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The effects of nucleotides on MutS-DNA binding kinetics clarify the role of MutS ATPase activity in mismatch repair

Emily Jacobs-Palmer and Manju M. Hingorani*

Wesleyan University, Molecular Biology and Biochemistry Department, Middletown, CT 06459

SUMMARY

MutS protein initiates mismatch repair with recognition of a non-Watson-Crick base pair or base insertion/deletion site in DNA, and its interactions with DNA are modulated by ATPase activity. Here we present a kinetic analysis of these interactions, including the effects of ATP binding and hydrolysis, reported directly from the mismatch site by 2-aminopurine fluorescence. When free of nucleotides, the *T. aquaticus* MutS dimer binds a mismatch rapidly ($k_{\text{ON}} = 3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) and forms a stable complex with a half-life of 10 seconds ($k_{\text{OFF}} = 0.07 \text{ s}^{-1}$). When one or both nucleotide-binding sites on the MutS•mismatch complex are occupied by ATP, the complex remains fairly stable, with a half-life of 5–7 seconds ($k_{\text{OFF}} = 0.1–0.14 \text{ s}^{-1}$), although MutS_{ATP} becomes incapable of (re-)binding the mismatch. When one or both nucleotide-binding sites on the MutS dimer are occupied by ADP, the MutS•mismatch complex forms rapidly ($k_{\text{ON}} = 7.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) and also dissociates rapidly, with a half-life of 0.4 seconds ($k_{\text{OFF}} = 1.7 \text{ s}^{-1}$). Integration of these MutS DNA-binding kinetics with previously described ATPase kinetics reveals that: a) in the absence of a mismatch, MutS in the ADP-bound form engages in highly dynamic interactions with DNA—perhaps probing base pairs for errors; b) in the presence of a mismatch, MutS stabilized in the ATP-bound form releases the mismatch slowly—perhaps allowing for onsite interactions with downstream repair proteins; c) ATP-bound MutS then moves off the mismatch—perhaps as a mobile clamp facilitating repair reactions at distant sites on DNA—until ATP is hydrolyzed (or dissociates) and the protein turns over.

INTRODUCTION

The DNA mismatch repair system functions to reduce mutation rates (to $\sim 1 \times 10^{-9}$ per replication cycle) mainly by correcting errors incorporated into DNA during replication and recombination. The resultant increase in genome stability is a defense against carcinogenesis; defects in mismatch repair predispose humans to hereditary non-polyposis colorectal cancer and are linked to cancers of many other tissues as well ^{1, 2}. Mismatch repair is initiated by MutS (in *E. coli* and other prokaryotes) or Msh (MutS homologs in eukaryotes) protein dimers that recognize mispaired or unpaired bases in the DNA duplex (henceforth collectively referred to as mismatches). Following mismatch recognition, MutS/Msh signal DNA repair, which involves excision of the error-containing strand past the mismatch followed by DNA resynthesis and ligation. MutL protein dimers (Mlh and Pms homologs in eukaryotes) help coordinate MutS/Msh actions with those of downstream repair proteins, including helicase, and exonuclease that help catalyze DNA excision, and DNA replication proteins—polymerase, clamp and clamp loader, and ligase—that help catalyze DNA synthesis ^{3–5}. *E. coli* and related

*Corresponding Author Contact information: Phone: (860) 685-2284, Fax: (860) 685-2141, Email: mHINGORANI@WESLEYAN.EDU

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bacteria also contain MutH endonuclease, which is stimulated by MutS and MutL to nick the daughter DNA strand specifically for initiation of excision; in other prokaryotes and eukaryotes, MutL proteins appear to provide endonuclease activity⁶. Both MutS and MutL proteins utilize ATP binding and hydrolysis to drive their actions in DNA mismatch repair.

Crystal structures of *E. coli* and *T. aquaticus* MutS proteins in complex with different mismatches have provided detailed snapshots of the interactions between MutS and DNA, and information on potential allosteric links between the DNA binding and ATPase sites^{7–11}. Important features of the protein-DNA complex include insertion of a phenylalanine residue (e.g., *T. aquaticus* Phe 39) from one subunit of the dimer into the mismatch site where it stacks against the unpaired/mispaired base (Figure 1A). A nearby glutamate residue (Phe-X-Glu motif), which forms a hydrogen bond with the unpaired/mispaired base, appears to aid MutS selectivity for mismatched DNA^{12, 13}. Over the past few years, many structural and biochemical analyses have shown that the MutS-mismatch interaction triggers dramatic changes in both DNA and protein; most prominently, the DNA is kinked at the mismatch site by about 60° towards the major groove, resulting in disruption of the stacking and pairing interactions of bases next to the mismatch^{7, 8}, and the MutS ATPase mechanism is altered, resulting in severe suppression of ATP hydrolysis^{14, 15}.

Kinetic analysis of *T. aquaticus* MutS, *S. cerevisiae* Msh2-Msh6, and *E. coli* MutS ATPase activities has revealed that in the absence of DNA, and in the presence of matched DNA, these proteins bind and hydrolyze ATP rapidly, with the rate-limiting step in the reaction occurring after phosphate release and likely related to ADP release from the active site^{14–17}. When MutS is bound to a mismatch, ATP still binds rapidly to the protein, but ATP hydrolysis is suppressed (30-fold for *T. aquaticus* MutS and 10-fold for *S. cerevisiae* Msh2-Msh6), and now becomes the rate-limiting step in the reaction^{14, 15}. Such fundamental alterations in the reaction kinetics indicate tight coupling between MutS DNA-binding and ATPase activities and, as with other ATP-fueled proteins, it is likely that the transient formation and decay of nucleotide-bound and -free forms of MutS in the reaction define its actions on DNA during mismatch repair.

