Contribution of Msh2 and Msh6 subunits to the asymmetric ATPase and DNA mismatch binding activities of Saccharomyces cerevisiae Msh2–Msh6 mismatch repair protein

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Contribution of Msh2 and Msh6 subunits to the asymmetric ATPase and DNA mismatch binding activities of *Saccharomyces cerevisiae* Msh2–Msh6 mismatch repair protein

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Abstract

Previous analyses of both *Thermus aquaticus* MutS homodimer and *Saccharomyces cerevisiae* Msh2–Msh6 heterodimer have revealed that the subunits in these protein complexes bind and hydrolyze ATP asymmetrically, emulating their asymmetric DNA binding properties. In the MutS homodimer, one subunit (S1) binds ATP with high affinity and hydrolyzes it rapidly, while the other subunit (S2) binds ATP with lower affinity and hydrolyzes it at an apparently slower rate. Interaction of MutS with mismatched DNA results in suppression of ATP hydrolysis at S1—but which of these subunits, S1 or S2, makes specific contact with the mismatch (e.g., base stacking by a conserved phenylalanine residue) remains unknown.

In order to answer this question and to clarify the links between the DNA binding and ATPase activities of each subunit in the dimer, we made mutations in the ATPase sites of Msh2 and Msh6 and assessed their impact on the activity of the Msh2–Msh6 heterodimer (in Msh2–Msh6, only Msh6 makes base specific contact with the mismatch). The key findings are: (a) Msh6 hydrolyzes ATP rapidly, and thus resembles the S1 subunit of the MutS homodimer, (b) Msh2 hydrolyzes ATP at a slower rate, and thus resembles the S2 subunit of MutS, (c) though itself an apparently weak ATPase, Msh2 has a strong influence on the ATPase activity of Msh6, (d) Msh6 binding to mismatched DNA results in suppression of rapid ATP hydrolysis, revealing a "cis" linkage between its mismatch recognition and ATPase activities, (e) the resultant Msh2–Msh6 complex, with both subunits in the ATP-bound state, exhibits altered interactions with the mismatch.

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1. Introduction

DNA mismatch repair is an important, widely conserved mechanism for maintaining the integrity of genetic information over generations. This repair mechanism corrects base substitution and insertion/deletion mismatches that occur due to errors in DNA replication and recombination, as well as DNA lesions resulting from a variety of internal and external stresses. Repair initiates with MutS protein in prokaryotes, or MutS homologues in eukaryotes (e.g., Msh2–Msh6, Msh2–Msh3), binding the site of the mismatch in duplex DNA. This recognition event triggers excision of the error-containing DNA strand past the site of the mismatch, which is followed by DNA resynthesis and ligation to complete the repair process [1–3].

In addition to their mismatch recognition activity, MutS/Msh proteins also possess an ATPase activity that is essential for DNA repair [4–7]. ATP binding and hydrolysis appear to modulate the interactions between MutS/Msh and DNA as well as other proteins in the repair pathway; thus, understanding how MutS/Msh proteins utilize ATP is necessary for understanding how they function in DNA mismatch repair. Several model mechanisms have been proposed for MutS/Msh action upon mismatch recognition: (a) MutS/Msh proteins translocate on DNA, fuelled by ATP binding and hydrolysis, possibly to interact with other proteins on DNA and coordinate mismatch recognition with downstream events such as initiation of strand excision and DNA resynthesis [8–10]; (b) upon binding ATP MutS/Msh proteins form sliding clamps that diffuse freely on DNA, again, to contact downstream repair proteins and direct repair [11,12]; (c) MutS/Msh proteins utilize ATP binding and hydrolysis to modulate their interaction with DNA, while remaining at the mismatch to direct repair [13–17]. At present, experimental data
are available in support of each of these very different model mechanisms, therefore the investigation into MutS/Msh DNA binding and ATPase activities continues.

