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Functional significance of conserved residues in the phosphohydrolase module of *Escherichia coli* MutT protein

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ABSTRACT

***Escherichia coli* MutT protein hydrolyzes 8-oxo-7,8-dihydro-2′-dGTP (8-oxo-dGTP) to the monophosphate, thus avoiding the incorporation of 8-oxo-7,8-dihydroguanine (8-oxo-G) into nascent DNA. Bacterial and mammalian homologs of MutT protein share the phosphohydrolase module (MutT: Gly37→Gly59). By saturation mutagenesis of conserved residues in the MutT module, four of the 10 conserved residues (Gly37, Gly38, Glu53 and Glu57) were revealed to be essential to suppress spontaneous A:T→C:G transversion mutation in a *mutT*⁻ mutator strain. For the other six residues (Lys39, Glu44, Thr45, Arg52, Glu56 and Gly59), many positive mutants which can suppress the spontaneous mutation were obtained; however, all of the positive mutants for Glu44 and Arg52 either partially or inefficiently suppressed the mutation, indicating that these two residues are also important for MutT function. Several positive mutants for Lys39, Thr45, Glu56 and Gly59 efficiently decreased the elevated spontaneous mutation rate, as seen with the wild-type, hence, these four residues are non-essential for MutT function. As Lys38 and Glu55 in human MTH1, corresponding to the non-essential residues Lys39 and Glu56 in MutT, could not be replaced by any other residue without loss of function, different structural features between the two modules of MTH1 and MutT proteins are evident.**

INTRODUCTION

The *mutT* mutant of *Escherichia coli* is a well characterized mutator mutant with an increased spontaneous occurrence of unidirectional A:T→C:G transversion mutation (1–4). MutT protein catalyzes hydrolysis of an oxidized form of dGTP, 8-oxo-7,8-dihydro-2′-dGTP (8-oxo-dGTP) to the monophosphate form (5). The α subunit of *E.coli DNA* polymerase III inserts 8-oxo-dGTP into the nascent strand opposite adenine in the template, the efficiency being almost equal that of cytosine. During the next round of replication the polymerase may insert dCTP opposite previously incorporated 8-oxo-7,8-dihydroguanine (8-oxo-G), the result being A:T→C:G transversion mutation after another round of replication (5–7).

Lack of the *mutT* gene increases occurrence not only of this type of transversion mutation in the *E.coli* genome 1000-fold over the wild-type level (1–4), it also causes transcriptional errors, an event known as non-genomic mutation (8). MutT protein hydrolyzes 8-oxo-GTP, an oxidized form of a ribonucleotide GTP which can be inserted into transcripts by RNA polymerase (8). These findings indicate that a significant level of oxidation of dGTP/GTP occurs in *E.coli* cells and that MutT eliminates mutagenic nucleotides from nucleotide pools. Thus, MutT protein functions as a guardian for both DNA and RNA synthesis.

Genes for MutT homolog proteins with dGTPase or 8-oxo-dGTPase activity were identified in *Proteus vulgaris* and *Streptococcus pneumoniae,* bacteria distantly related to *E.coli* (9,10). All three MutT homolog proteins have a molecular mass ranging from 13 to 18 kDa, and the degree of identity ranges from 19 to 40%. Most of the identical residues are in a region corresponding to the 23-residue sequence from Gly37 to Gly59 of *E.coli* MutT (11). Structural analyses of the *E.coli* MutT protein revealed that the region containing this 23-residue sequence constitutes the active center of dGTPase, with a loop and an α-helix structure (12,13).

Mammalian cells also possess 8-oxo-dGTPase encoded by the *mutT* homolog gene, *MTH1*, and expression of MTH1 protein in *mutT*⁻ cells suppresses the increased occurrence of A:T→C:G transversion mutation (14–17). Despite functional identity, the MTH1 and MutT proteins share only 30 residues (23%), 14 of which constitute the conserved 23-residue module, while the other 16 residues are scattered throughout the molecules. We reported that the 23-residue sequences of human MTH1 and *E.coli* MutT proteins are exchangeable so that they constitute functionally equivalent modules for 8-oxo-dGTPase, designated as the phosphohydrolase module; however, the chimeric MTH1 protein with the 23-residue module of MutT is thermolabile in structure and function (18). Saturation mutagenesis of the module in MTH1 revealed that

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six non-conserved residues among MutT homologs are essential for MTH1 function, as are eight of the 10 identical residues (18,19). These results indicate that the phosphohydrolase module of the MutT and MTH1 proteins is functionally equivalent, yet each possesses unique features of function or structure.

To better define the unique features of the phosphohydrolase module of MutT, we carried out saturation mutagenesis of the 10 conserved residues in the module. We now provide the first evidence that six conserved residues are essential for MutT function while the other four can be replaced by other residues without loss of function. Among the four non-essential residues, Lys39 and Glu56 correspond to the essential residues Lys38 and Glu55 in MTH1. Different structural features between the two modules of MTH1 and MutT proteins are evident.

MATERIALS AND METHODS

Chemicals

[α-^{32}P]dGTP and ^{125}I-labeled protein A were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Restriction enzymes, T4 DNA polymerase, T4 DNA ligase and Klenow fragment of DNA polymerase I were obtained from Toyobo Co. (Osaka, Japan). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside and phenyl-β-D-galactopyranoside were obtained from Sigma (St Louis, MO). The altered sites II *in vitro* mutagenesis system was purchased from Promega (Madison, WI).

Bacterial strains

Escherichia coli strain CC101T [*ara*Δ(*lacI-proB*)*XIII mutT::Km*] carrying an F *lacI*$^{-}$*Z*$^{-}$*proB*$^{+}$ episome was used for analysis of MutT protein and for measurement of mutation frequency (15). *Escherichia coli* strain JM109 [*endA1*, *recA1*, *gyrA96, thi-1, hsdR17* (*rk*$^{-}$, *mk*$^{-}$), *relA1, supE44*, λ^{-}, Δ(*lac-proAB*), (*F*$^{-}$, *traD36, proA*$^{+}$*B*$^{+}$, *lacI*^{q}*Z* Δ*M15*)] and ES1301 (*mutS*$^{-}$) [*lacZ53, mutS201::Tn5, thyA36, rha-5, metB1, deoC*, *IN* (*rrn-rrnE*)] were purchased from Promega and used for saturation mutagenesis.

