Bound Nucleotide Controls the Endonuclease Activity of Mismatch Repair Enzyme MutL*

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DNA mismatch repair corrects mismatched base pairs mainly caused by replication error. Recent studies revealed that human MutL endonuclease, hPMS2, plays an essential role in the repair. However, there has been little biochemical analysis of the MutL endonuclease. In particular, it is unknown for what the MutL utilizes ATP binding and hydrolyzing activity. Here we report the detailed functional analysis of Thermus thermophilus MutL (ttMutL). ttMutL exhibited an endonuclease activity that decreased on alteration of Asp-364 in ttMutL to Asn. The biochemical characteristics of ttMutL were significantly affected on ATP binding, which suppressed nonspecific DNA digestion and promoted the mismatch- and MutS-dependent DNA binding. The inactivation of the cysteinyl residues in the C-terminal domain resulted in the perturbation in ATP-dependent regulation of the endonuclease activity, although the ATP-binding motif is located in the N-terminal domain. Complementation experiments revealed that the endonuclease activity of ttMutL and its regulation by ATP binding are necessary for DNA repair in vivo.

In living cells, a great amount of DNA damage arises as a result of errors during DNA replication, genetic recombination, and other processes (1). Because the accumulation of such damage can result in various genetic diseases, many DNA repair systems have evolved to remove any lesions. One of these systems is the mismatch repair (MMR),2 which is conserved throughout all organisms (2). It is known that inactivation of MMR in humans has been implicated in over 90% of hereditary nonpolyposis colorectal cancers (3). The mechanism of MMR has been well characterized in Escherichia coli, and the reconstituted system has been established (4). The early reactions in the E. coli MMR are performed by the MutHLS system (5, 6), which consists of three proteins, MutS, MutL, and MutH. In this system, a mismatched base in double-stranded DNA (dsDNA) is recognized by a MutS dimer. A MutL dimer interacts with the MutS-mismatched DNA complex and stabilizes its complex, and then the MutH endonuclease is activated by MutL. MutH nicks the unmethylated strand at the hemi-methylated GATC site to provide an entry point for the excision and to direct the repair to the new DNA strand. To complete the repair, the strand containing the error is removed by helicases and exonucleases, and a new strand is synthesized by DNA polymerase III helozyme and ligase. Homologues of E. coli MutS and MutL exist in the majority of organisms (Fig. 1A), suggesting that MMR is a common repair mechanism among those species. However, despite the prevalence of the MMR system, no homologue of E. coli MutH has been identified in the majority of organisms (7; Fig. 1A). Therefore, in organisms lacking mutH, the MMR had not been well understood.

The research for the primary reactions in the eukaryotic MMR relies on homologues of bacterial MutS and MutL. Many studies on the mammalian MMR have shown that a strand discontinuity serves as a signal that directs the repair to one strand of the mismatched heteroduplex (2, 4). The discontinuities in the newly synthesized strands such as the 3’ ends or termini of Okazaki fragments may designate which strand is to be repaired. For biochemical characterization of MMR, the nicked circular heteroduplex has been used as a substrate containing a strand discontinuity. It has been shown that the excision system in MMR selects the shorter path from a nick to a mismatch (8). Therefore, the distinction between 5’- and 3’-directed MMR should exist. Interestingly, 5’- to 3’-exonuclease activity of Exo I is required for both 5’- and 3’-directed removal (9, 10). Recently, Modrich and co-workers (11, 12) explained how 3’-directed excision is performed by the 5’- to 3’-exonuclease activity of ExoI. They demonstrated that a human MutL homologue, MutLα (MLH1-PMS2 heterodimer), is a latent endonuclease that incises on both sides of a mismatch, and the 5’- to 3’-exonuclease activity of ExoI removes the DNA segment spanning the mismatch. They also showed that MutLα, in the presence of manganese ions, incises a supercoiled homoduplex without other MMR enzymes, and a DQHA(Xγ)2E(Xγ)2E motif in the PMS2 subunit of the MutLα heterodimer is a probable endonuclease active site.

There has been little biochemical analysis of a full-length MutL homologous to eukaryotic PMS2. Especially, it has been unknown for what the MutL homologues utilize as ATP-hydrolysis energy. Although the C-terminal regions of MutL homologues show a variety of constructions, MutL proteins from mutH-less bacteria have a C-terminal region homologous

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2 The abbreviations used are: MMR, mismatch repair; GdnHCl, guanidine HCl; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; AMPNP, adenosine 5’-(β,γ-imino)triphosphate; DTT, dithiothreitol; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); OC, open circular; CCC, covalently closed circular.

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to that of eukaryotic PMS2 (Fig. 1, C and E). Here we report the detailed functional analysis of ttMutL, including the analysis of its endonuclease activity. Proteins from *Thermus thermophilus* are heat-stable and suitable for physiochemical examination. This is the first study of the biochemical characteristics of a bacterial MutL homologous to human PMS2. In particular, the suppression of nonspecific digestion of homoduplex by binding of ATP is emphasized. This result may answer the question how cells regulate the endonuclease activity of MutL (13).

