Mutational Analysis of the MutH Protein from Escherichia coli*

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Site-directed mutagenesis was performed on several areas of MutH based on the similarity of MutH and PvuII structural models. The aims were to identify DNA-binding residues; to determine whether MutH has the same mechanism for DNA binding and catalysis as PvuII; and to localize the residues responsible for MutH stimulation by MutL. No DNA-binding residues were identified in the two flexible loop regions of MutH, although similar loops in PvuII are involved in DNA binding. Two histidines in MutH are in a similar position as two histidines (His-84 and His-85) in PvuII that signal for DNA binding and catalysis. These MutH histidines (His-112 and His-115) were changed to alanines, but the mutant proteins had wild-type activity both in vivo and in vitro. The results indicate that the MutH signal for DNA binding and catalysis remains unknown. Instead, a lysine residue (Lys-48) was found in the first flexible loop that functions in catalysis together with the three presumed catalytic amino acids (Asp-70, Glu-77, and Lys-79). Two deletion mutations (MutHΔ224 and MutHΔ214) in the C-terminal end of the protein, localized the MutL stimulation region to five amino acids (Ala-220, Leu-221, Leu-222, Ala-223, and Arg-224).

MutH is the endonuclease in the methyl-directed mismatch repair system (1), which, along with MutS and MutL (and bound ATP), initiates the repair process (2). MutS binds specifically to mismatched bases or deletion/insertion loops of one to four nucleotides (3–5). MutL appears to be a helper protein; it interacts with other proteins to increase their DNA binding or converts them to the active form (6). MutL appears to activate MutH for DNA binding and cutting the unmethylated strand of hemimethylated DNA based on endonuclease assays (7, 8). In Escherichia coli, mismatch repair takes place shortly after replication before the DNA becomes fully methylated (the newly synthesized daughter strand is unmethylated). MutH in the ternary complex binds to a hemimethylated d(GATC) site, recognizes the asymmetry of the unmethylated N6 position of the d(A) (9, 10), andcleaves 5′ to the d(G) in the unmethylated strand (11). If both strands are unmethylated, MutH is able to cleave both sites independently (dissecting after the first d(GATC) cut, with a second binding and cleavage event) leaving a four-base overhang (2). Although MutS is thought to bind heteroduplex DNA first, followed by MutL and MutH, it is unclear what the binding order is for these proteins. It is also unclear in the ternary complex whether MutH acts as a monomer or (like restriction endonucleases) as a dimer. The purified protein with a calculated molecular mass of 25.5 kDa is a monomer in solution.

Recently, the structure of the MutH protein was solved (11). It was described as resembling a clamp with a large cleft dividing the protein into two subdomains. From the three structures analyzed, it was shown that the two subdomains adopt multiple conformations that correlate with movement in the C-terminal end of the protein. This suggests that the C-terminal-exposed helix is a possible site through which MutL stimulation occurs. It was postulated that DNA lies in the bottom of the cleft in close proximity to residues Asp-70, Glu-77, and Lys-79, which would constitute the active site for catalysis. The catalytic sequence of ΔX79-80(E/D)XK was proposed for MutH based on the close resemblance to the structure of the restriction endonuclease PvuII (with a root mean square deviation of 2.3 over 83 pairs of Co atoms) (11). Both enzymes share a common core motif with a number of other restriction enzymes (BamHI, EcoRV, and EcoRI) (12, 13). These restriction enzymes are grouped according to their similarity in structure as well as function (PvuII and EcoRV are in one group and BamHI and EcoRI are in another). It was surprising that MutH is closer in structure to PvuII than BamHI, because PvuII approaches DNA from the minor groove and makes contacts in the major groove by reaching around the DNA with flexible loops to produce blunt ends. BamHI, however, approaches DNA from the major groove where the base-specific contacts are made to produce cuts with a four-base overhang. In addition, BamHI has a recognition site with d(GATC) as the central bases and cuts 5′ to the d(G). There is no structure available of MutH complexed with its cognate DNA, so it is not known from which direction MutH approaches the DNA and whether it acts as a monomer or dimer in the repair complex. Given the close similarity in structure between MutH and PvuII, we altered amino acids in MutH based on the corresponding functional regions of PvuII.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—KM54 (ΔmutH461:Cam) was constructed by electroporation of strain KM22 (Δ(recC ptr recB recD)::Cam red Kan) (20) with a recombinant PCR product as described by Murphy et al. (14), using the following oligonucleotides: mutH2, ATCATCGAGCTCCACCAGCTGCAAGAGAACCATT; mutH2, TGGCGATCAA-CTCTATGTCGGCGCCTGTTGGGACATGATCAGATCCGAA; mutH2, AAGGCGAGAATTTGACCGCCTTGTTTCTGTAGCATCGATGCAAGAATCCATT; mutH2, TAATCGAGATGCGCCGATGGCGTTGCGAAATA. GM7586 was generated by P1 transduction of GM4244 (CC106 (15)) with a lysate grown on KM54 and selecting for chloram,