Plasmids

Plasmid pHS:MutT was constructed by inserting an 877 bp *Eco*RI–*Bam*HI fragment from pMA106 containing the entire *mutT* gene (20), into the *Aat*II–*Bam*HI site of pBR322. Plasmid pALTER-1 was obtained from Promega and used for saturation mutagenesis. Plasmid pALTER:MutT-EM, used as template for saturation mutagenesis, was constructed as follows. An *Eco*RI–*Mlu*I fragment (159 bp) of *mutT* gene containing the sequence for the 23-residue module of MutT protein, was introduced at the *Eco*RI–*Sal*I site of pALTER-1 (Fig. 1). To construct plasmid pYN3103:TrpE-MutT encoding TrpE–MutT fusion protein, the *Nde*I–*Bam*HI fragment of *mutT* gene was subcloned into the *Pst*I–*Bam*HI site of pYN3103:TrpE (21). The *Nco*I–*Bam*HI fragment encoding the TrpE–MutT fusion protein was subcloned into the *Nco*I–*Bam*HI site of pET3d, in which a coding region can be transcribed from the T7 phage promoter (22), resulting in pET3d:TrpE-MutT.

Oligonucleotide primers

Oligonucleotide primers (27–37mer) carrying every possible combination of nucleotides for a target codon (Table 1) were obtained from Espec Oligo Service Corporation (Tsukuba, Japan), and were used as primers for saturation mutagenesis. In the case of Thr45, a negative mutant was isolated with the primer carrying an amber mutation at this site.

Table 1. Primers for negative and positive mutant screening of MutT

Target residues	Primer	Sequence
37Gly	1	TTC AAT TTT ACC **NNN** GGG AAA CTC CAG
38Gly	2	CAT TTC AAT TTT **NNN** GCC GGG AAA CTC
39Lys	3	TTC ACC CAT TTC AAT **NNN** ACC GCC GGG AAA CTC
44Glu	4	CTG TTC CGG CGT **NNN** ACC CAT TTC AAT
45Thr	5	CGC CTG TTC CGG **NNN** TTC ACC CAT TTC
45Thr	5Amb	CGC CTG TTC CGG **CTA** TTC ACC CAT TTC
52Arg	6	TTC CTG AAG TTC **NNN** CAC CAC CGC CTG
53Glu	7	TTC TTC CTG AAG **NNN** ACG CAC CAC CGC
56Glu	8	GG GGT AAT CCC GAC TTC **NNN** CTG AAG TTC ACG CAC CA
57Glu	9	G GGG GGT AAT CCC GAC **NNN** TTC CTG AAG TTC ACG C
59Gly	10	TTG GGG GGT AAT **NNN** GAC TTC TTC CTG

N, mixture of equal amounts of A, T, C and G.

Saturation mutagenesis

Saturation mutagenesis was done for the plasmid pALTER:MutT-EM, using the mutagenesis primers listed in Table 1, and according to the Promega technical manual for altered sites II *in vitro* mutagenesis system, but with the modifications described in Figure 1 (18,19). Mutagenized DNA was digested with a set of restriction enzymes, *Eco*RI and *Mlu*I, and subcloned into pHS:MutT. A mixture of plasmids pHS:MutT(G37X), for example, carrying mutations at the codon for Gly37 thus obtained was applied to *E.coli* CC101T and cultured on agar medium containing minimal A salts, 0.2% glucose, 0.05% phenyl-β-D-galactopyranoside and 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. After incubation for 5 days at 37°C, papillae formation was evident, and appropriate samples were used for further analyses. An appropriate negative mutant for each residue was used as a template for the second cycle of saturation mutagenesis, the objective being to obtain positive mutants. Nucleotide sequence of each mutant was determined using Dye terminator Cycle Sequencing FS Ready Kits and the model ABI373A automated DNA sequencer (PE Applied Biosystems).

Fluctuation test

Bacteria were grown overnight in LB medium in the presence of 50 μg/ml ampicillin, and aliquots containing about 100 cells were inoculated into 5 ml of LB medium containing 50 μg/ml ampicillin and cultured at 37°C for 48 h. Mutant frequencies were determined by plating aliquots of cultures on normal and 0.4% lactose-containing minimal A medium plates with 50 μg/ml ampicillin. Three or more independent experiments were done, and mutation rates were calculated (23,24).

