Evidence for ATP-dependent Structural Rearrangement of Nuclease Catalytic Site in DNA Mismatch Repair Endonuclease MutL

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The DNA mismatch repair (MMR) system greatly contributes to genome integrity via the correction of mismatched bases that are mainly generated by replication errors. Postreplicative MMR excises a relatively long tract of error-containing single-stranded DNA. MutL is a widely conserved nicking endonuclease that directs the excision reaction to the error-containing strand of the duplex by specifically nicking the daughter strand. Because MutL apparently exhibits nonspecific nicking endonuclease activity in vitro, the regulatory mechanism of MutL has been argued. Recent studies suggest ATP-dependent conformational and functional changes of MutL, indicating that the regulatory mechanism involves the ATP binding and hydrolysis cycle. In this study, we investigated the effect of ATP binding on the structure of MutL. First, a cross-linking experiment confirmed that the N-terminal ATPase domain physically interacts with the C-terminal endonuclease domain. Next, hydrogen/deuterium exchange mass spectrometry clarified that the binding of ATP to the N-terminal domain induces local structural changes at the catalytic sites of MutL. C-terminal domain. Finally, on the basis of the results of the hydrogen/deuterium exchange experiment, we successfully identified novel regions essential for the endonuclease activity of MutL. The results clearly show that ATP modulates the nicking endonuclease activity of MutL via structural rearrangements of the catalytic site. In addition, several Lynch syndrome-related mutations in human MutL homolog are located in the position corresponding to the newly identified catalytic region. Our data contribute toward understanding the relationship between mutations in MutL homolog and human disease.

The DNA mismatch repair (MMR) system greatly contributes to the replication fidelity of DNA by correcting the mismatched bases generated by the errors of DNA polymerases (1–3). The early reactions of eukaryotic MMR are performed mainly by MutSα (MSH2/MSH6 heterodimer) and MutLα (PMS2/MLH1 and PMS1/MLH1 heterodimers in Homo sapiens and Saccharomyces cerevisiae, respectively). It is reported that hereditary dysfunctions of these proteins are a major cause of Lynch syndrome (also known as hereditary non-polyposis colorectal cancer) in humans (4, 5). MutSα recognizes the mismatched bases and stimulates MutLα, which has a latent nicking endonuclease activity that introduces the entry or termination point of the subsequent excision reaction (6–8). MutLα directs MMR to the daughter strand by specifically nicking the discontinuous strand in cooperation with replication protein A as well as the loaded form of proliferating cell nuclear antigen in the presence of ATP (7). After the error-containing strand is excised by EXO1, DNA polymerase δ resynthesizes the strand to complete the repair reaction.

The MMR systems of the majority of bacteria except Escherichia coli and other γ-proteobacteria are believed to resemble the eukaryotic system (8). The majority of bacteria possess MutS and MutL dimers whose fundamental characteristics are similar to those of human MutSα and MutLα, respectively. Bacterial MutL homologs show high amino acid sequence similarity to the PMS2 subunit of human MutLα, which includes the endonuclease motif, DQHAX2EEXE, and the zinc-binding motif, CPHGRP, in the C-terminal domain (CTD) (6, 9, 10). To date, it has been confirmed that MutL proteins from Thermus thermophilus, Aquifex aeolicus, Neisseria gonorrhoeae, and Bacillus subtilis possess nicking endonuclease activity (9, 11–13).

Because MutL homologs need to incise the distal site from the mismatch, their nicking endonuclease activity itself has no mismatch, sequence, or structure specificity (6, 9, 11, 12, 14). This indicates that the nicking endonuclease activity of MutL homologs should be activated by other components only when they are required (15). Previous studies suggest that this regulatory mechanism involves the ATP-dependent conformational and functional changes of MutL (9, 16). MutL homologs are members of the GHKL ATPase/kinase superfamily proteins and contain the Bergerat ATP-binding fold in their N-terminal domain (NTD) (17–19). The GHKL superfamily proteins are known to undergo large conformational changes upon ATP binding and/or hydrolysis that are essential for their molecular and cellular functions (20). ATP binding suppresses the non-specific nicking endonuclease activity of bacterial MutL in an NTD- and CPHGRP motif-dependent manner (9, 11). On the
other hand, it was also determined that the endonuclease activity of relatively high concentrations of MutL is enhanced by ATP (12), implying that ATP hydrolysis promotes the endonuclease activity. However, physiological concentrations of MutL stably bind ATP without any detectable hydrolyzing activity (9). Therefore, it is expected that interactions with other MMR proteins define the timing of ATP hydrolysis to achieve mismatch- and daughter strand-specific incision.

Because the ATP binding and endonuclease catalytic sites are located in the NTD and CTD of MutL (11, 21), respectively, interdomain interaction is speculated. Atomic force microscopy analysis also suggested that ATP-induced conformational changes of MutL involve the association between the NTD and CTD (16). Recently, we prepared the NTD and CTD of *A. aeolicus* MutL (aqMutL) separately and confirmed that the NTD possesses DNA binding ability and stimulates the endonuclease activity of the CTD (21), suggesting the direct interaction between the NTD and CTD. Interestingly, substituting the cysteine in the CPHGRP zinc-binding motif with an alanine abolishes the NTD-dependent activation of the CTD (21). The interdomain interaction-induced stimulation of the CTD may be directly or indirectly mediated by the zinc ion in the CPHGRP motif.

