Interaction between the Msh2 and Msh6 Nucleotide-binding Sites in the Saccharomyces cerevisiae Msh2-Msh6 Complex*5

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Indirect evidence has suggested that the Msh2-Msh6 mispair-binding complex undergoes conformational changes upon binding of ATP and mispairs, resulting in the formation of Msh2-Msh6 sliding clamps and licensing the formation of Msh2-Msh6-Mlh1-Pms1 ternary complexes. Here, we have studied eight mutant Msh2-Msh6 complexes with defective responses to nucleotide binding and/or mispair binding and used them to study the conformational changes required for sliding clamp formation and ternary complex assembly. ATP binding to the Msh6 nucleotide-binding site results in a conformational change that allows binding of ATP to the Msh2 nucleotide-binding site, although ATP binding to the two nucleotide-binding sites appears to be uncoupled in some mutant complexes. The formation of Msh2-Msh6-Mlh1-Pms1 ternary complexes requires ATP binding to only the Msh6 nucleotide-binding site, whereas the formation of Msh2-Msh6 sliding clamps requires ATP binding to both the Msh2 and Msh6 nucleotide-binding sites. In addition, the properties of the different mutant complexes suggest that distinct conformational states mediated by communication between the Msh2 and Msh6 nucleotide-binding sites are required for the formation of ternary complexes and sliding clamps.

DNA mismatch repair (MMR)2 recognizes and corrects mispaired nucleotides that arise in DNA as a result of errors during DNA replication and chemical damage to DNA and DNA precursors and during the formation of heteroduplex recombinational intermediates (1–5). A key early step in MMR is the recognition of the mispaired base in the DNA. In bacteria, mispaired bases are recognized by the MutS homodimer (2, 3, 6–9). Eukaryotes possess multiple MutS homologs that form two heterodimers, Msh2-Msh6 and Msh2-Msh3, which have distinct but partially overlapping mispair-binding specificities (4, 10–14). Binding of both mispaired DNA and ATP converts MutS, as well as Msh2-Msh6 and Msh2-Msh3, into a form that is capable of recruiting other MMR proteins and triggering downstream events in MMR (15–23).

Structures of truncated forms of bacterial MutS (2, 6) and human Msh2-Msh6 (24) in complex with DNA mispairs have been determined by x-ray crystallography. MutS homodimers bind mispaired DNA and form asymmetric rings around the DNA in which only one subunit contacts the mispaired base (2, 6, 7, 25, 26). This basic structure is conserved in Msh2-Msh6, with Msh6 being the mispair-contacting subunit (24, 27–29). Consistent with known structures, challenging the mispair-bound forms of MutS, Msh2-Msh6, and Msh2-Msh3 with ATP converts them to a sliding clamp form that slides freely along the DNA but is trapped on an end-blocked DNA (15, 16, 21, 30, 31). In contrast, the base pair-bound form undergoes direct dissociation from the DNA when challenged with ATP (21, 32). Taken together, these observations suggest that mispair binding may license an ATP binding-dependent conformational change that results in conversion to the sliding form. Furthermore, only the mispair-bound forms of MutS and Msh2-Msh6 form ATP-dependent ternary complexes with MutL and Mlh1-Pms1, respectively, which facilitate subsequent steps in MMR (15–17, 21, 23, 33–35). Attempts to understand the ATP-bound conformations of these complexes by soaking ATP and ATP analogs into the MutS-mispair crystals have thus far failed to induce conformational changes expected from the biochemical characterization of these complexes, possibly because of restraints placed on the proteins by the crystal lattice (2, 6, 24–26). Thus, the nature of the ATP binding-induced conformational changes that link mispair recognition with conversion to the sliding form and/or the form that is competent for ternary complex formation is presently unknown.

The interactions between the bacterial and eukaryotic mispair-binding proteins and ATP are probably best understood for the Msh2-Msh6 complex (32). In the absence of DNA, the Msh6 nucleotide-binding site has high affinity for ATP and low affinity for ADP, whereas the Msh2 nucleotide-binding site has lower affinity for ATP and higher affinity for ADP. ATP binding at the Msh6 nucleotide-binding site results in reduced affinity for ADP in the Msh2 nucleotide-binding site. In the absence of mispair binding, the ATP in the Msh6 nucleotide-binding site is rapidly hydrolyzed, and the resulting ADP dissociates. Thus, in solution, Msh2-Msh6 binds and hydrolyzes ATP, resulting in a form that contains ADP bound in the Msh2 nucleotide-binding site (32). This ADP-bound form of Msh2-Msh6 can bind both base pairs and mispairs (21, 29, 30, 32, 36). Interestingly, pre-binding of ATP under non-hydrolyzing conditions (absence of Mg2+ ) or the presence of the non-hydrolyzable analog ATPγS prevents binding of Msh2-Msh6 to any DNA (21, 30, 32, 36). Once Msh2-Msh6 is bound to base-paired DNA, ATP binding
causes Msh2-Msh6 to directly dissociate from DNA (21). In contrast, when Msh2-Msh6 is bound to a mispair, ATP hydrolysis at the Msh6 site is inhibited, which then favors binding of ATP at the Msh2 site (32, 37, 38). A similar dual ATP-bound state can be achieved with ATPγS (21, 32). Binding of ATP or ATPγS converts mispair-bound Msh2-Msh6 into the sliding clamp form (21, 30, 32, 34). A mispair-bound, ATP-bound form of the mispair-binding complexes is also competent for assembling Mlh1-Pms1 ternary complexes (17, 21, 32), although it has not been definitively established whether the sliding conformation and the Mlh1-Pms1-binding conformation are the same.