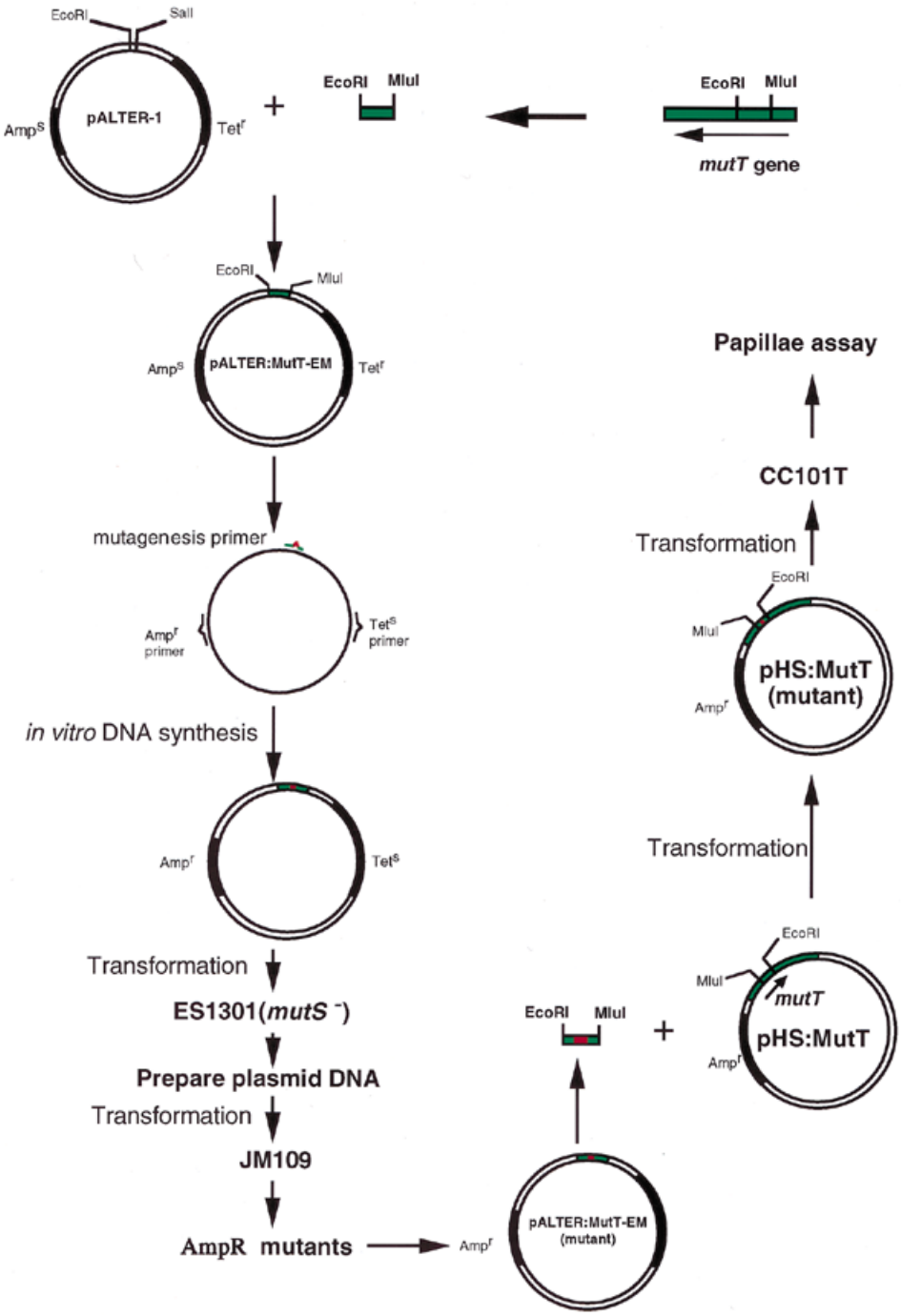

Figure 1. Schematic representation of saturation mutagenesis. After alkaline denaturation of pALTER:MutT-EM DNA, mutagenic primers Amp^r and Tet^s were annealed with the denatured DNA, and the complementary strand was synthesized with T4 DNA polymerase and sealed with T4 DNA ligase. The resulting double-stranded DNA was applied to *E.coli* ES1301 cells (*mutS*$^-$) and the cells were grown on an LB plate containing 50 µg/ml ampicillin. Plasmid DNA extracted from these cells was applied to *E.coli* JM109 cells and ampicillin-resistant (Amp^r) colonies were isolated. An *Eco*RI–*Mlu*I fragment of the mutagenized plasmid DNA was inserted into the *Eco*RI–*Mlu*I site of pHS:MutT to replace the corresponding wild-type fragment, and recombinant DNA was applied to *E.coli* CC101T (*mutT*$^-$, *lacZ*$^-$) cells. In the figure, DNA fragments with wild-type sequence of the *mutT* gene are shown in green, and those with mutation(s) are in red.

Western blotting

Bacterial crude extracts, prepared as described (18), were subjected to 15% SDS–polyacrylamide gel electrophoresis (PAGE), and then western blotting, which was done as described (25), using anti-MutT.

Preparation of anti-MutT antibody

Insoluble TrpE–MutT fusion protein was prepared from *E.coli* BL21 (DE3) cells carrying pET3d:TrpE-MutT and subjected to SDS–12.5% PAGE, as described (26). A region of the gel containing the fusion protein was excised (~100 µg/injection),

emulsified with adjuvant (Titer Max, Vaxel Inc., Norcross, GA) and injected into a Japanese white rabbit. Sera were obtained 2 weeks after the eighth injection of booster and antibodies were purified using TrpE–MutT–Sepharose and TrpE–Sepharose columns (21,25). The TrpE–MutT–Sepharose column, to which the anti-serum against TrpE–MutT was loaded, was washed using 10 mM sodium phosphate buffer (pH 7.6). The bound antibodies were then eluted, using buffer E (0.2 M glycine–HCl pH 2.5, 0.1 M NaCl, 0.1% Triton-X100), and the eluted fraction was dialyzed against Tris-buffered saline (10 mM Tris–HCl pH 7.6, 150 mM NaCl) and applied onto the TrpE–Sepharose column. The flow-through fractions were collected and designated as anti-MutT.

8-Oxo-dGTPase activity

8-Oxo-dGTPase activity was measured as described (19,27). One unit of 8-oxo-dGTPase was defined as the amount of enzyme that produced 1 pmol/min of 8-oxo-dGMP at 30°C. Relative specific activity of mutant MutT protein was calculated based on the relative content of each mutant protein in the extract, as determined by western blotting.

RESULTS

Saturation mutagenesis of the 10 conserved residues in the phosphohydrolase module of the *E.coli* MutT protein

Residues in the 23-residue module of MutT were classified into three groups; conserved, semi-conserved and non-conserved, as based on conservation among six MutT family proteins (Fig. 3). To better understand the roles of the conserved residues in the phosphohydrolase module of *E.coli* MutT, saturation mutagenesis of 10 residues in the module was done, in combination with negative and positive mutant screening, using mutagenesis primers that contain a completely degenerated codon for each residue (Fig. 1; Table 1).

First, we isolated mutants of pHS:MutT that lost the capacity to suppress the mutator phenotype of *E.coli* strain CC101T, and which were designated as negative mutants (Table 2). Next, we used as a template one of the negative mutants for each residue in the second round of saturation mutagenesis, the objective being to isolate positive mutants that acquired the capability to suppress the increased occurrence of $LacZ^+$ reversion in CC101T cells. As shown in Table 3, mutation rates of CC101T cells harboring negative mutants, used as templates for the second mutagenesis, were as high as that of the cells with vector pBR322. We used nonsense mutants as templates for the second mutaganesis in case of negative mutants of six residues (Lys39, Glu44, Thr45, Glu53, Glu56 and Gly59), because neither MutT protein nor 8-oxo-dGTPase activity was detected in CC101T cells harboring these mutants. Expression levels of the negative mutant proteins with missense mutation were lower than that of wild-type MTH1, and low 8-oxo-dGTPase activity was detectable in only a few cases (Fig. 2; Table 3).

Negative and positive mutants isolated for each amino acid residue are summarized in Table 2. Five or more different negative mutants for residues Gly37, Gly38, Glu53 and Glu57 were isolated, but fewer negative mutants, including nonsense ones were isolated for residues Lys39, Glu44, Thr45, Arg52, Glu56 and Gly59. No positive mutant, except for each true revertant, was isolated for the former residues, indicating that residues Gly37, Gly38, Glu53 and Glu57 cannot be replaced with any other residue without loss of function. On the other hand many different positive mutants were obtained for the latter, suggesting that residues Lys39, Glu44, Thr45, Arg52, Glu56 and Gly59 can be replaced without losing function.