**EXPERIMENTAL PROCEDURES**

**Culture Conditions for T. thermophilus HB8—*T. thermophi- lus* HB8 was grown at 70 °C in TR medium: 0.4% tryptone (Difco), 0.2% yeast extract (Oriental Yeast, Tokyo, Japan), and 0.1% NaCl (pH 7.5) (adjusted with NaOH). The TR medium was supplemented with 0.4 mM CaCl₂ and 0.4 mM MgCl₂ (TT medium) when used for the transformation experiments. To make plates, 1.5% gellan gum (Wako, Osaka, Japan), 1.5 mM CaCl₂, and 1.5 mM MgCl₂ were added to the TR medium (metals are necessary to solidify the gellan gum).

**Gene Disruption**—The 5′- and 3′-terminal 300-bp regions of *ttmutS* and *ttmutL* were amplified by PCR from a *T. thermophi- lus* genomic DNA plasmid. A thermostable kanamycin resistance gene, *HTK*, was also amplified by PCR from pUC18/HTK plasmid DNA (14). The three DNA fragments were fused by the fusion-PCR method (15) using LA Taq, and then cloned into the pT7Blue vector to obtain the pT7Blue::HTK and pT7Blue::ttmutL plasmids for *ttmutS* and *ttmutL* gene disruption. The transformation of *T. thermophilus* HB8 or HB27 was carried out according to the procedures described by Hashimoto et al. (16). Transformants were stored at −80 °C.

**Estimation of the Spontaneous Mutation Rate**—The spontaneous mutation rate of *T. thermophilus* HB8 or HB27 was estimated based on the frequency of streptomycin-resistant strains (Str₆) measured by means of the modified Luria-Delbrück fluctuation test (17). Streptomycin is an antibiotic agent that binds to the 30 S ribosomal subunit and inhibits translation of mRNA (18). A single amino acid substitution in streptomycin-binding site of the ribosomal protein S12 or a point mutation in 16 S rRNA can lead to the acquisition of streptomycin resistance. Wild type, *ttmutS*-deficient, and *ttmutL*-deficient *T. thermophi- lus* HB8 were cultured in 3 ml of TR medium at 70 °C for 12 h. The cultures were diluted 1:60 with 3 ml of TR medium and then shaken at 70 °C for 6 h (~1 × 10⁸ cells/ml). These cultures were spread on plates containing 20 μg/ml streptomycin. The same cultures were diluted 1:10³ with TR medium and then spread on drug-free plates. The plates were incubated at 70 °C for 15 h. The frequency of Str₆/pter ₁₀⁸ cells was calculated from the numbers of colonies formed on the streptomycin-containing and drug-free plates.

**Construction of Expression Plasmids**—The pET-3a/*ttmutS*-expression plasmid was constructed as described previously (19). DNA fragments expressing ttMutL and *Aquifex aeolicus* MutL (aqMutL) were generated by PCR using the *T. thermophi- lus* and *A. aeolicus* genomic DNAs as templates, respectively. The following pairs of primers were used for amplification of each fragment: 5′-ATATCATATGATTCGTCTTTACATTACCCGTTACG-3′ and 5′-ATATAGATTTTTATGTTGCTGGGTTAGAG- GTGCTTCC-3′ (ttMutL), and 5′-ATATCATATGTTTGTAA- GTTACCTTCCTC-3′ and 5′-ATATAGATTTTTATGTTGCTGGGTTAGAG- GTGCTTCC-3′ (aqMutL). The forward and reverse primers contained Ndel and BglII restriction sites, respectively (underlined). The amplified *ttmutL* and *aqmutL* fragments were ligated into the Ndel and BamHI sites of pET-11a (Novagen) and pET-21a (Novagen), respectively. The pET-11a/*ttmutL* plasmid was used as a template to generate D364N *ttmutL* using a QuickChange site-directed mutagenesis (Stratagene) with the following pairs of primers: 5′-TACGTGGTT- GAACCAGCATGCGGCGC-3′ and 5′-GGCGGCATGCTGTGGTT- CACCCAGTA-3′ (D364N). Sequence analysis revealed that the constructions were error-free.