Recent studies from several research groups, including our own, have revealed clear differences between the ATP binding and hydrolysis activities of the two subunits in the MutS/Msh dimer [18–21]. For instance, in *Thermus aquaticus* MutS, one subunit binds nucleotide (ATP)S with about 10-fold higher affinity than the other subunit (K_D = 3 μM versus 27 μM). Also, the high-affinity subunit hydrolyzes ATP at 30-fold faster rate than the low-affinity subunit (100 s^-1 versus 0.2–0.3 s^-1 at 40 °C) [18]. These differences are striking especially since MutS is a homodimer; however, they are in accord with known differences in the DNA binding activities of the two MutS subunits (e.g., conserved phenylalanine and glutamate residues from only one subunit undergo base stacking and hydrogen bonding interactions with the mismatch, respectively) [22,23]. In fact it appears that the asymmetry in the ATPase sites is linked to asymmetry in the interactions of the two subunits with DNA [24,25]. Consistent with this hypothesis, binding of mismatched DNA to MutS specifically suppresses the catalytic activity of the high-affinity subunit, such that the rate of ATP hydrolysis is reduced from 10 to 0.3 s^-1 [18]. The exact nature and function of asymmetry in the MutS dimer is not clear yet, but the characteristic appears to be important for DNA mismatch repair as it is conserved among a variety of organisms. For instance, subunits of the *E. coli* MutS homodimer also exhibit differences in their interactions with nucleotides and with mismatched DNA [20,21]. The eukaryotic Msh2–Msh6 heterodimer is no different, as the subunits bind nucleotides with differing affinities [19,26]; only one subunit catalyzes rapid ATP hydrolysis (*Saccharomyces cerevisiae* Msh2–Msh6: 2–3 s^-1 at 20 °C) [19], and only Msh6 contains the conserved phenylalanine residue that can make specific contact with the mismatch in DNA [27,28]. As in the case of *T. aquaticus* MutS, mismatched DNA binding strongly suppresses the activity of the rapid ATP-hydrolyzing subunit in *S. cerevisiae* Msh2–Msh6 (the rate constant decreases from 2–3 to 0.1–0.2 s^-1 at 20 °C) [19]. It is not known yet which of the two subunits, Msh2 or Msh6, catalyzes rapid ATP hydrolysis and, therefore, which one’s activity is altered so dramatically following mismatch recognition by Msh2–Msh6.

Previous studies have probed the ATPase activity of both Msh2 and Msh6 subunits by mutating conserved residues in their active sites for ATP binding and hydrolysis. The results confirmed that the ATPase activities of both Msh2 and Msh6 are required for DNA mismatch repair, and also highlighted differences between the two subunits [6,29,30]. Thus, the effects of mutating the Walker A motif (GxxxGKxGK), which coordinates the phosphate groups of ATP, and Walker B motif (DExx), which coordinates the Mg^2+ ion for catalysis, differed depending on whether Msh2 or Msh6 was changed. Substitution of the conserved Walker A glycine with aspartate, or Walker B glutamate with alanine, in Msh6 reduced the ATPase activity of *S. cerevisiae* Msh2–Msh6 to a greater extent than did identical mutations in Msh2 [6,29]. Similar results were obtained with a Walker A lysine to arginine mutation in human Msh6 versus Msh2 [30]. All these studies indicated that the Msh6 subunit contributes “more” than Msh2 to the overall ATPase activity of Msh2–Msh6. However, since the ATPase experiments were all performed in the steady state regime, i.e., they measured the rate-limiting step following ATP hydrolysis, the exact contribution and role of each subunit’s ATP binding and hydrolysis activity in the Msh2–Msh6 ATPase mechanism, including the identity of the subunit that catalyzes rapid ATP hydrolysis, remains unknown.