Table 2. Substitutions at conserved amino acid sites of MutT

Wild type		Negative mutants		Positive mutants	
Amino acid	Codon	Amino acid	Codon	Amino acid	Codon
Gly37	GGC	Val	GTC(5), GTT	Gly	GGC(7)
		Ala	GCC(3)		
		Cys	TGC		
		Thr	ACG(2)		
		Asp	GAC		
		Arg	CGC*		
Gly38	GGT	Ala	GCT(4)	Gly	GGT(9)
		Pro	CCT		
		Asp	GAT		
		Arg	CGT(2)*		
		His	CAT		
Lys39	AAA	Ochre	TAA(8)*	Lys	AAA
		Pro	CCA	Ala	GCA
				Leu	CTA
				Gln	CAA
				Ser	TCA(2)
				Tyr	TAC
				Thr	ACA
				Glu	GAA
Glu44	GAA	Ochre	TAA(5)*	Glu	GAA(2)
		Opal	TGA	Leu	TTA
		Pro	CCA(2), CCC	Cys	CAA(2)
		Ile	ATC	Gln	TGC
				Tyr	TAC
				Asp	GAC
				Arg	AGA
				His	CAC(2)
Thr45	ACG	Amber	TAG*	Leu	TTA, TTG
				Trp	TGG(2)
				Gln	CAG(4)
				Ser	TCG
				Tyr	TAT(2)
				Arg	CGG
Arg52	CGT	Pro	CCT(16)*, CCC	Arg	CGT
				Ala	GCG
				Leu	CTA, CTC, CTT
				Ser	TCT(2), AGT
				His	CAC(3)
Glu53	GAA	Ochre	TAA(4)*	Glu	GAA(7)
		Val	GTA(2), GTC		
		Ala	GCC, GCA		
		Phe	TTC		
		Leu	CTA		
		Asp	GAT, GAC		
		Ser	TCT		
Glu56	GAA	Ochre	TAA*	Glu	GAA
		Arg	CGC(4)	Val	GTA, GTG
		Lys	AAA	Ala	GCC
				Gln	CAA(3)
				Ser	TCC, TCT, TCA
				Cys	TGC(2)
				Thr	ACC
Glu57	GAA	Val	GTC	Glu	GAA, GAG(3)
		Ala	GCC(2)*, GCA		
		Pro	CCC		
		Gly	GGC		
		Gln	CAA(3)		
		Asp	GAC		
		Arg	CGA		
		His	CAC		
Gly59	GGG	Amber	TAG*	Ala	GCG
		Val	GTG(2)	Leu	TTG
				Phe	TTC
				Gln	CAG(2)
				Ser	TCG(2)
				Cys	TGC
				Tyr	TAT
				Glu	GAG
				Arg	AGG
				Lys	AAG

Numbers in parentheses indicate the number of independent clones obtained.
*Clones used as template for isolation of positive mutants.

Our data clearly show that negative and positive mutant screening is a self-complementary approach, because more negative mutants were obtained for all essential residues and for which only a true revertant was obtained, as a positive mutant.

In conclusion, four of the 10 conserved residues of the phosphohydrolase module of *E.coli* MutT are essential for function capable of suppressing the elevated A:T→C:G transversion in *mutT*$^-$ cells.

Table 3. Biological and biochemical properties of negative mutants of MutT

Amino acid residue	Residue in mutant	Mutation Rate (x10 $^{-6}$)	(ratio) [a]	MutT Protein (ratio) [b]	8-oxo-dGTPase Specific activity [c]	(ratio) [d]
Gly-37	Arg*	9.87 ± 0.78	(9400)	0.485	ND[e]	-
	Val	7.85 ± 0.16	(7480)	0.110	ND	-
	Ala	6.88 ± 0.90	(6550)	0.537	ND	-
	Cys	8.86 ± 1.71	(8440)	0.933	ND	-
	Thr	9.14 ± 1.08	(8700)	0.453	ND	-
	Asp	7.01 ± 0.93	(6680)	0.064	ND	-
Gly-38	Arg*	7.86 ± 3.86	(7490)	—[f]	—	—
	Ala	6.02 ± 0.36	(5730)	—	—	—
	Pro	8.90 ± 0.41	(8480)	—	—	—
	Asp	5.86 ± 2.54	(5580)	—	—	—
	His	6.70 ± 0.71	(6380)	—	—	—
Lys-39	Ochre*	5.76 ± 1.68	(5490)	0.106	ND	-
	Pro	4.42 ± 0.16	(4200)	0.877	7.24	(0.04)
Glu-44	Ochre*	8.04 ± 0.06	(7660)	ND	-	-
	Opal	4.87 ± 0.57	(4640)	—	—	—
	Pro	5.22 ± 0.53	(4970)	ND	-	-
	Ile	0.98 ± 0.06	(933)	ND	-	-
Thr-45	Amber*	8.50 ± 1.38	(8100)	—	—	—
Arg-52	Pro*	9.64 ± 1.01	(9180)	—	—	—
Glu-53	Ochre*	9.96 ± 0.24	(9490)	—	—	—
	Val	9.99 ± 1.20	(9510)	—	—	—
	Ala	10.6 ± 1.09	(10100)	—	—	—
	Phe	8.77 ± 0.49	(8350)	—	—	—
	Leu	6.59 ± 1.02	(6280)	—	—	—
	Asp	7.97 ± 0.01	(7590)	—	—	—
	Ser	7.73 ± 1.25	(7360)	—	—	—
Glu-56	Ochre*	12.4 ± 0.88	(11800)	—	—	—
	Arg	6.79 ± 2.50	(6470)	—	—	—
	Lys	4.54 ± 0.35	(4320)	—	—	—
Glu-57	Ala*	7.84 ± 1.69	(7480)	—	—	—
	Val	14.9 ± 1.48	(14200)	—	—	—
	Pro	4.56 ± 0.54	(4340)	—	—	—
	Gly	14.6 ± 0.45	(13900)	—	—	—
	Gln	2.54 ± 1.20	(2420)	—	—	—
	Asp	14.8 ± 0.05	(14100)	—	—	—
	Arg	14.0 ± 0.10	(13300)	—	—	—
	His	13.3 ± 0.13	(12700)	—	—	—
Gly-59	Amber*	14.3 ± 0.82	(13600)	—	—	—
	Val	1.11 ± 0.14	(1060)	—	—	—
pHS-mutT(wild)		1.05 ± 0.36x10 $^{-3}$	(1.00)	1.00	174	1.00
vector pBR322		4.44 ± 1.76	(4230)	ND	ND	-

[a]Relative ratio of each mutation rate to that of wild-type.
[b]Amount of MutT in crude extracts from cells expressing each mutant protein was determined by quantitative western blotting and the ratio is relative to that of the wild-type MutT.
[c]Specific activity of 8-oxo-dGTPase in crude extract prepared from cells expressing each mutant is shown as U/mg crude protein per relative MutT protein, determined by western blotting.
[d]Ratio of mutant MutT specific activity relative to that of wild-type MutT.
[e]ND, not detected
[f]—, not determined.
*Clones used as template for isolation of positive mutants.