**Expression and Purification of Enzymes—*E. coli* BL21(DE3) (Novagen) was transformed with pET-11a/*ttmutL* and then grown at 37 °C in 1.5 liters of YT medium containing 50 μg/ml ampicillin. When the density of cultures reached 4 × 10⁹ cells/ml, isopropyl β-D-thiogalactopyranoside was added to 0.4 μM. The cells were grown at 37 °C for 4 h after induction and harvested by centrifugation. The cells were then lysed by sonication in buffer I (20 mM Tris-HCl (pH 8.0), and 500 mM NaCl) and then heated to 55 °C for 10 min. After centrifugation at 48,000 × g for 60 min, ammonium sulfate was added to the supernatant to 1.05 M. The precipitant was resuspended in buffer I and then diluted with 20 mM Tris-HCl (pH 8.0) to 0.15 M NaCl. The resulting solution was loaded onto a HiTrap Hep- arin column (5 ml) (GE Healthcare) previously equilibrated with buffer II (20 mM Tris-HCl (pH 8.0), and 150 mM NaCl) using an AKTA explorer (GE Healthcare). The column was washed with 10 ml of buffer II and then eluted with a 50 ml gradient of 0.15–1 M NaCl in buffer II. The fractions containing ttMutL were detected by SDS-PAGE and concentrated using a Vivaspin concentrator (Vivascience, Hanover, Germany). The concentrated solution was applied to a HiLoad 16/60 Superdex 200-pg column (GE Healthcare) previously equilibrated with buffer I and eluted with the same buffer. D364N ttMutL was overexpressed by the same method as for wild type ttMutL and purified by a HiTrap heparin column chromatography, ammonium precipitation, and HiLoad 10/30 Superdex 200-pg column chromatography.

aqMutL was overexpressed by the same method as for ttMutL. Cells were lysed in 20 mM Tris-HCl (pH 8.0) containing 200 mM NaCl and heat-treated at 70 °C for 10 min. After cen- trifugation, the buffer for the supernatant was substituted with buffer III (50 mM NaP₄, (pH 7.0) and 1.5 M ammonium sulfate). The solution was loaded onto a Resource PHE column (6 ml) (GE Healthcare) previously equilibrated with buffer III. The column was washed with 15 ml of buffer III and eluted with a 50-ml gradient of 1.5 to 0 M ammonium sulfate in buffer III. The buffer for the protein solution was substituted with buffer V (10 mM NaP₄, (pH 7.0)) using a HiPrep 26/10 desalting column (GE Healthcare) and applied to a Resource S column (6 ml) (GE Healthcare) previously equilibrated with buffer V. The column was washed with buffer V and eluted with a 50-ml gradient of 0–1 M NaCl in buffer V. The fractions containing aqMutL were concentrated, then loaded onto a HiLoad 16/60 Superdex 200-pg column, and eluted with buffer I. ttMutS was overexpressed and purified as...
described previously (19). N-terminal sequencing of the purified proteins revealed the expected sequences for them. Protein solutions were stored at 4 °C.

Limited Proteolysis—Limited proteolysis was performed according to the procedure reported previously (20). In short, an aliquot of 4 μM ttMutL was reacted with various concentrations of trypsin. The concentrations of trypsin are indicated in the figure legends. The reaction was performed in buffer consisting of 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM dithiothreitol (DTT), 5 mM MgCl₂, and 2 mM ADP or AMPPNP at 25 °C for 10 min, and stopped by the addition of an equal volume of SDS-containing dye (125 mM Tris-HCl (pH 6.8), 10% β-mercaptoethanol, 4% SDS, 10% sucrose, and 0.01% bromphenol blue). The digests were separated by 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue.

Size-exclusion Chromatography—Size-exclusion chromatography was performed at 25 °C on a Superdex 200 HR column (1 × 30 cm; GE Healthcare) using an AKTA system. ttMutL (4 μM) was incubated in buffer consisting of 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM DTT, 5 mM MgCl₂, and 2 mM AMPPNP or ADP at 37 °C for 10 min. Then the resulting protein solution was loaded on the Superdex 200 HR column and eluted at the flow rate of 0.5 ml/min in 50 mM Tris-HCl (pH 7.5), 300 mM KCl, 1 mM DTT, and 5 mM MgCl₂. The elution profile was monitored by recording the absorbance at 280 and 260 nm. The Superdex 200 HR column was calibrated using apoferritin (443,000 Da), β-amyase (200,000 Da), alcohol dehydrogenase (150,000 Da), thyroglobulin (66,900 Da), albumin (66,000 Da), carbonic anhydrase (29,000 Da), and cytochrome c (12,400 Da).

ATPase Assay—Hydrolysis of ATP was measured by means of an enzyme-coupled spectrophotometric assay (21). The assay was performed at 25 °C because the enzymes used to couple the reactions were not thermostable. The change in absorbance at 340 nm was measured with a Hitachi spectrophotometer model U-3000 (Hitachi, Tokyo, Japan). ATP was reacted with 1 μM ttMutL or 100 nM ttMutS in buffer consisting of 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 2 mM phosphoenolpyruvate, 0.32 mM NADH, 25 units/ml pyruvate kinase (Sigma), and 25 units/ml lactate dehydrogenase (TOYOBO, Osaka, Japan). The concentrations of ATP are indicated in the figure legends.

