Structure of Human MTH1, a Nudix Family Hydrolase That Selectively Degrades Oxidized Nucleoside Triphosphates*

Masaki Mishima, a Yusunari Sakai, b Noriyuki Itoh, a Hiroyuki Kamiya, a Masato Furuichi, b Masayuki Takahashi, a Yuiko Yamagata, a Shigenori Iwai, b Yusaku Nakabeppu, b, g and Masahiro Shirakawa b, j

From the aGraduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan, bDivision of Neurogenomic Functions, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Fukuoka 812-8582, Japan, cGraduate School of Pharmaceutical Sciences, Osaka University, 1-3 Machikaneyama, Toyonaka, Osaka 560-8531, Japan, dGraduate School of Integrated Science of Yokohama City University, Suchihiro-cho 1-7-26, Tsurumi, Yokohama 230-0045, and eGradue School of Medical Sciences, RIKEN, 1-7-22 Suchihiro, Tsurumi, Yokohama, Kanagawa 230-0045, Japan

Oxygen radicals generated through normal cellular respiration processes can cause mutations in genomic and mitochondrial DNA. Human MTH1 hydrolyzes oxidized purine nucleoside triphosphates, such as 8-oxo-dGTP and 2-hydroxy-dATP, to monophosphates, thereby preventing the misincorporation of these oxidized nucleotides during replication. Here we present the solution structure of MTH1 solved by multidimensional heteronuclear NMR spectroscopy. The protein adopts a fold similar to that of Escherichia coli MutT, despite the low sequence similarity between these proteins outside the conserved Nudix motif. The substrate-binding pocket of MTH1, deduced from chemical shift perturbation experiments, is located at essentially the same position as in MutT; however, a pocket-forming helix is largely displaced in MTH1 (9 Å) such that the shape of the pocket differs between the two proteins. Detailed analysis of the pocket-forming residues enabled us to identify Asn 93 as one of the key residues in MTH1 for discriminating the oxidized form of purine, and mutation of this residue modifies the substrate specificity. We also show that MTH1 catalyzes hydrolysis of 8-oxo-dGTP through nucleophilic substitution of water at the β-phosphate.

Cellular DNA continually suffers assault from exogenous and endogenous agents that cause a wide variety of DNA modifications. Such modifications are often detrimental to the cell, leading to mutagenesis and carcinogenesis. Numerous enzymes have the important task of maintaining the integrity of DNA. These enzymes are generally well conserved from bacteria to humans.

Oxygen radicals, which are spontaneously generated during normal cellular metabolism or by ionizing radiation or various chemicals, often attack nucleic acids, thereby generating modified bases in DNA (1, 2). Among these modified bases, the most abundant species, 8-oxo-7,8-dihydroguanine (8-oxo-G),1 can pair with both cytosine and adenine with almost equal efficiency and consequently can induce A:T to C:G and G:C to T:A transversion mutations (3–5).

Organisms are equipped with elaborate mechanisms to counter the mutagenic effects of 8-oxo-G. In Escherichia coli, two glycosylases encoded by the mutM and mutY genes function to prevent mutation caused by the presence of 8-oxo-G in DNA. MutM protein removes 8-oxo-G paired with cytosine, whereas MutY protein removes adenine paired with 8-oxo-G (6–9). To prevent further mutation through the presence of 8-oxo-dGTP, MutT hydrolyzes 8-oxo-dGTP to its monophosphate form, thereby preventing the oxidized purine from being misincorporated into genomic DNA. The importance of this enzyme has been underscored by the observation that deficiency of the mutT gene increases the occurrence of A:T to C:G transversion mutations 1000-fold (10, 11). The mechanism concerning the coordinated action of MutM, MutY, and MutT, which constitute the so-called “GO system,” has been well characterized in prokaryotes. Protein factors with enzymatic activities similar to those of MutM, MutY, and MutT have been identified in mammals; however, these enzymes show limited sequence similarity to their prokaryotic counterparts (12, 13).

The mammalian counterpart of MutT, MutT homolog-1 (MTH1), is induced after proliferative activation (14), and is predominantly localized in the cytoplasm and mitochondria (15). Mice lacking the Mth1 gene exhibit an increase in the

* This work was supported in part by grants-in-aid for scientific research (to M. S.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by 21st Century COE Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the Japan Science and Technology Agency (to M. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1IRY) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

To whom correspondence may be addressed. Tel.: 81-92-642-6800; Fax: 81-92-642-6791; E-mail: ysakai@ierc.kyushu-u.ac.jp.