Positive mutants for the conserved residue Lys39

Seven different positive mutants were obtained from the negative mutant K39ochre for the conserved Lys39 residue as well as the true revertant (Tables 2 and 4). Among the positive mutants, the lowest mutation rate of LacZ$^+$ reversion in CC101T was attained by expression of a positive mutant K39Q, the mutation rate of 0.77×10^{-9} being slightly lower than that attained with wild-type MutT. A positive mutant K39Y showed the highest mutation rate, 3.72×10^{-9}, that is, over 3-fold higher compared to findings with wild-type MutT. Mutation rates attained by the other positive mutants were in the following order: Tyr>Ala>Leu>Glu>Thr>Ser>Gln.

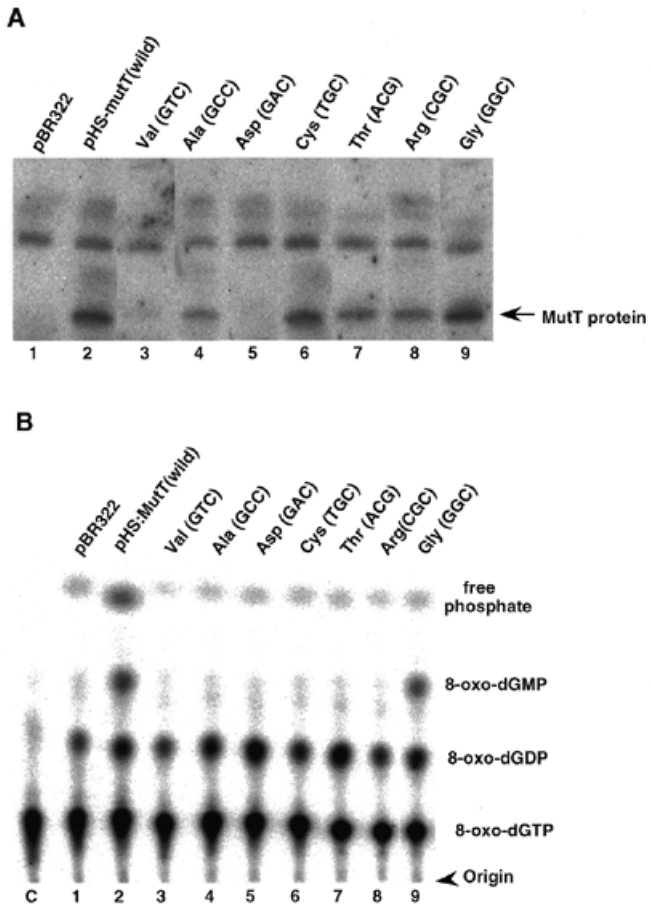

Figure 2. (**A**) Western blotting analysis of mutant MutT proteins. *Escherichia coli* CC101T (*mutT*⁻) cells carrying plasmids with mutation at residue 37 were grown overnight at 37°C and crude extracts were prepared. Crude extracts (15 µg of protein) were subjected to 15% SDS–PAGE, and then to western blotting with anti-MutT combined with ^{125}I-labeled protein A. The blot was air-dried, exposed to an imaging plate for 90 min and the resulting data were processed using a BAS 2000 Bio-image analyzer (Fuji Photo Film Co. Ltd, Tokyo, Japan). The arrow indicates 15 kDa MutT protein. Lane 1, pBR322 vector; lane 2, pHS:MutT; lanes 3–8, negative mutants for the residue Gly37 as indicated; lane 9, true revertant. (**B**) 8-Oxo-dGTPase assay for MutT mutant proteins. Crude extracts (2.0 µg of protein) prepared from CC101T cells carrying plasmids with mutation at residue 37 were subjected to 8-oxo-dGTPase assay. Lanes 1–9, as in (A); lane C, a control reaction without extract.

As shown in Table 5, the amount of all mutant proteins of the residue Lys39 expressed in CC101T cells was 47–97% of the level of the wild-type, and all possess a specific activity of 8-oxo-dGTPase comparable to 55–90% of the level of wild-type MutT.

Thus, the different positive mutants for the conserved residue Lys39 retained sufficient levels of both expression and capability to hydrolyze 8-oxo-dGTP and efficiently suppress the elevated A:T→C:G transversion in *mutT*⁻ cells.

Positive mutants for the conserved residue Glu44

Seven positive mutants from the negative mutant E44ochre were obtained for the conserved residue Glu44, in addition to the true revertant (Tables 2 and 4). Among the positive mutants, the lowest mutation rate of LacZ⁺ reversion in CC101T was attained by expression of a positive mutant E44D, and the mutation rate of 5.34×10^{-9} was 5-fold higher than that attained by wild-type MutT. A positive mutant E44R showed the highest mutation rate of 9.32×10^{-8}, that is, 89-fold higher compared to wild-type MutT, and is still 1/48 of that in *mutT*⁻ cells with the vector itself. Mutation rates attained by the other positive mutants were in the following order: Arg>Leu>Tyr>His>Cys>Gln>Asp. These results indicate that suppression of elevated A:T→C:G transversion by these positive mutants is partial and inefficient.

