH-dATPase nor 8-oxo-dGTPase activity was detected in the extracts containing ΔC12, ΔC16, or ΔC25-hMTH1. In comparison with wild type hMTH1, ΔC3-hMTH1 retained either comparable or higher levels of the specific activities with both substrates, whereas ΔC4-hMTH1 retained 63 and 68% the levels of those for wild type with 2-OH-dATP and 8-oxo-dGTP, respectively, and those for ΔC5 or ΔC6-hMTH were further decreased to 11 and 18%, or 1.1 and 1.2%, respectively. The ratio of the specific activity of 2-OH-dATPase to that of 8-oxo-dGTPase in ΔC5-hMTH1 was 0.59, and is smaller in yellow. Asterisks indicate 14 amino acid residues of hMTH1 essential for 8-oxo-dGTPase in phosphohydrolase modules (28, 32). The residues conserved between hMTH1 and the C-terminal region of hMYH (amino acid residues of 341–501) are boxed in black lines (34). Amino acid residues of MTH1 with red letters are those in which chemical shift perturbations were observed. Secondary structures of hMTH1 are shown above the sequence (26). αi.2, α-helix 1.2; βα-βG, β-strand A-G. B, secondary structures of MutT, hMTH1-Ec, and hMTH1. In hMTH1-Ec protein, the 23 residues of phosphohydrolase module of hMTH1 (Gly-36 to Gly-58) was replaced by the corresponding region of MutT (Gly-37 to Gly-59) (28). C, 2-OH-dATPase and 8-oxo-dGTPase activities of MutT, hMTH1-Ec, and hMTH1. 10 μl of reaction mixtures containing 20 μM 2-OH-dATP or 8-oxo-dGTP were incubated at 30 °C for 4 min with crude extracts (10 ng of total protein) prepared from BL21T cells expressing MutT, hMTH1-Ec, or hMTH1. The reaction products were analyzed by HPLC as described under “Experimental Procedures.” Separation of products from 2-OH-dATP (gray lines) and 8-oxo-dGTP (black lines) are shown. The arrowhead indicates 8-oxo-dGMP, whereas the asterisks indicate 2-OH-dAMP.