Here we report pre-steady state analysis of the ATPase activities of wild type and mixed wild type/Walker A/B mutant heterodimers of Msh2–Msh6, carried out in order to answer questions such as: (a) which subunit catalyzes rapid ATP hydrolysis and which one has the apparently slower activity? (b) does ATP binding and/or ATP hydrolysis by Msh2 influence ATP binding and/or ATP hydrolysis by Msh6, and vice versa? (c) how is Msh2–Msh6 ATPase activity linked to mismatch recognition, given that only Msh6 makes base specific contacts with the mismatch? The answers reveal complex coordination between Msh2 ATPase and Msh6 activities that is likely important for Msh2–Msh6 function in DNA mismatch repair.

2. Materials and methods

2.1. DNA and nucleotides

Synthetic oligodeoxyribonucleotides (37-nucleotide template and G-T complement) were purchased from Integrated DNA Technologies, purified by denaturing polyacrylamide gel electrophoresis, and annealed to prepare a G/T mismatch-containing duplex, as described [19]. PET11a vector was purchased from Novagen and pLANT ZbiPK was a gift from Michael O’Donnell (The Rockefeller University) [31]. Radioactive nucleotides ([α-35S]-ATP, [γ-32P]-ATP, and [32P]-ATP) were purchased from Perkin-Elmer Life Sciences, and non-radioactive nucleotides were purchased from Sigma Chemical Co. DNA was labeled with [35S]-ATP as described previously [19].

2.2. Proteins

Point mutations were introduced in *MSH2* and *MSH6* genes (contained in pET11a or pLANTZbiPK vectors) using overlap-extension PCR or the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing the entire gene. Mixed wild type–mutant Msh2–Msh6 dimers were co-expressed and purified from *E. coli* as described previously for wild type Msh2–Msh6 [19]. Restriction enzymes and T4 polynucleotide kinase were purchased from New England Biolabs.

2.3. Nucleotide and DNA binding assays

ATP-binding to Msh2–Msh6 was measured by nitrocellulose membrane binding assays as described previously [19]. Briefly, the membranes (Schleicher and Schuell) were washed with 0.5N NaOH and equilibrated in binding buffer (50 mM Tris–HCl, pH 8.0, 1 mM MgCl2, 5% glycerol), Msh2–Msh6 (2 μM) was incubated with 200 μM ATP plus 0.3 μCi [35S]-ATP for 15 min at 25 °C (15 μl reactions in binding buffer, 1.10 mM final NaCl concentration). Ten microliters of each reaction was filtered through the membrane and 1 μl was spotted onto a separate membrane to measure total nucleotide in the reaction. The molar amount of nucleotide bound to protein was determined and plotted versus nucleotide concentration. The binding isotherms were fit to equations describing 1:1 or 2:1 binding of ligands to macromolecules [18].

Dissociation of ATP from Msh2–Msh6 was measured by incubating Msh2–Msh6 (2 μM) with 200 μM ATP plus 1 μCi [γ-32P]-ATP in the binding buffer for 30 s at 25 °C (110 mM final NaCl concentration), followed by addition of 5 mM MgCl2–ATP chase and filtration of 10 μl aliquots through the membrane at 30 s intervals (up to 5 min). The molar amount of nucleotide bound to the pro-
tein was determined and plotted versus time of chase. The data describing decay of the protein–nucleotide complex were fit to a single exponential equation.

Interaction between Msh2–Msh6 protein complexes and mismatched DNA was also measured by nitrocellulose membrane filtration assays as described previously [19]. Msh2–Msh6 (1 μM) was incubated with 32 P-labeled DNA (0.1 μM) in the binding buffer (15 μl reaction) with varying NaCl concentration (0–300 mM), in the absence or presence of ATP (5 μM ATP) for 10 min at 4 °C. Ten microliters of each reaction was filtered through the membrane and the molar amount of DNA bound to protein determined and plotted versus NaCl concentration.