Recent studies have also revealed asymmetry in the ATPase activity of the two subunits in the MutS dimer^{14, 15, 18–22}, which correlates with asymmetry in their DNA binding activity; only one subunit provides Phe and Glu residues for base-specific interactions with the mismatch^{7, 8, 23}. Only one subunit (S1 in MutS, Msh6 in Msh2-Msh6) binds ATP with high affinity and hydrolyzes it rapidly while the other subunit (S2 in MutS, Msh2 in Msh2-Msh6) binds ATP weakly and appears to hydrolyze it slowly, when MutS is alone or with matched DNA. Mismatched DNA inhibits rapid ATP hydrolysis by S1/Msh6 but not the slow ATPase activity of S2/Msh2. It is clear that the two subunits' ATPase activities are linked, but exactly how ATP binding, hydrolysis, and product release are coordinated between the two is still under investigation^{18, 20}. The subunit asymmetry potentially increases the complexity of coupling between the DNA binding and ATPase activities of MutS, as there are now up to nine possible nucleotide-bound and -free forms of the dimer whose formation and decay could influence its actions on DNA during mismatch repair.

We continue to address this complex problem, and present here the kinetics of MutS-DNA interactions measured with 2-aminopurine positioned adjacent to an extra base in duplex DNA as a fluorescent reporter for MutS binding to mismatched DNA. Direct measurements of MutS binding and releasing the mismatch under various conditions reveal striking ATP and ADP-dependent changes in the interactions and, importantly, facilitate convergence of multiple model pathways currently under debate to describe how MutS uses ATP for DNA mismatch repair.

RESULTS

The series of experiments described here measure the kinetics of *T. aquaticus* MutS interactions with mismatched DNA, using an in-solution assay that directly reports interaction of MutS Phe 39 with an unpaired base in the duplex. Stopped-flow kinetic experiments performed with 2-aminopurine (2-AP)-labeled DNA and corresponding equilibrium anisotropy experiments with 5-(6)-carboxytetramethylrhodamine (TAMRA)-labeled DNA reveal novel and intriguing effects of nucleotides on MutS-DNA interactions, and clarify how this protein might use its ATPase activity to find and bind mismatches and to signal DNA repair.

2-aminopurine reports MutS binding to a mismatch in DNA

2-AP is an isomer of adenine that forms a base pair with thymine and emits fluorescence when excited between 310–320 nm. It has been used extensively as a probe for nucleic acid structure and protein-nucleic acid interactions because of its functional equivalence with adenine²⁴, fluorescence sensitivity to base stacking²⁵, and high signal-to-noise ratio due to minimal absorbance of radiation by proteins and DNA at 315 nm. We incorporated 2-AP into 23-mer duplex DNAs next to a thymine that is either paired with adenine (2-AP:AT) or is a +1 base insertion (2-AP:+T), and assayed the interactions of these duplexes with *T. aquaticus* MutS; all experiments were performed at 40 °C, the lowest temperature at which the protein exhibits stoichiometric ATPase activity¹⁵, and at 50 mM NaCl concentration. According to the *T. aquaticus* MutS•+T DNA structure, interaction of Phe 39 with the +T site is accompanied by a sharp bend in the DNA⁷, which should disrupt base-stacking interactions of the adjacent 2-AP base and perhaps make it more solvent accessible, and thereby relieve quenching of its fluorescence within the duplex (Figure 1A). Indeed, emission spectra of the DNAs reveal an approximately 4-fold increase in 2-AP:+T DNA fluorescence in the presence of MutS (Figure 1B); an insignificant change occurs in 2-AP:AT DNA fluorescence in the presence of MutS, consistent with the known MutS selectivity for mismatched *versus* matched DNA⁴. Titration of 2-AP:+T DNA with increasing concentrations of MutS yields a binding isotherm with equilibrium constant $K_D = 15 \pm 2.5$ nM for the interaction (Figure 1C). There is no detectable change in fluorescence for 2-AP:AT DNA even at MutS concentrations as high as 1 M (Figure 1C, data not shown). Consistent with our hypothesis that the increase in 2-AP:+T fluorescence is related to interaction of Phe 39 with the mismatch site, titration of the DNA with a mutant protein in which Phe 39 is replaced by Ala (MutS F39A)²⁶ does not change its fluorescence intensity (Figure 1C).

While the crystal structures of MutS•mismatch complexes show DNA in a bent conformation^{7, 8}, other studies indicate that the DNA can adopt less bent/unbent conformations as well^{27, 28}, and the “repair-active” form of the complex is still ambiguous^{3, 4}. In order to determine whether the change in 2-AP fluorescence reports the initial MutS DNA-binding step (and perhaps a related change in DNA conformation) or reports only a particular change in DNA conformation (achieved after an initial MutS DNA-binding step), we performed complementary experiments that measure the interaction more directly by changes in anisotropy of TAMRA-labeled DNA²⁸. DNAs of the same sequence as the 2-AP duplexes, except with adenine instead of 2-AP and with one strand 3' end-labeled with TAMRA, were titrated with increasing concentrations of MutS under the same conditions as above. Figure 1D shows the binding isotherm for TAMRA:+T, which yields $K_D = 25 \pm 1.7$ nM, similar to that obtained with 2-AP:+T DNA (Figure 1C, $K_D = 15$ nM). Moreover, no change in anisotropy could be detected for TAMRA:AT matched DNA nor for TAMRA:+T DNA with MutS F39A (Figure 1D). These data confirm that 2-AP fluorescence reports selective MutS binding to mismatched DNA, and that the signal depends on Phe 39 interaction with the mismatch site.

In order to confirm that 2-AP:+T serves as a suitable mismatched DNA substrate for MutS, the 2-AP-labeled DNAs were tested for their ability to modulate MutS ATPase activity. Pre-

steady state kinetic data for ATP hydrolysis and phosphate release show that MutS (1 M dimer), alone or with 2-AP:AT DNA, hydrolyzes one ATP molecule per dimer rapidly at $9.2 \pm 0.8 \text{ s}^{-1}$, followed by slow turnover at 0.2 s^{-1} (Figure 1E). In contrast, when MutS is bound to 2-AP:+T DNA, ATP hydrolysis is suppressed by 30-fold to 0.3 s^{-1} . These data are almost identical to those reported previously with unlabeled +T and matched DNAs, and confirm the coupling between MutS mismatch recognition and ATPase activities¹⁵.

