Dominant *Saccharomyces cerevisiae* msh6 Mutations Cause Increased Mispair Binding and Decreased Dissociation from Mispairs by Msh2-Msh6 in the Presence of ATP*

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A previous study described four dominant *msh6* mutations that interfere with both the Msh2-Msh6 and Msh2-Msh3 mismatch recognition complexes (Das Gupta, R., and Kolodner, R. D. (2000) *Nat. Genet.* 24, 53–56). Modeling predicted that two of the amino acid substitutions (G1067D and G1142D) interfere with protein-protein interactions at the ATP-binding site-associated dimer interface, one (S1036P) similarly interferes with protein-protein interactions and affects the Msh2 ATP-binding site, and one (H1096A) affects the Msh6 ATP-binding site. The ATPase activity of the Msh2-Msh6-G1067D and Msh2-Msh6-G1142D complexes was inhibited by GT, +A, and +AT mispairs, and these complexes showed increased binding to GT and +A mispairs in the presence of ATP. The ATPase activity of the Msh2-Msh6-S1036P complex was inhibited by a GT mispair, and it bound the GT mispair in the presence of ATP, whereas its interaction with insertion mispairs was unchanged compared with the wild-type complex. The ATPase activity of the Msh2-Msh6-H1096A complex was generally attenuated, and its mispair-binding behavior was unaffected. These results are in contrast to those obtained with the wild-type Msh2-Msh6 complex, which showed mispair-stimulated ATPase activity and ATP inhibition of mispair binding. These results indicate that the dominant *msh6* mutations cause more stable binding to mispairs and suggest that there may be differences in how base base and insertion mispairs are recognized.

Mismatch repair (MMR)

MR proteins has been made in *Escherichia coli* (for selected reviews, see Refs. 2 and 4–6), where MMR has been reconstituted with the MutH, MutL, MutS, and UvrD (helicase II) proteins along with DNA polymerase III holoenzyme, DNA ligase, single-strand DNA binding protein, and one of the exonucleases: exonuclease I, exonuclease VII RecJ protein, or the recently implicated exonuclease X (10). In eukaryotes, MMR is known to require homologs of the MutS and MutL proteins as well as DNA polymerase δ, proliferating cell nuclear antigen, replication factor C, replication factor A, and exonuclease I, although other MMR proteins remain to be identified or definitively shown to act in MMR (for selected reviews, see Refs. 2–6).

Central to MMR is the bacterial MutS protein and its eukaryotic homologs Msh2, Msh3, and Msh6. In bacteria, MutS appears to function as a dimer that recognizes mispaired bases in DNA and coordinates the mismatch repair reaction (11–14). In eukaryotes, the MutS equivalents are the Msh2-Msh6 (MutSα) and Msh2-Msh3 (MutSβ) heterodimers, which have different but overlapping mismatch recognition specificity (15–19). The observation that the unique subunits of these heterodimers (Msh3 and Msh6) determine mismatch recognition specificity rather than the common subunit (Msh2) suggested that the heterodimers and mismatch repair recognition are asymmetric in nature (3, 4, 15). Studies on the interaction between ATP and the Msh complexes or MutS bound to a mispair have indicated that a conformational change induced by ATP binding results in the formation of a ring-like complex that is clamped on the DNA and can slide along it (20–24). These views were borne out by the crystal structure of MutS bound to a mispaired base, demonstrating an induced fit mode of binding resulting in the formation of a protein ring in which only one monomer is primarily responsible for interaction with the mispaired base (12, 13, 25). Mispair recognition by MutS involves stacking of a Phe residue onto a ring of the mispaired base, kinking of the DNA facilitated by a Glu residue that contacts the DNA, and other specific amino acid-DNA contacts. Interactions between mispair and ATP binding are evident in the crystal structure. Genetic studies have shown that many of the residues critical for mispair binding of MutS are conserved in Msh6 and are required for mismatch recognition, supporting the asymmetric view of mispair recognition by both MutS and Msh2-Msh6 (26–32). However, mispair recognition by the Msh complexes cannot entirely mimic that by MutS because the *msh6*-E339A mutation has little effect on MMR, whereas the equivalent *E. coli* mutation (mutS-E38A) eliminates MMR (29, 32). In addition, Msh3 clearly lacks the equivalent of *E. coli* Phe66 that is critical for mispair binding by MutS (12, 13, 27).