In this study, we examined the interdomain interaction- and ATP binding-induced conformational changes in aqMutL by using hydrogen/deuterium exchange mass spectrometry (DXMS). The results indicated that the interdomain interaction influences the large part of the CTD including the DQHA<sub>2</sub>EX<sub>3</sub> endonuclease motif and that ATP binding results in the loss of flexibility in the DQHA<sub>2</sub>EX<sub>3</sub> and CPHGRP motifs. Furthermore, we identified the C-terminal region of the NTD and the C-terminal α-helix of the CTD as regions whose deuterium incorporation decreases upon ATP binding. Subsequent biochemical studies verified that the conserved 5 C-terminal residues of the NTD are essential for the stimulation of the CTD and that Arg-423 of the C-terminal α-helix is required for the nicking endonuclease activity. These results provide the molecular basis for the intramolecular regulatory mechanism of MutL endonuclease.

**EXPERIMENTAL PROCEDURES**

*Expression and Purification of Proteins—*Full-length aqMutL, aqMutL NTD<sub>310</sub> (residues 1–310 of aqMutL), and CTD<sub>425</sub> (residues 316–425 of aqMutL) were expressed and purified as described previously (21). DNA fragments expressing aqMutL NTD<sub>310</sub> (residues 1–310 of aqMutL), aqMutL CTD<sub>419</sub> (residues 316–419 of aqMutL), and CTD<sub>413</sub> (residues 316–413 of aqMutL) were generated by PCR using PET-11a/aqmutL (9) as a template. The forward and reverse primers used for amplification were as follows: 5′-ATATCATATGTTTGAAGGTA-TCATCTCCC-3′ and 5′-TATAAGATCTATTTCTTTCTTTCCCCGGAG-3′ (BEX Co., Tokyo, Japan), 5′-ATATCATATGTTTGAAGGTA-TCATCTCCC-3′ and 5′-TATAAGATCTATTTCTTTCTTTCCCCGGAG-3′ (BEX Co.), and 5′-TATAAGATCTATTTCTTTCTTTCCCCGGAG-3′ (BEX Co.).

The forward and reverse primers contained NdeI and BglII sites, respectively (underlined). The amplified aqmutL NTD<sub>310</sub>, CTD<sub>419</sub>, and CTD<sub>413</sub> fragments were ligated into the NdeI and BamHI sites of pET-11a (Novagen, Madison, WI) to yield pET-11a/aqmutL NTD<sub>310</sub> pET-11a/aqmutL CTD<sub>419</sub>, and pET-11a/aqmutL CTD<sub>413</sub> plasmids, respectively. Sequence analysis revealed that the constructions were error-free. The pET-11a plasmid carrying the gene for the R423A mutant of aqMutL CTD<sub>425</sub> was constructed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The forward and reverse primers were 5′-GGATCTTTATACGTAATTCCGGCTACTTTC-TCATATTTCTCCC-3′ and 5′-GAGGGAAATATACGTAATTCCGGCTACTTTC-3′ (BEX Co.), respectively. The pET-11a plasmid carrying the gene for the K199Q/K200Q mutant of aqMutL NTD<sub>315</sub> was also constructed using the QuikChange mutagenesis kit (Stratagene).

The forward and reverse primers were 5′-AGGAAACCCTGG-AAAATGCGCAAGTCAGTTTAAAGGAAAGGA-3′ and 5′-TTATTCCTTTTTAAATTTGCGCAAGTCAGTTTACG-3′ (BEX Co.), respectively. Sequence analysis revealed that the construction was error-free.