Several research groups have reported that the DNA-dependent steady state ATPase rate of MutS proteins varies with salt concentration, with maximal activity observed at 100 – 150 mM NaCl or KCl depending on the protein^{14, 17, 29, 30}. The basis for this salt effect on the ATPase mechanism is not resolved yet, although it may be linked to weaker interactions between MutS and DNA at higher salt, as reported earlier²⁹. We find that the K_D for MutS-mismatch binding is 250 nM at 150 mM NaCl, or about 10-fold weaker than at 50 mM NaCl ($K_D = 15 - 25 \text{ nM}$, Figure 1C, 1D), but MutS is highly selective for mismatched DNA *versus* matched DNA at both salt concentrations. Therefore, all equilibrium and kinetic experiments were performed at 50 mM NaCl concentration, under optimal conditions for high affinity MutS-mismatch interaction, and key experiments were repeated at 150 mM NaCl to mimic conditions in previous reports (150 mM NaCl data are shown in Supplementary Figure 1). As noted earlier, the K_D for MutS-mismatch interaction is weaker at high salt concentration, mainly because of a decrease in the apparent rate of association. All the other rates differ only slightly, and more importantly, the effects of nucleotides on MutS-DNA interactions (discussed below) are identical at both low and high salt concentrations.

MutS binds a mismatch rapidly and selectively to form a stable complex

MutS-induced changes in 2-AP:+T fluorescence were measured next in a stopped-flow instrument to determine the kinetics of the interaction. Consistent with the equilibrium data shown above, mixing 2-AP:+T DNA with MutS resulted in a rapid increase in 2-AP fluorescence over time (Figure 2A, $k_{\text{observed}} = 0.53 \pm 0.005 \text{ s}^{-1}$ at 200 nM MutS from a single exponential fit). No change in fluorescence was detectable when either 2-AP:AT DNA was mixed with MutS or 2-AP:+T DNA was mixed with mutant MutS F39A (Figure 2A). Titration of 2-AP:+T DNA with increasing concentrations of MutS yielded a linear increase in the observed binding rate, and an apparent bimolecular association rate constant $k_{\text{ON}} = 3 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for the interaction (Figure 2B). Next, the rate of dissociation of MutS from the mismatch was measured by mixing MutS and 2-AP:+T DNA with 133-fold excess unlabeled +T DNA (containing adenine instead of 2-AP) as a trap for any free MutS (Figure 2C). As the MutS•2-AP:+T complex dissociates over time, the decrease in fluorescence signal fit to a single exponential yields a dissociation rate constant $k_{\text{OFF}} = 0.07 \pm 0.0003 \text{ s}^{-1}$ ($t_{1/2} = 10$ seconds). The ratio of k_{OFF} and k_{ON} yields $K_D = 23 \text{ nM}$ for the MutS-mismatch interaction, very close to that measured in equilibrium conditions for both 2-AP and TAMRA-labeled DNAs (Figures 1C, 1D, $K_D = 15 - 25 \text{ nM}$). The relatively fast on-rate and slow off-rate indicate that MutS binds mismatched DNA rapidly and with high affinity to form a stable binary complex in the absence of nucleotides.

ATP binding to MutS does not trigger its rapid release from a mismatch, but does disrupt its ability to bind mismatches

ATP has a marked effect on MutS interactions with mismatched DNA, as revealed by experiments performed with wildtype MutS in the presence of the non-hydrolysable ATP analog, ATP γ S, and with an ATP hydrolysis-deficient Walker A site mutant MutS E663A in the presence of ATP (characterization of the MutS E663A mutant, shown in Supplementary Figure 2, reveals that the mutant protein binds mismatched DNA selectively with $K_D = 19.6 \pm 3.6 \text{ nM}$ similar to wildtype MutS, but catalyzes ATP hydrolysis at a rate of 0.02 s^{-1} *versus* 9.2 s^{-1} for wildtype MutS). Figure 3A shows the association kinetics of MutS mixed with 2-

AP:+T DNA in the presence of ATP γ S. As ATP γ S concentrations increase, the interaction between MutS and DNA appears to be blocked completely; Figure 3B shows similar data for MutS E663A mixed with 2-AP:+T DNA and ATP. A plot of the decrease in MutS•2-AP:+T signal amplitude *versus* nucleotide concentration indicates complete loss of the complex at saturating ATP γ S/ATP, and the data fit to a hyperbola yield $K_{1/2} = 1.7 \pm 0.5$ M for ATP γ S and $K_{1/2} = 0.8 \pm 0.1$ M for ATP effects on MutS and MutS E663A, respectively (Figure 3C). A complementary anisotropy experiment measuring the effect of ATP γ S on MutS-TAMRA:+T interaction confirms the loss of binding, and yields a similar $K_{1/2} = 0.7 \pm 0.1$ M for the ATP γ S effect (Figure 3D). These data confirm that upon binding ATP with high affinity, MutS attains a conformation in which it is unable to form a stable complex with (short linear) mismatched DNA.

The impact of ATP on dissociation of a MutS•+T DNA complex was measured by mixing MutS or MutS E663A bound to 2-AP:+T with saturating concentrations of ATP γ S or ATP, respectively, and 133-fold excess unlabeled +T DNA trap. The decrease in fluorescence signal fit to a single exponential revealed that on binding ATP γ S/ATP, MutS and MutS E663A release the mismatch at a fairly slow rate, $k_{\text{OFF}} = 0.14 \pm 0.001$ s $^{-1}$ ($t_{1/2} = 5$ seconds) and 0.1 ± 0.001 s $^{-1}$ ($t_{1/2} = 7$ seconds), respectively, similar to that in the absence of nucleotides (Figure 2C, $k_{\text{OFF}} = 0.07$ s $^{-1}$); note: we have reported previously that ATP binds rapidly to MutS under these conditions¹⁵. Thus, ATP binding to MutS already in complex with a mismatch does not trigger rapid dissociation of the complex, but once that occurs, ATP-bound MutS cannot rebind the mismatch.