To whom correspondence may be addressed. Tel.: 81-45-508-7213; Fax: 81-45-508-7361; E-mail: shirakawa@tsurumi.yokohama-cu.ac.jp.

1 The abbreviations used are: 8-oxo-G, 8-oxo-7,8-dihydroguanine; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum correlation spectroscopy; r.m.s.d., root mean square deviations; AMPPCP, α,β-methyleneadenosine triphosphate; ADP-ribose pyrophosphatase; 2-OH-dATP, 2-hydroxy-dATP.
occurrence of spontaneous carcinogenesis in liver and, to a lesser extent, in lung and stomach, suggesting that an accumulation of 8-oxo-dGTP, which is a substrate of MTH1, may trigger malignant transformation in vivo (16). The detrimental effect of 8-oxo-dGTP in eukaryotes is also implied from the observation that an increase in the accumulation of 8-oxo-G in DNA, together with an increase in the expression of MTH1, is found not only in human cancer tissue (17, 18) but also in degenerating neurons, as determined by immunohistochemical analyses (19, 20).

MTH1, but not E. coli MutT, can hydrolyze in vitro nucleotide triphosphates containing oxidized adenine such as 2-hydroxy-dATP (2-OH-dATP), 8-oxo-dATP, and 8-oxo-rATP, in addition to those containing oxidized guanines such as 8-oxo-dGTP and 8-oxo-rGTP (21, 22). Furthermore, MTH1 exhibits higher enzymatic activity for 2-OH-dATP than for 8-oxo-dATP, 2-OH-rATP, 8-oxo-dATP, and 8-oxo-tide triphosphates containing oxidized adenine such as 2-hydroxy-1-thio-β-D-galactopyranoside when the absorbance at 660 nm reached 0.45. Cells were harvested 4 h after protein induction and resuspended in 50 mM Tris buffer (pH 7.5) containing 500 mM KCl, 10 mM dithiothreitol, and 1 mM EDTA. The suspension was lysed by sonication and ultracentrifuged, and the resultant supernatant was loaded onto a DEAE-Sephrose column. The flow-through fraction was collected, concentrated by ammonium sulfate saturation, and passed through a phenyl-Sepharose column (Amersham Biosciences). Finally, the protein was purified by passage through a Sephacryl S-100 (Amersham Biosciences) gel filtration column. Protein concentrations were estimated by using the molar absorption coefficients at 280 nm (ε280 = 2.6 × 104 M−1 cm−1).

Sequence Alignment—The primary sequences of human MTH1 and MutT were manually aligned on the basis of the fitted tertiary structures of human MTH1 and MutT (Protein Data Bank code 1MUT), assuming insertions and gaps. The sequence of mouse MTH1 is highly homologous to that of human MTH1 and contains the same number of amino acids. Thus, no alignment process was needed, and the murine sequence was fitted to the human MTH1 sequence with no insertions and gaps. Manual alignment procedures were performed by the BioEdit program (33).

Monitoring Isotope Shifts—The MTH1-catalyzed hydrolysis of 8-oxo-dGTP and the isotope shift of the reaction products were monitored by 31P NMR, essentially using the procedure of Mildvan and co-workers (31). The reaction mixture, containing 1 mM 8-oxo-dGTP, 0.6 μM MTH1, 50 mM Tris buffer (pH 9.0), 10% 2H2O and 0, 24, or 48% 18O2, was incubated at 30 °C. After 120 min, the reaction was stopped with excess EDTA. One-dimensional 31P NMR spectra were obtained at 202.45 MHz on a Bruker DMR 500 spectrometer equipped with a broad band 5-mm probe.

Structure Determination—Purified MTH1 was dissolved in 50 mM potassium phosphate buffer (pH 6.9) containing 20 mM KCl, 0.1 mM EDTA, and 1 mM dithiothreitol in either 95% H2O, 5% 2H2O or 99.8% 18O2. The final concentration of the protein was adjusted to 1.7 mM and placed in 5-mm diameter micro-NMR cells (Shigemi, Inc.) for multidimensional NMR spectroscopy.