*E. coli* Rosetta-gami(DE3) (Novagen) was transformed with pET-11a/aqmutL CTD<sub>419</sub>, CTD<sub>413</sub>, and CTD<sub>425</sub> R423A and subsequently cultivated at 37°C in 1.5 liters of YT medium (0.8% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Oriental Yeast, Tokyo, Japan), and 0.5% (w/v) NaCl) containing 50 μg/ml ampicillin. When the density of the cultures reached 4 × 10<sup>8</sup> cells/ml, isopropyl β-D-thiogalactopyranoside (Wako, Osaka, Japan) was added to a final concentration of 0.1 mM. The cells were grown at 37°C for 4 h after induction and subsequently harvested by centrifugation. The cells were lysed by sonication in buffer I (20 mM Tris-HCl (pH 8.0) and 50 mM NaCl) and heated at 70°C for 10 min. After centrifugation at 48,000 × g for 60 min, the resultant supernatant was loaded onto an SP-Sepharose column (40 ml) (GE Healthcare) pre-equilibrated with buffer I. The flow-through fraction was collected and loaded onto a SuperQ TOYOPEARL column (40 ml) ( Tosoh, Tokyo, Japan) pre-equilibrated with buffer I. The flow-through fraction was collected, and ammonium sulfate was added to a final concentration of 1 M. The solution was loaded onto a TOYOPEARL-Phenyl column (40 ml) (Tosoh) equilibrated with buffer I containing 1 M ammonium sulfate. The column was washed with 100 ml of buffer I containing 1 M ammonium sulfate and then eluted with a 300-ml gradient of 1–0 M ammonium sulfate in buffer I. The fractions containing aqMutL CTDs were detected by SDS-PAGE and concentrated with a Vivaspin concentrator (Vivasience, Hanover, Germany). The concentrated solution was applied to a Superdex 75 HR column (24 ml; GE Healthcare) pre-equilibrated with buffer I using an ÄKTAl Chromatography system (GE Healthcare). aqMutL NTD<sub>310</sub> and NTD<sub>315</sub> K199Q/K200Q were overexpressed and purified using the same procedure as that used for aqMutL NTD<sub>315</sub> (21). The molar extinction coefficients of aqMutL NTD<sub>315</sub>, NTD<sub>310</sub>, CTD<sub>425</sub>, CTD<sub>419</sub>, CTD<sub>413</sub>, CTD<sub>423</sub> R423A, and NTD<sub>315</sub> K199Q/K200Q were calculated to be 11,760, 11,760, 19,000, 17,500, 16,100, 19,000, and 11,760 M<sup>−1</sup> cm<sup>−1</sup>, respectively, at an absorption maximum of ~278 nm as described previously (23).
Small Angle X-ray Scattering Experiment—Small angle x-ray scattering (SAXS) experiments were performed at the RIKEN Structural Biology Beamline (BL45XU) at SPring-8, Sayo-Gun, Hyogo, Japan. To remove any impurities, prior to the measurement, the protein sample was purified by chromatography on a Superdex 75 HR column (1 × 30 cm; GE Healthcare) in freshly prepared running buffer containing 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl. After filtration through an Amicon ultracentrifugal filter device (22,000 Da, Millipore, Billerica, MA), 0.81, 1.40, 1.89, 2.39, and 5.65 mg/ml aqMutL CTD_{325} and 2.29, 4.59, 6.88, 9.17, and 11.46 mg/ml aqMutL NTD_{315} were subjected to SAXS measurements. The x-ray wavelength and the sample-to-detector distance were 0.9 Å and 2,530 mm, respectively. The scattering profiles of the protein samples and the buffer were collected at 20 °C using an imaging plate detector, R-Axis IV++ (Rigaku, Tokyo, Japan), with an exposure time of 60 s. After correcting the image for distortions and sensitivities, the scattering data were normalized by the x-ray intensity and the data collection time. Scattering intensities (I(s)) were determined at multiple protein concentrations using the equation $S = 4 \sin \theta / \lambda$, where $2 \theta$ is the scattering angle and $\lambda$ is the wavelength of the incident beam. The background scattering intensity of the buffer was subtracted from the scattering intensity of the protein sample by using PRIMUS (24). The scattering curves were then extrapolated to zero concentration, and the radius of gyration ($R_g$) was determined by a Guinier plot on PRIMUS. The GNOM program (25) was used to generate the pair distribution function ($P(r)$) through the indirect Fourier transform of the scattering curve. The output file was used to generate an ab initio model with the DAMMIN program (26). The obtained model was processed using the DAMAVER package (27) following the procedure described previously (28).

Protein Cross-linking Coupled with Mass Spectrometry—The 17.5 μM aqMutL CTD was preincubated with 4 μM NTD at 37 °C for 10 min before the addition of the cross-linker. The protein mixture was incubated with 5, 10, 20, or 40 μM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) (Dojindo, Kumamoto, Japan) in 20 mM HEPES-KOH (pH 7.5), 30 or 100 mM NaCl, and 0.5 mM ZnCl₂ at 37 °C for 45 min. The reaction products were separated on 12.5% SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue R-250 (Tokyo Chemical Industry, Tokyo, Japan). The cross-linked product was excised from the gel, digested by trypsin, separated with an EASY-nLC system (Proxeon, Odense, Denmark), and analyzed with a micrOTOF-QII instrument (Bruker Daltonics, Billerica, MA) as described previously (21).

Hydrogen/Deuterium Exchange Coupled with Mass Spectrometry—For mass spectrometry (MS), full-length aqMutL and aqMutL CTD_{325} solutions were dialyzed against 50 mM Tris-HCl (pH 7.5). Pepsin was obtained from Sigma, and D₂O (99.9 atom % deuterium) and acetic acid-d₆ (99 atom % deuterium) were purchased from EURISO-TOP (Saint-Aubin, France) and IsoTec USA Inc. (Miamisburg, OH), respectively. All other chemicals were of analytical grade.

The DXMS experiment was performed using a modified version of a procedure described previously (29–31). The hydrogen/deuterium exchange reactions of both full-length and aqMutL CTD for these apo and ATP binding states were started by mixing 180 μL of D₂O solution with 20 μL of protein solutions (100 μM full-length aqMutL or 350 μM CTD including 50 mM magnesium acetate, 0 or 2 mM ATP, and 50 mM Tris-HCl (pH 7.5)) in three tubes for each state at 10 °C. The pH of the mixture was 7.5, and the atomic ratio of hydrogen to deuterium was 1:9. Proteins in three tubes were incubated for three different time sets: (i) 0.33, 1, 2.5, 5, 13, or 30 min; (ii) 0.33, 1.5, 3, 7, 17, or 45 min; and (iii) 0.66, 2, 4, 10, 22, or 60 min. After each incubation time, 20 μL of the reaction mixture was taken out and immediately quenched by the addition of 2 μL of 20% acetic acid with an atomic ratio of hydrogen to deuterium of 1:9. The deuterated protein samples were digested with 2 μL of 0.1% pepsin (Sigma) dissolved in an H₂O/D₂O mixture (hydrogen:deuterium, 1:9) for 2 min at pH 2.8 and 0 °C and frozen by liquid nitrogen. The frozen sample was mixed with saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid and loaded on a sample plate at 7 pascals. Three minutes after removing the deuterated fragment from the freezer, the plate was set up on a MALDI-TOF MS Voyager DE PRO system (Applied Biosystems, Foster City, CA) at 10⁻⁵ pascal. Peptides enclosed in α-cyano-4-hydroxycinnamic acid crystals were ionized with an N₂ laser at 337 nm and accelerated at an accelerating voltage of 25 kV with a pulse delay time of 300 ns. Peptic fragments were identified by MALDI tandem time-of-flight (TOF/TOF)-MS Proteomics Analyzer 4700 (Applied Biosystems). The deuterium incorporation of residues 389–410 was determined by the mass difference between the 389–416 and 410–416 fragments.