It should be noted that previous reports of rapid MutS dissociation from the mismatch following addition of ATP/ATP γ S to the reaction were based on experiments performed under steady state or equilibrium conditions^{31–34}, which are unlikely to yield accurate rates of association and dissociation for ligand-macromolecule interactions. In this case it is likely that in the absence of nucleotides, MutS can release and rebind the mismatch during the experiment and thus the MutS•mismatch complex appears “stable” over time, whereas in the presence of ATP, MutS is unable to rebind the mismatch and thus the MutS•mismatch complex appears “unstable” over time.

ADP binding to MutS does trigger its rapid release from a mismatch, but the protein retains its ability to bind mismatches

ADP also has a dramatic effect on MutS interactions with mismatched DNA. Fig. 4A shows the association kinetics of MutS mixed with 2-AP:+T DNA in the presence of ADP. With increasing ADP concentrations, both the amplitude and the rate of the single exponential describing the kinetics change, indicating that on binding ADP, MutS attains a new equilibrium for its interactions with mismatch DNA. A plot of the amplitude *versus* ADP concentration shows nearly 50 % loss in MutS•+T DNA complex at saturating ADP, and the data fit to a hyperbola yield $K_{1/2} = 2.4 \pm 0.6$ M for the ADP effect on MutS (Figure 4B). Complementary equilibrium anisotropy measurements of MutS•TAMRA:+T complex titrated with ADP also show approximately 40 % loss of complex, and yield $K_{1/2} = 1.3 \pm 0.4$ M for the ADP effect (Figure 4C). These data confirm that upon binding ADP with high affinity, MutS suffers a loss in affinity for mismatched DNA.

Next, Figure 4D shows the dissociation kinetics of MutS•2-AP:+T complex when mixed with saturating ADP concentration and 133-fold excess trap DNA. The data fit to a single exponential show that ADP binding triggers rapid release of MutS from the mismatch at $k_{\text{OFF}} = 1.74 \pm 0.03$ s $^{-1}$ ($t_{1/2} = 0.4$ seconds), which is 25-fold faster than in the absence of nucleotides (Figure 2C, $t_{1/2} = 10$ seconds). These data explain the change in the MutS DNA-binding equilibrium in the presence of ADP; a plot of association rates *versus* ADP concentrations yields maximum $k_{\text{observed}} = 3.2$ s $^{-1}$ (Figure 4A, data not shown) and, given

$k_{\text{OFF}} = 1.74 \text{ s}^{-1}$, the apparent k_{ON} is calculated as $7.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ ($k_{\text{observed}} = k_{\text{ON}}[\text{MutS}] + k_{\text{OFF}}$). This association rate constant is only slightly faster than that measured in the absence of nucleotides (Figure 2B, $k_{\text{ON}} = 3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$), but since k_{OFF} is much faster (1.74 s^{-1} versus 0.07 s^{-1}), the fraction of MutS•2-AP:+T DNA complex in the reaction is lower when ADP is present in the reaction. The ratio of k_{OFF} and k_{ON} yields $K_D = 0.227 \text{ M}$ for MutS-DNA interaction, consistent with $K_D = 0.232 \pm 0.06 \text{ M}$ obtained by titration of 2-AP:+T DNA with increasing amounts of MutS in the presence of 150 M ADP (data not shown), and indicates approximately 10-fold reduction in the affinity of ADP-bound MutS for DNA compared with MutS alone (Figures 1C, 1D, $K_D = 15 - 25 \text{ nM}$). Thus, upon binding ADP with high affinity, MutS attains a conformation in which it can both rapidly bind and rapidly release DNA—a much more dynamic interaction than observed for MutS in the absence of nucleotides or in the presence of ATP.

DISCUSSION

Mismatched base pair or base insertion/deletion-containing DNA is less thermodynamically stable than matched DNA and exhibits increased conformational flexibility at the mismatch site, which is proposed to facilitate its recognition by MutS for DNA repair³⁵; for example, poor stacking interactions between a mismatched or unpaired nucleotide and its neighbors can favor insertion and stacking of the MutS Phenylalanine residue at the site³⁶. We have shown previously that in the absence of DNA and in the presence of matched DNA, both *T. aquaticus* MutS and *S. cerevisiae* Msh2-Msh6 bind ATP, hydrolyze it, and release phosphate rapidly, and thus can exist predominantly in an ADP-bound conformation (MutS_{ADP})^{14, 15}; the reaction occurs at one of the two ATPase sites on the dimer, and this asymmetry will be discussed further below. Our current data reveal that *T. aquaticus* MutS_{ADP} can rapidly bind and insert Phe 39 into mismatched DNA (Figure 4A, $k_{\text{ON}} = 7.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$), but MutS_{ADP} also dissociates rapidly from DNA, making the interaction highly unstable (Figure 4D, $t_{1/2} = 0.4$ seconds). We have not been able to detect MutS binding to matched DNA, but low-affinity interaction between the two has been reported previously^{28, 31, 32}; thus, it is possible that MutS_{ADP} rapidly binds and releases both matched and mismatched DNA, and that its brief contact with the short matched duplexes in our assays is not favorable for detection. We propose that in the ADP-bound conformation MutS examines base pairs by repeated, transient contacts via the Phe residue—in a sense probing the DNA—in order to detect mismatch sites.