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§ The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase(s); Amp, ampicillin; Cam, chloramphenicol; Rif, rifampicin; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); PNK, T4 polynucleotide kinase.
phenolic resistance. Isogenic strains GM4244 and GM7586 (GM4244 ΔmutH461::Cam) were used for the in vivo screen. Strain GM5856 (hisd1717 endA1 thi-1 spoT1 rifB1 supE44 mutH71::Tn5) was also used for the overexpression of wild-type MutH and the mutant proteins. Strain GM5862 (16) was used for the overexpression of wild-type MutL, pBAD18 (21) and pBAD244 (20) (10 ml each). The 215 and 225 using the Sculpture mutagenesis kit (Amersham Pharmacia Biotech). The wild-type and all mutant plasmids were sequenced with the restriction enzymes NbI and HindIII. The fragment was obtained by digestion of pTX417 with the restriction enzymes XbaI and HindIII. The full-length product was then sequenced by the DNA Sequencing Facility, Iowa State University. Both strands of wild-type MutH and the promoter region were sequenced. We used the mutH gene in GenBank, accession number U61361. The site-directed PCR products were identified by sequencing with the outside flanking primers used in the PCR reactions. Both strands and the promoter region of all mutant plasmids were sequenced.

Preparation of Histidine-tagged MutH—The wild-type and all mutant proteins were purified in the same manner. For each strain, a 20-ml LB-Amp culture was grown overnight from a single colony. One liter of LB-Amp medium was inoculated with the 20-ml overnight culture and allowed to grow with shaking at 37 °C to an A$_{600}$ of 1.0. Arabinopectic (Difco) was added to a final concentration of 0.2%, and the culture was induced for 2 h at 37 °C. Cells were harvested by centrifugation for 20 min at 5000 rpm, washed with water, and stored frozen (-80 °C). Cell lysis was achieved with 4 ml of reconstituted buffer (20 mM Hepes (pH 7.4), 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride), and the cells were lysed using a French pressure cell. The extract was then sonicated (20 pulses from a Tekmar sonicator) and centrifuged for 30 min at 15,000 rpm to remove cell debris. The supernatant was applied to a 4-ml Fast Flow Chelating Sepharose (Amersham Pharmacia Biotech) column charged with 100 ml of water. The column was then washed with 20 ml of reconstituted buffer, containing 100, 150, 250, and 400 mM imidazole (10 ml each) were used to elute the MutH protein. The 250 mM fraction contained the MutH protein and was dialyzed against two changes of 20 mM Hepes (pH 7.4), 300 mM NaCl, and 0.1 mM EDTA. The MutH concentration was determined by measuring the A$_{600}$ (1.0 = 0.67 mg/ml). MutH was at least 95% pure as determined by a SDS-PAGE Coomassie Brilliant Blue-stained gel.