Several models have been proposed for how MutS and the Msh complexes coordinate MMR. In one model, MutS binding
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results in the assembly of a higher order complex of proteins that translocate along the DNA to coordinate with the MutH endonuclease activity, which is then activated to initiate MMR (22, 33). The MutS complex structure likely facilitates this translocation. In a second model, the MutS complex and the MutH incision protein recognize their binding sites independently, and loops facilitate the interaction between the two complexes and other proteins, leading to the transactivation of MutH and hence MMR (34). Finally, it has been suggested that the Msh complexes and MutS undergo a conformational change upon ATP binding, converting them to a clamp structure. The clamp then slides along the DNA and signals other proteins involved in MMR (20, 21, 24, 35). Integral to these models is the ability of the MutS or Msh complex to interact with and, in some cases, activate other proteins that function in MMR. Many such interactions have been characterized, including the interaction of MutL and the Mlh1-Pms1 (Pms2 in humans) complexes and other proteins, leading to the transactivation of MutH and hence MMR (34). It has not, however, shed much light on the overall mechanism of MMR and, in particular, the mechanism of eukaryotic MMR.

We have been performing a number of genetic studies in Saccharomyces cerevisiae aimed at better understanding the mechanism of MMR. In a previous study, we described novel mutations in MSH6 that appeared not only to inactivate the Msh2-Msh6 complex but also to inactivate the Msh2-Msh3 complex (26). Here, we describe the biochemical properties of four of these mutant Msh2-Msh6 complexes. Three of them form a much more stable complex with a mispaired base in the presence of ATP compared with the wild-type complex. In the case of the fourth mutant complex, a mispaired base is unable to activate the ATPase activity of the mutant complex to the extent seen for the wild-type complex. These results support the view that dynamic interactions of Msh2-Msh6 at a mispair are critical for MMR.

MATERIALS AND METHODS

DNA Binding Analysis—Total internal reflection measurements using the IAsys Auto Plus system (Affinity Sensors) were performed to monitor the binding of wild-type and mutant Msh2-Msh6 protein complexes to dsDNA substrates in the absence of ATP. The IAsys Auto Plus system (Affinity Sensors) contained two reaction cells coated with biotin, which were subsequently bound with excess streptavidin (Prozyme). Unbound streptavidin was removed from the cuvette by extensive washing with PBST buffer. One µg of the indicated annealed dsDNA substrate containing a single 3′-biotin was added to the streptavidin-coated reaction cells, and excess unbound single-stranded DNA was removed by washing with PBST buffer. Reaction cells were then equilibrated with binding buffer (25 mM HEPES (pH 7.8), 5 mM MgCl2, 1 mM dithiothreitol, 2% glycerol, and 0.001% IGEPAL 40). Twenty µg of Msh2-Msh6 proteins were added to the cells. Protein binding was monitored for 3 min or until saturation was achieved (26). To stop the reactions, 400 µl of 10% (w/v) activated charcoal (Nor A) in 40 mM NaHPO4 was added, and the samples were incubated on ice for 10 min. The tubes were then centrifuged at 14,000 rpm in a tabletop centrifuge to sediment the charcoal, and the radioactivity present in duplicate 100-µl portions of the supernatant was determined using the Bradford assay (Bio-Rad) and gel filtration chromatography on Polybuffer Exchanger 94 resin, single-stranded DNA-cellulose, and Q-Sepharose essentially as previously described (53); and the final protein preparation was frozen in aliquots with liquid nitrogen. All protein preparations contained an equimolar ratio of Msh2 and Msh6 subunits and were >98% pure. Protein concentrations were determined using the Bradford assay (Bio-Rad) and gel filtration protein size standards (Bio-Rad) as protein concentration standards. The yield from 50 g of cells ranged from 0.7 to 1.8 mg.

ATPase Assays—ATPase activity was measured following a previously published method (55, 56) in reactions (20) consisting of 25 mM HEPES (pH 7.8), 10 mM MgCl2, 0.1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.001% IGEPEAL 40, 75 µg/ml acetylated bovine serum albumin (Promega), 250 µM [γ-32P]ATP, and the indicated dsDNA substrates at 200 nM. The reactions were started by addition of the Msh2-Msh6 complex (wild-type or mutant Msh6) to 120 nM, followed by incubation at 30 °C for the times indicated in individual experiments. To stop the reactions, 400 µl of 10% (w/v) activated charcoal was added to 40 mM NaHPO4, and the samples were incubated on ice for 10 min. The tubes were then centrifuged at 14,000 rpm in a tabletop centrifuge to sediment the charcoal, and the radioactivity present in duplicate 100-µl portions of the supernatant was determined using the liquid scintillation counting (Beckman LS6000SC). All values were corrected for the value obtained for a no-protein control that had been incubated for the same time as the experimental reaction.