Titration of SH Groups—The experiments were performed according to the procedure reported previously (22). A 135-μl aliquot of ttMutL solution at a protein concentration of 7 μM in the presence or absence of 3 μM guanidine hydrochloride (GdnHCl) was mixed with 15 μl of freshly prepared 500 μM 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) in a buffer containing 200 mM Tris-HCl (pH 8), 1 mM EDTA, and 500 mM KCl at 25 °C, and its absorbance was measured at 412 nm against a freshly prepared reagent blank with a Hitachi spectrophotometer model U-3010 (Hitachi, Tokyo, Japan). The molar extinction coefficients of reduced DTNB at pH 8 in the presence and absence of 3 μM GdnHCl were assumed to be 14,100 and 13,900 M⁻¹ cm⁻¹, respectively.

Gel Shift Assay—Synthesized 80-mer ssDNA (Bex, Tokyo, Japan), 5’-CGCAACGCGACAGGAACCTTCGAGGGATCCG-TTCTAGGAGCGCTGCTACGGAAGCTTCTCGAGG-TTCTGTCGCTTCCG-3’, was radiolabeled at the 5’ end with [γ-³²P]ATP using polynucleotide kinase (Takara, Kyoto, Japan). The 5’-³²P-labeled ssDNA was annealed to complementary ssDNA (Bex) (5’-CGCAACGCGACAGGAACCTTCG-AGAAGCTTCCGGGTAGXGCGGCTTGTAGGAGCGGATCCCTGAGGTTTCCTTGCTTGCCG-3’) to obtain dsDNA containing a matched or mismatched base pair (X = A and G for perfectly matched dsDNA and G and T mismatched dsDNA, respectively). The 20 nM ³²P-labeled ssDNA or dsDNA was incubated with various concentrations of ttMutL in 80 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM DTT, 0.2 mg/ml bovine serum albumin, and 2 mM AMPPNP, ATP, or ADP for 30 min at 25 °C for 30 min. The concentrations of ttMutL used are indicated in the figure legends. The reaction mixture (10 μl) was loaded onto a 12 or 6% polyacrylamide gel and then electrophoresed in 1× TBE buffer (89 mM Tris borate and 2 mM EDTA). The gel was dried and placed in contact with an imaging plate. The bands were visualized and analyzed using a BAS2500 image analyzer (Fuji Film, Tokyo, Japan). Dissociation constants (K₀) were determined by fitting the percentage of shifted signals to all signals (% shifted) to Equation 1 using the software Igor 4.03 (WaveMatrics, Lake Oswego, OR).

\[
\text{shifted} = 100\left(\frac{[S]}{[S]_0}\right) = 100\left(\frac{K_0 + [E]_0 + [S]_0}{[E]_0 + [S]_0 + K_0^2 - 4[S]_0[E]_0}\right)^{1/2} / (2[S]_0) \quad \text{(Eq. 1)}
\]

Nuclease Assay Using Plasmid DNA—The assay was performed by modifying the procedure reported previously (23). The 50 ng/μl supercoiled plasmid DNA (pUC19) was incubated with 100 nM enzyme in 80 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MnCl₂, and 1 mM DTT in the presence or absence of 5 mM ATP, AMPPNP, or ADP at 55 °C for 30 min unless otherwise noted in the figure legends. In the metal dependence experiment, 5 mM MgCl₂, NiCl₂, ZnCl₂, CoCl₂, or CaCl₂ was added in place of MnCl₂. The reactions were stopped by adding 5× loading buffer (5 mM EDTA, 1% SDS, 50% glycerol, and 0.05% bromphenol blue). When the effects of adenine nucleotides were examined, reactions were stopped when the initial rate was maintained. The reaction solutions were then loaded onto a 1.0% agarose S (Takara) gel containing 0.5× TBE buffer and 10 μg/ml ethidium bromide and electrophoresed in the same buffer. DNA fragments stained with ethidium bromide were detected under UV light at 254 nm. The amounts of incised and unreacted DNA were determined using the ImageJ software, which is a freely available image processing and analysis program developed at the National Institutes of Health.

Complementation Study—The isocitric acid dehydrogenase promoter from T. aquaticus YT1 and the thermostable hygromycin resistance gene was provided by Dr. Yoshinori Koyama (National Institute of Advanced Industrial Science and Technology) and cloned into EcoRI-HindIII site of pMK18 vector (24) to create pMK18::Hyg. The pET-11a/ttmutL, pET-11a/D364N ttmutL, and pET-11a/C496A ttmutL were digested by XbaI and HindIII and then ligated into the complement site of the pMK18::Hyg plasmid to generate pMK18::Hyg::WT, pMK18::Hyg::D364N, and pMK18::Hyg::C496A plasmids encoding the wild type and two mutant ttMutL. These ttmutL genes were designed to be polycistronically expressed with the hygromycin resistance gene from the isocitric acid dehydro-
genase promoter. These plasmids were then transformed into *T. thermophilus* HB27, and the spontaneous mutation rates of the transformants were estimated.