Another critical question is: “How does MutS_{ADP} signal repair once it finds a mismatch?” The following information appears key to answering this question: a) the Fishel research group has reported that MutS_{ADP} (hMsh2-hMsh6) binding to a mismatch triggers rapid ADP dissociation¹⁷; b) we have reported previously that in the presence of a mismatch *T. aquaticus* MutS binds ATP rapidly ($k_{\text{ON}} = 0.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) and hydrolyzes it slowly ($k = 0.3 \text{ s}^{-1}$ versus 10 s^{-1} when alone or in the presence of matched DNA)¹⁵; c) we show here that in this ATP-bound conformation (MutS_{ATP}) the protein remains stably in complex with the mismatch (Figure 3E, $t_{1/2} = 5-7$ seconds, in the absence of ATP hydrolysis). We therefore hypothesized that an initial unstable interaction between ADP-bound MutS and the mismatch could be stabilized in the presence of ATP. Figure 5A shows that when MutS is pre-incubated with 2-AP:+T DNA and 8 M ADP—enough for all MutS to bind at least one ADP¹⁵ and approximately 50 % protein•mismatch complex present at equilibrium (Figures 4B and 4C)—and then mixed with 150 M ADP and 133-fold excess +T DNA trap, the remaining MutS_{ADP}•2-AP:+T complex dissociates rapidly, at $k_{\text{OFF}} = 1.56 \pm 0.07 \text{ s}^{-1}$ ($t_{1/2} = 0.45$ seconds), as expected from the results in Figure 4D ($k_{\text{OFF}} = 1.74 \text{ s}^{-1}$ in the presence of ADP). However, when the same reaction is performed with 150 M ATPγS instead of 150 M ADP, the rate of dissociation of the complex slows dramatically to $k_{\text{OFF}} = 0.17 \pm 0.003 \text{ s}^{-1}$ ($t_{1/2} = 4$ seconds), as expected for MutS in the ATP-bound conformation (Figure 3E, $t_{1/2} = 5-7$ seconds). Thus, it appears that when

MutS_{ADP} encounters a mismatch, ATP can quickly replace ADP in the active site, and this ADP-ATP exchange stabilizes MutS_{ATP} on the mismatch.

The role of ATP in driving MutS actions during DNA mismatch repair has been controversial; most data indicate loss of MutS binding to the mismatch in the presence of ATP/ATP_γS, with particularly compelling data suggesting that MutS_{ATP} moves away from the mismatch in the form of a mobile clamp on DNA^{32, 33, 37}. Other data indicate that in the presence of ATP/ATP_γS, MutS binds MutL to form a ternary protein•DNA complex at the mismatch^{31, 38, 39}. Thus, one model proposes that ATP binding triggers rapid movement of MutS away from the mismatch for interaction with downstream repair proteins such as MutL, while another proposes that MutS stays at the mismatch for interaction with downstream repair proteins. Our kinetic analysis indicates that when ATP binds to MutS_{FREE}•+T complex or replaces ADP in MutS_{ADP}•+T complex, it does not trigger rapid release of MutS from the mismatch; indeed, MutS_{ATP}•+T ($t_{1/2}$ = 5–7 seconds) has almost the same lifetime as MutS_{FREE}•+T ($t_{1/2}$ = 10 seconds). We have measured the lifetime of the MutS_{ATP}•+T complex in the absence of ATP hydrolysis—with ATP_γS or an ATPase mutant of MutS—however, under normal reaction conditions MutS_{ATP}•+T catalyzes ATP hydrolysis at 0.3 s^{-1} , thus the ATP-bound state of MutS could have a $t_{1/2}$ closer to 3 seconds (note: it is not clear yet how the two MutS subunits contribute to this 0.3 s^{-1} ATPase rate, therefore the mismatch-binding, high affinity nucleotide-binding subunit may remain in an ATP-bound state even longer; see discussion of MutS subunit asymmetry below). Thus, there appears to be time enough for MutL to bind MutS_{ATP}•+T and form a stable complex at the mismatch site. On the other hand, unlike the MutS_{FREE} or MutS_{ADP} species, MutS_{ATP} soon attains a conformation in which it is unable to interact with the mismatch (pre-incubation of MutS with ATP blocks its binding to DNA; Figures 3A and 3B)^{32, 37}. These data are consistent with MutS_{ATP} forming a closed clamp-like structure that moves away from the mismatch and cannot access it again until ATP is hydrolyzed or dissociates from the protein. The function of such a mobile MutS clamp may be to deliver proteins (e.g., MutL) and/or activate proteins at distant sites for DNA repair. We have also shown that when MutS is not in contact with a mismatch it hydrolyzes ATP rapidly^{14–16}, which would help recycle the protein to an ADP-bound conformation that can again search for mismatches. Thus our study provides quantitative data to bolster key steps in multiple model pathways of DNA mismatch repair currently under discussion, and facilitates their convergence^{3, 4}. The next step is to measure the kinetics of MutS interactions with nucleotides, as well as MutS ATPase activity in the presence of MutL in order to determine how this protein contributes to the mechanism of initiation of DNA mismatch repair.

The question of asymmetry within the MutS dimer is also important when considering how the protein uses its ATPase activity to recognize mismatches and initiate repair. Several research groups, including ours, have shown that there is asymmetry within the MutS dimer for both ATPase and mismatch binding activities^{7, 8, 15, 18–20, 23, 40}. One MutS subunit, S1 (Msh6 in Msh2-Msh6) is known to bind ATP with high affinity and hydrolyze it rapidly in the absence of DNA; the same subunit inserts Phe into the mismatch and is stabilized in the ATP-bound state¹⁸. In our current study, the apparent equilibrium constants for ATP/ATP_γS and ADP effects on MutS-DNA interactions are close to 1–2 M (Figures 3C, 3D, 4B and 4C), similar to K_D values determined previously for the high-affinity nucleotide binding site on the dimer and 5–10 fold tighter than for the other site^{14, 15, 18}. Together these data suggest that ADP binding at the high-affinity site promotes dynamic interactions of MutS with DNA and, when MutS encounters a mismatch, exchange of ADP for ATP first stabilizes MutS on DNA and then promotes its movement away from the mismatch. The role of ATP binding and hydrolysis at the other subunit, S2 (Msh2 in Msh2-Msh6) is not entirely clear yet, although there is ample evidence for coordination between the activities of the two subunits^{18, 20}. For example, ATP binding at S1 influences ADP binding at S2²⁰, which in turn facilitates formation of the S1_{ATP}-S2_{ATP} state when MutS binds a mismatch^{15, 20}. At the mismatch,

while the rapid ATPase activity of S1 is suppressed, the slow ATPase activity of S2 appears unchanged, which is important for MutS turnover^{15, 18}. Ongoing measurements of the affinities, rates, and order of nucleotide binding, hydrolysis, and dissociation at each MutS subunit, as well as experiments with mixed mutant/wild type Msh2-Msh6 heterodimers, will further clarify the role each MutS subunit ATPase in DNA mismatch repair.