Preparation of Histidine-tagged MutL—The His-tagged MutL protein was purified as described previously (6), except that the transformation mixture of GM5862 with pMQ393 was incubated for 90 min after heat shock and then added to 50 ml of YT-Cam medium for overnight incubation at 37 °C. The step fractions used for elution from the Sepharose column were one 100 mM, two 150 mM, and one 400 mM fraction. The 225 using the Sculpture mutagenesis kit (Amersham Pharmacia Biotech). The wild-type and all mutant plasmids were sequenced with the restriction enzymes NbI and HindIII. The fragment was obtained by digestion of pTX417 with the restriction enzymes XbaI and HindIII. The full-length product was then sequenced by the DNA Sequencing Facility, Iowa State University. Both strands of wild-type MutH and the promoter region were sequenced. We used the mutH gene in GenBank, accession number U61361. The site-directed PCR products were identified by sequencing with the outside flanking primers used in the PCR reactions. Both strands and the promoter region of all mutant plasmids were sequenced.

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Based on the MutH crystal structure, it was suggested that the C-terminal tail region is contacted by MutL to stimulate the MutH endonuclease activity (7, 11). To test this possibility, two deletion mutants were made with the creation of amber stop codons. MutH Δ214 and MutH Δ224 (the number of the last residue present in the protein) were made to narrow down the area where MutL might stimulate MutH activity.

**In Vivo Screening of the Mutant Plasmids**—After checking that there were no secondary mutations in each of the mutant MutH plasmids, they were transformed into strains GM4244 (wild-type) and GM7586 (ΔmutH::Cam). The null mutation in mutH was constructed, because the commonly used mutH471::Tn5 mutation is not completely defective in our assays.  

The mutant MutH plasmids in GM4244 were tested for a dominant-negative phenotype (mutator phenotype in a wild-type strain) by the same method as the *in vivo* screen described under “Experimental Procedures.” None of the mutant plasmids gave a dominant-negative phenotype (data not shown).

For the complementation testing in strain GM7586, the control (GM7586 versus pMQ402 in GM7586) show that the wild-type MutH protein causes a 200-fold reduction in the frequency to rifampicin resistance, indicating efficient complementation of the chromosomal ΔmutH::Cam allele (Table I). The vector plasmid (pBAD18) causes a small reduction in the mutation frequency and doesn’t interfere with the complementation testing. No alterations in mutation frequency were seen in GM4244 with the control plasmids (pBAD18 and pMQ402).

Very few of the MutH site-directed mutants showed a mutator phenotype. Plasmid Δ214-containing cells show a strong mutator phenotype along with mutants K48A and G49A. These three were chosen for further biochemical studies. A few others were also chosen to validate the screen and for the following specific reasons. The D47A mutant was used because of the Asp-34 analogous position in the MutHII structure that recognizes the d(A) in the recognition sequence for *Pvu*II (23). The H115A and H112A mutants were used because of the signaling properties of the *Pvu*II histidines (His-84 and His-85) described earlier. The Δ224 mutant was used to test the hypothesis that the C-terminal end is where MutL stimulation of MutH activity occurs.

The steady-state level of MutH produced by wild-type and mutant constructs was determined by Western blotting (New England BioLabs). All of the mutant plasmids produced comparable amounts of MutH to the wild-type plasmid in strain GM3856 (data not shown).

**Endonuclease Assay**—This assay tests for the endonuclease function of the MutH protein. The product of the reaction is a nick in the MR1 covalently closed circular molecule, which has only one (dGATC) site. In the absence of MutL, increasing amounts of the wild-type MutH protein were added to the duplex until complete nicking was achieved (Fig. 2A and Fig. 3A). The addition of 2.5–3.0 pmol of wild-type protein resulted in complete nicking of the substrate. The results of the endonuclease assay for the remaining three mutants (K48A, G49A, and Δ214) are shown below the wild-type in Fig. 2A. K48A has no detectable endonuclease activity and G49A activity is decreased ~30-fold (Fig. 3A). D47A, H112A, H115A, and Δ224 MutH proteins gave the same result as wild-type and were not tested further (data not shown). These results were in agreement with the *in vivo* screen, because the mutation frequencies in cells with these proteins (D47A, H112A, and H115A) were similar to the mutation frequency of the wild-type MutH protein in strain GM7586 (ΔmutH::Cam). A circular dichroism