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Overproduction and Purification of Msh2-Msh6 Complexes—Msh2 was coexpressed with wild-type or mutant MSH6 genes under the control of a GAL10 promoter. The Msh2 expression vector pDRK534 contains the GAL10-Msh2 fusion on a 2µ URA3 plasmid (54). The pDRK945-GAL10-Msh6-WT (where WT is wild-type), pDRK942-GAL10-Msh6-S1036P, pDRK581-GAL10-Msh6-G1067D, pDRK582-GAL10-Msh6-H1096A, and pDRK944-GAL10-Msh6-G1142D plasmids all consist of two DNA fragments joined to each other: an HincII-BglII fragment containing the C-terminal coding sequence of Msh6 (the BglII site is located at base pair 855 of MSH6) and the BlpII-HindIII fragment containing the GAL10 promoter fused to the N-terminal coding sequence of Msh6 (the BlpII site is located at base pair 585 of MSH6) and the BlpII-HindIII fragment of MSH6 containing the C terminus of the gene containing the allele of interest. The C-terminal fragments were obtained following the wild-type Msh6 plasmid pDRK941, and the mutant fragments were obtained from pDRK828-Msh6-S1036P, pDRK737-Msh6-G1067D, pDRK721-Msh6-H1096A, and pDRK495-Msh6-G1142D (26). All of the plasmids were sequenced to ensure that they contained mutations other than the desired mutation. The overexpression strains were cultured by cotransforming the S. cerevisiae strain KY2418 (MATa ura3-52 leu2-1 his3200 pep4-4 his3-I3116 TRP1 can1 msh2::HIS3 msh6::HO) and wild-type and mutant Msh2-Msh6 proteins were purified from cell extracts of galactose-induced cells. Briefly, cells were grown at 30 °C in a fermentor (New Brunswick Scientific) at 10 liters of synthetic complete dropout medium lacking uracil and leucine and containing 3% glycerol and 0.02% lactate to an A600 of 0.8. The production of Msh2 and Msh6 proteins was induced by addition of galactose to a final concentration of 2% for 10 h. Galactose-induced cells (50 g) suspended in an equal volume of buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, and 0.01% IGEPEAL 20 were lysed with glass beads (Sigma) in a bead beater (Biospec Products, Inc., Bartlesville OK); the Msh2-Msh6 complexes were purified by sequential chromatography on Polybuffer Exchanger 94 resin, single-stranded DNA-cellulose, and Q-Sepharose essentially as previously described (53); and the final protein preparation was frozen in aliquots with liquid nitrogen. All protein preparations contained an equimolar ratio of Msh2 and Msh6 subunits and were >98% pure. Protein concentrations were determined using the Bradford assay (Bio-Rad) and gel filtration protein size standards (Bio-Rad) as protein concentration standards. The yield from 50 g of cells ranged from 0.7 to 1.8 mg.

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Molecular Modeling—The molecular models for the Msh2-Msh6 complex were based on the structural coordinates of *E. coli* and *Thermus aquaticus* MutS protein (12, 13, 34). The ribbon diagram rendition was created with MOLSCRIPT and RASTER3D, whereas the stick diagram was created with XFIT and RASTER3D (58–60).

RESULTS

Structural Effects of MSH6 Amino Acid Substitutions—In a previous study, we described a genetic screen for mutations in *MSH6* that cause an increase in the rate of accumulating frameshift mutations when the mutation is present at the chromosomal *MSH6* locus or when present on a 2μ vector in an otherwise wild-type strain (26). The phenotype caused by the chromosomal alleles is a stronger mutator phenotype than caused by null mutations in *MSH6* (15, 17) and was suggested to result from the formation of a mutant Msh2-Msh6 complex that was capable of interfering with the function of the Msh2-Msh3 complex (26). The dominant phenotype observed on high copy also suggested that the mutant Msh2-Msh6 complexes formed might be capable of titrating out downstream components of the MMR pathway. Molecular modeling based on the MutS structure (12, 13) indicates that three of the dominant *msh6* mutations that cause the strongest phenotypes (S1036P, G1067D, and G1142D) alter amino acid residues in the region of S1036P, these substitutions do not change residues previously thought to be critical for ATP binding or hydrolysis, but rather affect the Msh2-Msh6 interface and might affect the previously thought to be critical for ATP binding or hydrolysis, but rather affect the Msh2-Msh6 interface and might affect the conformational transitions predicted to occur at this interface in response to ADP-ATP exchange (61–63).