FIG. 1. Phosphohydrolase module in hMTH1 is not involved in the differential recognition of oxidized purine nucleotides. A, comparison of the structures of human (h), mouse (m), and rat (r) MTH1 and MutT. The conserved residues among MTH1 and MutT are shaded in gray. The phosphohydrolase modules of MutT and MTH1 are shaded in yellow. Asterisks indicate 14 amino acid residues of hMTH1 essential for 8-oxo-dGTPase in phosphohydrolase modules (28, 32). The residues conserved between hMTH1 and the C-terminal region of hMYH (amino acid residues of 341–501) are boxed in black lines (34). Amino acid residues of MTH1 with red letters are those in which chemical shift perturbations were observed. Secondary structures of hMTH1 are shown above the sequence (26). αi.2, α-helix 1.2; βα-βG, β-strand A-G. B, secondary structures of MutT, hMTH1-Ec, and hMTH1. In hMTH1-Ec protein, the 23 residues of phosphohydrolase module of hMTH1 (Gly-36 to Gly-58) was replaced by the corresponding region of MutT (Gly-37 to Gly-59) (28). C, 2-OH-dATPase and 8-oxo-dGTPase activities of MutT, hMTH1-Ec, and hMTH1. 10 μl of reaction mixtures containing 20 μM 2-OH-dATP or 8-oxo-dGTP were incubated at 30 °C for 4 min with crude extracts (10 ng of total protein) prepared from BL21T cells expressing MutT, hMTH1-Ec, or hMTH1. The reaction products were analyzed by HPLC as described under “Experimental Procedures.” Separation of products from 2-OH-dATP (gray lines) and 8-oxo-dGTP (black lines) are shown. The arrowhead indicates 8-oxo-dGMP, whereas the asterisks indicate 2-OH-dAMP.
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The C-terminal region of hMTH1 is essential for maintaining its stable conformation. A, primary structures of mutant hMTH1 with various C-terminal deletions. Wt, wild type hMTH1; ΔC3-ΔC25, mutant hMTH1 proteins with various C-terminal deletions from 3 to 25 residues. Amino acid residues constituting α2, βF, and βG are shaded. The amino acid residues conserved between hMTH1 and C-terminal region of hMYH (amino acid residues 477–501) are shown in bold letters (34). The residues replaced by alanine are underlined. B, expression and solubility of mutant hMTH1 proteins. Whole cell extracts (0.1 μg of protein/lane) prepared from BL21T cells expressing wild type and ΔC-hMTH1 (upper panel), and materials recovered in the insoluble fractions (lower panel) were subjected to Western blotting with pAb78B3B, and signal intensities of bands for hMTH1 were quantified and shown (arbitrary units (AU)). Ratio of hMTH1 recovered in the insoluble fraction is shown in parentheses. C, quantification of ΔC-hMTH1 proteins recovered in the soluble fractions. Amounts of mutant hMTH1 recovered in the soluble fractions were determined by Western blotting with pAb78B3B. Lanes 1–4, purified hMTH1 protein (8, 4, 2, and 1 ng/lane); lane 5, wild type; lane 6, ΔC3; lane 7, ΔC4; lane 8, ΔC5; lane 9, ΔC6; lane 10, ΔC12; lane 11, ΔC16; lane 12, ΔC25-hMTH1. Extracts loaded were 50 ng of total protein for lanes 5–8, and 500 ng for lanes 9–12. Amounts of wild type, ΔC3, ΔC4, ΔC5, ΔC6, ΔC12, ΔC16, and ΔC25-hMTH1 proteins in the soluble fractions were estimated to be 65.2, 67.2, 30.4, 18.0, 6.58, 4.16, 1.15, and 1.88 ng/lane, respectively. D, thermostability of 2-OH-dATPase activity of mutant hMTH1. Extracts from cells expressing wild type, ΔC3, ΔC4 or ΔC5-hMTH1 were incubated at 55 or 65 °C for 10 min, and the residual activities of 2-OH-dATPase in heated extracts were determined. The relative activities in comparison to those in the nonheated extracts are shown. E, thermostability of 2-OH-dATPase and 8-oxo-dGTPase in mutant hMTH1 proteins with single amino acid substitutions in the C-terminal region. Residual activities of 2-OH-dATPase (solid lines) or 8-oxo-dGTPase (dotted lines) after 10 min of heat treatments were determined using crude extracts containing wild type (circle), V156A (square), and L150A (triangle) mutant hMTH1. The relative activities to those without heat treatment are shown.

The C-terminal Region of hMTH1 Is Essential for Maintaining Its Stable Conformation—To uncover the essential function of the C-terminal region in hMTH1, we further examined the thermostabilities of the mutants described above. Crude extracts containing wild type, ΔC3, ΔC4, or ΔC5-hMTH1 were incubated at 55 or 65 °C for 10 min, and remaining activities of 2-OH-dATPase in each extracts were determined at 30 °C. As shown in Fig. 2D, wild type hMTH1 retained 94 and 49% of the activity in the untreated extracts after treatment at 55 and 65 °C, respectively. ΔC3-hMTH1 retained the wild type level of residual activity (89%) after treatment at 55 °C, whereas the level decreased to 20% of the control after treatment at 65 °C. The 2-OH-dATPase activities in the extracts containing ΔC4 and ΔC5-hMTH1 decreased to 34 and 21% of the controls by treatment at 55 °C, and to 8.7 and 7.9% by treatment at 65 °C, respectively.

The seven mutants with alanine substitution also exhibited
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| Clone     | Specific activity 2-OH-dATP | Specific activity 8-oxo-dGTP | Ratio  
|-----------|---------------------------|-----------------------------|--------
| Wild type | 302.6 (1.00)              | 308.7 (1.00)                | 0.98   |
| pET8c     | 0.0 (0.00)                | 0.0 (0.00)                  | —      |
| ΔC5       | 311.7 (1.03)              | 369.2 (1.20)                | 0.84   |
| ΔC4       | 191.0 (0.63)              | 210.5 (0.86)                | 0.91   |
| ΔC5       | 31.9 (0.11)               | 54.3 (0.18)                 | 0.59   |
| ΔC6       | 3.4 (0.01)                | 3.6 (0.01)                  | 0.94   |
| ΔC12      | 0.0 (0.00)                | 0.0 (0.00)                  | —      |
| ΔC16      | 0.0 (0.00)                | 0.0 (0.00)                  | —      |
| ΔC25      | 0.0 (0.00)                | 0.0 (0.00)                  | —      |
| V156A     | 281.7 (0.93)              | 269.8 (0.87)                | 1.04   |
| T155A     | 231.9 (0.77)              | 249.1 (0.81)                | 0.93   |
| D154A     | 334.8 (1.11)              | 391.4 (1.27)                | 0.86   |
| V153A     | 236.4 (0.78)              | 179.2 (0.58)                | 1.32   |
| E152A     | 227.0 (0.75)              | 226.8 (0.73)                | 1.00   |
| R151A     | 218.1 (0.72)              | 231.2 (0.75)                | 0.94   |
| L150A     | 169.4 (0.56)              | 148.2 (0.48)                | 1.14   |