In this study, we have used Saccharomyces cerevisiae to analyze eight MMR-deficient Msh2-Msh6 complexes for defects in ATP binding, sliding clamp formation, and Msh2-Msh6-Mlh1-Pms1 ternary complex assembly and for ATP binding-induced conformational changes. The results of this study have allowed us to directly detect conformational changes induced by ATP binding to the Msh6 nucleotide-binding site and to relate the ATP-binding properties of the Msh2 and Msh6 nucleotide-binding sites to conformational changes and the ability of Msh2-Msh6 to form sliding clamps and ternary complexes with Mlh1-Pms1.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Genetic Manipulations**—S. cerevisiae was grown on standard medium, either yeast extract/peptone/dextrose or complete supplement mixture medium (United States Biological), lacking specific amino acids to select for plasmid markers and/or Lys⁺ revertants. Escherichia coli strains used for the propagation of plasmids were grown on LB medium containing antibiotics to select for plasmids as appropriate. All S. cerevisiae strains used for genetic tests are isogenic derivatives of the same parental S288C strain (39). The wild-type strain RDKY 3590 has the genotype MATα ura3-52 leu2Δ1 trp1Δ63 hom3-10 lys2::InsE-A10, and the strains used for complementation studies were RDKY 3688 (MATα ura3-52 leu2Δ1 his3Δ200 hom3-10 lys2::InsE-A10 msh2Δ::hisG) and RDKY 4234 (MATα ura3-52 leu2Δ1 trp1Δ63 his3Δ200 hom3-10 lys2::InsE-A10 msh3Δ::hisG msh6Δ::hisG). Plasmids pRDK361-ARS-CEN-URA3-MSH2 and pRDK439-ARS-CEN-LEU2-MSH6 were from our laboratory collection and were derived from parental vectors pRS315 and pRS316, respectively. The dual Msh2-Msh6 E. coli expression plasmid pET11a-Msh2-Msh6 was from Dr. Manju Hingorani (38). Derivatives of these plasmids containing the indicated MSH2 and MSH6 alleles were made by site-directed mutagenesis and are described in supplemental Table S1. All of the genetics methods used in the described studies, including those used for site-directed mutagenesis, verification by DNA sequencing, and evaluation of mutator phenotypes by patch tests, were exactly as described previously (11, 29, 40, 41).

**Proteins**—Wild-type and mutant Msh2-Msh6 complexes were purified from E. coli as described (21, 38, 42, 43). Mlh1-Pms1 was purified by sequential chromatography over two HiTrap heparin columns (GE Healthcare) linked in series, anti-FLAG antibody resin (Sigma), and an SP-Sepharose column (GE Healthcare) (21) as described in detail under supplemental “Experimental Procedures.” LacI protein was generously provided by Kathleen Matthews (Rice University).

**Partial Proteolysis**—To determine whether ATP binding affects the conformation of Msh2-Msh6, 1 μg of wild-type or mutant Msh2-Msh6 was incubated with 0–500 ng of trypsin with or without 100 μM ATPγS in reaction buffer (25 mM Tris, pH 8.0, 110 mM NaCl, 4 mM MgCl₂, 0.01% IGEPAL, 2 mM dithiothreitol, 2% glycerol) in a final volume of 10 μl for 1 h at room temperature. Reactions were stopped by the addition of phenylmethylsulfonyl fluoride to a final concentration of 10 mM. The proteolysis products were separated by SDS-PAGE on a 4–15% gel (Bio-Rad), and the resulting gel was silver-stained. Western blotting using rabbit polyclonal antibodies to S. cerevisiae Msh2 or Msh6, which were raised in this laboratory, was performed to detect and differentiate Msh2 and Msh6.

**UV Cross-linking Experiments**—Nucleotide binding was measured by UV cross-linking exactly as described previously (32, 44). ATP-binding reactions were performed in the absence of Mg²⁺, and ADP-binding reactions were performed in the presence of Mg²⁺.