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2 T. Loh, K. C. Murphy, and M. G. Marinus, unpublished data.
TABLE I
In vivo complementation screen for mutant MutH proteins

| Strain          | Colonies on media containing Ampicillin | Rifampicin | Mutation frequency |
|-----------------|---------------------------------------|------------|-------------------|
| GM4244          | 280                                   | 42         | 4 ± 1             |
| GM7586          | 235                                   | 1559       | 675 ± 121         |
| pBAD15/GM4244   | 261                                   | 16         | 1 ± 1             |
| pBAD15/GM7586   | 362                                   | 1447       | 546 ± 52          |
| pMG402/GM4244   | 108                                   | 0          | 0                 |
| pMG402/GM7586   | 232                                   | 41         | 5 ± 1             |
| P41A            | 215                                   | 24         | 8 ± 0             |
| E42A            | 203                                   | 10         | 3 ± 0             |
| N43A            | 217                                   | 14         | 4 ± 1             |
| L44A            | 239                                   | 32         | 8 ± 0             |
| K45A            | 213                                   | 9          | 3 ± 1             |
| R46A            | 223                                   | 6          | 2 ± 1             |
| D47A            | 244                                   | 9          | 2 ± 1             |
| K48A            | 242                                   | 2178       | 560 ± 47          |
| G49A            | 269                                   | 1502       | 330 ± 95          |
| L59A            | 303                                   | 20         | 5 ± 1             |
| G60A            | 246                                   | 15         | 4 ± 1             |
| S62A            | 347                                   | 5          | 1 ± 1             |
| G64A            | 286                                   | 18         | 5 ± 1             |
| S65A            | 214                                   | 14         | 4 ± 1             |
| K66A            | 214                                   | 23         | 6 ± 1             |
| P67A            | 252                                   | 27         | 7 ± 1             |
| E68A            | 280                                   | 18         | 4 ± 1             |
| Q69A            | 239                                   | 16         | 3 ± 0             |
| H115A           | 171                                   | 11         | 2 ± 0             |
| H115A           | 207                                   | 21         | 10 ± 1            |
| Δ214            | 199                                   | 2234       | 1123 ± 38         |
| Δ224            | 175                                   | 6          | 3 ± 1             |

*a* The number of colonies in this column are from a 10^-6 dilution of the 1-ml overnight culture. These numbers are used to calculate the total number of cells present in the culture: colony number × (volume of dilution/volume plated) × 10^8.

*b* The number of colonies in this column are from a 10^-6 dilution of the 1-ml overnight culture. These numbers are used to calculate the total number of cells present in the culture: colony number × (volume of culture/volume plated).

In the presence of MutL and ATP, 1000-fold less MutH is needed to achieve the same results as without MutL (Figs. 2B and 3B). The cleavage of mutants K48A and G49A are shown below the wild-type in Fig. 2B. Although G49A is partially stimulated by MutL (it is increased 10-fold from the unstimulated activity), its activity is reduced about 200-fold with the stimulated wild-type protein (Fig. 3B). K48A shows the most dramatic increase in MutL-stimulated nicking, and is reduced only 3-fold relative to the wild-type protein (Fig. 3C). MutL is unable to increase the activity of the G49A mutant as much as that for K48A. There was no MutL stimulation seen for Δ214 in this assay, and its activity is decreased about 15-fold from the unstimulated wild-type MutH protein (Fig. 3D). It would appear then to have a 15,000-fold decrease in activity compared with the stimulated MutH protein. Because the reduction in endonuclease activity of the mutant proteins could be due to decreased binding ability, binding assays were done as described below.

**Band Shift Assay**—In this assay the specific binding of the MutH protein to DNA is tested. The substrate is a linear 36-mer duplex of DNA with one d(GATC) site. The MutH binding is specific, because it is not seen with the same linear substrate that has a d(GGTC) site in place of the d(GATC) site (data not shown). Without MutL, the wild-type protein gives a complete band shift with the appearance of a transient band between the free DNA and bound DNA (Fig. 4A). Both mutant proteins, K48A and G49A, gave results similar to the wild-type (data not shown). The Δ214 mutant protein appeared to initiate a binding-like activity but failed to achieve a final shifted position (Fig. 4A).