NMR spectra were acquired at 30 °C on a Bruker DMR500, DRX600, or DRX800 NMR spectrometer. Chemical shifts were referenced to 4,4-dimethyl-4-silapentane-1-sulfonate. All multidimensional NMR spectra were acquired in a phase-sensitive mode employing a States-TPP1 or Rance-Kay manner (34). The water flip-back method (35) was employed in several experiments, starting from amide proton magnetization. Lorentz-to-Gauss transformations or shifted sine-bell window functions were applied to the NMR data before zero-filling and Fourier transformation. Mirror image or forward-backward linear prediction was used in indirect time domains. All spectra were processed by the NMRPipe package (36), and analyzed by Pipp (36) and Sparky (37). 13C and 15N were obtained from standard multidimensional NMR methods (38). The isotopologues of all leucine and valine residues were assigned in a stereospecific manner from the HSQC spectrum of a 15% 13C-enriched sample (39). Hydrogen bond restraints were derived from correlations through 1H→15N, couplings (40, 41), which were observed in TROSY-HNCO experiments. Well-dispersed side-chain carbonyl 13C resonances of glutamate and aspartate residues were assigned by an H/C/CO2 experiment (42). Interproton distances were derived from two-dimensional NOESY, 15N-NOESY-HSQC, four-dimensional 13C/15N-edited HMBC-NOE-NOESY-HSQC, and four-dimensional 13C/15C/13C/15C-edited HMCO-NOE-NOESY-HSQC (38). NOE were grouped into three upper distance ranges, 2.8, 3.5, and 5.0 (3.2, 4.2, and 5.0) Å for NOEs involving NH protons, corresponding to strong, medium, and weak peak intensities. NOE intensities were calibrated by using known distances in regular secondary structure elements, and standard pseudo-atom distance corrections were applied to upper bound constraints involving methyl and methylene protons. Aromatic ring protons were represented as a (2r−6)−15 1H sum (43).
Backbone dihedral angles were estimated from vicinal coupling constants \( \{\delta_{2J_{NN}}, \delta_{3J_{NN}}\} \) obtained from HNHA experiments (58). The dihedral \( \phi \) and \( \psi \) angles derived from TALOS (44) were used in a final refinement step. If the deviation predicted by TALOS was less than 20°, the value was set to 20°. The program DYNAMA version 1.5 (45) was used at the stage of structural constraint collection. Finally, an ensemble of 100 MTH1 structures was calculated with the program CNS version 1.0 (46). The calculated structures were analyzed by PROCHECK-NMR (47) and MOLMOL (48), and the graphics were created by MOLMOL, Ribbons (49), or GRASP (50).

Chemical Shift Perturbation—H and \( ^{13}N \) amide resonance changes in uniformly \( ^{15}N \)-labeled MTH1 were monitored after the addition of an equimolar amount of 8-oxo-dGDP and 2-Oh-dADP to 20 mM HEPES buffer (pH 6.8) containing 20 mM KCl, 0.1 mM protein, 1 mM EDTA, 1 mM dithiothreitol, and 5% \( \text{H}_2\text{O} \). The \( ^{15}N^{2}H \) HSQC spectra were obtained at 30 °C. Changes in signal intensities and chemical shifts were analyzed by Sparky (37). For each cross-peak, the normalized weight average shift difference, \( \delta_{\text{av}}/\delta_{\text{max}} \), was calculated (51, 52). The weighted average shift difference, \( \delta_{\text{av}} \), was calculated as \( (\delta_{15N} + (\delta_{12C})^2)/2; \) where \( \delta_{15N} \) and \( \delta_{12C} \) represent the differences in ppm between the free and perturbed chemical shifts. The \( \delta_{\text{av}} \) value represents the maximum observed weighted average shift difference. Changes in signal intensity were also evaluated by the ratio of the intensity differences caused by perturbation and the reference spectrum (\( I_{\text{ref}} - I_{\text{mut}} \))/\( I_{\text{ref}} \), where \( I_{\text{ref}} \) and \( I_{\text{mut}} \) represent the signal intensities in the reference and perturbed spectrum, respectively.

RESULTS

Structure Determination of MTH1—Recombinant MTH1 was overexpressed in \( \text{E. coli} \), isotopically labeled, and purified by chromatography on hydrophobic and gel filtration columns. The elution profile derived from gel filtration chromatography suggests that MTH1 exits as a monomer when purified to homogeneity (data not shown). The \( ^{15}N^{2}H \) HSQC spectrum of MTH1 gave a highly dispersed pattern of cross-peaks, suggesting that the whole protein molecule adopts a stable tertiary conformation in solution (data not shown).