Table 4. Biological properties of positive mutants of MutT

Amino acid residue	Residue in mutant	Mutation Rate (x10 $^{-9}$)	(ratio) [a]
Lys-39	Gln	0.77 ± 0.52	0.73
	Ser	1.16 ± 0.11	1.10
	Thr	1.28 ± 0.17	1.22
	Glu	1.30 ± 0.32	1.24
	Leu	1.86 ± 0.46	1.77
	Ala	2.30 ± 0.91	2.19
	Tyr	3.72 ± 0.45	3.54
Glu-44	Asp	5.34 ± 1.75	5.09
	Gln	6.54 ± 1.47	6.23
	Cys	21.0 ± 4.56	20.0
	His	31.7 ± 12.0	30.2
	Tyr	39.3 ± 13.9	37.4
	Leu	52.4 ± 15.9	49.9
	Arg	93.2 ± 32.4	88.8
Thr-45	Tyr	0.87 ± 0.23	0.83
	Ser	0.90 ± 0.25	0.86
	Gln	1.24 ± 0.60	1.18
	Arg	1.30 ± 0.47	1.24
	Trp	1.78 ± 0.55	1.70
	Leu	2.03 ± 0.79	1.93
Arg-52	Ala	8.48 ± 2.50	8.08
	His	11.3 ± 0.05	10.8
	Ser	12.9 ± 0.54	12.3
	Leu	20.1 ± 1.50	19.1
Glu-56	Val	1.14 ± 0.47	1.09
	Gln	3.78 ± 1.21	3.60
	Ala	15.2 ± 2.80	14.5
	Ser	20.0 ± 10.7	19.0
	Cys	37.9 ± 3.74	36.1
	Thr	70.6 ± 22.8	67.2
Gly-59	Cys	0.69 ± 0.19	0.66
	Gln	1.25 ± 0.31	1.19
	Ala	1.54 ± 0.76	1.47
	Leu	1.56 ± 0.40	1.49
	Glu	2.61 ± 0.10	2.49
	Phe	2.92 ± 0.31	2.78
	Arg	2.95 ± 0.40	2.81
	Tyr	3.62 ± 1.30	3.45
	Ser	5.19 ± 1.39	4.94
	Lys	9.46 ± 3.31	9.01
pHS-mutT (wild)		1.05 ± 0.36	1.00
pBR322		4.44 ± 1.76 x 10 ^{3}	4230

[a]Relative ratio of each mutation rate to that of wild-type.

As shown in Table 6, the amount of all mutant proteins expressed in CC101T cells was low, and that of E44D, the highest among them, was 13.8% of the level of the wild-type. We could only determine the relative specific activity of 8-oxo-dGTPase for mutants E44D and E44Y as values exceeded 80%

of the level of the wild-type MutT. Although the amounts of four positive mutant proteins (E44Q, E44C, E44H, and E44R) expressed in CC101T cells was likely to be under the detection limit by anti-MutT, substantial levels of 8-oxo-dGTPase activity was detected in extracts, suggesting that these mutant proteins have higher specific activity of 8-oxo-dGTPase than does the wild-type.

Table 5. Immunoreactive MutT protein content and 8-oxo-dGTPase activity in positive mutants at the Lys39 site

Amino acid substitution	Codon	Relative content of immunoreactive MutT protein [a]	8-oxo-dGTPase activity		8-oxo-dGTPase specific activity	
			(u/mg) [b]	(ratio) [c]	(b/a) [d]	(ratio) [e]
Gln	CAA	0.803	80.4	(0.462)	100	(0.575)
Glu	GAA	0.725	69.4	(0.399)	95.7	(0.550)
Ser	TCA	0.801	139	(0.799)	174	(1.00)
Thr	ACA	0.965	137	(0.787)	142	(0.816)
Leu	CTA	0.472	59.7	(0.343)	127	(0.730)
Ala	GCA	0.677	98.7	(0.567)	146	(0.839)
Tyr	TAC	0.879	137	(0.787)	156	(0.897)
pHS-MutT(wild)		1.00	174	(1.00)	174	(1.00)
pBR322		ND [f]	ND	(-)	-	(-)

[a]Amount of MutT protein in crude extracts from cells expressing each mutant protein was determined by quantitative western blotting and relative ratio to the amount of wild-type MutT is shown.
[b]Activity of 8-oxo-dGTPase in crude extracts prepared from cells expressing each mutant protein is shown as U/mg crude protein.
[c]Ratio of mutant MutT activity relative to that of wild-type MutT.
[d]Specific activity of 8-oxo-dGTPase in crude extracts prepared from cells expressing each mutant is shown as U/mg crude protein per relative MutT protein, determined by western blotting.
[e]Ratio of mutant MutT specific activity relative to that of wild-type MutT.
[f]ND, not detected.

Table 6. Immunoreactive MutT protein content and 8-oxo-dGTPase activity in positive mutants at the Glu44 site

Amino acid substitution	Codon	Relative content of immunoreactive MutT protein [a]	8-oxo-dGTPase activity		8-oxo-dGTPase specific activity	
			(u/mg) [b]	(ratio) [c]	(b/a) [d]	(ratio) [e]
Asp	GAC	0.138	19.6	(0.112)	142	(0.816)
Gln	CAA	ND [f]	39.8	(0.229)	-	(-)
Cys	TGC	ND	22.8	(0.131)	-	(-)
His	CAC	ND	19.0	(0.109)	-	(-)
Tyr	TAC	0.062	12.2	(0.070)	197	(1.13)
Leu	TTA	0.097	ND	(-)	-	(-)
Arg	AGA	ND	37.2	(0.214)	-	(-)
pHS-MutT(wild)		1.00	174	(1.00)	174	(1.00)
pBR322		ND	ND	(-)	-	(-)

[a–f]As in Table 5.

Thus, different positive mutants for conserved residue Glu44 were obtained; however, they retained a limited level of capability to suppress the elevated A:T→C:G transversion in *mutT*⁻ cells, probably because structural stability was inadequate. The residue Glu44 is apparently an essential residue for MutT function.

Positive mutants for the conserved residue Thr45

Six different positive mutants were obtained from the negative mutant T45amber for the conserved Thr45 residue, although the true revertant was not isolated in this case (Tables 2 and 4). Among the positive mutants, the lowest mutation rates were attained by expression of two mutants, T45Y and T45S, with mutation rates of 0.87×10^{-9} and 0.9×10^{-9}, respectively, which were lower than that attained by wild-type MutT. A positive mutant T45L showed the highest mutation rate, 2.03×10^{-9}, that is, ~2-fold higher compared to wild-type MutT. Mutation rates attained by the other positive mutants were in the following order: Leu>Trp>Arg>Gln>Ser>Tyr.

Thus, different positive mutants for the conserved residue Thr45 we obtained retained the capability to suppress the elevated A:T→C:G transversion in *mutT*⁻, hence, Thr45 is non-essential for MutT function.