To compare the hydrogen/deuterium exchange kinetics of digestion fragments consisting of different numbers of residues, the molecular weight of each fragment, $M_r$, at exchange time $t$ was normalized to the exchanged fraction of main-chain amide protons, $D_r$, using the equation

$$D_r = (M_r - M_{\text{side chain}}^{100\%})/(M_r^{\text{theo}} - M_{\text{side chain}}^{100\%})$$

(Eq. 1)

where $M_{\text{theo}}$ and $M_{\text{side chain}}^{100\%}$ are the theoretical molecular weights of each fragment and its side chains, respectively, assuming a complete exchange of protons to deuterium. $D_r$ was determined using the centroid mass of each isotopic distribution. Because of the extremely high deuterium concentration (hydrogen:deuterium, 1:9), the hydrogen/deuterium exchange of each amide proton would follow first-order kinetics at constant pH and temperature (11). Therefore, the hydrogen/deuterium exchange time course was analyzed using the equation

$$D_t = D_\infty - A \exp(-k_{\text{ex}}t)$$

(Eq. 2)

where $D_t$ and $D_\infty$ are the fractions of deuterium incorporation at exchange times $t$ and infinity, respectively; $A$ is the fraction of exchangeable protons that can be detected during the exchange time; and $k_{\text{ex}}$ is the apparent first-order rate constant of the hydrogen/deuterium exchange reaction. The observed $k_{\text{ex}}$ value represents the average of all the exchange rates of the different amide protons. The background exchange of hydrogen for deuterium during manipulations was calculated as <0.03% by using 100% deuterated melittin.
DXMS Revealed ATP-dependent Structural Change of MutL

Nicking Endonuclease Assay—The nicking endonuclease assay was performed as described previously with some modifications (32). The supercoiled pT7Blue plasmid DNA was prepared by using a Wizard Plus SV Miniprep kit (Promega). The 10 ng/μl supercoiled plasmid DNA was incubated with various concentrations of freshly prepared aqMutL CTD in 100 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MnCl₂, 0.5 mM ZnCl₂, and 1 mM dithiothreitol at 55 °C for 30 min unless otherwise noted in the figure legends. The reactions were stopped by adding 5× loading buffer (5 mM EDTA, 1% SDS, 50% glycerol, and 0.05% bromphenol blue). The reaction solutions were loaded onto a 1.0% agarose (Takara, Shiga, Japan) gel containing 0.5 mM EDTA buffer (89 mM Trisborate and 2 mM EDTA) and electrophoresed in the same buffer. The gel was stained with ethidium bromide, and DNA fragments were detected under UV light at 254 nm. Therefore, we created the model structures of NTD₃₁₅ (residues 1–315) and CTD₄₂₅ (residues 316–425) of aqMutL by a homology modeling methodology using the crystal structures of E. coli MutL NTD (18) (Protein Data Bank code 1B63) and B. subtilis MutL CTD (13) (Protein Data Bank code 3DKD) as template structures. The aqMutL NTD and CTD exhibit over 30% amino acid sequence similarities with E. coli MutL NTD and B. subtilis MutL CTD, respectively. Because the E. coli MutL CTD lacks the DQHAX₃EX₄E and CPHGRP motifs (6), we used the crystal structure of B. subtilis MutL CTD (13) as a template structure to predict the structure of aqMutL CTD₄₂₅. These similarities are sufficient to build reliable model structures using homology modeling (33). The generated model structures of aqMutL NTD₃₁₅ and CTD₄₂₅ were almost identical to those of the template structures. To examine the validity of these model structures, we performed the SAXS experiments on aqMutL NTD₃₁₅ and CTD₄₂₅ (supplemental Fig. S1). The bead model structures created from the SAXS precisely matched the predicted structures (Fig. 1, A and B). In this study, we utilized these predicted structures to discuss the results of DXMS and other experiments.