NMR resonance assignments were obtained by measuring the double and triple resonance NMR spectra of \( ^{15}N \)- and \( ^{15}N,^{13}C \)-labeled protein samples. Assignments for all of the main-chain resonances, except for those of Met1, were obtained from the CBCANH, CBCA(CO)NH, HN(CA)CO, and HNCO spectra. Side-chain assignments were essentially obtained from C(CO)NH, HC(CO)NH, HCCH-TOSY, and four-dimensional HC(CO)NH spectra. By combining the three-dimensional HCCH-TOSY spectra with the four-dimensional HC(CO)NH spectra, we obtained far less ambiguous correlations between side-chain \( ^{13}C \) and \( ^{2}H \) nuclei, which led to reliable side-chain assignments. Most of the aromatic \( ^{1}H \) resonances were obtained from two-dimensional NOESY and \( ^{13}C \)-edited NOESY spectra.

The structure of MTH1 was determined from more than 2100 distance and torsion angle constraints, including those for 46 hydrogen bonds that were directly detected through \( ^{3}\text{J}_{\text{HCNC}} \) couplings across the bonds in the TROSY-HNCO spectrum. Of these hydrogen bonds, 32 were identified in regions of regular secondary structure, such as antiparallel and parallel \( \beta \)-sheets and \( \alpha \)-helices, and 14 were found in regions with no secondary structure. This direct observation of hydrogen bonds was extremely useful in the initial steps of structure determination, because it enabled us to establish unambiguously the topology of the secondary structure, in particular that of the \( \beta \)-strands. Furthermore, it was also helpful for identifying irregular patterns of hydrogen bonding in the \( \beta \)-sheet regions, which are generally difficult to determine by other NMR techniques, such as measuring the rate of amide proton exchange with solvent or NOE patterns (see below). The backbone of the final 30 structures derived from the NMR data (Fig. 1A) shows that the atomic coordinates throughout the protein molecule are well defined except for the N- and C-terminal residues. The root mean square deviations (r.m.s.d.) for the averaged structure of the well defined region were 0.44 Å for the backbone and 0.99 Å for all heavy atoms (residues 5–153). The statistics of the structures are shown in Table I.

Structure of MTH1—MTH1 adopts an \( \alpha+\beta \) fold with four long loops consisting of seven \( \beta \)-strands and two \( \alpha \)-helices as follows: \( \beta\beta\alpha \) (residues 5–11), \( \beta\beta\beta\beta \) (67–74), \( \beta\delta\beta\beta \) (80–87), \( \beta\delta\beta\delta \) (125–139), \( \alpha\delta \) (44–58), and \( \alpha\beta \) (120–130) (Fig. 1B and Fig. 2). The main frame of the fold is a curled mixed \( \beta \)-sheet made up of five strands. Whereas most of the residues on the convex side of the sheet form a continuous solvent-accessible surface, those on the concave side are packed against helix \( \alpha\delta \), loop 4 (L4) linking \( \alpha\delta \) and \( \beta\delta \), and loop 1 (L1) linking \( \alpha\beta \) and \( \beta\delta \), all of which form the main body of the protein. In addition, a lobe made up of helix \( \alpha\delta \) and an antiparallel two-stranded \( \beta \)-sheet is attached to the main body at strand \( \alpha\beta \), which forms the edge of the mixed \( \beta \)-sheet. As a consequence, a deep and narrow pocket is formed between this additional lobe and the main body of the protein; this pocket is surrounded by walls comprising helix \( \alpha\delta \), loop L1, and the mixed sheet (Fig. 1B and Fig. 2).

The hydrogen bonds directly observed through \( ^{3}\text{J}_{\text{HCNC}} \) couplings across the bonds reveal the presence of a \( \beta \)-bulge between strands C and D in the core \( \beta \)-sheet. In the TROSY-HNCO spectrum used to detect these \( ^{3}\text{J}_{\text{HCNC}} \) couplings, a correlation was observed between the carbonyl group of Val65 and the amide group of Val67, whereas the amide group of Gly68 showed no detectable correlations. These observations indicate that a hydrogen bond is formed between Val65 and Val67, establishing a small \( \beta \)-bulge structure with a bend at the main-chain position of Gly68 (Fig. 1C).

The Nudix motif, which is located at residues 37–59, is composed of an amphipathic helix of about three turns (\( \alpha1 \)) and a preceding loop (L1) that adopts a well defined hairpin-like structure (Fig. 1B and Fig. 3). This loop contains a type II \( \beta \)-turn formed by residues 40–43, which is characterized by positive \( \psi \) and \( \phi \) angles of Gly42 (\( \phi = 108 \pm 32^\circ \) and \( \psi = 43 \pm 13^\circ \)), and is stabilized by a hydrogen bond formed between the main-chain amide group of Gln40 and the side-chain carboxyl group of Glu43. Both Glu43 and Gly42 are conserved in all members of the Nudix enzyme family, suggesting the importance of this \( \beta \)-turn. Notably, an \( ^{3}\text{J}_{\text{HCNC}} \) coupling across this hydrogen bond was detected in the TROSY-HNCO experiment, which unambiguously confirms its presence.