Analysis of the ATPase Activity of the Wild-type and Mutant Msh2-Msh6 Complexes—To begin to understand the interaction of the Msh2-Msh6 complex with ATP and DNA, a time course of ATP hydrolysis and a titration of ATP hydrolysis versus NaCl concentration for the wild-type complex were performed. Experiments carried out in the absence of DNA or in the presence of an oligonucleotide duplex with or without a +A insertion/deletion mispair are shown in Fig. 2. The results of...
these experiments show that the wild-type complex is an ATPase in the absence of DNA and that this ATPase activity is stimulated by DNA without a mispair and further stimulated by DNA with a mispair. Stimulation of the ATPase by the +A mispair showed a salt optimum of ∼100 mM NaCl. Similar results were obtained when the oligonucleotide duplex contained a GT mispair (data not shown). The effect of DNA without a mispair or with a +A mispair on the ATPase activity of Msh2-Msh6 in the presence of 100 mM NaCl was examined in seven independent experiments using two different Msh2-Msh6 preparations and incubation times of 20, 30, and 40 min. The observed -fold stimulation of ATPase activity by DNA either without a mispair or with a +A mispair versus no added DNA was 2.94 ± 0.27 and 4.79 ± 0.44, respectively, and the -fold stimulation by DNA with a +A mispair versus DNA without a mispair was 1.64 ± 0.20. Similarly, the -fold stimulation of ATPase activity due to adding 100 mM NaCl to the reactions versus no NaCl (5 mM due to reaction components) was 1.15 ± 0.06, 0.96 ± 0.06, and 1.53 ± 0.02 in the absence of DNA or in the presence of DNA without a mispair or with a +A mispair, respectively. These results are in contrast with previously published results for the S. cerevisiae Msh2-Msh6 complex, where mispair stimulation was either not observed, or a +A mispair stimulated the ATPase activity by 25%, but only at NaCl concentrations of 300–500 mM (28, 64–66). However, our results are similar to published results for the human Msh2-Msh6 complex (56).

To analyze the ATPase activity of the mutant proteins, ATPase assays were performed under conditions in which the ATPase activity was linear with respect to time of incubation and protein concentration. Two independent experiments were performed in which the ATPase activity of the wild-type and mutant proteins was measured in the absence of DNA and in the presence of an oligonucleotide duplex that was fully base-paired or that contained a GT mispair or a +A, +AT, or +ATGC insertion/deletion mispair. The absolute ATPase activity for each experimental condition for one of the experiments is shown in Fig. 3; the second experiment yielded essentially the same results (data not shown). The ratio of ATPase activity in the presence of the different mispaired base-containing DNA substrates to ATPase activity with fully base-paired DNA and the ratio of ATPase activity in the presence of fully base-paired DNA to ATPase activity in the absence of DNA were then calculated for both experiments. The average value for each ratio and the associated error are presented in Table I. In the case of the wild-type complex, the base-paired duplex stimulated ATP hydrolysis ∼3-fold. The GT, +A, and +AT duplexes further stimulated the reaction, consistent with the genetic view that these mispairs are recognized by the Msh2-Msh6 complex (3, 4, 15, 17, 53, 67). The +ATGC duplex did not stimulate the reaction above that seen with the base-paired duplex, consistent with the genetic view that 4-base insertion/deletion mispairs are not well recognized by the Msh2-Msh6 complex (3, 4, 15, 17, 53, 67).

All four mutant proteins had significant ATPase activity in the absence of DNA that ranged from 55 to 188% of the activity of the wild-type protein. These results indicate that the amino acid substitutions did not greatly affect the intrinsic ATPase activity of the Msh2-Msh6 complex. This is consistent with the fact that three of the amino acid substitutions (S1036P, G1067D, and G1142D) affect residues that are generally not thought to be critical for ATP binding and/or hydrolysis, whereas one of the amino acid substitutions (H1096A) likely affects hydrolysis by the Msh6 ATP-binding site, but not by the Msh2 ATP-binding site. However, all of the mutant protein complexes showed significantly altered ATPase activity in the presence of DNA with or without a mispaired base. The mutant proteins fell into three basic groups in regard to the effect of DNA on ATPase activity. The Msh2-Msh6-H1096A complex showed a reduced stimulation by DNA compared with the wild-type complex, and the mutant complex showed a modestly reduced, although still significant, relative stimulation by mispaired bases compared with the wild-type complex. These data indicate that the ATPase activity of the Msh2-Msh6-H1096A complex is generally attenuated in response to DNA, even though it binds DNA and recognizes mispaired bases (see below). The Msh2-Msh6-S1036P and Msh2-Msh6-G1067D ATPase activity was increased by some (but not all) of the DNA substrates and not to the extent of the wild-type complex. Addition of the GT mispair substrate almost completely inhibited the ATPase activity of these two complexes, whereas the +A and +AT mispairs stimulated the ATPase activity of these complexes relative to DNA without a mispair. The extent of stimulation by the +A and +AT mispairs was the same for the Msh2-Msh6-S1036P and wild-type complexes, whereas the stimulation of the Msh2-Msh6-G1067D complex was not as high as seen for the wild-type complex. Finally, the Msh2-Msh6-G1142D ATPase activity showed a different response to added DNA. DNA without a mispair did not stimulate the ATPase activity, and the GT, +A, and +AT mispairs inhibited the ATPase activity relative to DNA without a mispair; in this case, the relative inhibition by the +A and +AT mispairs was close to that seen with the GT mispair.