2.4. ATPase assays

Steady state ATPase assays were performed with Msh2–Msh6 (1 μM) and 500 μM ATP + 2 μCi [α-32P]ATP in reaction buffer (50 mM Tris–HCl, pH 8.0, 5 mM MgCl2, 4 mM DTT) at 30 °C (30 μl reaction); all ATPase assays were performed at 110 mM final NaCl concentration. Five microliters of the reaction were quenched after varying times with 5 μl of 0.5 M EDTA, and the amount of [α-32P]ADP formed was analyzed by PEI-cellulose TLC (EM Science) with 0.6 M potassium phosphate buffer, pH 3.4. The molar amount of ADP formed was plotted versus time and the data fit to a linear equation. The slope of the line divided by Msh2–Msh6 concentration yielded the $k_{cat}$ for the reaction.

Pre-steady state assays for ATP hydrolysis were performed on a quench-flow instrument (KinTek Corp., Austin, TX) as described previously [19]. Briefly, 16 μl of 4 μM Msh2–Msh6 (±6 μM DNA) was mixed with 16 μl of 1 mM ATP + 2 μCi [α-32P]ATP and quenched with 35 μl of 0.7 M formic acid after varying times (0.08–15 s), followed by TLC and data analysis, as above (final concentrations: 2 μM Msh2–Msh6, 500 μM ATP, and 3 μM DNA). The data were fit to a linear equation or an exponential + linear equation, as appropriate.

3. Results

3.1. Mutations in the conserved Walker A motif, but not Walker B motif, disrupt nucleotide binding to Msh2 and Msh6

In order to quantify the contributions of Msh2 and Msh6 subunits to the ATPase activity of the S. cerevisiae Msh2–Msh6 dimer, we decided to prepare mutant versions of the proteins that were deficient in either ATP binding (and therefore hydrolysis) or only ATP hydrolysis activity. In doing so, we were guided by previous studies indicating that mutation of the Walker A lysine often disrupts ATP binding to proteins [30], while mutation of the Walker B glutamate appears to specifically disrupt ATP hydrolysis [32,33]. Four mixed wild type–mutant heterodimers – Msh2K694A–Msh6WT, Msh2WT–Msh6K988A, Msh2E768A–Msh6WT, and Msh2WT–Msh6E1062A – were over-

![Diagram](image-url)

**Fig. 1.** Msh2, Msh6 Walker A and B motifs: (A) ADP, Mg2+, and SO4 2− bound in the conserved ATP binding and hydrolysis site of Thermus aquaticus MutS. (B) Purified mixed wild type–mutant Msh2–Msh6 heterodimers, containing mutations in Msh2 or Msh6 Walker A (Msh2: K694A, Msh6: K988A) and Walker B (Msh2: E768A, Msh6: E1062A) sites.
expressed and purified from *E. coli* in milligram quantities, to enable accurate measurement of the stoichiometry of nucleotide binding and the kinetics of ATP binding, hydrolysis, and product release (Fig. 1).

Previously, nitrocellulose membrane filtration assays had revealed that two molecules of ATP-S (a non-hydrolyzable ATP analog) bind per Msh2–Msh6 dimer, indicating that the recombinant protein purified from *E. coli* is fully active for nucleotide binding [19] (all protein preparations are free from nucleotide contaminants; Supplemental Fig. S1). Fig. 2A shows nucleotide binding and the kinetics of ATP binding, hydrolysis, and product enable accurate measurement of the stoichiometry of nucleotide binding and steady state ATPase activities: (A) wild type Msh2–Msh6 (kcat = 0.008 s−1) and Msh2WT–Msh6K988A (kcat = 0.008 s−1); although, Msh2WT–Msh6K988A activity is still above the baseline whereas Msh2K694A–Msh6WT activity is at the baseline and appears to be shut down completely.