Values are shown as (× 10^2 units/μg of MTH1). 1 unit of enzyme activity equals the capability to produce 1 pmol of 8-oxo-dGMP or 2-OH-dAMP per min. Data for each specific activity were reproducible with at least two times of determination.

a Relative ratio of the specific activities hydrolyzing 2-OH-dATP and 8-oxo-dGTP.

b Values in parentheses are the relative activities in comparison to those of wild type protein.

c —, not determined.

Next, the initial velocities of 2-OH-dATP or 8-oxo-dGTP hydrolysis by purified preparations of wild type and mutant hMTH1 were determined in the presence of various concentrations of substrates (up to 20 μM), and Lineweaver-Burk plots were obtained from these data. Under the standard reaction condition with 1 μM enzyme at 30 °C, plots consistent with the Michaelis-Menten type reaction were obtained for all mutants as well as for wild type hMTH1, with 2-OH-dATP and 8-oxo-dGTP (data not shown). The obtained kinetic constants are summarized in Table III. The ratio of k_{cat}/K_m, which represents the relative efficiency of the reaction, was calculated to be 3.30 or 1.71 for 2-OH-dATPase or 8-oxo-dGTPase of wild type hMTH1, respectively, thus confirming that hMTH1 hydrolyzes 2-OH-dATP more efficiently than 8-oxo-dGTP as reported previously (22). The k_{cat}/K_m values for 2-OH-dATPase and 8-oxo-dGTPase of F27A mutant were 1.82 and 0.62, respectively, whose levels are equivalent to 55 and 36% of the values for wild type, thus indicating that its 8-oxo-dGTPase activity was more severely affected by alanine substitution for Phe-27 than was its 2-OH-dATPase. In the case of W117A, ~10% levels of 2-OH-dATPase and 8-oxo-dGTPase activities compared with wild type hMTH1 were detected in the crude extracts, whereas no activity was detected in the purified preparation. Purified D119A-hMTH1 retained an 18% level of the relative efficiency of wild type 8-oxo-dGTPase; however, it had no 2-OH-dATPase activity as seen in the crude extracts.

From these results it is concluded that Phe-27, Trp-117, and Asp-119 are involved in the catalytic functions of hMTH1, in greater or lesser degrees. Specifically, Trp-117 is essential for both the 2-OH-dATPase and 8-oxo-dGTPase activities, whereas Asp-119, on the other hand, is only indispensable for the 2-OH-dATPase activity.

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1 M. Mishima, Y. Sakai, N. Ito, H. Kamiya, M. Furuchi, M. Takahashi, S. Iwai, Y. Nakabeppu, and M. Shirakawa, manuscript in preparation.

2 Y. Sakai, M. Furuchi, H. Kamiya, and Y. Nakabeppu, unpublished results.
the presence of various concentrations of 8-oxo-dGTP (0–8584 M) plotted. Relative activities of 2-OH-dATPase to those without 8-oxo-dGTP were identified, and specific activities for 2-OH-dATPase and 8-oxo-dGTPase were determined from its fluorescence analysis were 0.1 M for 2-OH-dATP under the same conditions, we concluded that 2-OH-dATP has no inhibitory effect on the hydrolysis of 8-oxo-dGTP by the two Asp-119 mutants. The hydrolysis of 2-OH-dATP (10 μM) by wild type hMTH1 was inhibited to 50% of the control in the presence of 17 μM 8-oxo-dGTP, whereas the W117Y mutant efficiently hydrolyzed 2-OH-dATP even in the presence of 100 μM 8-oxo-dGTP and its initial velocity was 35% of the control. The direct measurement of the affinity between nucleoside triphosphates and hMTH1 requires an assay condition in which the binding of the substrate to hMTH1 can be analyzed without undergoing hydrolysis of the substrate. We previously reported that 8-oxo-dGTPase activity was almost completely inhibited in the presence of Cd²⁺ (37). We recently found that the fluorescence emission from the tryptophan residues of hMTH1 protein peaked at 350 nm, thus corresponding to the fluorescence of rather exposed tryptophan residues. In the presence of CdCl₂, the addition of 8-oxo-dGTP to the solution containing hMTH1 decreased the fluorescence to 40% of the control with a slight red shift in the emission maximum, thus indicating the formation of a stable complex and the absence of any significant hydrolysis of the nucleotide. Based on this observation, we developed a method for the quantitative measurement of the affinity of hMTH1 for its substrates. The fluorescence of W117A protein did not change after the addition of 100 μM 8-oxo-dGTP or 2-OH-dATP, thus indicating that the majority of quenched fluorescence was derived from Trp-117.³