Interaction of the Wild-type and Mutant Msh2-Msh6 Complexes with Mispaired Bases—The mispair recognition properties of the mutant Msh2-Msh6 complexes were then investigated using an IAsys biosensor method. The method was calibrated by analyzing the binding properties of the wild-type complex (Fig. 4). In the absence of a nucleotide cofactor, the wild-type complex was found to bind duplex DNA without a mispair to a low extent and to bind GT and +A mispairs to a significantly greater extent; somewhat greater binding was observed with the GT mispair relative to the +A mispair. Addition of ADP to the binding buffer reduced the binding to the GT and +A mispairs by ∼10 and ∼40%, respectively. In contrast, addition of ATP to the binding buffer reduced the binding to the GT and +A mispairs by ∼90%. Next, the wild-type complex was allowed to associate with the three different substrates in the absence of nucleotide cofactor and then allowed to dissociate in the absence of nucleotide cofactor or in the presence of ADP or ATP (Fig. 5). ADP modestly increased the dissociation from the duplex and GT substrates and more significantly increased the dissociation from the +A substrate. In all cases, ATP markedly increased the dissociation of the Msh2-Msh6 complex. Indeed, relative to no nucleotide in the
dissociation buffer, ATP reduced the time for half-dissociation from the GT and +A substrates from 375 to 4 s and from 107 to 4 s, respectively. We did not observe that binding of Msh2-Msh6 to a mispair in the presence of ATP yielded a form resistant to ATP-induced dissociation (data not shown), as has been observed for *E. coli* MutS (23). The binding of Msh2-Msh6 as a function of protein concentration in the absence of nucleotide and the presence of ATP was determined to calculate the various binding and dissociation constants that describe binding; Fig. 6 shows an example of the analysis of mispair binding versus protein concentration (Msh2-Msh6-S1036P binding to the GT substrate is shown), and Table II lists the relevant affinity constants. This analysis confirmed that the relative binding affinity of the different DNA substrates in the absence of nucleotide was duplex/HT/H1001 GT, consistent with a 10–20-fold greater affinity for a mispair relative to a base pair. This analysis also showed that ATP reduced the binding affinity for a mispair to the level seen for DNA without a mispair.

To characterize the mispair-binding properties of the mutant Msh2-Msh6 complexes, a comparison of the association phase of binding to the duplex, GT, and +A substrates was performed in the absence of added nucleotide and in the presence of ADP or ATP (Fig. 4). Binding of the Msh2-Msh6-H1096A complex to the three different DNA substrates in the absence of nucleotide

| Msh6 protein | +DNA−DNA (wild-type) | GC−DNA | GT/GC | +A/GC | +AT/GC | +ATGC/GC |
|--------------|-----------------------|--------|-------|-------|--------|----------|
| Wild-type    | 2.8 ± 0.12            | 1.15 ± 0.01 | 1.71 ± 0.05 | 1.6 ± 0.1 | 0.92 ± 0.03 |
| S1036P       | 0.55 ± 0.05           | 2.2 ± 0   | 0.25 ± 0.003 | 1.61 ± 0.05 | 1.5 ± 0.05 | 0.91 ± 0.05 |
| G1067D       | 1.47 ± 0.11           | 1.29 ± 0.01 | 0.23 ± 0   | 1.26 ± 0.01 | 1.31 ± 0.05 | 0.97 ± 0.03 |
| H1096A       | 0.65 ± 0.01           | 1.78 ± 0.17 | 1.12 ± 0.02 | 1.33 ± 0.02 | 1.24 ± 0.05 | 0.91 ± 0.04 |
| G1142D       | 1.81 ± 0.07           | 0.78 ± 0.01 | 0.48 ± 0.004 | 0.71 ± 0.03 | 0.77 ± 0.09 | 1.04 ± 0.03 |

**Fig. 4.** Biosensor analysis of the association of wild-type and mutant Msh2-Msh6 complexes with dsDNA and dsDNA containing a GT mispair or a +A insertion. Shown is the association phase over 5 min for DNA binding reactions performed with the indicated Msh2-Msh6 preparations and DNA substrates under standard conditions as described under "Materials and Methods." The concentration of wild-type (WT) and mutant Msh2-Msh6 complexes used was 50 nM. The black lines indicate the absence of nucleotide in the binding buffer; the red lines indicate the presence of 250 μM ATP in the binding buffer, and the blue lines indicate the presence of 250 μM ADP in the binding buffer.