**RESULTS**

The MutL Homologue in *T. thermophilus*—First, we examined the effect of *ttmutL* disruption on the spontaneous mutation rate of *T. thermophilus* to confirm the involvement of *ttMutL* in DNA repair. High frequency mutation to a streptomycin-resistant strain was observed when *ttmutS* was disrupted (Fig. 1B). Because *ttmutS* complements the high mutagenicity of *mutS*-disrupted *E. coli* and purified *ttMutS* preferably binds to mismatched heteroduplexes (19), *ttMutS* should function as a mismatch-recognition protein just like *E. coli* MutS. The same high frequency level was observed when *ttmutL* was disrupted (Fig. 1B). These results indicate that *ttmutL* is responsible for MMR in *T. thermophilus* HB8.

Amino acid sequence comparison revealed that the N-terminal 40-kDa region of *ttMutL* is highly homologous to that of *E. coli* (Fig. 1C). It is known that this N-terminal region of MutL homologues contains four sequence motifs uniquely conserved in Gyrase b/Hsp90/MutL ATPase family proteins (25) and functions in adenine nucleotide binding and DNA binding (25, 26). In contrast, there are obvious differences among their C-terminal regions (Fig. 1C). Although eukaryotic PMS2 proteins have a sequence homologous to the C-terminal 20-kDa region of *ttMutL* (Fig. 1E), there seems to be a large insertion between the N-terminal and C-terminal regions of PMS2. To determine the organization of the structural domains of *ttMutL*, we performed limited proteolysis of *ttMutL*. *ttMutL* was digested with trypsin under mild conditions to generate four major fragments with masses of 37, 35, 27, and 24 kDa, which were designated as F1, F2, F3, and F4 (Fig. 1D). To identify the
cleavage sites, the N-terminal amino acid sequence of each material was determined. Both the F1 and F2 materials started at Met-1, which is the N-terminal residue of the intact protein. In addition, both the F3 and F4 materials started at Ala-320, indicated by an arrow in Fig. 1E. These results suggest that the cleavage occurred in the inter-domain region predicted on the basis of amino acid sequence comparison, i.e. ttMutL consists of N-terminal 40-kDa and C-terminal 20-kDa domains.

**ttMutL Undergoes Conformational Change upon ATP Binding**—The recombinant ttMutL was purified and subjected to size-exclusion chromatography to analyze self-association and adenine nucleotide-binding properties. The elution profile showed a single peak at an elution volume of 11.2 ml corresponding to a molecular mass of about 130 kDa (Fig. 2A, upper panel). Because the molecular weight of the monomeric ttMutL calculated from amino acid sequence is about 60 kDa, ttMutL should exist in a dimeric form in solution. It has been reported that the N-terminal 40-kDa region of *E. coli* MutL is in a monomeric form in the absence of adenine nucleotides and undergoes ATP binding-dependent dimerization. The elution profile monitored as to the absorbance at 260 nm showed that no nucleotide co-existed with ttMutL in the solution, indicating that ttMutL exists as a dimer even in the absence of adenine nucleotides. We examined the effects of ADP and AMPPNP on the self-association of ttMutL. It was confirmed that the pH perturbation caused by the addition of adenine nucleotides was negligible under the conditions used in this study (data not shown). As shown in Fig. 2A, middle and lower panels, ADP did not affect the elution profile, whereas AMPPNP caused a slight delay of the elution. The profiles recorded as to the absorbance at 260 nm showed that ttMutL tightly bound to AMPPNP but not ADP. Based on the molar extinction coefficients of ttMutL (ε = 280, 32,000 and 16,000 M⁻¹ cm⁻¹, respectively) and AMPPNP (ε = 280, 2,310 and 15,400 M⁻¹ cm⁻¹, respectively), it was found that the eluted ttMutL was carrying equimolar AMPPNP. Because the elution buffer contained no adenine nucleotides, it is expected that the affinity of ttMutL to AMPPNP is extremely high. However, the delay of elution was very slight; therefore we can say that there was no change in the self-association state but that some kind of conformational change of ttMutL was enhanced on the binding of AMPPNP. It was also found that ATP has the same effect on the self-association property of ttMutL as AMPPNP (data not shown).

Because ATP hydrolysis activity of ttMutL was slight in the absence of a mismatch and other MMR enzymes (Fig. 2B), the majority of ATP-bound ttMutL species will stably exist under the conditions used here.

The ATP-dependent conformational change was also confirmed by the experiment in which we examined the effects of adenine nucleotides on the limited proteolysis of ttMutL. As shown in Fig. 2C, ttMutL mixed with AMPPNP exhibited higher resistance to trypsic digestion than ttMutL alone, suggesting that AMPPNP binding triggered the conformational change of ttMutL. Consistent with the results of gel filtration analysis, the addition of ADP did not affect the resistance of ttMutL to digestion. Thus, ttMutL existed as a dimeric form that preferably binds to ATP. In addition, ATP affected on the reactivities of the SH groups in ttMutL (Fig. 2D). The C-terminal domain of ttMutL has three cysteinyl residues at positions 465, 489, and 496 (Fig. 1E). ttMutL is completely reduced with 5 mM dithiothreitol in the presence of 5 mM GdnHCl. After dithiothreitol used had been removed, the time course of the reaction of the SH groups with DTNB was followed at 412 nm. In the presence of 3 mM GdnHCl, the SH groups were titrated within the
Regulation of a Nonspecific Endonuclease