We see that both Walker A and B mutations in Msh6 almost completely inactivate Msh2–Msh6, suggesting that this subunit is the predominant ATPase in the dimer; it does remain possible that Msh2 is also a robust ATPase, but its optimal activity requires that Msh6 bind and/or hydrolyze ATP. It is also not clear why, if the ATP binding (and therefore hydrolysis) activity of Msh2 is knocked out (Msh2K694A–Msh6WT; Fig. 2A), Msh6 remains catalytically active, albeit to a lesser extent than wild type Msh2–Msh6 (Fig. 2B), but if Msh2 can bind ATP but not hydrolyze it (Msh2E768A–Msh6WT; Fig. 2A), Msh6 activity is affected much more severely (Fig. 2B). In order to understand the ATPase mechanisms of the two subunits, and how they might be linked, in greater detail than possible by steady state analysis, we assayed the mixed dimers under pre-steady state conditions, as described in the next section.

![Fig. 2. Effects of Walker A and B site mutations on Msh2–Msh6 ATP binding and steady state ATPase activity (A) wild type Msh2–Msh6 (●), Msh2K694A–Msh6WT (○), and Msh2WT–Msh6K988A (□), bind two ATP-S molecules per dimer, indicating that the Walker B site mutation does not disrupt nucleotide binding activity. In contrast, Msh2K694A–Msh6WT (□) and Msh2WT–Msh6K988A (○) bind only one ATP-S molecule per dimer, indicating that the Walker A site mutation does disrupt nucleotide binding activity; (B) steady state assays indicate a substantial reduction of Msh2–Msh6 ATPase activity resulting from either mutation in Msh6, curiously, only the Walker B mutation in Msh2 appears to cause such a striking reduction in Msh2–Msh6 ATPase activity.](image)
3.2. Msh6 is responsible for the rapid ATP hydrolysis activity of Msh2–Msh6 heterodimer

Wild type Msh2–Msh6 is known to bind at least one ATP molecule at a fast rate (0.1 \( \mu \text{M}^{-1} \text{s}^{-1} \)), hydrolyze it and release the phosphate product at a fast rate (2 s\(^{-1} \)), and then undergo slow catalytic turnover (0.1–0.2 s\(^{-1} \)) [19], as shown here in Fig. 3A (2 \( \mu \text{M} \) Msh2–Msh6 in the reaction; exponential burst amplitude = 2 \( \mu \text{M} \), burst rate constant = 1.4 s\(^{-1} \), linear rate = 0.36 \( \mu \text{M} \text{s}^{-1} \) or \( k_{\text{cat}} = 0.18 \text{s}^{-1} \)). The mixed wild type–Walker A mutant Msh2 K694A –Msh6 WT, in which the ATP binding activity of Msh2 is knocked out, still catalyzes a burst of ATP hydrolysis at a rate constant of 1.8 s\(^{-1} \), followed by a linear ATPase rate at 0.38 \( \mu \text{M} \text{s}^{-1} \) (Fig. 3B; Msh2 K694A –Msh6 WT ATPase rate can be expressed as \( k_{\text{cat}} = 0.38 \text{ divided by 2 = 0.19 s}^{-1} \)). In contrast, Msh2 WT –Msh6 K988A, in which the ATP binding activity of Msh6 is knocked out, does not display any significant burst activity and the data are best fit by a linear ATPase rate at 0.1 \( \mu \text{M} \text{s}^{-1} \) (Fig. 3C). Together, these data suggest that the Msh6 subunit is responsible for the rapid ATP hydrolysis activity of the Msh2–Msh6 dimer. Data from experiments with mixed wild type–Walker B mutant heterodimers support the above conclusion—Msh2 WT–Msh6 K988A catalyzes a burst of ATP hydrolysis at 2 s\(^{-1} \) (Fig. 3D); however, Msh2 K694A –Msh6 WT hydrolyzes ATP at a much slower linear rate of 0.1 \( \mu \text{M} \text{s}^{-1} \) (Fig. 3E). The residual ATPase rate of both Msh6 mutant-containing dimers may reflect inherently slow Msh2 activity or indicate that ATP binding and/or hydrolysis by Msh6 is necessary for optimal Msh2 activity.