The band shift assay for MutH in the presence of MutL was complicated, because MutL shows nonspecific DNA binding at concentrations above 9 pmol to approximately the same position in the gel as the MutH band. The amount of MutL, therefore, was lowered until it no longer shifted the DNA substrate. The assay with MutL and wild-type MutH caused the band shift to start a little sooner (compare at 38 pmol), but it finished at the same concentration as without MutL (Fig. 4B). The K48A mutant was like wild-type and the G49A mutant bound a little tighter than wild-type. The Δ214 mutant was not stimulated by MutL and gave the same result as without the added protein (not shown).

From these experiments, the apparent dissociation constants (K_d) for the proteins and the substrate were calculated (Fig. 5). The binding affinity for the wild-type MutH protein increased...
at least 16-fold when the MutL protein was present (Fig. 5B). K48A was as proficient as the wild-type protein in binding ability, but the activity in the endonuclease assay was reduced, so it has a catalytic defect that MutL is able to overcome. G49A bound 2- to 4-fold tighter than wild-type, which may or may not be a significant contribution to the overall ability of the protein to function. Because Δ214 did not achieve the full band shift position, the apparent dissociation constant was not calculated. Although MutL has an effect on MutH binding to the DNA (about 16-fold), the greatest effect is on catalysis (1000-fold stimulation). The apparent $K_{f}$ values of the wild-type and mutant proteins and their substrate are given in Table II.

**DISCUSSION**

Methyl-directed mismatch repair is initiated by a mismatch in the DNA followed by formation of a ternary complex containing the MutS (with bound ATP), MutL, and MutH proteins. A defect in the ability to function correctly of any one of these three proteins causes a mutator phenotype. In this paper, that phenotype was used as a screen to identify defective MutH proteins. Four site-directed mutants having a wild-type mutation frequency to rifampicin resistance (D47A, H112A, H115A, and Δ224) displayed normal MutH activity in vitro. Subsequent biochemical assays confirm a defect in MutH activity in these mutant proteins. Four site-directed mutants having a wild-type mutation frequency to rifampicin resistance (D47A, H112A, H115A, and Δ224) displayed normal MutH activity in vitro. These results indicate that the in vivo screen was sensitive enough to detect defective MutH proteins. It takes very few copies of the protein (estimated at 34 monomers per cell) to achieve full repair activity (26). The copies of protein from the plasmid are probably much higher than those normally in the cell. Under these conditions an
impaired protein might do much better in the in vivo screen than in the in vitro testing. Therefore an estimated 10-fold reduction in activity of MutH would probably not have been detected.

The qualitative results between the biochemical and in vivo assays agree fairly well. Mutant Δ214 had the highest mutation frequency of the mutants tested in the in vivo screen and had the highest reduction in endonuclease activity in the presence of MutL. Mutants G49A and K48A somewhat follow this pattern with G49A having the lower mutation frequency and achieving complete nicking in the endonuclease assay (with increased protein amounts).

Although the stimulation of MutH activity by MutL has been shown at the endonuclease level (7), the ability of MutL to stimulate the DNA binding ability of MutH has not been demonstrated. The amount of MutL (9 pmol) in the band shift assay appears to be adequate for binding all of the MutH (0.1 pmol) monomers present. Yet the amounts of MutH needed to achieve a full band shift remain the same (Fig. 4). One conclusion from the data is that the MutL contribution to MutH DNA binding is marginal. The differences in the $K_d$ values (Table II) were about 16-fold. This is significant, but it adds very little compared with the 1000-fold difference MutL makes in catalysis. MutL’s presence makes it a little easier for MutH to start binding to the DNA, but overall binding isn’t affected that much.