**Surface Plasmon Resonance Analysis**—Experiments analyzing the interaction between the Msh2-Msh6 complex and mispaired DNA were performed with a Biacore T100 instrument (GE Healthcare) essentially as described previously (21, 44, 45) using an IAsys instrument and adapted for use with a Biacore T100 instrument. DNA substrates 236 bp in length with biotin conjugated to one end and a centrally located GT mispair were constructed as described (21, 41, 46). Approximately 20 ng of DNA (140 response units) was conjugated to a flow cell of a streptavidin-coated Biacore SA chip. In the unblocked sliding experiments, 50 nM wild-type or mutant Msh2-Msh6 and 250 μM ATP were flowed over the chip at 20 μl/min for 120 s in reaction buffer. In the end-blocked sliding experiments, 30 nM LacI was flowed over the chip at 20 μl/min for 120 s in reaction buffer before and during Msh2-Msh6 binding. Dissociation of Msh2-Msh6 from the DNA end was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside to the sample. The DNA-coated surface was regenerated with a 20-μl pulse of 3 M NaCl. Data were analyzed with BIAevaluation 3.1 software. Reference subtraction was made from an unmodified flow cell. All experiments were performed at 25 °C.

The formation of Msh2-Msh6-Mlh1-Pms1 ternary complexes on mispaired DNA was also analyzed with a Biacore T100 instrument essentially as described previously (21, 44, 45). The minus Mlh1-Pms1 control was performed similarly to the end-blocked sliding experiment described above; 30 nM LacI was flowed over the chip, followed by 20 μl of 20 nM Msh2-Msh6, 30 nM LacI, and 250 μM ATP and then 40 μl of 20 nM Msh2-Msh6, 30 nM LacI, and 250 μM ATP. In the plus Mlh1-Pms1 experiments, the third injection contained 20 nM Msh2-Msh6, 40 nM Mlh1-Pms1, 30 nM LacI, and 250 μM ATP. Selected ternary complex formation experiments were also performed in which 2 μM ATP was substituted for 250 μM ATP.
Partial Proteolysis Reveals That Msh6 Undergoes Conformational Changes upon Binding ATPγS—To monitor nucleotide-induced conformational changes in the Msh2-Msh6 complex, we incubated wild-type Msh2-Msh6 with increasing concentrations of trypsin in the presence or absence of ATPγS in reactions containing Mg^{2+}, and the digestion of Msh2-Msh6 was monitored by SDS-PAGE (Fig. 2) as well as by Western blotting with Msh2- and Msh6-specific antibodies (supplemental Fig. S3). In the absence of ATPγS, Msh6 was extensively degraded at low trypsin concentrations, whereas Msh2 was degraded only at higher trypsin concentrations (>100 ng/reaction). Msh6 degradation correlated with the appearance of three species of ∼90, 80, and 75 kDa that were derived from Msh6 as indicated by Western blotting. As the trypsin concentration increased (≥50 ng/reaction), the 90- and 80-kDa species were degraded, and the 75-kDa species remained until much higher trypsin concentrations (>150 ng/reaction). We have shown previously that the 90-kDa species corresponds to an N-terminal Msh6 truncation product that is missing the first 441 residues (43). Msh2 degradation correlated with the appearance of lower molecular weight species, many of which were detected with the anti-Msh2 antibody (supplemental Fig. S3). The addition of 100 μM ATPγS in the presence of Mg^{2+} resulted in protection of Msh2-Msh6 from digestion by trypsin in three ways (Fig. 2). First, at low trypsin concentrations, only the 90-kDa species was present, and at higher trypsin concentrations, both the 90- and 80-kDa Msh6 species were present and remained highly resistant to digestion with trypsin. Second, the 75-kDa Msh6 species, which is derived from the 90- and 80-kDa species, was not formed. Finally, Msh2 remained intact at intermediate trypsin concentrations, and at higher trypsin concentrations, it was cleaved into fragments in the size range of 40–50 kDa that remained highly resistant to degradation and were not seen at all in the absence of ATPγS. Identical digestion patterns were observed with ATPγS in the absence of Mg^{2+} or when ATPγS was replaced with ATP in the absence of Mg^{2+} (data not shown).

ATPγS titration experiments were performed to determine whether the observed changes in protease sensitivity resulted in a strong loss of function but do not cause a dominant phenotype like that caused by the equivalent msh6 mutations (29).
from filling of only the high affinity ATP-binding site in Msh6 or from filling of both of the ATP-binding sites in the Msh2-Msh6 complex. At 0.5 μM ATPγS and 10 ng of trypsin, we observed substantial protection of Msh2 as well as protection of the 90- and 80-kDa Msh6 species and reduced formation of the 75-kDa Msh6 species. Maximal protection was seen in the presence of 1 μM ATPγS (Fig. 3), which correlates with filling of only the high affinity Msh6 ATP-binding site (32). Importantly, the level of protection did not increase further at higher ATPγS concentrations (Fig. 3), suggesting that filling of the Msh2 site with ATP and consequentially any conformational changes required to convert Msh2-Msh6 to a sliding clamp are not revealed by this proteolysis assay.