Structural Comparison of MTH1 and MutT—Although the sequence identity between human MTH1 and \( \text{E. coli} \) MutT is as low as 9.3% outside the Nudix motif (residues 37–59 of MTH1), the overall folds of these proteins resemble one another (Fig. 4A). In particular, the central part of MTH1, comprising \( \beta \)-strands \( \beta\alpha \), \( \beta\delta \), and \( \beta\delta \) and the Nudix helix (\( \alpha1 \)), shows high structural similarity to the corresponding part of MutT. This is reflected by the r.m.s.d. between MTH1 and MutT (PDB code 1MUT) of 1.8 Å over 39 Ca coordinates for residues in these regions (MTH1 residues 4–12, 43–58, 67–72, and 80–87).

The largest difference between the structures of MTH1 and MutT is the presence of a \( \beta \)-hairpin comprising strands \( \beta\delta \) and \( \beta\delta \) and their connecting loop in MTH1, which is absent in MutT (Fig. 4A). Strand \( \beta\delta \) is connected to \( \beta\delta \) through a main-chain hydrogen bond network, resulting in the formation of a continuous five-stranded \( \beta \)-sheet made up of strands \( \beta\delta \), \( \beta\delta \), \( \beta\delta \), and \( \beta\delta \). Given that residues from the additional \( \beta \)-hairpin make extensive hydrophobic contacts with those from helix \( \alpha\delta \), the orientation of this helix and the N-terminal half of loop L1 relative to strands \( \beta\delta \), \( \beta\delta \), and \( \beta\delta \) in MTH1 differs from the orientation of the corresponding regions in MutT. The helix is
displaced by $-9 \, \text{Å}$ at the position of the N-terminal residue of helix $\alpha$II. Previous analyses have shown that deleting the additional strands $\beta F$ and $\beta G$ totally abolishes both the 2-OH-dATPase and the 8-oxo-dGTPase activity of MTH1 (53).

Chemical Shift Perturbation Experiments—To identify the substrate-binding site of MTH1, we carried out chemical shift perturbation experiments in which we monitored the cross-peaks of the $^{15}\text{N}-^{1}\text{H}$ HSQC NMR spectrum by using $^{15}\text{N}$-labeled MTH1. As substrate analogs, we used 8-oxo-dGDP and 2-OH-dADP, which act as specific inhibitors of the 8-oxo-dGTPase and 2-OH-dATPase activity with $K_i$ values of 0.51 and 0.22 $\mu\text{M}$, respectively (22). Hydrolysis of these analogs by MTH1 is extremely slow, especially in the absence of magnesium ions (22). We therefore performed the chemical shift perturbation experiments using these oxidized diphosphates in the absence of magnesium so that the substrate-enzyme complex would be stable during the measurements.

Upon mixing MTH1 with 8-oxo-dGDP, large broadening and/or relatively small changes in chemical shift were observed for some signals in the $^{15}\text{N}-^{1}\text{H}$ HSQC spectrum (Fig. 5A). Most of the signals exhibited intermediate exchange on the NMR time scale during the titration. After mixing in an equimolar amount of substrate, the addition of more 8-oxo-dGDP caused no further changes in the HSQC spectra, suggesting that the complex is formed at a 1:1 molar ratio (data not shown). The observed spectral changes were specific to 8-oxo-dGDP because titration with dGTP and dGDP caused no detectable changes in the spectra (data not shown). Most of the residues that showed a large normalized weighted average shift difference ($\delta_{\text{ave}}/\delta_{\text{max}} > 0.4$) or broadening ($I_{\text{ref}} - I_{\text{per}}/I_{\text{ref}} > 0.4$ of the original values) upon titration were confined to the inside or rim of a pocket that is formed between the five-stranded mixed sheet, helix $\alpha$II, and the hairpin loop (Fig. 5B), suggesting that this pocket may be the nucleotide-binding site. The inner surface of the pocket is dominated by hydrophobic residues, most signals of which in the $^{15}\text{N}-^{1}\text{H}$ HSQC spectrum were affected by nucleotide binding (Fig. 5B and Fig. 6A).