Subtle differences among the four mixed heterodimers reveal some links between the ATPase mechanisms of Msh2 and Msh6. For instance, although Msh6 hydrolyzes ATP at a fast rate in both Msh2 mutant-containing dimers, Msh2 K694A–Msh6 WT can undergo catalytic turnover (Fig. 3B; linear rate = 0.38 \( \mu \text{M} \text{s}^{-1} \)) while Msh2 E768A–Msh6 WT apparently cannot (Fig. 3D; linear rate = 0.02 \( \mu \text{M} \text{s}^{-1} \)). This difference is likely related to the fact that the Msh2 K694A mutant does not bind nucleotide and Msh2 E768A clearly does (Fig. 2A). A filter binding chase assay measuring \( [\text{ADP}] \)–32P dissociation from these proteins shows that Msh2 E768A–Msh6 WT undergoes very stable binding to nucleotide, with no dissociation detectable over several minutes (Fig. 3F; wild type Msh2–Msh6 ATP \( k_{\text{off}} \geq 0.1 \text{s}^{-1} \)). This nucleotide may be unhydrolyzed ATP retained at the Msh2 E768A active site, possibly stabilized by the loss of negative charge repulsion between the wild type glutamate residue and \( \gamma \) phosphate of ATP. It is also possible that the stably bound nucleotide is ADP, produced by ATP hydrolysis at the Msh6 active site. We do not favor this possibility as extraction of the bound nucleotide from Msh2 E768A–Msh6 WT followed by chromatographic anal-
Fig. 4. Msh2–Msh6 binding to mismatched DNA results in suppression of ATP hydrolysis at the Msh6 subunit (— no DNA, — G:T); similar to (A) wild type Msh2–Msh6, the rapid ATP hydrolysis activity of mutant Msh2–wild type Msh6 dimers, (B) Msh2 K694A –Msh6 WT and (D) Msh2 E768A –Msh6 WT, is inhibited by mismatched DNA. The residual activity of wild type Msh2–mutant Msh6 dimers, (C) Msh2 WT –Msh6 K988A and (E) Msh2 WT –Msh6 E1062A, is relatively insensitive to the presence of mismatched DNA.

ysis indicates that it is mostly in ATP form (data not shown). In either case, it appears that the Msh2 active site must be empty (as in Msh2K694A–Msh6WT) for Msh6 to undergo catalytic turnover. It should be noted that for both Msh2K694A–Msh6WT and Msh2E768A–Msh6WT, the amplitude of the burst phase is about half that of wild type Msh2–Msh6 (0.8 μM instead of 2 μM). The reason for this partial loss of ATP hydrolysis activity is not clear, as the proteins display the correct stoichiometry for ATP binding (Fig. 2A). The two subunits in a MutS dimer have composite catalytic sites with one subunit contributing residues to the active site of the other [13,22,25], and it is possible that the linkage is such that perturbation of the Msh2 site can potentially knock out Msh6 activity; so, while some fraction of the mixed wild type–mutant heterodimers can retain optimal Msh6 activity, the rest may well not.