The dissociation constants (Kₐ) for wild type hMTH1 determined from its fluorescence analysis were 0.1 μM for 2-OH-dATP and 0.08 μM for 8-oxo-dGTP in the presence of 1 mM CdCl₂ (Table IV), thus indicating that hMTH1 has high affinities to 2-OH-dATP and 8-oxo-dGTP even with Cd²⁺. A mutant protein, F27A, has slightly higher Kₐ values for both 8-oxo-dGTP (0.5 μM) and 2-OH-dATP (0.4 μM) than does wild type, thus indicating that the residue Phe-27 partly contributes the high affinity of hMTH1 to both substrates. The fluorescence of D119A did not change after the addition of 50 μM 2-OH-dATP, whereas its Kₐ for 8-oxo-dGTP was determined to be 0.5 μM. We thus concluded that D119A mutant protein completely lost its affinity toward 2-OH-dATP but still retained its affinity toward 8-oxo-dGTP.

**DISCUSSION**

The major conclusions of the present study are as follows. First, the phosphohydrolase module, a catalytic domain conserved among MutT family proteins, is not involved in the selective recognition of 2-OH-dATP and 8-oxo-dGTP. Second, the C-terminal region of hMTH1, a unique structure conserved among MutT family proteins, plays critical roles. The alteration of the reaction kinetics for mutant hMTH1 shown in Table III, however, reflect the total effect on the sequential steps of the catalytic reaction from the formation of an enzyme-substrate complex to the dissociation of products, thus indicating that our results are not sufficient to conclude that the residues Trp-117 and Asp-119 are indeed involved in the recognition or binding of substrates by hMTH1. To clarify this point, two substrates, 2-OH-dATP and 8-oxo-dGTP, were simultaneously incubated with each mutant protein, and the inhibitory effect of each substrate on the other was determined by measuring its initial velocity for the hydrolysis of the latter. The inhibitory effects of 2-OH-dATP on the hydrolysis of 8-oxo-dGTP by wild type, D119A, and D119N mutants are summarized in Fig. 3B. The initial velocities for the hydrolysis of 8-oxo-dGTP (10 μM) by wild type and F27A decreased to 42 and 44% of the control in the presence of 10 μM 2-OH-dATP, respectively. On the other hand, the 8-oxo-dGTPase activities of D119A and D119N mutants were not altered in the presence of 2-OH-dATP up to 100 μM. Because the 8-oxo-dGTPase activity of wild type hMTH1 decreased to 73% of the control in the presence of 100 μM dATP under the same conditions, we concluded that 2-OH-dATP has no inhibitory effect on the hydrolysis of 8-oxo-dGTP by the two Asp-119 mutants.

**Differential Interaction of Mutant hMTH1 with 2-OH-dATP and 8-Oxo-dGTP**—Tyrosine substitution for residue Trp-117 in hMTH1 selectively abolished the 8-oxo-dGTPase activity, whereas alanine substitution for residue Asp-119 completely abolished only the 2-OH-dATPase activity, thus strongly suggesting that hMTH1 recognizes 2-OH-dATP and 8-oxo-dGTP in a differential manner, in which residues Trp-117 and Asp-119 play critical roles. The alteration of the reaction kinetics for mutant hMTH1 shown in Table III, however, reflect the total effect on the sequential steps of the catalytic reaction from the formation of an enzyme-substrate complex to the dissociation of products, thus indicating that our results are not sufficient to conclude that the residues Trp-117 and Asp-119 are indeed involved in the recognition or binding of substrates by hMTH1.