Partial proteolysis was also performed in the presence of Mg2+ with mutant complexes in which the Msh6 ATPase site (Msh2-Msh6-G1142D, Msh2-S742P-Msh6, and Msh2-T773D-Msh6) or an intersite position (Msh2-R730C-Msh6) was affected (Fig. 2). The first three complexes were selected for analysis because assessment of ATP binding by UV cross-linking (see below) suggested that they were defective in ATP binding to the Msh6 nucleotide-binding site, and the latter complex was selected as a control. The digestion patterns of these complexes were the same as those of wild-type Msh2-Msh6. These mutants therefore undergo the same ATPγS-induced conformational changes in the presence of 100 μM ATPγS and Mg2+ as wild-type Msh2-Msh6; we also confirmed that 2 μM ATPγS induced protease protection of Msh2-Msh6-G1142D and Msh2-T773D-Msh6 (data not shown). However, this result seems inconsistent with the observation that the Msh2-Msh6-G1142D and Msh2-T773D-Msh6 complexes were defective in binding ATP in the Msh6 nucleotide-binding site when ATP binding was assessed by UV cross-linking (see below) (Table 1). The proteolysis and cross-linking experiments differed in that the former was performed in the presence of Mg2+, whereas the latter was performed in the absence of Mg2+ to prevent ATP hydrolysis. Therefore, to determine the role that Mg2+ plays in the ATPγS-induced conformational change in Msh2-Msh6, the partial proteolysis reactions were repeated in the absence of Mg2+ (Fig. 4). The digestion pattern of the wild-type Msh2-Msh6 complex was not affected by the absence of Mg2+ either with or without ATPγS or by the absence of Mg2+ when ATP was substituted for ATPγS (data not shown). In contrast, the digestion patterns of the Msh2-Msh6-G1142D and Msh2-T773D-Msh6 complexes were different from those of wild-type Msh2-Msh6 in the absence of Mg2+. Here, the presence of Mg2+ resulted in ATPγS-dependent protection of the 90- and 80-kDa species, whereas in the absence of Mg2+ the 90- and 80-kDa species were not protected by ATPγS, and the 75-kDa species was formed (Fig. 4). In addition, the digestion pattern of the Msh2-Msh6-G1142D complex was the same when ATP was substituted for ATPγS in the absence of Mg2+ (data not shown). Thus, the Msh6 proteins in these mutant complexes require Mg2+ to undergo the ATPγS-induced conformational change, suggesting that the Msh6-G1142D and Msh2-T773D amino acid substitutions destabilize ATP binding, potentially through electrostatics, and therefore require Mg2+ to stabilize binding of ATP to the Msh6 site. The Msh2-Msh6-S742P complex appeared to be partially protected from digestion by trypsin in the presence of ATPγS and absence of Mg2+.
ATP-induced Conformational Changes in Msh2-Msh6

Mutant Msh2-Msh6 Complexes Have Different ATP-binding Defects—To investigate the effects of the Msh2 and Msh6 amino acid substitutions on nucleotide binding at the high affinity Msh6 and low affinity Msh2 ATP-binding sites (32), we used UV cross-linking to determine the affinities of the eight different mutant Msh2-Msh6 protein complexes for ATP under non-hydrolyzing conditions (absence of Mg^{2+}) and for ADP in the presence of Mg^{2+} (Table 1) as described previously (32, 44). The intersite mutant complexes Msh2-R730C-Msh6 and Msh2-Msh6-R1024C bound ATP at the Msh2 and Msh6 sites with affinities that were similar to those of wild-type Msh2-Msh6. The amino acid substitutions affecting the Msh2 sites with affinities that were similar to those of wild-type Msh2-Msh6 and Msh2-Msh6-R1024C bound ATP at the Msh2 and Msh6 sites with affinities that were similar to those of wild-type Msh2-Msh6. The amino acid substitutions affecting the Msh2 sites with affinities that were similar to those of wild-type Msh2-Msh6 and Msh2-Msh6-R1024C bound ATP at the Msh2 and Msh6 sites with affinities that were similar to those of wild-type Msh2-Msh6.

In contrast, each of the three mutations affecting residues near the Msh6 ATP-binding site caused distinct defects. The Msh2-T773D substitution strongly reduced binding to the Msh6 ATP-binding site but did not affect binding to the Msh2 ATP-binding site. The Msh6-G1142D substitution caused a strong reduction in binding of ATP to both the Msh6 and Msh2 nucleotide-binding sites. Finally, the Msh2-S742P substitution caused only a small reduction in binding of ATP to the Msh6 nucleotide-binding site but caused a strong reduction in binding of ATP to the Msh2 nucleotide-binding site. It should be noted that the proteolysis experiments discussed above indicate that the ATP-binding defects at the Msh6 nucleotide-binding sites of the Msh2-Msh6-G1142D and Msh2-T773D-Msh6 complexes likely result from a Mg^{2+} dependence of ATP binding by these mutants. Unfortunately, ATP binding cannot be assessed by UV cross-linking in the presence of Mg^{2+} because the ATP is hydrolyzed too rapidly. The results with the msh2-T773D mutation indicate that ATP binding to the high affinity Msh6 ATP-binding site is not required for ATP binding to the lower affinity Msh2 site. However, the results with the msh6-G1142D and msh2-S742P mutations suggest that conformational changes linking the Msh6 and Msh2 ATP-binding sites can affect ATP binding by the Msh2 site. All three of these mutant complexes had wild-type affinities for binding of ADP to the Msh2 nucleotide-binding site (Table 1).