The DNA Binding Activity of ttMutL Was Suppressed by the Binding of ATP—We performed an electromobility shift assay to examine the DNA binding activity of ttMutL. The results showed that ttMutL can bind to 80-bp dsDNA with a dissociation constant of 420 nM (Fig. 3, A, left panel, and B). Interestingly, the nonspecific DNA binding of ttMutL was suppressed by the addition of 2 mM AMPPNP (Fig. 3, A, right panel, and B). In the presence of AMPPNP and magnesium ions, the dissociation constant for 80-bp dsDNA is estimated to be over 5 μM. The same result was obtained when AMPPNP was substituted by ATP (Fig. 3B). On the other hand, there was no detectable effect of ADP (Fig. 3B). These observations were consistent with the results obtained on size-exclusion chromatography and indicate that the ATP-bound form of ttMutL is different from that of the nucleotide-free form in both structural and functional aspects. The ssDNA binding ability of ttMutL was also examined using 80-mer ssDNA as a substrate. As shown in Fig. 3, C and D, ttMutL bound weakly to ssDNA. The dissociation constants for the 80-mer ssDNA in the presence and absence of AMPPNP were over 2 and 4 μM, respectively. ADP did not affect the ssDNA binding ability of ttMutL (data not shown). Unlike E. coli MutL (27), ttMutL preferably bound to dsDNA rather than to ssDNA. The dsDNA preference of the
ttMutL exhibited the endonuclease activity—The endonuclease activity of ttMutL was then examined using the covalently closed circular (CCC) form of plasmid DNA as a substrate. The CCC of plasmid DNA is often used in in vitro experiments to confirm a latent endonuclease activity of DNA repair and recombination enzymes. For example, MutH (28), RAD1/RAD10 (29), MutLα (11), and other endonucleases incise it in vitro without lesions due to the absence of competitors and inhibitors (11, 30). As shown in Fig. 4A, in the presence of manganese ions, ttMutL relaxed the CCC to generate the nicked open circular form (OC) of plasmid DNA, indicating that ttMutL possesses nicking endonuclease activity. The level of the activity increased according to the reaction temperature from 25 to 70 °C. It is known that enzymes from *T. thermophilus* often perform best at about 70 °C.

To identify the residue involved in the catalysis, we generated the D364N and C496A mutants. Asp-364 corresponds to Asp-699 in human PMS2, whose substitution to Asn results in the loss of the endonuclease activity (11). Cys-496 is located in the region highly conserved in MutL homologues from all organisms (Fig. 1E); however, it is unknown for what activity this region is responsible. Although D364N mutant retained DNA binding activity just as wild type (Fig. 4, B and C), the endonuclease activity was undetectable (Fig. 4D), indicating that Asp-364 takes part in the catalytic step of the endonuclease reaction. On the other hand, C496A retained the endonuclease activity, although the velocity was slightly weaker than that of wild type (supplemental Fig. 1A). The metal ion specificity of C496A nuclease activity was the same as that of wild type (supplemental Fig. 1B).

In the absence of a divalent cation, ttMutL did not show the endonuclease activity (data not shown). We found that nickel and cobalt ions could substitute for manganese ion, whereas magnesium, zinc, and calcium ions cannot (Fig. 4E). The manganese ion concentration dependence and salt concentration dependence are shown in Fig. 4, F and G, respectively. It was revealed that a millimolar concentration of manganese ions is sufficient for the nuclease activity and that higher concentrations of potassium ions inhibit the activity.
We examined the complementation of a high mutability phenotype of \( \text{tt}\)mutL-deficient strain with wild type, D364N, and C496A \( \text{tt}\)mutL genes. As shown in Fig. 4H, the introduction of Circles, D364N mutant \( \text{tt}\)MutL; triangles, wild type \( \text{tt}\)MutL; D, nuclease assaying of the D364N mutant. The reactions were performed at 37 °C, and the enzyme concentrations are indicated at the top of the figure. E, divalent cation dependence. The CCC of plasmid DNA was reacted with \( \text{tt}\)MutL in buffer containing 25 mM KCl, 5 mM MnCl\(_2\), and 5 mM MgCl\(_2\). The percentage of OC as to all plasmid DNA was plotted against the \( \text{AMPPNP} \) concentration. The data are the means of five experiments with standard deviations. F, effects of adenine nucleotides on stabilization of the MutS-dsDNA complex by \( \text{tt}\)MutL. The substrate 80-bp G-T mismatched \( \) or perfectly matched \( \) dsDNA was incubated with 50 nM \( \text{tt}\)MutL and 200 nM \( \text{tt}\)MutS in the presence or absence of 5 mM adenine nucleotide at 37 °C for 60 min and 60 °C for 10 min. Then the mixtures were subjected to 6% native-PAGE.