**Fig. 5.** Biosensor analysis of the dissociation of wild-type and mutant Msh2-Msh6 complexes from dsDNA and dsDNA containing a GT mispair or a +A insertion. Association of the indicated Msh2-Msh6 preparations with the indicated DNA substrates was performed in the absence of nucleotide in the binding buffer for 5 min exactly as described in the legend to Fig. 4. The protein solution was replaced with binding buffer, and dissociation was monitored over 4 min. The black lines indicate the absence of nucleotide in the dissociation buffer; the red lines indicate the presence of 250 μM ATP in the dissociation buffer; and the blue lines indicate the presence of 250 μM ADP in the dissociation buffer. WT, wild-type.
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Fig. 6. Protein concentration dependence of the association of the Msh2-Msh6-S1036P complex with dsDNA containing a GT mispair. A, association of the different concentrations of Msh2-Msh6-S1036P with the GT substrate in the absence of nucleotide was performed exactly as described in the legend to Fig. 4. B, shown is a plot of $K_{on}$ versus Msh2-Msh6-S1036P concentration for the data in A calculated using GraFit5 software.

TABLE II

Binding constants for the Msh2-Msh6 and Msh2-Msh6-S1036P complexes

| Binding conditions        | $K_D$ (nM) | $k_a$ (μM$^{-1}$ s$^{-1}$) | $k_d$ (s$^{-1}$) |
|---------------------------|------------|-----------------------------|-----------------|
| GC DNA substrate          |            |                             |                 |
| Msh2-Msh6                 | 61 ± 2     | 0.365 ± 0.054               | 0.022 ± 0.004   |
| Msh2-Msh6 + ATP           | 190 ± 5    | 0.051 ± 0.018               | 0.010 ± 0.002   |
| Msh2-Msh6-S1036P          | 56 ± 3     | 0.529 ± 0.094               | 0.030 ± 0.007   |
| Msh2-Msh6-S1036P + ATP    | 200 ± 19   | 0.324 ± 0.232               | 0.065 ± 0.020   |
| GT DNA substrate          |            |                             |                 |
| Msh2-Msh6                 | 21 ± 3     | 0.265 ± 0.027               | 0.006 ± 0.002   |
| Msh2-Msh6 + ATP           | 107 ± 5    | 0.116 ± 0.035               | 0.012 ± 0.003   |
| Msh2-Msh6-S1036P          | 9 ± 2      | 0.362 ± 0.019               | 0.003 ± 0.001   |
| Msh2-Msh6-S1036P + ATP    | 29 ± 1     | 0.361 ± 0.024               | 0.011 ± 0.002   |
| +A DNA substrate          |            |                             |                 |
| Msh2-Msh6                 | 41 ± 1     | 0.363 ± 0.034               | 0.015 ± 0.003   |
| Msh2-Msh6 + ATP           | 175 ± 8    | 0.137 ± 0.065               | 0.024 ± 0.005   |
| Msh2-Msh6-S1036P          | 34 ± 1     | 0.426 ± 0.031               | 0.014 ± 0.002   |
| Msh2-Msh6-S1036P + ATP    | 121 ± 6    | 0.274 ± 0.090               | 0.033 ± 0.007   |

or in the presence of ADP or ATP was essentially indistinguishable from that of the wild-type protein. Binding of the Msh2-Msh6-S1036P complex to the three substrates in the absence of nucleotide or in the presence of ADP was similar to that of the wild-type complex. However, addition of ATP had a significantly different effect on binding of the Msh2-Msh6-S1036P complex compared with the wild-type complex. Binding of the Msh2-Msh6-S1036P complex to the GT mispair was not inhibited by ATP in comparison with almost complete inhibition of the wild-type complex. Surprisingly, binding of the Msh2-Msh6-S1036P complex to the +A mispair was almost as inhibited by ATP as binding of the wild-type complex (36% of steady-state binding with ATP versus without ATP for Msh2-Msh6-S1036P compared with 32% for the wild-type complex). The binding properties of the Msh2-Msh6-S1036P complex were verified by determining the various association and dissociation constants for mispair binding (Table II). These results show that, in the absence of nucleotide, the Msh2-Msh6-S1036P complex has a similar binding affinity for DNA without a mispair or with a GT or +A mispair compared with the wild-type complex in the absence of nucleotide. In contrast, a high binding affinity of the Msh2-Msh6-S1036P complex for a GT mispair was observed in the absence of nucleotide or in the presence of ATP, whereas ATP reduced the binding affinity of the Msh2-Msh6-S1036P complex for DNA without a mispair or with +A mispair, similar to the results obtained with the wild-type complex. Binding of the Msh2-Msh6-G1067D complex to the GT mispair was also not inhibited by ADP or ATP, and binding of the Msh2-Msh6-G1067D complex to the +A mispair was not inhibited by ADP and was only partially inhibited by ATP (66% of steady-state binding with ATP versus without ATP for Msh2-Msh6-G1067D compared with 32% for the wild-type complex). The effect of nucleotides on the binding of the Msh2-Msh6-G1142D complex was somewhat different. ADP did not inhibit binding to either the GT or +A mispair, whereas ATP caused a similar partial inhibition of binding to both the GT and +A substrates, but the level of binding seen in the presence of ATP was significantly higher than that seen for the wild-type complex (44% + ATP/−ATP for Msh2-Msh6-G1142D versus 21% without ATP).
for the wild-type complex for the GT substrate and 65% + ATP/−ATP for Msh2-Msh6-G1142D versus 32% for wild-type complex for the +A substrate).