Mutant Msh2-Msh6 Complexes Are Defective in Sliding Clamp Formation—To monitor sliding clamp formation, we modified an established method to evaluate binding of Msh2-Msh6 to a DNA duplex containing a central mispaired base with or without a LacI protein-mediated end block for use with a Biacore T100 instrument (21, 44, 45). Briefly, wild-type Msh2-Msh6 was flowed over mismatched DNA that was immobilized at one end to a streptavidin-coated chip in the presence of ATP.

![FIGURE 3. ATPγS-induced conformational change in Msh2-Msh6 results from filling the high affinity Msh6 nucleotide-binding site. Wild-type Msh2-Msh6 was incubated with 10 ng of trypsin in the presence of Mg^{2+}, and the indicated concentrations of ATPγS. A, silver-stained gel; B, Western blot with an anti-Msh6 antibody to detect Msh6 proteolytic products; C, Western blot with an anti-Msh2 antibody to detect Msh2 proteolytic products.](image)

**TABLE 1**

Nucleotide-binding affinities for Msh2-Msh6 proteins

All ATP-binding reactions were carried out in the absence of Mg^{2+}, and all ADP-binding reactions were carried out in the presence of Mg^{2+}.

| Protein and subunit | Nucleotide | K_d (μM) |
|---------------------|-----------|----------|
| Msh2-Msh6 | ATP | 0.14 ± 0.01 |
| Msh2-Msh6 | ATP | 41.6 ± 10.8 |
| Msh2-Msh6 | ADP | 1.4 ± 0.7 |
| Msh2-R730C-Msh6 | ATP | 0.3 ± 0.1 |
| Msh2-Msh6-G1142D | ATP | 64.7 ± 3.7 |
| Msh2-Msh6-G1067D | ATP | 2.5 ± 0.1 |
| Msh2-S742P-Msh6 | ATP | 1.7 ± 0.2 |
| Msh2-T773D-Msh6 | ATP | >200 |
| Msh2-Msh6-G1067D | ATP | 0.23 ± 0.06 |
| Msh2-Msh6-S1036P | ATP | >200 |
| Msh2-Msh6-R1024C | ATP | 0.4 ± 0.1 |
| Msh2-Msh6-G1142D | ATP | >200 |
| Msh2-Msh6-G1142D | ATP | 2.3 ± 0.5 |
| Msh2-Msh6-G1142D | ATP | 3.8 ± 0.2 |

* These data are the average of two independent experiments.

* Affinities reported as >200 μM were difficult to measure due to low signal-to-background ratios.
After hydrolyzing the ATP to ADP, Msh2-Msh6 binds the mismatch (21, 32, 36). After exchange of ADP for ATP, wild-type Msh2-Msh6 forms a sliding clamp that slides off the DNA end (21, 30, 36, 44), which gives rise to a moderate level of steady-state binding (Fig. 5). The lac operator site is present at the free end of the immobilized DNA. When LacI is added, it binds the operator site and acts as a block to prevent dissociation of Msh2-Msh6 from the DNA end, giving rise to a higher level of steady-state Msh2-Msh6 binding than in the absence of LacI (Fig. 5) (21). Upon the addition of isopropyl $\beta$-D-thiogalactopyranoside, LacI is released from the operator site, and the accumulated Msh2-Msh6 slides off of the free DNA end, resulting in reduced steady-state Msh2-Msh6 binding comparable with that seen in the absence of LacI (Fig. 5) (21).

Like wild-type Msh2-Msh6, complexes containing each of the four Msh2 mutations or the Msh6-R1024C mutation all showed higher binding to the blocked mispaired DNA substrate compared with the blocked base-paired DNA substrate in the presence of ATP, indicating that these complexes retained specificity for mispaired DNA (supplemental Fig. S4). However, all of the mutant complexes displayed lower absolute levels of binding to both end-blocked and unblocked mispaired DNA substrates relative to wild-type Msh2-Msh6 in the presence of ATP (Fig. 5 and supplemental Fig. S4). The mutant complexes also responded differently than the wild-type complex to the LacI end block (Fig. 5). The addition of LacI did not increase the steady-state levels of binding of these mutant complexes relative to those seen for binding to the unblocked substrate, and the addition of isopropyl $\beta$-D-thiogalactopyranoside to release the LacI end block did not cause significant dissociation of the mutant Msh2-Msh6 complexes from end-blocked mispaired substrates (Fig. 5). These results are consistent with the mutant complexes failing to undergo the ATP-induced conformational change to the sliding clamp form, as seen for the previously studied dominant mutant complexes containing the Msh6-S1036P, Msh6-G1067D, and Msh6-G1142D substitutions (42, 44).