To summarize, we now have a fairly detailed view of MutS actions in signaling mismatch repair—depicted in Figure 5B with emphasis on the well-defined ATPase activity of the high-affinity nucleotide binding site. We have shown that in the absence of a mismatch (a), MutS_{FREE} binds ATP (b), hydrolyzes it, and releases Pi rapidly to form MutS_{ADP} (c), which recycles to form MutS_{FREE} again. When MutS_{FREE} encounters DNA containing a mismatch, it binds the mismatch site rapidly and with high affinity (d). MutS_{FREE}•mismatch also binds ATP rapidly, but ATP hydrolysis is suppressed and the stable MutS_{ATP}•mismatch complex (e) becomes predominant. When MutS_{ADP}—which is a predominant species in the absence of a mismatch (c)—encounters DNA containing a mismatch, it also binds the mismatch site rapidly (f) but the complex is very unstable; the nature of this interaction leads us to propose that in the ADP-bound conformation MutS makes transient contacts with base pairs to probe for mismatch sites in DNA. Notably, in the MutS_{ADP}•mismatch complex (f), ADP can be replaced readily by ATP and MutS_{ATP}•mismatch (e) is again predominant. That both pathways, starting from MutS_{FREE} or from MutS_{ADP}, favor formation of the MutS_{ATP}•mismatch complex (e) suggests a central role for this intermediate in the repair reaction. In time, MutS_{ATP} releases the mismatch (b, g), apparently in a closed conformation that precludes rebinding until ATP is hydrolyzed or dissociates from the protein. In this conformation, MutS could move on DNA as a sliding clamp, as proposed previously, and deliver proteins or activate proteins at sites distant from the mismatch, and thereby couple mismatch recognition to excision of the error-containing strand.

Our findings also illustrate how MutS ATPase activity enhances the specificity of mismatch repair, as stabilization of MutS_{ATP} on DNA, which appears to be a key step in the reaction, occurs only at the site of a mismatch. Future kinetic experiments will address the fate of MutS_{ATP} in the context of downstream components of the DNA repair reaction.

MATERIALS AND METHODS

DNA, proteins, and other reagents

DNAs were purchased from Integrated DNA Technologies Inc. with no modifications, with 2-aminopurine (2-AP) incorporated 3' to the +T insertion and corresponding A:T site, or with a 3'-amino linker for labeling with 5-(6)-carboxytetramethylrhodamine (TAMRA; Invitrogen). The sequences were: Ap+T: 5'-GCGCGACGGTATApTAGCTGCCGG-3' (T denotes +T insertion and Ap denotes 2-AP); Ap+Tcomplement: 5'-CCGGCAGCTATTACCGTCGCGC-3'; ApATcomp: 5'-CCGGCAGCTATATACCGTCGCGC-3'; in corresponding unlabeled DNAs and TAMRA-labeled +T DNA (+T-3'NH₂), 2-aminopurine was replaced by adenine. All DNAs were purified by denaturing PAGE on 20 % acrylamide/6 M Urea gels, followed by electro-elution and ethanol precipitation. +T-3'NH₂ was labeled with TAMRA as described previously, with some modifications⁴¹. Briefly, in the dark, 800 g TAMRA was dissolved in dimethylsulfoxide and reacted with 400 g DNA in 0.1 M sodium tetraborate, pH 8.5 buffer (final volume 400 l), for 16 hours at 25 °C. Excess dye was extracted with water-saturated butanol, followed by gel-filtration (5 ml P6 resin column; BioRad) in 10mM Tris-HCl, pH 8.0, 0.1mMEDTA, separation of labeled from unlabeled DNA by denaturing PAGE, elution by diffusion into 10 mM Tris-HCl, pH 8.0, and ethanol precipitation. DNA concentrations were determined by absorbance at 260 nm (TAMRA contribution to +T-3'NH₂ absorbance at 260 nm was <10%). Duplex DNA stocks were prepared by annealing complementary single strands (1:1.15 ratio

labeled:unlabeled) with heating to 95 °C for 2 min and slow cooling to 25 °C in 20 mM Tris-HCl, pH 7.0, 100 mM NaCl. Duplex DNAs were analyzed by non-denaturing PAGE to confirm >95 % annealed product. *T. aquaticus* wildtype MutS, F39A and E663A mutants were purified from *E. coli* as described previously¹⁵. Phosphate binding protein (PBP) was purified and labeled with N-[2(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) as described previously⁴². ATP, ATP γ S, ADP, Purine Nucleoside Phosphorylase (PNPase) and Phosphate standard solution were purchased from Sigma Chemicals Co. 7-Methylguanosine (7-MEG) was purchased from R. I. Chemical, Inc.