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the wild type *mutL* gene resulted in the reduction of the mutability in the *mutL*-deficient strain equal level to that of the wild type strain. In contrast, the D364N *mutL* gene did not complement the phenotype, strongly indicating that the endonuclease activity of *mutL* is required for the DNA repair *in vivo*. Interestingly, C496A *mutL* gene also could not complement the phenotype, although purified C496A *mutL* retained the endonuclease activity. This result is consistent with the fact that the amino acid sequence around Cys-496 is highly conserved in MutL homologues (Fig. 1E).

**The Endonuclease Activity of *mutL* Was Negatively Regulated by the Binding of ATP**—The effects of adenine nucleotides on *mutL* endonuclease activity were also examined. As shown in Fig. 5, A–D, AMP-PNP and ATP inhibited the nonspecific DNA digestion by *mutL*, whereas ADP did not. These results are consistent with that of the DNA binding assay. When DNase I, a nonspecific endonuclease, was used as a negative control, no detectable effect of adenine nucleotides on the rate of incision was observed (data not shown). Because it has been reported that the endonuclease activity of human MutLα is slightly enhanced by the addition of 0.5 mM ATP under the conditions of a low salt concentration (11), we performed the same experiment in buffer containing a low concentration of salt. Fig. 5E shows that a relatively high concentration of AMP-PNP was needed to suppress the endonuclease activity of *mutL* under such conditions. As mentioned above, the binding of AMP-PNP caused a decrease in the affinity of *mutL* to dsDNA. Generally, ionic bonds between the phosphate backbone of DNA and basic amino acid residues of a protein are responsible for the binding of the protein to its substrate DNA, and the interaction is strengthened under the condition of low salt concentration. Therefore, the small effect of AMP-PNP in Fig. 5E is quite comprehensible. The differences in the effects of adenine nucleotides between *mutL* and human MutLα would arise from the experimental conditions, including the salt concentration. According to the report by Kondo et al. (31), the intracellular concentrations of free metal ions (mainly sodium and potassium ions) were about 100 mM in *T. thermophilus*. Therefore, as to the assay of an enzyme from *T. thermophilus*, the experimental condition used was similar to the intracellular ionic condition.

Furthermore, the effects of adenine nucleotides on stabilization of a *mutS*-mismatch complex by *mutL* were analyzed. As shown in Fig. 5F, a large stable dsDNA-protein complex was detected when a G-T mismatched dsDNA was incubated with *mutS*, *mutL*, and AMP-PNP (lane 10), whereas only a small complex was detected when the dsDNA was incubated without *mutL* (lane 5). Small and large complexes are thought to be *mutS*-dsDNA and *mutS*-*mutL*-dsDNA complexes, respectively. The stabilization of a G-T mismatched dsDNA-protein complex by *mutL* was more effective than that of a perfectly matched dsDNA-protein complex (Fig. 5F, compare lanes 10 and 20). Because gel filtration analysis revealed that *mutL* tightly binds to AMP-PNP and that the conditions used in this experiment involved an excess of AMP-PNP, it is expected that *mutL* existed as an AMP-PNP-bound form during the experiment. An AMP-PNP-bound form of *mutL* can specifically interact with a mismatch-bound *mutS* and stabilize it. In other words, the binding of ATP will promote the mismatch- and MutS-dependent DNA binding of *mutL*. In this experiment, hydrolyzable ATP apparently had no effect on the stabilization of the complex. This might be because of the nature of the substrate linear dsDNA whose termini were not blocked. It has been reported that the binding and hydrolysis of ATP by MutS lead to the formation of a sliding clamp of MutS and dissociation from mismatched DNA (32, 33). Therefore, the addition of ATP causes the apparent dissociation of MutS from a substrate DNA when nonblocked substrates are used. Then the same experiment was performed in the presence of a bifunctional cross-linker, glutaraldehyde, which cross-links two amino groups of protein and DNA that are in close proximity to each other. Not only AMP-PNP but also ATP induced the formation of a large DNA–protein complex in the presence of the cross-linker (supplemental Fig. 2), indicating that ATP-bound form of *mutL* also interacts with *mutS*-mismatched DNA complex.