Positive mutants for the conserved residue Arg52

Four different positive mutants were obtained from the negative mutant R52P for the conserved Arg52 residue, in addition to the true revertant (Tables 2 and 4). Among the positive mutants, the lowest mutation rate of LacZ⁺ reversion in CC101T was attained by expression of a positive mutant R52A, with a mutation rate of 8.48×10^{-9} which was 8-fold higher than that attained by wild-type MutT. A positive mutant R52L showed the highest mutation rate, 2.01×10^{-8}, that is, 19-fold higher compared to wild-type MutT. Mutation rates attained by the other positive mutants were in the following order: Leu>Ser>His>Ala.

While different positive mutants for the conserved residues Arg52 were obtained, they retained only a limited capability to suppress the elevated A:T→C:G transversion in *mutT*⁻ cells. Residue Arg52 is apparently an important residue for MutT function.

Positive mutants for the conserved residue Glu56

Six different positive mutants were obtained from the negative mutant E56ochre for the conserved Glu56 residue, in addition to the true revertant (Tables 2 and 4). Among the positive mutants, the lowest mutation rate of LacZ⁺ reversion in CC101T was attained by expression of a positive mutant E56V, and the mutation rate of 1.14×10^{-9} was as low as that attained by wild-type MutT. CC101T cells with a positive mutant E56T showed the highest mutation rate, 7.06×10^{-8}, that is, 67-fold higher compared to findings with the wild-type and is 1/63 of that in *mutT*⁻ cells with the vector itself. Mutation rates attained by the other positive mutants were in the following order: Thr>Cys>Ser>Ala>Gln>Val.

Among the different positive mutants for the conserved residue Glu56, some retained an efficient or slightly lower capability to suppress the elevated A:T→C:G transversion in *mutT*⁻ cells, hence residue Glu56 is apparently non-essential.

Positive mutants for the conserved residue Gly59

Ten different positive mutants were obtained from the negative mutant G59amber for the conserved Gly59 residue, though no true revertant was isolated (Tables 2 and 4). Among the positive mutants, the lowest mutation rate of LacZ⁺ reversion in CC101T was attained by expression of a positive mutant G59C, and the mutation rate of 6.91×10^{-10} was lower than that attained with wild-type MutT. CC101T cells with a positive mutant G59K exhibited the highest mutation rate, 9.46×10^{-9}, that is, 9-fold higher compared to those with wild-type. Mutation rates

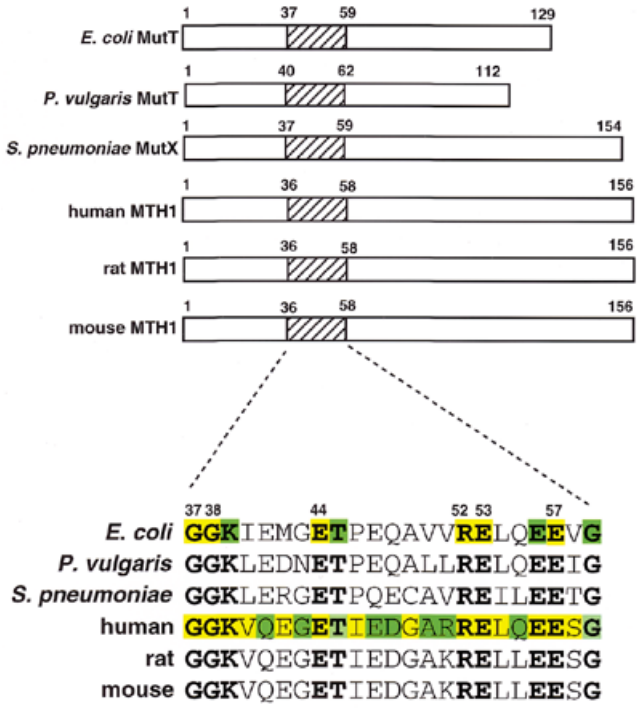

Figure 3. Comparison of phosphohydrolase modules in MutT family proteins and a summary of negative and positive mutant screening for the 23-residue module of MutT. The 23-residue modules from *E.coli* MutT, *P.vulgaris* MutT and *S.pneumoniae* MutX, and human, rat and mouse MTH1 proteins shown above are aligned in the lower panel. In *E.coli* MutT, residues 37–45 constitute loop I and residues 46–59 constitute α-helix I. Conserved amino acid residues are indicated by bold letters. Residues in MutT (this work) and MTH1 (18,19) in the yellow boxes indicate those that could not be replaced by any other residue without losing function, and those in the green boxes represent those that can be replaced by other residues.

attained with the other positive mutants were in the following order: Lys>Ser>Tyr>Arg>Phe>Glu>Leu>Ala>Gln>Cys.

Of the different positive mutants for the conserved residue Gly59, some retained levels sufficient to suppress the elevated A:T→C:G transversion in *mutT*⁻; thus, residue Gly59 is non-essential for MutT function.

DISCUSSION

Our main conclusion based on findings in the present study is that in the phosphohydrolase module of MutT, four of the 10 conserved residues, Lys39, Thr45, Glu56 and Gly59 are non-essential for MutT function (Fig. 3). The two residues, Lys39 and Glu56 in MutT correspond to Lys38 and Glu55 in human MTH1 that could not be replaced by any other residue without loss of function (18); thus, different structural features between the two modules of MTH1 and MutT proteins are evident.

The 23-residue sequence in *E.coli* MutT constitutes the active center for dGTPase activity (12,28), and perhaps also for 8-oxo-dGTPase activity. The module consists of loop I (Gly37→Thr45) and α-helix I (Pro46→Gly59) (29). The α-helix I in MutT is apparently amphipathic and MutT has two hydrophobic cores, one consisting of Val50, Val51, Leu54 from the helix I clustering with Phe65 and Leu67 from loop II, Val87 from β-strand D and Trp95 from loop III (29). Interactions between the phosphohydrolase module and other secondary structures may be important to support stability or to provide functional moieties for the active center. It was proposed that residues Gly38, Glu56 and Glu57 in the module of MutT are involved in coordination of enzyme-bound metal (Mg^{2+}), Glu57 provides the largest contribution (factor $>10^5$) to the catalytic power of MutT enzyme and residues Glu53, Arg52 and Lys39 are involved in the catalytic reaction, each residue contributing a factor of $10^{4.7}$, $10^{3.0}$ and $10^{0.9}$, respectively, to catalytic power (12,13,30).

The data presented herein are largely consistent with the proposed mechanism of the MutT pyrophosphohydrolase reaction by Harris *et al*. (30), because residues Gly37, Gly38, Glu44, Arg52, Glu53 and Glu57 cannot be replaced by any other residue without loss of function, and are therefore essential for MutT function. The essential residues are likely to contribute to a factor of $>10^3$ to the catalytic power of MutT. However, Lys39 and Glu56, whose contribution to MutT catalytic power is a factor of $10^{0.9}$ and $10^{1.4}$, respectively, can be replaced by other residues without loss of function, thus indicating that the MutT reaction may differ somewhat from the proposed mechanism, as discussed below.