³ M. Takahashi, F. Maraboeuf, Y. Sakai, H. Yakushiji, M. Mishima, M. Shirakawa, S. Iwai, H. Hayakawa, M. Sekiguchi, and Y. Nakabeppu, manuscript in preparation.
of D119A. It is thus concluded that Asp-119 is the amino acid and 2-OH-dATP does not quench the tryptophanyl fluorescence.

8-oxo-dGTPase activity that is not inhibited by 2-OH-dATP at all, indicating a purine ring by their substrate binding pocket have an electrostatic effect on stacking amino acid residues with aromatic side chains present in a conformationally to the increasing amounts of protein in bound form.

A plane formed by C8-OH, maximize the favorable interaction between the purine ring of 8-oxoG and the indole ring of tryptophan or tyrosine in its binding pocket may be sufficient for hMTH1 to bind to 2-OH-A, whereas the indole ring of tryptophan might serve as an essential structure for its recognition of 8-oxoG. In general, tryptophan and other amino acid residues, especially for 2-OH-dATP and 8-oxo-dGTP.

The residue Trp-117 is essential for the recognition of 8-oxo-dGTP. A plane formed by an aromatic ring of tryptophan or tyrosine in its binding pocket may be sufficient for hMTH1 to bind to 2-OH-A, whereas the indole ring of tryptophan might serve as an essential structure for its recognition of 8-oxoG. In general, tryptophan and other amino acid residues with aromatic side chains present in a substrate binding pocket have an electrostatic effect on stacking a purine ring by their π-π interactions (38). It is likely that the unique structural features of 8-oxoG, N7-H/C8=O or N7/C8-OH, maximize the favorable interaction between the purine and indole rings. In the case of the W117Y mutant, the electrostatic interaction between the purine ring of 8-oxoG and the electron-rich aromatic ring of the tyrosine residue seems to be weak or rather repulsive, thus resulting in a selective loss of interaction with 8-oxo-dGTP.

Fig. 4A depicts a model for the substrate binding pocket of hMTH1 and amino acid residues, which gave rise to apparent perturbations in chemical shifts in the presence of 2-OH-dADP and 8-oxo-dGDP. 1 D119A and D119N mutants retain only the 8-oxo-dGTPase activity that is not inhibited by 2-OH-dATP at all, and 2-OH-dATP does not quench the tryptophanyl fluorescence of D119A. It is thus concluded that Asp-119 is the amino acid residue that is directly involved in the selective recognition of 2-OH-A. The D119N mutant also exhibits the same features as D119A, thus indicating that the carboxyl group of Asp-119 plays

in mammalian MTH1 is essential for preserving its conformational stability. Finally, residues Trp-117 and Asp-119 in hMTH1 are essential for its specific recognition of the substrates, especially for 2-OH-dATP and 8-oxo-dGTP.

Selective Recognition of 2-OH-dATP and 8-Oxo-dGTP by hMTH1—The residue Trp-117 is essential for the recognition of both 2-OH-dATP and 8-oxo-dGTP by hMTH1 and likely contributes to its binding to the two substrates, because the W117A mutant has no catalytic activity for the two substrates but the W117Y mutant retains an intact 2-OH-dATPase activity that is poorly inhibited by 8-oxo-dGTP. A plane formed by an aromatic ring of tryptophan or tyrosine in its binding pocket may be sufficient for hMTH1 to bind to 2-OH-A, whereas the indole ring of tryptophan might serve as an essential structure for its recognition of 8-oxoG. In general, tryptophan and other amino acid residues with aromatic side chains present in a substrate binding pocket have an electrostatic effect on stacking a purine ring by their π-π interactions (38). It is likely that the unique structural features of 8-oxoG, N7-H/C8=O or N7/C8-OH, maximize the favorable interaction between the purine and indole rings. In the case of the W117Y mutant, the electrostatic interaction between the purine ring of 8-oxoG and the electron-rich aromatic ring of the tyrosine residue seems to be weak or rather repulsive, thus resulting in a selective loss of interaction with 8-oxo-dGTP.