**The Cysteinyl Residue in the C-terminal Domain Was Responsible for the ATP-induced Functional Change of**
**Regulation of a Nonspecific Endonuclease**

*ttMutL*—Interestingly, the modification of cysteinyl residues in the C-terminal domain of *ttMutL* with DTNB caused the decrease in the efficiency of ATP-dependent suppression of its nonspecific endonuclease activity (Fig. 6, A and B), although the ATP-binding motif is located in the N-terminal domain. The substitution of Cys-496 in *ttMutL* with an Ala residue also brought about the same change in the efficiency (Fig. 6C). It is also revealed that the DNA binding activity of C496A is independent of the presence of AMPPNP unlike wild type (Fig. 6, D and E). The requirement of the C-terminal domain for the ATP-induced functional change of MutL is consistent with the previous report that the DNA binding activity of NhPMS2 is not affected by the binding of ATP (26). These results indicate that the cysteinyl residue in the C-terminal domain is responsible for the regulation of the endonuclease activity. As mentioned above, C496A *ttmutL* gene could not complement the hyper-mutability phenotype of *ttmutL*-deficient strain (Fig. 4H). The ATP-dependent regulation of the endonuclease activity of *ttMutL* will be essential for DNA repair in *vivo*. Further experiments revealed that C496A did not undergo AMPPNP-dependent conformational change (Fig. 6F), although it retained the strong affinity for AMPPNP (Fig. 6G). It is intriguing how the C-terminal cysteinyl residue is involved in the ATP-induced structural and functional change. To uncover this, structural analysis of a complex of a full-length MutL endonuclease with substrate DNA is necessary.

* *A. aeolicus* MutL Also Exhibited the Endonuclease Activity That Is Suppressed by the Binding of ATP.—To know whether MutL homologue from another organism also shows the ATP-dependent functional change, we also analyzed the biochemical characteristics of MutL from a hyperthermophilic bacterium, *A. aeolicus*, which grows at over 90 °C. Although the *A. aeolicus* MutL (aqMutL) contains residues corresponding to Asp-364 and Cys-496 of *ttMutL*, it lacks about 90 amino acid residues corresponding to the central region of the C-terminal domain of *ttMutL* (Fig. 1E). Gel filtration analyses revealed that the purified recombinant aqMutL, like *ttMutL* and unlike *E. coli* MutL, is in a dimeric form in the absence of adenine nucleotides (data not shown). We assume that this self-association property is common for MutL proteins from *mutH*-less organisms and depends on the C-terminal domain of MutL. As shown in Fig. 7, *A* and *B*, aqMutL relaxed the supercoiled plasmid DNA at an extremely high temperature in a manganese, nickel, zinc, or cobalt ion-dependent manner. It is also confirmed that the DNA binding and endonuclease activities are suppressed by AMPPNP or ATP but not by ADP (Fig. 7, *C* and *D*). These results suggest that the characteristic ATP binding-dependent functional change is a general feature of MutL proteins in *mutH*-less organisms.

**DISCUSSION**

In this study, the endonuclease activities of MutL from *mutH*-lacking bacteria, *T. thermophilus*, and *A. aeolicus* were confirmed. These results suggest that not only eukaryotic but also bacterial MutL have endonuclease activity, and the model established for human MMR (11) could be universal for organisms lacking *mutH*. Human MutLα incises supercoiled plasmid DNA even in the absence of a mismatch, strand discontinuity, and other MMR enzymes (11). Therefore, it can be speculated that, in *vivo*, the endonuclease activity of a MutL homologue is suppressed until mismatch recognition and that other MMR enzymes promote the mismatch-provoked incision. In this study, the ATP-dependent conformational change of bacterial MutL is described. This is consistent with the recent report that an ATPase cycle modulates the conformational states of *Saccharomyces cerevisiae* MutLα (34). The ATP-dependent conformational change would be a common feature among MutL homologues from all organisms. We also clarified that the ATP binding significantly affected the DNA binding and nucleolytic activities of bacterial MutL. In our *in vitro*
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experiments, the concentration of AMPPNP or ATP needed for the sufficient inhibition of the nonspecific endonuclease activity of ttMutL was 3–5 mM. The bacterial intracellular concentration of ATP has been estimated to be over 3 mM (35). In addition, ttMutL tightly bound to AMPPNP, when it was mixed with 2 mM ATP (Fig. 2A). From these results, we suspect that binding of ATP abolished the endonuclease activity of bacterial MutL to protect DNA from nonspecific degradation in vivo until mismatch recognition and matchmaking by other MMR enzymes are completed. Such a notion is supported by the results of complementation studies that showed that the ATP-dependent functional change of ttMutL is necessary for DNA repair in living cell (Fig. 4H). The ATP-bound form of MutL might specifically interact with a mismatch-MutS complex and then hydrolyze ATP to bring about the downstream reactions, including the mismatch-promoted strand break (Fig. 7E). The experiments concerned with the mismatch-cleavage reaction should be necessary for further understanding of the mechanism.

It is noteworthy that we could detect the ATP-induced conformational change by monitoring the reactivities of the cysteinyl residues (Fig. 2D), although the ATP-binding site is located in the N-terminal domain. In addition, the inactivation of the C-terminal cysteinyl residues caused the perturbation in the ATP-induced conformational and functional change (Fig. 6) indicating that the conserved cysteinyl residues would play significant roles in the ATP-dependent regulation of the endonuclease activity of ttMutL. It is known that cysteinyl residues often form a metal-binding motif, such as a zinc finger. A metal-binding motif in the C-terminal domain of ttMutL might function in sensing signals of ATP binding and the interaction with other MMR enzymes.

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