Mutant Msh2-Msh6 Complexes Exhibit Different Defects in Assembly of Ternary Complexes with Mlh1-Pms1—To determine whether the mutant Msh2-Msh6 complexes are capable of forming ternary complexes with Mlh1-Pms1, Msh2-Msh6 was flowed over end-blocked mismatched DNA substrate in the presence of ATP, followed by a mixture containing Msh2-Msh6 and ATP with or without Mlh1-Pms1. These conditions included ATP, which is required for ternary complex formation, and the LacI end block to prevent aberrant ternary complexes from forming on the DNA ends (21, 32, 44, 45). The level of ternary complex formation was determined by subtracting the Msh2-Msh6-only curve from the Msh2-Msh6/Mlh1-Pms1 curve (Fig. 6, insets). Wild-type Msh2-Msh6 formed ternary complexes as evidenced by the large difference between the curves observed in the presence and absence of Mlh1-Pms1 (Fig. 6A).

We confirmed that the Msh2-Msh6-R1024C mutation sensitized the Msh2-Msh6 complexes to ADP-induced dissociation. ATP is required for the proper conformational change of Msh2-Msh6, and its absence results in premature dissociation from the DNA end. The presence of ATP allows Msh2-Msh6 to form a sliding clamp on the DNA. The results of this study suggest that the conformational change required for Msh2-Msh6 to form a sliding clamp is mediated by ATP and is essential for the proper function of the complex in mismatch repair.
for ternary complex formation, whereas the Msh2-Msh6-G1142D complex was proficient (data not shown) (44). The Msh2-R730C-Msh6 (Fig. 6B) and Msh2-T773D-Msh6 (Fig. 6D) complexes formed ternary complexes with Mlh1-Pms1, whereas the Msh2-Msh6-R1024C complex (Fig. 6F) appeared to be partially defective in ternary complex formation. The Msh2-S742P-Msh6 (Fig. 6C) and Msh2-G855D-Msh6 (Fig. 6E) complexes were defective in the formation of ternary complexes. Interestingly, complexes containing the Msh2-S742P, Msh2-T773D, Msh6-S1036P, and Msh6-G1067D mutants, all of which were defective in ternary complex formation (Fig. 6) (44), also all had reduced affinity for ATP in the Msh2 ATP-binding site relative to that seen for wild-type Msh2-Msh6 (Table 1).

**DISCUSSION**

The detailed biochemical analysis of eight MMR-deficient Msh2-Msh6 complexes with various defects in ATP binding, sliding clamp formation, and Msh2-Msh6-Mlh1-Pms1 ternary complex assembly (Table 2) supports several key conclusions. First, binding of ATP to the Msh6 nucleotide-binding site results in conformational changes that are communicated to...
ATP-induced Conformational Changes in Msh2-Msh6

TABLE 2
Summary of the defects caused by the eight Msh2-Msh6 mutations studied

| Amino acid substitution | Msh6-G1067D | Msh6-S1036P | Msh2-G855D | Msh6-G1142D | Msh2-S742P | Msh2-T773D | Msh6-R1024C | Msh2-R730C |
|-------------------------|-------------|-------------|------------|-------------|------------|------------|------------|------------|
| Site                    | Msh2        | Msh2        | Msh2       | Msh6        | Msh6       | Msh6       | Msh6       | Msh2       |
| Msh2 ATP binding        | Normal      | Normal      | Defective  | Normal      | Normal     | Normal     | Normal     | Normal     |
| Msh2 ATP binding        | Normal      | Normal      | Defective  | Defective   | Normal     | Normal     | Normal     | Normal     |
| Msh2 ADP binding        | Normal      | Defective   | Normal     | Normal      | Normal     | Normal     | Normal     | Normal     |
| Sliding clamp formation  | Normal      | Defective   | Normal     | Normal      | Normal     | Normal     | Normal     | Normal     |
| Mlh1-Pms1 ternary       | Normal      | Normal      | Normal     | Normal      | Normal     | Normal     | Normal     | Normal     |
| complex formation       | Normal      | Normal      | Normal     | Normal      | Normal     | Normal     | Normal     | Normal     |

Defect classes are as follows: I, failure of the Msh2 nucleotide-binding site to bind ATP and disruption of conformational changes required for both ternary complex and sliding clamp formation; II, possibly reduced affinity of the Msh2 nucleotide-binding site for ATP and disruption of conformational changes required for sliding clamp formation; III, disruption of conformational change required for sliding clamp formation.

Defect class III IV II III III

Msh2 ADP binding Normal Normal Normal Normal Normal Normal Normal Normal Normal

Msh6 ATP binding Normal Normal Normal Defective Normal Normal Normal Normal Normal

Site Msh2 Msh2 Msh2 Msh6 Msh6 Msh6 Intersite Intersite

Defective Defective Defective Defective Defective Defective Defective Defective

ATP binding requires Mg²⁺.

This site has 10-fold reduced affinity for ATP and saturates with ATP at 30-fold lower concentrations than those required to fill the Msh2 site.

It is not known if this site binds ATP in the presence of Mg²⁺.

This site has slightly reduced affinity for ATP, but the affinity is within the range of wild-type affinity for ATP.

the Msh2 ATP-binding site; however, binding of ATP to both sites can be uncoupled. Second, conversion of Msh2-Msh6 to the sliding clamp form appears to require ATP binding to both sites as well as communication between the two ATP-binding sites. Third, ternary complex formation appears to require ATP binding only at the Msh6 nucleotide-binding site. Finally, sliding clamp formation and ternary complex formation can be uncoupled.