The interaction of the mutant Msh2-Msh6 complexes with mispaired bases was further characterized by measuring the dissociation of the complexes from the different DNA substrates (Fig. 5). As discussed above, ADP and, to a much greater extent, ATP induced the dissociation of the wild-type Msh2-Msh6 complex from the GT and +A substrates. As already pointed out, ATP decreased the half-time of dissociation from the GT and +A substrates from 375 to 4 s and from 107 to 4 s, respectively, compared with no nucleotide in the dissociation buffer. The dissociation behavior of the Msh2-Msh6-H1096A complex was essentially the same as that of the wild-type complex for all three substrates. In the case of the Msh2-Msh6-S1036P complex bound to the GT mispair, dissociation in the presence of no nucleotide or ADP was essentially indistinguishable from that of the wild-type complex, whereas ATP-induced dissociation was much slower, with a half-time of 74 s compared with a half-time of 4 s for the wild-type complex. In the case of the Msh2-Msh6-S1036P complex bound to the +A mispair, the dissociation behavior in the absence of nucleotide or in the presence of ADP or ATP was virtually indistinguishable from that of the wild-type complex. The dissociation of the Msh2-Msh6-G1067D complex from both the GT and +A substrates in the presence of ATP were 140 and 23 s compared with 4 and 4 s for the wild-type complex, respectively. In addition, dissociation of Msh2-Msh6-G1067D from the +A mispair did not go to completion and reached a stable plateau at ~40% of the protein bound compared with >90% dissociation for the wild-type complex. The dissociation behavior of the Msh2-Msh6-G1142D complex resembled that of the Msh2-Msh6-G1067D complex, with some differences. These differences were that the Msh2-Msh6-G1142D complex dissociated more slowly in the presence of ADP than in the absence of nucleotide; and in the presence of ATP, it dissociated more rapidly than the Msh2-Msh6-G1067D complex, but more slowly than the wild-type complex; the half-times of dissociation from the GT and +A substrates in the presence of ATP were 9 and 9 s compared with 4 and 4 s for the wild-type complex, respectively. In addition, a fraction of the Msh2-Msh6-G1142D complex remained stably associated with both the GT (20% bound) and +A (20% bound) mispairs in the presence of ATP. Overall, these data support the conclusion that the Msh2-Msh6-S1036P, Msh2-Msh6-G1067D, and Msh2-Msh6-G1142D complexes form more stable complexes with mispaired bases compared with the wild-type Msh2-Msh6 complex under physiological conditions in which ATP is present.

**DISCUSSION**

In a previous study, we described mutations in MSH6 that behave as if they interfere with both Msh6- and Msh3-dependent MMR (26). It was suggested that these mutations result in mutant Msh2-Msh6 complexes that either bind to mispairs in a way that occludes access by Msh2-Msh3 or sequester other MMR proteins or protein complexes, rendering mismatch-bound Msh2-Msh3 nonfunctional for MMR. In the present study, we have analyzed the ATPase activity, mismatch-binding specificity, and effect of ADP and ATP on binding to and dissociation from mispaired bases for three of the complexes encoded by mutants with the strongest phenotypes observed and for one encoded by a mutant with a weaker phenotype. All of the mutant proteins appeared to bind mispaired bases with normal affinity and to discriminate them from base pairs. In addition, all of the mutant complexes retained intrinsic ATPase activity. However, all of the mutant complexes appeared to have some defect in the modulation of mispair binding and dissociation by ATP and the modulation of ATP hydrolysis by mispaired bases. In contrast to the wild-type complex, the ATPase activity of three of the mutant complexes (Msh2-Msh6-S1036P, Msh2-Msh6-G1067D, and Msh2-Msh6-G1142D) was inhibited by the presence of one or more types of mispaired bases; each of these mutant complexes showed increased binding to one or more types of mispaired bases in the presence of ATP; and the mispair-bound complex was refractory to dissociation upon addition of ATP to an extent that depended on the type of mispaired base. The mispair-binding properties of the Msh2-Msh6-S1036P, Msh2-Msh6-G1067D, and Msh2-Msh6-G1142D complexes are in some ways similar to those of the *S. cerevisiae* and human Msh2-Msh6 complexes containing G987D and K1140R substitutions, respectively, which alter a conserved residue of the P-loop of the Msh6 ATP-binding site required for ATP hydrolysis (66, 68). These two mutant complexes show higher levels of steady-state binding to a mispaired base in the presence of ATP than seen for the wild-type complex, although not as high as seen for the mutant or wild-type complexes in the absence of ATP. It was not determined whether the amino acid substitutions alter the association or dissociation kinetics or both, although in the case of the human mutant complex, the effect was attributed to decreased binding of ATP. However, although these latter two mutations are loss-of-function mutations, in neither case has it been demonstrated that they interfere with Msh2-Msh3 function, in contrast to the four novel msh6 mutations characterized here.