The mutational changes made in MutH were based upon a comparison with the PvuII enzyme where detailed structure-function data are available. Mutational analysis of the amino acids in the flexible arms would show whether or not MutH has any functional similarity to PvuII (Fig. 1). In PvuII there are a few residues present that are involved in DNA binding or the recognition of the binding site, such as an aspartate (Asp-34) that would be comparable to Asp-47 in MutH (23). No one residue in the flexible arms of MutH was found to make an important base recognition DNA contact so that, when it is disrupted, a mutator phenotype results. Minor contacts in the DNA such as phosphate binding residues may not result in a mutator phenotype and therefore may not be seen through this in vivo screen. The amino acids chosen for this study are shown in the structure of MutH (Fig. 6).

The conserved histidines found in both PvuII (His-84 and His-85) and MutH (His-112 and His-115) do not appear to have the same function. Changing the histidines (His-84 and His-85) in PvuII results in a loss of catalysis but not binding (25). Their movement upon DNA binding brings the catalytic residues into proper alignment for catalysis. When the DNA is methylated, the excess movement of these histidines ensures the catalytic residues cannot function. No loss of binding or catalysis resulted when the histidines were substituted with alanines in MutH. Therefore, from this work, the signal (for DNA binding and state of methylation) for MutH is unknown. The DNA binding domain for MutH is clearly not similar to the one in PvuII.

A lysine (Lys-48) was found in MutH that functions in catalysis. The lysine to alanine mutation (K48A) in MutH resulted in a protein devoid of activity in the absence of MutL. MutL may have a lysine or other residue that is able to give the ternary complex partial function. The function of lysine 48 in MutH is unclear. It is positioned within the BC loop (12) similar to the wild-type protein and shows similar in vitro activity. Therefore, it is unlikely that lysine 48 next to it is working in catalysis, then the DNA may be able to reach the phosphates of the DNA. If lysine 48 next to it is working in catalysis, then the DNA may be very close to these residues. This mutational change interferes with the proper functioning of the protein but is not informative about the mechanism of action.

The G49A mutant may have defects in catalysis because of its proximity to lysine 48. The extra methyl group may cause slight structural changes in the lysine 48 side chain that slows catalysis. When MutL is present, the mutant is able to achieve full nicking of the substrate with larger amounts of protein than the wild-type MutH. The DNA binding of this glycine mutant is slightly tighter than the wild-type. The methyl group of G49A may be able to reach the phosphates of the DNA. If lysine 48 next to it is working in catalysis, then the DNA may be very close to these residues. This mutational change interferes with the proper functioning of the protein but is not informative about the mechanism of action.

Two C-terminal deletion mutations (Δ214 and Δ224) were made to help identify the residues that contact and cause the stimulation of MutH by MutL. This idea is based on predictions from the structural model of MutH (11). The Δ224 functions similarly to the wild-type protein in vivo as well as in vitro, indicating that residues 225–229 at the C-terminal end of the protein are not required for MutL stimulation. The mutation frequency of the Δ214 mutant is elevated in vivo and shows decreased cleavage activity with some initial DNA binding capabilities in vitro. However, it is not able to be stimulated by MutL. This places the residues between 215 and 224 as being involved in activation by MutL. It has been shown that a deletion of 10 amino acids in the C-terminal region of MutH (Δ219) prevents MutL stimulation (7). That leaves the amino

### Table II

| MutL  | $K_d$ μM |
|-------|----------|
| +     | WT 4.2   |
| +     | K48A 9.8 |
| +     | G49A 1.0 |
| -     | WT 68.2  |
| -     | K48A 68.7|
| -     | G49A 27.4|

**Fig. 6. Location of the mutated residues in the MutH structure.** The ribbon diagram of MutH was made from the B monomer with the coordinates submitted by W. Yang (Protein Data Bank code 2AZ0) using MIDAS. The N and C termini are labeled. The residues tested from the site-directed mutagenesis (Asp-47, Lys-48, Gly-49, His-112, His-115) are displayed in red. Also, the last residue remaining from each of the deletion mutants is shown in red (Lys-214 and Arg-224).
acids 220 through 224 (sequence: ALLAR) as contacting MutL and stimulating MutH activity. Comparing the amino acid sequence of *E. coli* and *Hemophilus influenzae* MutH, there is one conserved amino acid: the leucine at position 222. Site-directed mutagenesis at each of the five positions (220–224) could reveal the most important residues for stimulation.

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