3.3. Contact between Msh6 and a mismatched base pair results in suppression of Msh6-catalyzed ATP hydrolysis

When Msh2–Msh6 is bound to mismatched DNA, its ATPase mechanism is altered such that instead of a step after ATP hydrolysis and phosphate release, a step before or at ATP hydrolysis becomes slow and rate limiting [19]. Thus, in the presence of a G:T mismatch there is no rapid burst of hydrolysis; instead, ATP is apparently hydrolyzed at a linear rate of 0.74 μM s⁻¹ (i.e., kₐₚ = 0.37 s⁻¹, Fig. 4A; an initial lag in the kinetic trace may be related to the length of the DNA substrate, Hingorani et. al., unpublished data). The same, slow ATPase rate in observed when Msh2–Msh6 interacts with G:T mismatch in an ADP-bound form (see Supplemental Fig. S2). The effect of mismatched DNA on Msh2 mutant–Msh6 wild type mixed dimers is similarly striking; the ATP hydrolysis rate constant of Msh2K694A–Msh6WT drops from 1.8 s⁻¹ to 0.12 μM s⁻¹ (Fig. 4B), and that of Msh2E768A–Msh6WT from 2 s⁻¹ to 0.04 μM s⁻¹ (Fig. 4D). These data confirm that mismatched DNA binding suppresses the activity of the subunit responsible for rapid ATP hydrolysis, which in this case is Msh6. In contrast, the residual activity of Msh2 wild type–Msh6 mutant mixed dimers does not change significantly in the presence of mismatched DNA. When bound to a G:T mismatch, Msh2WT–Msh6K988A and Msh2WT–Msh6E1062A hydrolyze ATP at 0.09 μM s⁻¹ (Fig. 4C) and 0.08 μM s⁻¹ (Fig. 4E), respectively, similar to their ATPase rates of 0.1 μM s⁻¹ in the absence of DNA (Fig. 3C and E). If this rate reflects inherently slow ATPase activity of Msh2, this subunit does not appear to be affected by mismatched DNA.

3.4. ATP binding to both Msh2 and Msh6 is necessary to alter the interaction between Msh2–Msh6 and mismatched DNA

MutS/Msh proteins bind mismatched DNA with high affinity and stability, but in the presence of ATP (and ATPγS), the interaction is altered and the protein dissociates from DNA if its ends are left unblocked [9,11,15]. MutS/Msh binding to DNA appears also to be sensitive to the concentration of NaCl in the reaction, as expected for protein–DNA interactions that involve sequence
Fig. 5. Msh2–Msh6 binding to mismatched DNA is altered when both subunits bind ATP (ATP/S): interaction of a G:T mismatch with (A) Msh2–Msh6, (D) Msh2E768A–Msh6WT, (E) Msh2WT–Msh6E1062A dimers (in which both subunits are capable of binding ATP) becomes highly sensitive to NaCl concentration in the presence of ATP/S vs. in the absence of ATP/S. In contrast, there is no significant change in the NaCl sensitivity of G:T binding to (B) Msh2K694A–Msh6WT and (C) Msh2WT–Msh6K988A dimers (in which one subunit is mutated for ATP binding activity), in the presence of ATP/S. (A) A control experiment shows background level interaction between Msh2–Msh6 and matched DNA at all NaCl concentrations. Therefore, in order to assess the impact of ATP site mutations on Msh2–Msh6 interactions with DNA, we analyzed the activity of wild type and mixed wild type–mutant dimers in the absence and in the presence of ATP/S, as a function of NaCl concentration. In Fig. 5A, data from nitrocellulose membrane filtration experiments reveal that at low NaCl concentrations, wild type Msh2–Msh6 binds a G:T mismatch preferentially over fully matched DNA, with or without ATP/S present in the reaction. As NaCl is increased, however, the difference between Msh2–Msh6 and ATP/S-bound Msh2–Msh6 becomes obvious. Thus, at 120 mM NaCl, 100% of the DNA is bound by Msh2–Msh6 in the absence of ATP/S while only about 30% remains bound in the presence of ATP/S. The difference in the $K_{1/2}$ values for the two isotherms provides a measure of the striking change in the interaction of Msh2–Msh6 with DNA upon binding ATP. $K_{1/2} = 200$ mM (–ATP/S) and 85 mM (+ATP/S). Disruption of nucleotide binding by a Walker A site mutation in either subunit impacts the link between the ATPase and mismatch binding activities of Msh2–Msh6. For Msh2264A–Msh6WT, the $K_{1/2}$ values are 200 mM (–ATP/S) and 145 mM (+ATP/S), indicating that the protein–DNA complex remains fairly resistant to NaCl if Msh2 is incapable of binding ATP (Fig. 5B). The effect is even more striking when Msh6 cannot bind ATP, as the binding isotherms for Msh2264A–Msh6E1062A are virtually identical in the absence or presence of ATP/S, with $K_{1/2}$ values of 190 mM (–ATP/S) and 80 mM (+ATP/S) (Fig. 5C). Subtle differences between the data for Msh2 and Msh6 Walker A/B mutants suggest that the Msh6 subunit has a more critical role in the link between the mismatch recognition and ATPase activities of Msh2–Msh6 (e.g., the Walker A site mutation in Msh6 has a greater stabilizing effect on the protein–DNA complex than the same mutation in Msh2). Nonetheless, ATP binding to both Msh2 and Msh6 is necessary to trigger a substantial change in the interaction between the heterodimer and mismatched DNA.