Chemical shift perturbation experiments were also performed in the absence of magnesium ions for the complex formed between 2-OH-dADP and MTH1. As observed for the
Table I

| Total number of distance constraints (NOE) | Bond length (Å) | Bond angle (°) | Improper torsion (°) | CNS potential energy (E_total) | PROCHECK Ramachandran plot (5–153) |
|------------------------------------------|-----------------|---------------|---------------------|-------------------------------|----------------------------------|
| 2135                                     | 0.99 Å          | 1.9%          | 1.9%                | 0.276 \(\text{cal} \)         | Residues in most favored regions: 74.3%         |
|                                          |                 |               |                     |                               | Residues in additional allowed regions: 23.0%         |
|                                          |                 |               |                     |                               | Residues in generously allowed regions: 2.61%         |
|                                          |                 |               |                     |                               | Residues in disallowed regions: 0.05%                |

8-oxo-dGDP titration, residues in the hydrophobic pocket showed a large, normalized, weighted average shift difference \(\delta_{\text{ave}}/\delta_{\text{max}} > 0.4\) or signal broadening \(\delta_{\text{ave}} = \delta_{\text{max}} > 0.4\) of the original values. This result suggests that 2-OH-dADP binds to the same pocket that 8-oxo-dGDP uses (Fig. 5B and Fig. 6A). More residues around the pocket exhibited signal perturbations after the addition of 2-OH-dADP than after that of 8-oxo-dGDP, suggesting that local conformation changes take place in the proximity of the pocket. It may be possible that the higher affinity of 2-OH-dADP, as compared with 8-oxo-dGDP, may cause larger structural effects on binding. Such a conformational change induced by substrate binding is observed in the pocket of MutT on the binding of 8-oxo-dGMP (54); helix I of MutT moves 4.5 Å toward the Nudix motif such that the pocket becomes narrower.

The nucleotide-binding pocket in MTH1 suggested by the chemical shift perturbation experiments is juxtaposed to exposed residues from the Nudix motif, such as Arg51, Glu52, Glu55, and Glu56 (Fig. 5B and Fig. 6A). MutT has a hydrophobic cleft at the same position, which has been shown to serve as the base-binding site in structures of its complex with a nonhydrolyzable analog, AMPCPP, and with 8-oxo-dGMP (54), a product of the hydrolysis reaction (Fig. 6C). Our chemical shift perturbation experiments and the structural similarity of MTH1 and MutT suggest that the pocket of MTH1 acts as the binding site for the base moiety of ligand nucleotides. In contrast, the Nudix motif of MTH1 probably binds to phosphate groups of the substrate indirectly via coordinated metals, as seen in the MutT-AMPCPP complex (55). The position of the Nudix motif relative to the nucleotide-binding pocket is similar in MutT and MTH1.

Although MTH1 and MutT seem to possess the base-binding pocket at the same position on the protein surface, their shapes are dissimilar, as shown in Fig. 6, A and B. The pocket in MTH1 is much deeper and narrower because of the presence in part of the pocket wall defined by residues Phe27, Asn33, Trp117, and Asp119 from loop L1 or L4 (Fig. 6, A and B). These residues on one side form the pocket on the other side and make contact with residues from the side chain of Arg51, Asp52, Glu55, and Glu56, which form a hydrogen bond that is important for the hairpin structure (see text), are shown in green.
Determination of the Site of Nucleophilic Attack on the Triphosphate—MTH1-mediated cleavage of the substrate Pa–Pβ bond is initiated by nucleophilic attack by water on either the α-phosphate (Pa) (Fig. 7A, scheme 1) or the β-phosphate (Pβ) (scheme 2). In order to determine which phosphate is attacked by water, we employed the isotope-labeling method of Mildvan and co-workers (29, 31). In this experiment, hydrolysis reactions are carried out in the presence of H2O enriched with 18O. Given that an oxygen atom of the water-attacked phosphate is substituted by that of water, the phosphate group of either 8-oxo-dGMP or pyrophosphate will be labeled with 18O, depending on whether Pa or Pβ is attacked by water. On the one hand, if nucleophilic substitution by water occurs at the Pa site, 18O-labeled 8-oxo-dGMP will be generated. On the other hand, if nucleophilic substitution occurs at the Pβ site, 18O-labeled pyrophosphate will be generated. The compound that is labeled can be easily determined from 31P NMR spectra of the reaction mixture, because the phosphorus attached to 18O will exhibit a chemical shift difference because of the isotope effect.