Lin *et al*. (13) reported that the δ-CH_2 of Lys39 on loop I interacts with the hydrophobic face of the ribose ring of α,β-methyleneadenosine 5′-triphosphate (AMPCPP), and its side chain ammonium group approaches an oxygen of α-phosphoryl group at a distance of 4.7 ± 1.1 Å. Mutation of Lys39 to Gln results in an 8-fold decrease in k_{cat} and a 5.3-fold increase in K_M(dGTP) in the Mg^{2+}-activated dGTPase reaction (28,30). Based on these data, it was suggested that Lys39 is positioned in the MutT reaction to interact electrostatically with the α-phosphoryl group and thereby facilitate departure of the nucleoside monophosphate (NMP) group.

In contrast to previous reports (28,30), we isolated the K39Q mutant as one of the positive mutants for K39ochre which completely suppresses the *mutT*⁻ mutator phenotype, and retains 58% of the level of specific activity for 8-oxo-dGTPase of wild-type MutT. Using saturation mutagenesis combined with negative and positive mutant screening, we isolated seven different positive mutants for Lys39, all of which are stable and retain >50% of the specific activity of wild-type MutT. We obtained only two negative mutants, K39ochre (eight clones) and K39P (one clone) from 152 clones examined, and the latter missense mutant, while stable, possesses little 8-oxo-dGTPase activity. Thus, it is likely that most amino acid residues, but not proline, may be able to replace Lys39 without loss of the potential to suppress the *mutT*⁻ mutator phenotype. Thus, the Lys39 residue is apparently not essential for MutT function. We wish to emphasize that this conclusion can be reached only by using saturation mutagenesis in combination with negative and positive mutant screening.

For catalytic analysis of MutT, we used 8-oxo-dGTP while other workers used dGTP as substrate (28,30), to which MutT protein exhibits a high K_M value (1100 μM), 2200-fold higher than that for 8-oxo-dGTP. With 8-oxo-dGTP, K39Q exhibits ~58% of the specific activity for wild-type; however, with dGTP its k_{cat} value was decreased 8-fold with a 5.3-fold higher

K_M value, which means that the specific activity may be <5% of the wild-type. We suggest that the MutT protein interacts with dGTP and 8-oxo-dGTP in a different manner, and that Lys39 has a more crucial role for dGTPase than for 8-oxo-dGTPase of MutT.

8-Oxo-dGTP but not dGTP appears to favor a *syn* conformation at the glycosidic bond (31), and it has been shown that AMPCPP in the quaternary MutT–Mg^{2+}–AMPCPP–Mg^{2+} complex takes a high *anti* torsional angle at the glycosylic bond, which is part of the a *syn* conformation near the *syn–anti* boundary, with interaction between Lys39 and the ribose (13). Lys39 may be involved in binding to dGTP or AMPCPP but not to 8-oxo-dGTP, thus contributing to dGTP hydrolysis. It is essential to analyze interactions between MutT and 8-oxo-dGTP or its non-hydrolyzable derivatives in order to delineate substrate recognition by MutT. Identification of a negative mutant, K39P MutT, suggests that substitution of Lys39 for proline altered its secondary structure because the proline residue assumes only two fixed angles in the polypeptide. While loop I seems flexible in structure with most amino acid residues, proline may destroy the flexibility, leading to loss of catalytic potential.

It has been proposed that Glu56 contributes to metal binding in the active quaternary complex and its contribution to the catalytic power of MutT enzyme is calculated to be a factor of $10^{1.4}$, as based on the data from mutants E56D or E56Q (30). In the present work, we isolated E56V as a positive mutant for Glu56. This mutant retained the full capability to suppress the increased mutation rates in *mutT*$^-$ cells, to the level of the wild-type. Since no positive mutant was isolated for the essential residue, Glu57, which is likely to contribute a factor of $>10^{5.0}$ to the catalytic power of MutT enzyme as a ligand-to-metal residue (30), we suggest that Glu56 is not crucial as a ligand-to-metal residue, or rather that valine at the 56th position can act as an efficient ligand-to-metal residue.

We reported that the chimeric MTH1 protein carrying the phosphohydrolase module of MutT is functional but thermolabile, suggesting that phosphohydrolase modules of MutT and MTH1 have different features regarding function or structure (18). The functional significance of each conserved residue in the module of MutT is mostly equivalent to that of MTH1 protein, because six and eight out of 10 conserved residues are essential for MutT and MTH1 function, respectively, and two non-essential residues, Thr45/Thr44 and Gly59/Gly58 are shared between the two (18,19). However, we found differences in the requirement of Lys38/Lys39 and Glu55/Glu56 for MTH1/MutT reaction, that is, these two residues are essential in human MTH1 but not in MutT, as summarized in Figure 3. This may be due to different semi- or non-conserved residues between MTH1 and MutT in or surrounding the modules, which may build up a different microenvironment for each phosphohydrolase module. We reported that some of the semi- or non-conserved residues in MTH1 are essential (18). Thus, there are different structural features between the phosphohydrolase modules of MTH1 and MutT proteins, and saturation mutagenesis of the semi- and non-conserved residues in the module of MutT protein is expected to reveal their molecular basis.

As shown in Figure 3, the residues in MTH1 corresponding to the Gly37, Gly38, Lys39, Glu44, Arg52, Glu53, Glu56 and Glu57 in MutT are essential in MTH1 (18,19). In our recent NMR analysis of MTH1 (N.Itoh, M.Mishima, Y.Nakabeppu and M.Shirakawa, unpublished results), we found that MTH1 essentially has a tertiary structure similar to that previously proposed based on computer analysis (32). We suggest that the proposed mechanism of pyrophosphohydrolase reaction for MutT (30) may be applicable to the MTH1 reaction. That is, Lys38 in MTH1 interacts electrostatically with the α-phosphoryl group of 8-oxo-dGTP and thereby facilitates the departure of the 8-oxo-dGMP group, or Glu55 in MTH1 plays a crucial role as a ligand-to-metal residue. To demonstrate this model of MutT or MTH1, it will be necessary to analyze protein complexes with 8-oxo-dGTP or 8-oxo-dGDP, a non-hydrolysable substrate of MTH1.

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