In previous studies, it was observed that the N terminus of Msh6 in the Msh2-Msh6 complex was highly sensitive to digestion with trypsin (43). Digestion first resulted in the loss of the unstructured N-terminal 251 amino acid residues, followed by digestion up to residue 441, leaving a stable 90-kDa C-terminal core of Msh6 complexed with apparently intact Msh2. Here, we found that, at higher trypsin concentrations, the C-terminal Msh6 core was cleaved at two additional sites, resulting in 80- and 75-kDa fragments. We have found that the 75- and 80-kDa species result from cleavage of an N-terminally truncated Msh6 species at two different sites between the Msh6 C terminus and the Msh6 ATP-binding site.³ ATP and ATPγS protected the 90- and 80-kDa species and prevented the formation of the 75-kDa fragment at concentrations expected to fill only the high affinity Msh6 nucleotide-binding site. These results support the view that binding of ATP in the Msh6 nucleotide-binding site induces a significant conformational change within the Msh2-Msh6 complex. The nature of this conformational change, suggested by examination of the crystal structure of the related Rad50 ATP-binding cassette ATPase in the ATP-bound form (48), is likely an open-to-closed transition of the Msh2-Msh6 ATPase domains relative to the ADP- and DNA-bound conformational states of the MutS and Msh2-Msh6 crystal structures (2, 6, 24–26). Modeling this closed state (Fig. 1 and supplemental Fig. S6) revealed that the ATP-binding cassette ATPase domain "signature motif" of one subunit made conserved interactions with ATP bound in the other subunit and revealed that the observed trypsin digestion sites were protected.

Using the available crystal structures of MutS and human Msh2-Msh6 (2, 6, 24–26), the local defects in the various symmetric mutants can be understood. The Msh2-S742P and Msh6-S1036P amino acid substitutions disrupt a key residue of the ATP-binding cassette ATPase domain signature motif that probably makes direct interactions with the ATP γ-phosphate when ATP is bound in the opposite subunit. The defects in Msh2-S742P-Msh6 and Msh2-Msh6-S1036P are consistent with defects in stabilization of the closed conformation of the ATPase domains. The strong Mg²⁺ dependence of ATP binding to the Msh6 nucleotide-binding site of the mutant Msh2-T773D-Msh6 and Msh2-Msh6-G1142D complexes is consistent with a simple model of destabilization of ATP binding in the Msh6 site by electrostatic repulsion. Despite this, Mg²⁺ is unable to mask either the defects caused by the equivalent Msh2-G855D and Msh6-G1067D amino acid substitutions in ternary complex formation or the defects caused by all four amino acid substitutions in the formation of the sliding clamp, suggesting that these amino acid substitutions cause additional defects. Finally, the intersite amino acid substitutions Msh2-R730C and Msh6-R1024C appear to be positioned to play roles in stabilizing the closed conformation.

Closing of the ATPase domains induced by binding of ATP to the Msh6 nucleotide-binding site is also consistent with evidence for communication between the high affinity Msh6 and low affinity Msh2 nucleotide-binding sites. The Msh6-G1142D amino acid substitution results in a defect in binding of ATP at both nucleotide-binding sites in the absence of Mg²⁺ even though this mutation does not alter an amino acid residue near the Msh2 nucleotide-binding site. The Msh2-S742P amino acid substitution, which disrupts a key residue of the signature motif predicted to interact with the γ-phosphate of Msh6-bound ATP, also appears to disrupt communication between the two nucleotide-binding sites, as it causes only a slight decrease in the affinity for binding of ATP by Msh6 but causes a substantial defect in ATP binding at the Msh2 nucleotide-binding site. The properties of these two mutations suggest that communication between the Msh6 and Msh2 nucleotide-binding sites is required for filling the Msh2 site with ATP. Importantly, the reciprocal amino acid substitutions at the Msh2 ATPase site (Msh2-G855D and Msh6-S1036P) have no effect on ATP binding by Msh6. This result suggests that these amino acid substitutions are unlikely to disrupt ATP binding at the Msh2 site by preventing ATP binding to the Msh6 site. Instead, they likely prevent a conformational change in the ATPase domains initiated by binding of ATP to the high affinity Msh6 site, and this inhibits ATP binding to the low affinity Msh2 site. In contrast, the Msh2-T773D amino acid substitution, which affects the Msh6 nucleotide-binding site and causes a strong defect in ATP binding only at the Msh6 nucleotide-binding site, is likely an open-to-closed transition of the Msh2-Msh6 ATPase domains. The strong Mg²⁺ dependence of ATP binding to the Msh6 nucleotide-binding site of the mutant Msh2-T773D-Msh6 and Msh2-Msh6-G1142D complexes is consistent with a simple model of destabilization of ATP binding in the Msh6 site by electrostatic repulsion. Despite this, Mg²⁺ is unable to mask either the defects caused by the equivalent Msh2-G855D and Msh6-G1067D amino acid substitutions in ternary complex formation or the defects caused by all four amino acid substitutions in the formation of the sliding clamp, suggesting that these amino acid substitutions cause additional defects. Finally, the intersite amino acid substitutions Msh2-R730C and Msh6-R1024C appear to be positioned to play roles in stabilizing the closed conformation.