MutS-DNA interactions at equilibrium

MutS interactions with 2-AP-labeled DNAs were measured on a FluoroMax-3 fluorometer (Jobin-Yvon Horiba Group; Edison, NJ). Emission scans of 0.1 M 2-AP:+T and 2-AP:AT duplexes were collected in 3 ml quartz cuvettes in 50 mM NaCl, 20 mM Hepes-NaOH, pH 7.7, 5 mM MgCl₂ (DNA binding buffer) at 40 °C, with and without MutS dimer (0.3 M), after 30 sec mixing and λ_{EX} = 315 nm. Background fluorescence from the buffer and MutS was subtracted from the raw data. Titrations of 0.01 M DNAs with 0 – 0.3 M MutS were performed under similar conditions, in the absence or presence of 150 M ATP γ S, ATP (with MutS E663A), or ADP, with emission recorded at 375 nm. The data were corrected for intrinsic MutS fluorescence by subtracting data from parallel experiments with unlabeled DNA. Fluorescence intensity was plotted *versus* MutS concentration and the apparent dissociation constant (K_D) for the interaction obtained by fitting the data to a quadratic equation:

$$[D \cdot M] = F_0 + [0.5(F_{\max} - F_0)\{(K_D + [D_t] + [M_t]) - [(K_D + [D_t] + [M_t])^2 - 4[D_t][M_t]]^{1/2}\}]$$

where $D \cdot M$ is the fraction of MutS•DNA, F_0 is 2-AP:+T fluorescence in the absence of protein and $F_{\max}$ is maximal fluorescence, and D_t and M_t are total molar concentrations of DNA and MutS, respectively. The data were fit by non-linear regression using KaleidaGraph (Synergy Software).

Anisotropy measurements of MutS-DNA interaction were performed under the same conditions as above by titrating 0.01 M TAMRA:+T and TAMRA:AT duplexes with 0 – 0.3 M MutS, using vertically polarized light at λ_{EX} = 555 nm, and calculating anisotropy from the emitted vertical (I_{VV}) and horizontal (I_{VH}) polarized fluorescence intensities at λ_{EM} = 582 nm ($I_{VV} - GI_{VH}/I_{VV} + 2GI_{VH}$; G is the calculated grating correction factor). The binding isotherms obtained from plots of observed anisotropy *versus* MutS concentration were fit to a quadratic equation as described above.

The effects of nucleotides on MutS binding to DNA were measured by 2-AP fluorescence and TAMRA-labeled DNA anisotropy under the same conditions as above, except at 23 °C and by titrating 0.01 M DNA and 0.2 M MutS with 0 – 150 M ATP γ S, ATP (with MutS E663A), or ADP. The data were plotted *versus* nucleotide concentration and fit to a hyperbola to determine the apparent binding constant, $K_{1/2}$.

Kinetics of MutS-DNA interactions

MutS interactions with DNA were measured on a KinTek SF-2001 stopped-flow (KinTek Corp.; Austin, TX). Experiments were performed at 40 °C in DNA binding buffer by mixing 40 l of 0.4 M wildtype MutS, F39A and E663A mutants (or 0 – 1 M for a titration experiment) rapidly with 40 l of 0.06 M 2-AP:+T or 2-AP:AT DNAs, exciting 2-AP at 315 nm and measuring fluorescence emission over time at >350 nm using a 350 nm long-pass cut-off filter. Five or more kinetic traces of 1000 data points each were averaged, and intrinsic MutS fluorescence from parallel experiments without DNA was subtracted from the raw data. The change in fluorescence intensity was normalized to the initial value and fit to a single

exponential to determine the apparent binding rate (k_{observed}). Experiments with nucleotides were performed similarly except 0 – 100 M ATP γ S, ATP (with MutS E663A), or ADP were added to both MutS and DNA.

MutS dissociation from DNA was measured by mixing 40 l of 0.4 M MutS, pre-incubated with 0.06 M 2-AP:+T DNA for 2 mins, with 40 l of 8 M unlabeled trap DNA in the absence of nucleotides or in the presence of 300 M ATP, ATP γ S, or ADP. Decrease in 2-AP fluorescence was measured over time, corrected for intrinsic MutS fluorescence, and fit to a single exponential to determine k_{OFF} . In order to measure the effect of ATP γ S on ADP-bound MutS•2-AP:+T, 40 l of 0.4 M MutS was pre-incubated with 0.06 M 2-AP:+T DNA and 8 M ADP for 2 minutes, then mixed with 40 l of 8 M unlabeled trap DNA and 300 M ADP or 300 M ATP γ S + 8 M ADP, and the data analyzed as described above.

ATPase assays

Stopped-flow phosphate (Pi) release assays using MDCC-labeled PBP were performed with wildtype MutS, F39A and E663A mutants, as described previously¹⁵. Briefly, 40 l of 2 M MutS dimer, in the absence or presence of 8 M DNA, was mixed with 40 l of 16 M MDCC-PBP and 1 mM ATP in 50 mM NaCl, 20 mM Hepes-NaOH, pH 7.7, 5 mM MgCl₂, at 40 °C, and the change in fluorescence upon MDCC-PBP binding to Pi measured by excitation at 425 nm and emission at > 450 nm. The kinetic traces were fit to a linear equation or an exponential + linear equation for burst kinetics:

$$[\text{Pi}] = (A_0 e^{-kt} + Vt + F_0) / m_{\text{Pi}}$$

where [Pi] corresponds to phosphate concentration, A_0 is the amplitude, k is the observed rate constant, V is the velocity of the linear phase, F_0 is the initial fluorescence intensity, and m_{Pi} is the slope of the Pi standard curve measured under the same conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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MutS interactions with DNA

Figure 1.

Selective interaction of MutS with +T mismatched DNA. (A) *T. aquaticus* MutS Phe 39 with +T DNA⁷ with 2-AP positioned 3' to the +T insertion. (B) Emission spectra of 2-AP:AT and 2-AP:+T DNA in the absence ($\circ$, $\square$) and presence ($\bullet$, $\blacksquare$) of MutS upon excitation at 315 nm show increase in fluorescence when MutS binds +T DNA. (C) Titrations of 10 nM 2-AP:AT ($\bullet$) and 2-AP:+T ($\blacksquare$) with 0–0.2 M MutS, and 2-AP:+T ($\diamond$) with MutS F39A reveal interaction only between wildtype MutS and +T DNA ($K_D = 15 \pm 2.5$ nM for MutS•+T complex). (D) Anisotropy measurements of 10 nM TAMRA-labeled AT ($\bullet$) and +T DNA ($\blacksquare$) titrated with 0–0.2 M MutS, and +T DNA ($\diamond$) with MutS F39A confirm high-affinity binding of MutS to mismatched DNA ($K_D = 25 \pm 1.7$ nM for MutS•+T complex). (E) 2-AP:+T suppresses ATP hydrolysis by MutS (1 M dimer) from 9.2 ± 0.8 s $^{-1}$ (no DNA or AT DNA) to 0.3 s $^{-1}$, while MutS F39A ATPase activity remains unaffected by +T DNA (1 M MutS dimer, 500 M ATP, 3 M DNA)

Kinetics of MutS interaction with DNA

Figure 2.