The altered mispair-binding dynamics of the Msh2-Msh6-G1067D and Msh2-Msh6-G1142D complexes for both GT and +A mispairs are consistent with the hypothesis that these two mutant complexes bind to these mispairs with increased stability and occlude the Msh2-Msh3 complex from interacting with insertion/deletion mispairs, resulting in a dominant phenotype (26). The Msh2-Msh6-S1036P complex showed altered binding dynamics with the GT mispair, but not with the +A mispair. Because the Msh2-Msh3 complex can function in the repair of +1-base insertion/deletion mispairs, but not base base mispairs, we hypothesize that the dominant behavior of the Msh2-Msh6-S1036P complex results from formation of an unusually stable complex with base base mispairs that sequesters other MMR components, limiting their ability to interact with Msh2-Msh3 (26). The mispair-binding dynamics of the Msh2-Msh6-H1096A complex were indistinguishable from those of the wild-type complex, although mispair stimulation of its ATPase activity was modestly attenuated. This suggests that some ATP-mediated downstream interaction may be partially defective in the mutant Msh6-H1096A complex.

A number of studies have indicated that binding of a MutS dimer or Msh2-Msh6 to a mispaired base causes a conformational change in the protein complex such that subsequent ATP binding results in a further conformational change, upon which the protein is released from the mispair (11, 20–24, 53, 67–69). Given this view, how then do the Msh2-Msh6-S1036P, Msh2-Msh6-G1067D, and Msh2-Msh6-G1142D complexes form stable complexes with mispaired bases in the presence of ATP? The data presented here indicate that these complexes should bind ATP and hydrolyze it to ADP because they each retain the intrinsic DNA-independent ATPase activity. Then the ADP-bound form binds the mispair (20, 21, 56), forming a complex in which either the ADP-ATP exchange is defective or the ADP-ATP exchange occurs, but the ATP-induced conformational change is insufficient to provoke rapid release from the mispair. Interestingly, at least for the Msh2-Msh6-G1067D and
Msh2-Msh6-G1142D complexes, ADP binding results in increased stability of the protein-mispair complex compared with the wild-type protein complex. This mode of mispair binding and altered mispair-binding dynamics is consistent with the observation that the S1036P, G1147D, and G1142D substitutions change amino acids located in the Msh2-Msh6 dimer interface adjacent to the ATP-binding sites (Fig. 1). Based on studies of related proteins such as Rad50 (61–63), these amino acid substitutions would be predicted to affect conformational changes across the interface induced by ADP-ATP exchange and to alter interactions with mispaired base-induced conformational changes communicated by the transmitter region between the mispair binding and ADP-ATP binding regions (12, 13, 26). The Msh2-Msh6-H1096A complex appears to differ from the other three mutant complexes, as it shows normal mispair binding and dissociation, but fails to show complete mispair activation of the ATPase. Reduced mispair activation of the ATPase is consistent with modeling based on the MutS structure indicating that the Msh6-H1096A substitution alters an amino acid that is likely important for ATP hydrolysis by the Msh2-ATP-binding site. These results suggests that the weak dominant phenotype caused by the Msh6-H1096A substitution likely stems from the reduced activation of the ATPase, which might in turn result in inappropriate interactions between Msh2-Msh6 and other MMR proteins.

The crystal structure of MutS bound to a mispaired base indicates that mispair recognition by MutS involves stacking of a Phe residue onto a ring of the mispaired base, kinking of the DNA facilitated by a Glu residue that contacts the DNA, and other specific amino acid-DNA contacts (12, 13, 25, 32, 34). The same mode of recognition was demonstrated for both base base mispairs and insertion/deletion mispairs. Genetic and biochemical studies have shown that Msh6 is the mispair recognition subunit of Msh2-Msh6 and is the functional equivalent of the Msh2-Msh3 complex. These results suggest that the weak dominant phenotype caused by the Msh6-H1096A substitution likely stems from the reduced activation of the ATPase, which might in turn result in inappropriate interactions between Msh2-Msh6 and other MMR proteins.

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S. cerevisiae msh6 Mutations Cause Increased Mispair Binding
Dominant *Saccharomyces cerevisiae msh6* Mutations Cause Increased Mispair Binding and Decreased Dissociation from Mispairs by Msh2-Msh6 in the Presence of ATP

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