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binding at this site, does not cause a defect in ATP binding at
the Msh2 nucleotide-binding site. This finding suggests that
communication between the two nucleotide-binding sites can be
uncoupled such that nucleotide binding at each site can occur independently.

The results presented here indicate that ATP concentrations
sufficient for binding of ATP to the Msh6 nucleotide-binding site
are sufficient for the formation of Msh2-Msh6-Mlh1-Pms1 ternary complexes. It is possible that this ATP requirement for
ternary complex formation reflects a requirement for ATP binding to
Mlh1-Pms1 rather than the Msh6 nucleotide-binding site; however, ATP binding by MutS and not by MutL is
required for the formation of MutS-MutL ternary complexes (15, 16), mutations affecting ATP hydrolysis by human Mlh1 and
Pms2 (homolog of S. cerevisiae Pms1) do not affect the formation of Msh2-Msh6-Mlh1-Pms2 ternary complexes (35), and
mutations that are predicted to prevent ATP binding by
Mlh1 and Pms1 individually cause only a small MMR defect
(49). Four (Msh6-S1036P, Msh6-G1067D, Msh2-G855D, and
Msh2-S742P) of the eight amino acid substitutions studied
corelated complete defects in ternary complex formation, and
each of these had relatively normal ATP binding at the Msh6 site
but substantially reduced ATP binding at the Msh2 site
(Tables 1 and 2). It seems likely that these mutations cause
defects both in ATP binding to the Msh2 nucleotide-binding site and in a conformational change required for ternary com-
plex formation that is driven by ATP binding to the Msh6 nucleotide-binding site. Three of the mutant complexes
(Msh2-T773D-Msh6, Msh2-R730C-Msh6, and Msh2-Msh6-
G1142D) were proficient in formation of Mlh1-Pms1 ternary complexes, and one (Msh2-Msh6-R1024C) was partially profi-
cient in ternary complex formation. The Msh2-T773D-Msh6
and Msh2-Msh6-G1142D complexes appeared to undergo the
same conformational change as wild-type Msh2-Msh6 in
response to ATP binding to the Msh6 nucleotide-binding site. Furthermore, the wild-type Msh2-Msh6, Msh2-T773D-Msh6,
and Msh2-Msh6-G1142D complexes (Msh2-Msh6-R1024C and Msh2-R730C-Msh6 were not tested) were proficient in ternary
complex formation at ATP concentrations predicted to
saturate only the Msh6 nucleotide-binding site. It therefore
seems likely that these amino acid substitutions do not disrupt
(Msh2-T773D, Msh6-G1142D, and Msh2-R730C) or partially disrupt (Msh2-R1024C) the conformational change required
for ternary complex formation that is driven by ATP binding
Msh6 nucleotide-binding site even though they appear to
disrupt the conformational changes required for sliding clamp
formation. Recently, Msh2 domain II was identified as contain-
ing the interaction interface between Msh2-Msh6 and Mlh1-
Pms1, and an isolated Msh2 domain II was shown to bind
Mlh1-Pms1 independently of nucleotide and mispaired DNA
(45). Thus, ATP binding to the Msh6 nucleotide-binding site
likely induces the conformational change that exposes the
Mlh1-Pms1 interface and mediates ternary complex formation.

Conversion of mispair-bound Msh2-Msh6 complexes to
sliding clamps has been suggested to require ATP binding to
both the Msh6 and Msh2 nucleotide-binding sites, based on
the ATP concentration dependence of sliding clamp formation (21, 32). Remarkably, all eight amino acid substitutions studied here
caused defects in sliding clamp formation, regardless of whether or not they affected ATP binding to the Msh6 or Msh2 nucleotide-binding site. This group included mutant com-
plexes that were competent for ATP’S-induced protease
sion of Msh6 and ATP-induced ternary complex formation
(Msh2-T773D, Msh6-G1142D, and Msh2-R730C). These
results suggest that the conformational changes involved in
sliding clamp formation are distinguishable from those changes
that can be monitored by limited trypsin proteolysis and tern-
ary complex formation, and thus, they indicate that ternary
complex formation and sliding clamp formation are separable.

One intriguing explanation for the sensitivity of sliding clamp
formation relative to other functions of Msh2-Msh6 is that slid-
ing clamp formation likely requires energy to drive dissociation of the Msh6 mispair-binding domain from the bound mispair
and as such may be more sensitive to subtle misalignments
and/or destabilization of the dual ATP-bound state of Msh2-
Msh6 (32) than are other conformational changes affecting
ATP binding at the Msh2 nucleotide-binding site or ternary complex formation. Moreover, these data also indicate that for-
formation of Msh2-Msh6 sliding clamp is critical for MMR, as
mutations encoding complexes that have defects only in sliding
complex formation (msh2-T773D, msh6-G1142D, msh2-R730C,
and msh6-R1024C) are MMR-defective in vivo.

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