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TABLE III

| Protein     | Substrates     | $V_{\text{max}}$ | $K_m$ | $k_{\text{cat}}$ | $k_{\text{cat}}/K_m$ |
|-------------|----------------|-----------------|-------|-----------------|----------------------|
|             |                | $s^{-1}$       | $\mu M$  | $s^{-1}$       | $s^{-1} \cdot \mu M^{-1}$  |
| Wild type   | 2-OH-dATP      | 20.0 (1.0)$^a$ | 8.7 (1.0) | 28.7 (1.0) | 3.30 (1.0)           |
|             | 8-Oxo-dGTP     | 14.5 (1.0)     | 15.1 (1.0) | 25.9 (1.0) | 1.71 (1.0)           |
| F27A        | 2-OH-dATP      | 8.25 (0.41)    | 17.0 (2.0) | 31.0 (1.1) | 1.82 (0.55)          |
|             | 8-Oxo-dGTP     | 3.72 (0.26)    | 40.3 (2.7) | 25.1 (0.97) | 0.62 (0.36)          |
| W117A       | 2-OH-dATP      | 0.00 (0.0)     |       |               |                      |
|             | 8-Oxo-dGTP     | 0.00 (0.0)     |       |               |                      |
| D119A       | 2-OH-dATP      | 3.71 (0.26)    | 30.2 (2.0) | 9.54 (0.37) | 0.31 (0.18)          |
|             | 8-Oxo-dGTP     | 8-Oxo-dGTP     | 8-Oxo-dGTP | 8-Oxo-dGTP | 8-Oxo-dGTP |

$^a$ Data in parentheses represent the relative values of each parameter in comparison to those of wild type hMTH1.

$^b$ —, not determined.
an important role for interaction with 2-OH-A (Fig. 4B).

2-OH-A, also known as isoguanine, a structural isomer of guanine, has an oxygen at C2 which takes a keto (C2=O) or enol (C2-OH) form, and a NH$_2$ group at C6, as shown in Fig. 4B. Usually, more than 90% of 2-OH-A takes the keto form in aqueous solution (39). From a DEAE-2SW column with a buffer of 75 mM sodium phosphate, pH 7.0, 20% acetonitrile 2-OH-dATP was eluted with a shorter retention time than dGTP and 8-oxo-dGTP, thus indicating that 2-OH-A is more positively charged than guanine and 8-oxoG (Fig. 1C). Moreover, it is likely that C2=O or C2-OH in 2-OH-A may not serve as an electron donor because 2-OH-A is electrochemically inert in contrast to 8-oxoG (40). Taking these facts into account, we propose that the second carboxyl group of Asp-119 forms two hydrogen bonds between N1-H and C6-NH$_2$ groups of 2-OH-A, as shown in Fig. 4B. This model is consistent with observations that hMTH1 possesses a weak affinity for dATP, a purine with C6-NH$_2$ and that hMTH1 does not hydrolyze purine nucleoside triphosphates without NH$_2$ groups at the C6 position such as dITP (N1-H, C2-H, C6=O) and dXTP (N1-H, C2=O, N3-H, C6-O). On the other hand, it is likely that the second carboxyl group of Asp-119 may also weakly interact with the C2-NH$_2$ group of 8-oxoG because D119A and D119N mutants showed a lower 8-oxo-dGTPase activity than wild type hMTH1.

Molecular Evolution of MutT Family Proteins—In E. coli MutT, a residue, Asn-119 was previously identified as a residue interacting with the base region of the substrate nucleotide (41). The C6-NH$_2$ group of adenine was reported to cause a chemical shift in the downfield NH proton of the side chain of Asp-119 to move upfield, whereas the dGTP caused a chemical shift of the downfield NH proton to move downfield. It is likely that the side chain NH$_2$ of Asn-119 is involved in substrate recognition by forming a hydrogen bond with C6=O of the guanine ring and by electrostatic repulsion with adenine, in which C6=O is replaced by C6-NH$_2$. As shown in Fig. 1A, the position of Asp-119 in hMTH1 is likely to correspond to that of Asn-119 in MutT in terms of both secondary and tertiary structure, thus suggesting that both play a similar role in the interaction with their substrates. In D119N mutant of hMTH1, acquisition of the side chain NH$_2$ group of Asn-119, which provides a force of repulsion with the C6-NH$_2$ of adenine, resulted in loss of capability to bind 2-OH-A and thus converted the mutant protein to a MutT type enzyme recognizing only 8-oxo-dGTP. This idea supports the proposed interaction between residue Asp-119 in hMTH1 and 2-OH-dATP as shown in Fig. 4B.