Direct Interaction between aqMutL NTD and CTD—Previously, we found that aqMutL NTD₃₁₅ stimulates the endonuclease activity of CTD₄₂₅ in a zinc-dependent manner (21). To further characterize the direct interaction between them, we performed a cross-linking experiment. It should be mentioned that inducively coupled plasma emission spectrometry revealed that the purified full-length aqMutL used in this study contained 0.8 zinc ion per subunit without the addition of exogenous zinc ions (data not shown). Protein solutions of aqMutL NTD₃₁₅, CTD₄₂₅, or NTD₃₁₅-CTD₄₂₅ mixture were incubated with DIDS, which has two equivalent isothiocyanate groups and cross-links between the two amino groups. At neutral pH, DIDS reacts effectively with the ε-amino group of lysine and slightly with the α-amino groups of the N termini of peptides. When NTD₃₁₅-CTD₄₂₅ mixture was incubated with DIDS, a cross-linked band with a molecular mass estimated to be about 50 kDa appeared (Fig. 1C, asterisk). The appearance of the band was dependent on both NTD₃₁₅ and CTD₄₂₅; its estimated molecular mass was consistent with the summation of the theoretical molecular masses of NTD₃₁₅ (36 kDa) and CTD₄₂₅ (13 kDa). The cross-linked molecule in the band was excised from the gel, digested with trypsin, and analyzed by nano-liquid chromatography-mass spectrometry. The results revealed that the cross-linked molecule included both the NTD and CTD (supplemental Fig. S3, A and 3B), supporting the direct physi-
ological interaction between them. The result shown in Fig. 1C was obtained under the condition of relatively low salt concentration. Although higher concentrations of salt seemed to weaken the appearance of the cross-linked molecule, cross-linking between NTD and CTD was detected in the presence of 100 mM NaCl (supplemental Fig. S2).

To identify the cross-linked site, we searched for the cross-linked peptide among the tryptic digests; however, no DIDS-modified fragment was found. There may have been an unexpected modification or interference of ionization of the peptide by DIDS. Nonetheless, we successfully detected over 90% of the tryptic fragments derived from NTD and CTD (supplemental Fig. S3, A and B). We listed the unidentified lysine-containing fragments as the putative cross-linked sites (supplemental Fig. S3C). DIDS-modified lysine loses its positive charge and is expected to no longer be recognized by trypsin because the aspartate residue (Asp-189) in the specificity pocket of trypsin cannot attract neutral residues (34). Therefore, the possible cross-linked residues would be Lys-109, Lys-199, or Lys-200 in the NTD and the α-imino group of Pro-316 in the CTD (supplemental Fig. S3, C and D). Consistent with this, we previously found that DIDS modifies the α-imino group of Pro-316 in CTD under the same conditions (21). Accounting for the length of DIDS (14 Å) and the predicted structure of aqMutL NTD and CTD, Lys-109 would be unable to be cross-linked to Pro-316. Therefore, we speculate that cross-linking occurred between Lys-199 or Lys-200 in the NTD and Pro-316 in the CTD.

To know the cross-linked site, we created the mutant NTD (K199Q/K200Q) in which Lys-199 and Lys-200 were substituted by Gln residues. The NTD K199Q/K200Q mutant was successfully overexpressed and purified by the same procedure as that used for wild-type NTD and CTD. MS confirmed that the band marked with an asterisk consists of peptide fragments of both CTD and NTD, showing the proper folding of the mutant. As a result, we observed no cross-linking between NTD (K199Q/K200Q mutant and CTD (Fig. 1E). These results indicate that Lys-199 and Lys-200 in the wild-type...
NTD<sub>315</sub> were cross-linked to the CTD<sub>425</sub> by DIDS. From these results, Lys-199 and Lys-200 are thought to be within 15 Å from Pro-316 when NTD interacts with CTD, and this can be used to predict the structure of full-length aqMutL.

**Interdomain Interaction-induced Conformational Change in aqMutL CTD**—Interaction between the aqMutL NTD and CTD is expected to be required for the regulation of the endonuclease activity of the CTD (21). DXMS was used to examine the interdomain interaction-induced conformational change of aqMutL (Fig. 2A). DXMS can measure the deuteration rate of the main-chain amides of proteins, which represents the solvent accessibility and secondary structure of each region in a protein (29, 35, 36). Full-length aqMutL and aqMutL CTD<sub>425</sub> were subjected to DXMS. The difference between the results of the full-length aqMutL and aqMutL CTD<sub>425</sub> is expected to reflect the NTD-induced conformational change of the CTD.

The protein samples were incubated in a deuterium-containing buffer for various periods, and the exchange was quenched by acetic acid-<sup>d</sup> in 90% deuterium oxide (pH 2.8). At pH 2.8, exchanges of all protons in amino acid side chains are expected to be completed instantaneously after addition of deuterium. Therefore, results can be simply interpreted as the deuterium incorporation of main-chain amides. The deuterated proteins were subjected to pepsin digestion, and the resultant peptic fragments were assigned by sequencing by MALDI-TOF/TOF MS. The MALDI mass spectrum of pepsin-digested aqMutL is shown in Fig. 2B. We identified six fragments comprising residues 315–330 (identified in aqMutL CTD<sub>425</sub>), 302–317 (in full-length aqMutL), 355–369 (in both), 389–410 (in both), 410–416 (in both), and 417–425 (in both), which cover more than 60% of the aqMutL CTD<sub>425</sub> sequence. Fig. 2C shows the time courses of deuterium incorporation in fragment 355–369.