The 31P spectra of reaction mixtures are shown in Fig. 7, A–C. In the absence of 18O-enriched water, the proton-decoupled 31P NMR of the mixture showed two signals at about 2.52 and −7.75 ppm, which were attributable to the phosphates of 8-oxo-dGMP and pyrophosphate, respectively (Fig. 7B). When the reaction was carried out in the presence of 48% H218O, a new peak appeared 0.012 ppm up field from the signal at −7.75 ppm (Fig. 7C). This chemical shift difference of 0.012 ppm is very similar to previously observed isotope effects caused by the incorporation of 18O into phosphate (31). Therefore, it can be concluded that the new signal is attributable to 18O-labeled pyrophosphate, and thus that Pβ of the substrate is attacked by water during the cleavage reaction. This cleavage site was further confirmed by carrying out the reaction in the presence of 24% H218O, where the intensity of the new signal decreased to nearly half of the value observed for the reaction in the presence of 48% H218O (Fig. 7D).

**DISCUSSION**

The Nudix Motif—Saturation mutagenesis study has shown that 14 of the 23 conserved residues in the Nudix motif (residues 39, 41, 43, 45, 48, 51, 52, 53, 55, 56, and 57) are essential for catalysis by MTH1 (56). Of these, Glu52, Glu55, and Glu56 form an acidic cluster on the Nudix helix in our structure determination of MTH1 (Fig. 6A). These residues probably function to coordinate the catalytic metal ion, because the same residues in two other Nudix enzymes, MutT (55) and ADP-
ribose pyrophosphatase (ADPRase) (57, 58), have been shown to bind the catalytic divalent metal. Two more acidic residues, Glu100 and Asp99, are located within the vicinity of these acidic residues (Fig. 3). Our preliminary mutational analyses have shown that replacing Glu100 with alanine causes a total loss of catalytic activity, whereas a mutant in which Asp99 is replaced with alanine retains 91 and 79% of the wild-type activity for the hydrolysis of 2-OH-dATP and 8-oxo-dGTP, respectively, indicating that Glu 100, but not Asp 99, is critical for catalytic activity. Structural determinations have shown that Glu 100 of E. coli ADPRase, Glu98 of MutT (55), and Glu142 of Mycobacterium tuberculosis ADPRase (58) are metal-coordinating residues located outside the Nudix motif. In relation to the metal-coordinating Nudix residues, the structural position of these residues is similar to that of Glu100 of MTH1. These observations suggest that Glu 100 of MTH1 functions as a metal-coordinating residue.

The moderately conserved hydrophobic residues in the Nudix motif correspond to residues Ala49, Leu53, and Leu59 of MTH1, which are located on the inner side of the helix, opposite the acidic metal-coordinating residues. These residues are important for packing the Nudix helix against the β-sheet and contribute to a hydrophobic core involving Thr8, Val10, and Val12 from strand βA, Phe86 from strand βD, and Phe35 from loop L1 (Fig. 3). Saturated mutagenesis study has shown that...
the β-sheet is further stabilized by hydrophobic contact between Leu$^{39}$ located at the C terminus of the Nudix motif and both Leu$^{19}$ from strand ββ and Val$^{12}$ from the C-terminal stretch of strand βA (Fig. 3).

In addition to the helix, the conformation of the preceding loop in the Nudix motif, located at residues 37–43, seems to be important for catalytic activity. This loop contains a β-turn at Gly$^{42}$, which is stabilized by a hydrogen bond formed between the side-chain carboxyl group of Glu$^{43}$ and the main-chain amide group of Gln$^{40}$. Both Gly$^{42}$ and Gln$^{43}$ are highly conserved among Nudix enzymes. Glu$^{43}$ has been shown to be essential in mutagenesis studies (57, 60). This residue is unlikely to bind the metal ion, as shown by previous structural studies of MutT-AMPCPP (55), E. coli ADPase (57), and M. tuberculosis ADPase (58); therefore, the hydrogen bond mediated by this residue is probably important for catalytic activity. The importance of Gly$^{42}$ has also been confirmed by mutagenesis. Replacement with either proline or threonine causes a reduction in the 8-oxo-dGTP-specific activity (to 32% of the wild-type activity) (56). The same mutagenesis study also highlighted the importance of other loop-forming residues, Val$^{39}$ and Ile$^{45}$, which cannot be replaced by another residue without loss of enzyme activity (56). These two residues make hydrophobic contact with each other, defining the β-hairpin conformation of the loop together with the β-turn-forming residues (Fig. 3).