Judging from similarities in the amino acid sequences and secondary structures between MutT and hMTH1, phosphohydrolase modules of hMTH1 and MutT, catalytic centers of the proteins, are very likely evolved from the same ancestral molecule (Fig. 1). On the other hand, each binding pocket, surrounded by poorly conserved residues may have different origins. To clarify this point, we replaced the Ala-118 or Asn-119 of MutT by aspartate and examined whether or not the mutants MutT could acquire 2-OH-dATPase activity. However, the A118D, N119D, and even A118D/N119D-MutT mutants retained the 8-oxo-dGTPase activity without acquiring any 2-OH-dATPase activity. These results lead to the conclusion that the structural alteration of substrate binding pocket that enables MutT to recognize 2-OH-dATP cannot be achieved by simply replacing its Ala-118 and Asn-119. The stacking interaction provided by aromatic side chains such as Trp-117 of hMTH1 may thus be necessary for 2-OH-dATPase activity, or the substrate-binding pocket of MutT may have evolved into a conformation highly adapted for 8-oxo-dGTP. This means that the origins of those for hMTH1 and MutT may be different.

Such a difference may explain why MutT has a 10-fold lower $K_m$ value for 8-oxo-dGTP compared with hMTH1 (8). Determining the pocket structure of hMTH1 for binding 2-OH-dATP and 8-oxo-dGTP will provide us with an essential clue for understanding its molecular function and evolution.

Cellular Dysfunction Caused by Oxidized Nucleotides and the Biological Roles of MTH1—It has been shown that the misincorporation potential of 8-oxo-dGTP appears to be very low for some DNA polymerases, such as E. coli DNA polymerase I and II (42), suggesting that the mutator phenotype of mutT strains of E. coli (4) is not related to errors caused by these DNA polymerases (42). Because the replicative DNA polymerase III seems to utilize 8-oxo-dGTP more efficiently than do others (8, 43), it has been suggested that the increased occurrence of spontaneous mutations in mutT-deficient E. coli cells is caused by more efficient incorporation of 8-oxo-dGTP by the DNA polymerase III (42). Hence, we emphasize that MutT or hMTH1 proteins is essential to achieve the high fidelity of DNA replication in vivo. Consistently, in mth1-deficient mice, the incidence of spontaneous carcinogenesis in lung, liver, and stomach increases up to severalfold in comparison to that in wild type mice (19). Furthermore, the increased accumulation of 8-oxo-dG in human cancerous tissues including brain tumors is generally coincidental with the increased expression of hMTH1 protein (44, 45). As well as for carcinogenesis, oxidative damage has been considered to be one of the major causes for neurodegenerative diseases, and we found the regional accumulation of 8-oxo-dG and altered expression of hMTH1 in patients with various neurodegenerative diseases. Most such cases are patients with Parkinson’s disease, in which we found that a significant increase of 8-oxo-dG accumulated in cytoplasm or mitochondria with coincidentally elevated expression of hMTH1 in the substantia nigral neurons (21). The expression levels of hMTH1 at entorhinal cortex have also been reported to be elevated in postmortem tissue specimens from patients with Alzheimer’s disease (20).

It is therefore very likely that oxidative damage to nucleotides or DNA by reactive oxygen species is implicated in the development and progress of cancer, neurodegeneration, or other age-associated diseases. However, the damaged molecules responsible for their molecular etiologies are still largely unknown. There are accumulating data suggesting the implication of 8-oxo-dG or 8-oxo-dGTP in these diseases because of the availability of methods including antibodies for its detection. However, concerning 2-OH-dATP, a major substrate of hMTH1, there are little data suggesting its biological involvement in these diseases, probably because of its difficulty in being detected. Two groups reported that mice lacking ogg1 gene encoding the 8-oxoG DNA glycosylase are not cancer-prone, although an increased accumulation of 8-oxo-dG in their genomic DNA was observed (46, 47). These observations suggest that the increased incidence of carcinogenesis in mth1-deficient mice was caused by the incorporation of 2-OH-dATP into DNA rather than 8-oxo-dGTP.

To evaluate the biological significance of 2-OH-dATP in vivo, we are developing transgenic mice expressing D119A-hMTH1 or W117Y-hMTH1, mutant proteins with either 8-oxo-dGTPase or 2-OH-dATPase activity alone. The establishment of transgenic mice expressing engineered hMTH1 proteins with altered substrate specificity will hopefully shed some light on the molecular etiology of carcinogenesis and neurodegeneration.

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