D<sub>0</sub> and D<sub>∞</sub> (the fractions of deuterium incorporation at exchange time 0 and infinity, respectively) were determined using Equation 2 and are shown in supplemental Table S1 and Fig. 3A. As shown in Fig. 3A, D<sub>0</sub> values for the fragments from full-length aqMutL (cyan bars) were all smaller than those for the fragments from CTD<sub>425</sub> (gray bars). Similarly, D<sub>∞</sub> values for full-length aqMutL (blue bars) were generally smaller than those for CTD<sub>425</sub> (black bars) except for the CPHGRP motif-containing fragment (green background). These results indicate that the solvent accessibility of a large part of the CTD was limited by the presence of the NTD; alternatively, the secondary structures of the main chain became more stable in the presence of the NTD. This result supports the interaction between the CTD and NTD, which is consistent with the results obtained in the CTD-NTD cross-linking experiment. It should be noted that the deuterium incorporations of the 355–369 fragment, which is the region containing the DQHAX<sub>3</sub>EX<sub>4</sub>E motif, was largely influenced by the NTD (Fig. 3A, cyan background), suggesting that the endonuclease activity can be modulated by the interaction with the NTD. Interestingly, the existence of the NTD also limited the deuterium incorporation of the C-terminal region of the CTD (410–416 and 417–425 fragments) (Fig. 3A). This region may be involved in the direct interaction with the NTD because the 410–425 region in the CTD faces the NTD in the predicted structure of full-length aqMutL (Fig. 1D).

**ATP-induced Conformational Changes of Full-length aqMutL**—The main purpose of this study was to determine the ATP-dependent conformational changes of aqMutL, which were also investigated by DXMS. aqMutL was preincubated with or without ATP and then mixed with deuterium solution. It was expected that aqMutL would stably bind to ATP during the hydrogen/deuterium exchange experiment because the K<sub>eq</sub>
value of MutL for ATP is known to be about 100 µM (18, 19). In fact, ATP (2 mM; the same concentration as that used in the hydrogen/deuterium exchange experiment) tightly bound to aqMutL and did not dissociate during gel filtration chromatography despite the absence of ATP in the running buffer (supplemental Fig. S5A). On the basis of the ratio of the absorbance intensities at 280 and 260 nm, it was determined that eluted aqMutL was carrying one ATP molecule per protein. Limited backgrounds, respectively. The gray background indicates the region whose incorporation ratio was limited by the addition of ATP although it is far from ATP-binding site.
FIGURE 5. Biochemical properties of C-terminal α-helix-deleted mutants of aqMutL CTD. 

A, multiple alignment of the amino acid sequences of the C-terminal regions of MutL endonucleases. The alignment was generated by using ClustalW (22) and GeneDoc. Numbers on the left and right indicate the distances from the protein N termini. Blue, red, and purple backgrounds indicate residues whose chemical characteristics are conserved in all, six, and five of the seven species presented here, respectively. The GenBank accession numbers of the sequences are as follows: AAB19236 (B. subtilis MutL), AAC07483 (A. aeolicus MutL), BAA87903 (T. thermophilus MutL), AAC50285 (H. sapiens MLH1), ABC86950 (S. cerevisiae MLH1), ABQ59090 (H. sapiens PMS2), and CAA95956 (S. cerevisiae PMS1). The locations of the secondary structural elements are shown above the sequence of B. subtilis MutL CTD whose crystal structure has been solved. The 413th and 419th amino acid residues in aqMutL CTD are indicated by arrows. Arg-423 of aqMutL is indicated by an asterisk.

B, it has been reported that the C-terminal α-helix of one subunit achieves close proximity to the metal-binding site of the other subunit in B. subtilis MutL CTD (13, 37) (Protein Data Bank codes 3KDK and 3NCV). The DQHA X 2E X 4E and CPHGRP motifs shown in light pink are thought to be involved in the binding of catalytic metal ions and regulatory zinc ions, respectively. The C-terminal α-helix includes the highly conserved arginine residue located at the site close to the DQHA X 2E X 4E and CPHGRP motifs. C, aqMutL CTD419 and CTD413 lack amino acids 420–425 (shown in green) of the C-terminal region, respectively. D, the nicking endonuclease activity of aqMutL CTD and its mutants. The indicated concentrations of CTD425, CTD419, and CTD413 were incubated with CCC at 55 °C for 15 min. E, size exclusion chromatography. One (light gray), two (gray), four (dark gray), or eight (black) μM CTD425 (upper panel), CTD419 (middle panel), or CTD413 (lower panel) was loaded onto a Superdex 75 HR column. The elution positions for some of the size markers are shown at the top of the figure. The apparent molecular masses corresponding to peaks a, b, c, d, and e are estimated to be about 30, 28, 12, 23, and 12 kDa, respectively. The theoretical masses of aqMutL CTD425, CTD419, and CTD413 are 13.3, 12.6, and 11.8 kDa, respectively. F, cross-linking experiment. CTD425, CTD419, and CTD413 (4 μM) were incubated with the indicated concentrations of cross-linker (DIDS) and subjected to SDS-PAGE.
DXMS Revealed ATP-dependent Structural Change of MutL

proteolysis confirmed that the effect of ATP on the conformational change of MutL was as large as that of AMP-PNP under the conditions in which the hydrogen/deuterium exchange experiments were performed (data not shown). Furthermore, no ATP hydrolysis by aqMutL was observed under these conditions (supplemental Fig. S5B). Thus, the following results of DXMS in the presence of ATP should reflect the effects of ATP binding on the structure of aqMutL.