The N-terminal residues of the hairpin loop in the Nudix motif, residues 37–40, create part of the rim of the hydrophobic pocket. Of these, Gly$^{37}$ is conserved across all Nudix enzymes and is essential for catalytic activity as it cannot be replaced without loss of activity (57). The Hα1 proton of this residue is in close proximity to the side chain of the metal-coordinating residue Glu$^{52}$ (Fig. 3). Therefore, replacement of Gly$^{37}$ with another amino acid probably alters the local structure of the catalytic site residues, thereby leading to a loss of enzyme activity.

Important Residues for Recognition of the Oxidized Bases—Previous mutational analyses suggest that two of the pocket-forming residues of MTH1, Trp$^{117}$ and Asp$^{119}$, contribute to substrate recognition (53). Replacement of Trp$^{117}$ with alanine significantly increases the $K_{m}$ for both 8-oxo-dGTP and 2-OHdATP (53). The involvement of this tryptophan residue has also been implied from fluorescence studies, which show that binding of 8-oxo-dGTP or 2-OH-dATP alters the intensity and wavelength of the fluorescence attributed to this tryptophan, indicating that it is located proximal to the binding site (59). The decrease in the fluorescence intensity of Trp$^{117}$ induced by 8-oxo-dGTP or 2-OH-dATP is larger than that induced by unoxidized nucleotides. In contrast to mutation of Trp$^{117}$, replacing Asp$^{119}$ with alanine exhibits different effects on the catalysis of 8-oxo-dGTP and 2-OH-dATP (53). The D119A mutant has about half of the wild-type activity for 8-oxo-dGTP but shows almost no activity for 2-OH-dATP, and the D119N mutant also has similar activities with the D119A, suggesting that the charged carboxyl group (COO$^{-}$) of the side chain but not C=O group is crucial for discriminating the 2-hydroxyadenine ring.

In addition to these key residues identified through previous mutagenesis, our structure determination suggested that Asn$^{33}$ is important for nucleotide binding because its side chain is exposed in the pocket (Fig. 6A). To examine whether Asn$^{33}$ is involved in nucleotide interactions, we replaced it with alanine or glutamate. The N33A mutation decreased catalytic activity toward 2-OH-dATP to 5% of the wild-type activity, whereas the N33E mutant showed a relative activity of 53%, suggesting that the presence of the side-chain C=O group at this position...
is also important for recognizing 2-OH-dATP. Intriguingly, the N33A mutant showed 14% of the wild-type 8-oxo-dGTP activity, whereas the N33E mutation totally abolished 8-oxo-dGTPase activity. The detrimental effect of this mutation on 8-oxo-dGTPase activity may be explained by a significant steric clash between the carboxyl group of the glutamate and 8-oxo-dGTP and charge repulsion between the carboxyl group of the glutamate.

In summary, these structural and mutagenesis studies suggest that the side chains of Asp119 and Asn33 play an essential role in 2-OH-dATP recognition, whereas the indole ring of Trp117 may be important for interacting with the purine bases of substrate nucleotides.

Conclusion—A characteristic feature of MTH1 is its dual specificity for nucleotides that contain 2-OH-adenine bases and those that contain 8-oxo-guanine bases, whose oxidation sites are located at the opposite side of the purine rings to each other. Together with previous mutagenesis studies, our present study has enabled us to identify the 2-OH-dATP- and 8-oxo-dGTP-binding pocket of MTH1. On the basis of the structure determined, we performed additional mutagenesis studies that revealed that Asn33 also plays important role in 2-OH-dATP recognition.

To our knowledge, this is the first report of the tertiary structure of an enzyme that is known to recognize oxidized adenine. Although more than 130 proteins that contain a Nudix motif have been identified thus far (30), detailed substrate interactions have been well characterized for only a few of these enzymes. Our work has shown that MTH1 is similar to, but distinct from, E. coli MutT, with respect to the structure of the substrate-binding pocket. Our study may provide a structural basis for investigating substrates of the remaining orphan Nudix enzymes, as well as substrates of proteins that recognize oxidized nucleotides.
Structure of Human MTH1, a Nudix Family Hydrolase That Selectively Degrades Oxidized Purine Nucleoside Triphosphates
Masaki Mishima, Yasunari Sakai, Noriyuki Itoh, Hiroyuki Kamiya, Masato Furuichi, Masayuki Takahashi, Yuriko Yamagata, Shigenori Iwai, Yusaku Nakabeppu and Masahiro Shirakawa

J. Biol. Chem. 2004, 279:33806-33815.
doi: 10.1074/jbc.M402393200 originally published online May 7, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402393200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 58 references, 21 of which can be accessed free at http://www.jbc.org/content/279/32/33806.full.html#ref-list-1