4. Discussion

Two key features of MutS/Msh function in DNA mismatch repair are the asymmetry and coordination between mismatch recognition and ATPase activities of the subunits in these dimeric proteins. The asymmetry is clearly evident from both structural and biochemical analyses of the proteins. Amino acids from each Msh subunit make distinctly different, sequence-independent contacts with mismatched DNA, and only one subunit uses a Phe-X-Glu motif for base-specific stacking and hydrogen bonding interactions with the mismatch [22,23,34]. Both subunits bind ATP, but with differing affinities, and their ATPase kinet-
Fig. 6. Model pathways for Msh2–Msh6 ATPase activity and interaction with mismatched DNA. (A) The ATPase activities of mixed wild type–mutant Msh2–Msh6 heterodimers indicate that the Msh6 subunit catalyzes ATP hydrolysis at a fast rate while Msh2 hydrolyzes ATP at a relatively slow rate. However, Msh6 activity appears to be maximal only when Msh2 can also bind and hydrolyze ATP. Also, catalytic turnover of Msh6 requires that Msh2 be in a nucleotide-free state. (B) ATP binding to Msh6 is necessary but not sufficient to alter Msh2–Msh6 interaction with DNA.
perturbed such that the nucleotide remains tightly bound to it (Msh2K694A–Msh6WT, Fig. 3F and 2A), we do not observe any Msh6 ATPase activity beyond the first turnover (Fig. 3D); i.e., Msh6 appears to be trapped in an inactive state following ATP hydrolysis and phosphate release. Apparently, ATP (or ADP?) binding to either Msh2 (Msh2K694A–Msh6WT) or Msh6 (Msh2WT–Msh6K988A) is disrupted, the protein–DNA complex becomes refractory to the effects of ATP/ATP5 relative to wild type Msh2–Msh6–DNA complex (Fig. 5). The data do suggest that ATP binding to Msh6 has a somewhat greater impact on Msh2–Msh6 interaction with mismatched DNA, and this is reflected in the schematic shown in Fig. 5B.

Several reports in the literature indicate that upon binding ATP, MutS/Msh proteins appear to release the mismatch by sliding away from the site [15,16]. But, ATP binding to MutS/Msh also facilitates formation of ternary complexes containing MutS and MutL proteins and the mismatch [12,15,16]. These two very different outcomes illustrate the dynamic character of MutS–DNA interactions, and highlight the need for continued kinetic analysis to better define the nature and timing of various MutS, MutL, and DNA binding/release events, and coupled ATP binding/hydrolysis events, in order to understand how MutS proteins signal DNA repair following mismatch recognition. Asymmetry within the MutS (and MutL) dimers adds yet one more layer of complexity to the mismatch repair system, but our study, along with others in the recent past, indicate that at least this aspect of the puzzle can be addressed effectively with the help of mixed wild type–mutant heterodimers [1].

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at 10.1016/j.dnarep.2005.08.016.

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