The ATP-induced difference in the hydrogen/deuterium exchange of full-length aqMutL is shown in supplemental Table S1 and Fig. 3, B and C; the difference is also mapped onto the predicted structure of full-length aqMutL as shown in Fig. 4A. In Fig. 4A, the regions whose deuterium incorporation was limited and enhanced by the addition of ATP are represented by cool and warm colors, respectively. In the NTD, we identified 12 fragments (supplemental Table S1), which cover over 60% of the aqMutL NTD_{315} sequence. It was obvious that ATP decreased both the D_0 and D_∞ (Fig. 3B, compare red line with blue line and salmon with cyan) of regions around the ATP-binding motif (Fig. 3B, orange). The ATP-induced differences in the ATP-binding motif are colored cyan or blue in Fig. 4A. This result is concordant with a previous report regarding the ATP-dependent conformational change of E. coli MutL NTD in which the disordered regions in the apostructure form ordered structures upon ATP binding (18). We also found an ATP-dependent decrease in the D_∞ for the C-terminal region of the NTD (Figs. 3B, gray, and 4A, colored yellow green or deep green). Because no ATP binding-induced structural change for this region was reported in the crystallographic analysis of E. coli MutL NTD (18), the decrease in the hydrogen/deuterium exchange of the 282–301 region of aqMutL may reflect the direct effect of the ATP binding-induced interdomain interaction (Fig. 4B). This notion is consistent with the predicted structure of the full-length aqMutL in which the C-terminal region of the NTD is exposed to the CTD (Fig. 4B). Interestingly, the DXMS experiment detected no significant change in deuterium incorporation of the 302–317 region of the NTD (Fig. 3B), although this region is expected to be closer to the CTD than the 282–301 region. Because DXMS monitors the environmental change of amide protons in the main chain, a stable secondary structure of a polypeptide often prevents the detection of interactions of the region. The 302–317 region may form a stable secondary structure such as an α-helix.

In the CTD (Fig. 3C), an ATP-dependent decrease in D_0 and D_∞ was detected for the DQHAX_3EX_4 motif-containing region (the 355–369 fragment) and the CPHGRP motif-containing region (the 389–410 fragment), respectively (compare red line with blue line, and salmon with cyan). These regions are colored green and blue, respectively, in Fig. 4A. Because D_0 reflects the environments of the molecular surface more directly than D_∞, these results may indicate that the DQHAX_3EX_4 motif- and CPHGRP-containing regions are directly and indirectly affected by the interaction of the CTD with the NTD, respectively. The previous structural analyses of MutL CTD clarified that DQHAX_3EX_4 is essential for the formation of the catalytic site (6, 9, 11). In addition, our previous results revealed the significance of the CPHGRP motif in the ATP-dependent regulatory mechanism of MutL endonuclease activity (9). Therefore, the present results can be considered evidence that ATP binding influences the endonuclease activity through the structural rearrangement of the catalytic site.

Intriguingly, DXMS also detected a significant ATP-dependent decrease in D_0 at the C-terminal region (fragments 410–416 and 417–425) of the CTD (Figs. 4C and 5). The ATP-induced decreases in deuterium incorporation will reflect the ATP-dependent direct interaction of this region with the NTD (Fig. 4B). This is also supported by the result that the existence of the NTD limits the incorporation in this region (Fig. 3A) and by the predicted structure in which the C-terminal region of the CTD faces the NTD (Fig. 4B). The C-terminal 417–425 region of aqMutL is predicted to form an α-helix. In the crystal structure of B. subtilis MutL, the C-terminal α-helix of one
Identification of Novel Region in CTD Required for Nicking Endonuclease Activity of MutL—Our DXMS experiment revealed the interdomain interaction- and ATP binding-dependent structural rearrangement in the C-terminal α-helix in the CTD endonuclease domain. To examine the involvement of the C-terminal α-helix (Fig. 5, A and B) in the endonuclease activity, we created C-terminal residue-deleted mutants of aqMutL CTD: CTD_{419} (residues 316–419 of aqMutL) and CTD_{413} (residues 316–413 of aqMutL); the deleted regions are indicated in Fig. 5, A–C. These mutants were successfully overexpressed in E. coli and purified to homogeneity (supplemental Fig. S6A). The CD spectra of CTD_{419} and CTD_{413} were nearly identical to that of wild-type CTD except for the decrease in molar ellipticity around 222 nm, which is consistent with the lack of the C-terminal α-helix (supplemental Fig. S6B). The temperature dependence of the mean residue molar ellipticity at 222 nm indicates that the two mutants are thermostable up to 95 °C similar to the wild type (supplemental Fig. S6C). Furthermore, inductively coupled plasma emission spectrometry measurements confirmed that CTD_{419} and CTD_{413} retain the Zn^{2+} binding ability as the wild type (supplemental Fig. S6D). We then tested the nicking endonuclease activity of aqMutL CTD mutants. To detect the nicking endonuclease activity, the covalently closed circular form of plasmid DNA (CCC) was used as a substrate. Nicking endonuclease activity converts CCC into an open circular form of plasmid DNA that can be easily separated from CCC and the linearized form by agarose gel electrophoresis (32). It is reported that Mn^{2+} facilitates the mismatch- and MutS-, clamp-, and clamp loader-independent incision of plasmid DNA by non-sequence-specific MutL endonuclease activity (6, 9, 11–13). As shown in Fig. 5D (upper panel), in the presence of Mn^{2+}, wild-type aqMutL CTD incised the plasmid DNA without mismatches and other MMR proteins. In contrast, CTD_{419} and CTD_{413} barely incised the plasmid DNA under conditions in which the wild-type CTD efficiently digested it (Fig. 5D, middle and lower panels). These results imply that the C-terminal α-helix is required for the exertion of the endonuclease activity of the CTD. However, the C-terminal α-helix of MutL CTD has been reported to be associated with the dimerization ability of MutL CTD (38–40). In fact, gel filtration chromatography revealed the relatively reduced dimerization ability of CTD_{425} compared with that of the wild type (Fig. 5E). Cross-linking experiments with DIDS also demonstrated the significantly reduced efficiency of CTD_{419} and CTD_{413} to cross-link dimeric molecules (Fig. 5F). Thus, at this stage, we cannot judge whether the C-terminal α-helix is involved in the catalysis from the results regarding CTD_{419} and CTD_{413}.  