MutS binds +T DNA rapidly to form a stable complex. (A) Stopped-flow traces of 30 nM 2-AP-labeled DNA mixed with 200 nM MutS reveal rapid binding of wildtype MutS to +T DNA ($k_{\text{observed}} = 0.53 \pm 0.005 \text{ s}^{-1}$), but no interaction between MutS and AT DNA or MutS F39A and +T DNA. (B) MutS concentration dependence of k_{observed} yields $k_{\text{ON}} = 3 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. (C) A pre-formed complex of 2-AP:+T (30 nM) and MutS (200 nM) mixed with 4 M unlabeled +T DNA trap yields $k_{\text{OFF}} = 0.07 \pm 0.0003 \text{ s}^{-1}$ for MutS•+T complex.

Effects of ATP on MutS interactions with DNA

A. ATP γ S effects on MutS-2-AP:+T binding B. ATP effects on MutS E663A-2-AP:+T binding C. Loss of 2-AP:+T binding with ATP γ S/ATP

D. Loss of TAMRA:+T binding with ATP γ S E. ATP γ S/ATP effects on MutS-2-AP:+T dissociation

Figure 3.

Slow ATP-induced loss of MutS interaction with a mismatch in DNA. (A) Increasing concentrations of ATP γ S (0 – 60 M) result in loss of 2-AP:+T (30 nM) and MutS (200 nM) binding. (B) A similar result is obtained with ATP hydrolysis-deficient MutS E663A with increasing ATP. (C) A plot of the changing amplitude *versus* ATP γ S or ATP concentration fit to a hyperbola yields $K_{1/2} = 1.7 \pm 0.5 \text{ M}$ or $0.8 \pm 0.1 \text{ M}$, respectively, for ATP γ S/ATP effects on MutS. (D) Anisotropy measurements of 10 nM TAMRA:+T DNA and 200 nM MutS titrated with 0–40 M ATP γ S confirm the loss of binding and yield $K_{1/2} = 0.7 \pm 0.1 \text{ M}$ for the nucleotide effect on MutS. (E) A pre-formed complex of 2-AP:+T (30 nM) and MutS (200 nM) mixed with 4 M unlabeled +T DNA trap and 150 M ATP γ S or ATP yields $k_{\text{OFF}} = 0.14 \pm 0.001 \text{ s}^{-1}$ and $0.1 \pm 0.001 \text{ s}^{-1}$ for MutS•+T and MutS E663A•+T complexes, respectively.

Effects of ADP on MutS interactions with DNA

Figure 4.

ADP-induced rapid DNA binding and release by MutS. (A) Presence of ADP (0–60 M) in the reaction with 30 nM 2-AP:+T and 200 nM MutS results in a decrease in amplitude and an apparent increase in the rate of MutS binding to +T DNA. (B) A plot of the changing amplitude *versus* ADP concentration fit to a hyperbola reveals a new, lower equilibrium concentration of MutS•+T complex (~ 50 %), and yields $K_{1/2} = 2.4 \pm 0.6 \text{ M}$ for the ADP effect on MutS. (C) Anisotropy measurements of 10 nM TAMRA:+T DNA and 200 nM MutS titrated with 0–80 M ADP confirm the decrease in MutS•+T complex and yield $K_{1/2} = 1.3 \pm 0.4 \text{ M}$ for the ADP effect on MutS. (D) A pre-formed complex of 2-AP:+T (30 nM) and MutS (200 nM) mixed with 4 M unlabeled +T DNA trap and 150 M ADP yields $k_{\text{OFF}} = 1.74 \pm 0.03 \text{ s}^{-1}$ for MutS•+T.

Summary of nucleotide effects on MutS-DNA interactions

Figure 5.

Implications of the nucleotide effects on MutS-DNA interaction in mismatch repair. (A) The effect of ATP on MutS_{ADP}•+T was tested by mixing pre-incubated 30 nM 2-AP•+T, 200 nM MutS, and 8 M ADP with 4 M +T DNA trap and 150 M ADP (■) or 150 M ATP γ S (◇), which yielded $k_{\text{OFF}} = 1.56 \pm 0.07 \text{ s}^{-1}$ and $0.17 \pm 0.003 \text{ s}^{-1}$, respectively, indicating that ATP rapidly replaces ADP on MutS and stabilizes MutS•+T complex. (B) In the absence of DNA (a), MutS_{FREE} can bind ATP (b), hydrolyze it and release Pi rapidly (*only the activity of the rapid ATP-hydrolyzing subunit is shown in this model) to form MutS_{ADP} (c). When MutS_{FREE} binds mismatched DNA, rapidly and with high affinity (d), ATP binding is fast but ATP hydrolysis is suppressed and MutS_{ATP}, which forms a stable complex with the mismatch (e), becomes predominant. MutS_{ADP} also binds mismatched DNA rapidly (f), but the interaction is unstable. However, the ADP can be replaced readily by ATP and mismatch-bound MutS_{ATP} (e) is again predominant. That both pathways lead to the MutS_{ATP}•mismatch complex suggests a key role for this species in the repair reaction. In time, MutS_{ATP} releases the mismatch (b, g), in a conformation that precludes rebinding until ATP dissociates or is hydrolyzed.