FIGURE 7. Function of 5 C-terminal residues of aqMutL NTD. A, amino acid sequence alignment of the C-terminal regions of NTDs from bacterial and eukaryotic MutL homologs. Black, gray, and light gray backgrounds indicate residues whose chemical characteristics are conserved in all, five, and four of the six species, respectively. The GenBank accession numbers of the sequences are as follows: ACZ50725 (E. coli MutL), AAA87031 (Mus musculus PMS2), and DAA15209 (Bos taurus PMS2). The asterisks indicate the PLLDV motif, which is found in mammalian and A. aeolicus MutL endonucleases. B, the DNA binding ability of aqMutL NTD_{310} was examined by electrophoretical mobility shift assay. Two hundred nanomolar 30-bp double-stranded DNA was incubated with various concentrations of NTD_{310} at 55 °C for 30 min and subjected to native PAGE. The concentrations of NTD_{310} are indicated at the top of the panel. DNA was visualized by staining with SYBR Gold. C, DNA binding assay for the various concentrations of NTD_{315} (circles) and NTD_{310} (triangles) was performed. The percentages of shifted signals to all signals were then determined by using CS Analyzer 3.0 and plotted against the protein concentration. The K_{d} values of NTD_{310} and NTD_{315} were determined by fitting the data to Equation 3 using Igor Pro 4.03. D, comparison of the CTD stimulating abilities of NTD_{310} and NTD_{315}. The nicking endonuclease activity of CTD_{425} was examined in the presence of various concentrations of NTD_{310} or NTD_{315}. The concentrations of CTD_{425}, NTD_{310}, and NTD_{315} are indicated at the top of the panel. OC, open circular form of plasmid DNA.
Novel Region in NTD Required for Stimulation of Endonuclease Activity of CTD—The DXMS experiment detected a significant ATP-dependent structural rearrangement in the C-terminal region of the NTD. Sequence alignment showed that this region of aqMutL containing the PLLDV motif is highly conserved also in the PMS2 subunit of mammalian MutL endonucleases but not in E. coli MutL (Fig. 7A, asterisk). To assess the function of this region, we created a deletion mutant of the NTD, the NTD310, which lacks the 5 C-terminal residues 311–315 (PIVDI). NTD310 was successfully overexpressed and purified using the same procedure as that for NTD315 (supplemental Fig. S8A). NTD310 was confirmed to retain secondary structures (supplemental Fig. S8B), thermostability (supplemental Fig. S8C), and DNA binding activity to the same extent as those of NTD315 (Fig. 7, B and C). The $K_d$ values of NTD310 and NTD315 for the 30-bp linear double-stranded DNA were 1.36 and 1.15 $\mu$M, respectively. Despite the functional similarities between NTD310 and NTD315, NTD310 did not enhance the nicking endonuclease activity of the CTD in contrast to NTD315 (Fig. 7D). These results clearly indicated that the PLLDV motif in the NTD is essential for the NTD-dependent activation of CTD. This region may be required for the direct interaction between the NTD and CTD. To the best of our knowledge, this is the first report concerning the function of the PLLDV motif.

Conclusions—In this study, the results of the DXMS experiments suggested the direct interaction of the PLLDV motif-containing region of the NTD with the nicking endonuclease active site comprising the DQHAX$_2$EX$_4$E motif and the C-terminal $\alpha$-helix regions of the CTD (Fig. 4). The structure of the region around the CPHGRP motif seem to be indirectly affected by interaction with the NTD (Fig. 4), implying that this motif is responsible for the stabilization of the interdomain interaction. Because the CD spectrum of aqMutL was not influenced by the presence of ATP (supplemental Fig. S9), the structural rearrangement caused by ATP binding would not involve a large change in the secondary structure. Future structural analyses (e.g. crystallographic analyses) of full-length MutL are necessary to describe the details of the ATP-dependent structural rearrangement.

Our DXMS experiment revealed that the highly conserved arginine residue (Arg-423 in aqMutL) in the C-terminal $\alpha$-helix is essential for the nicking endonuclease activity. Several mutations in the C-terminal $\alpha$-helix region of human MLH1, which includes the arginine residue corresponding to Arg-423 of aqMutL, are related to Lynch syndrome (39, 41) (Fig. 5A). Because eukaryotic MutL$\alpha$ contains a single catalytic site per dimer molecule, it is reasonable that the PMS2 subunit lacks the corresponding arginine residue. Together with these previous findings, our data presented in this study explain the relationship between the mutations and dysfunction of MMR in humans.

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