Large conformational changes in MutS during DNA scanning, mismatch recognition and repair signalling

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MutS protein recognizes mispaired bases in DNA and targets them for mismatch repair. Little is known about the transient conformations of MutS as it signals initiation of repair. We have used single-molecule fluorescence resonance energy transfer (FRET) measurements to report the conformational dynamics of MutS during this process. We find that the DNA-binding domains of MutS dynamically interconvert among multiple conformations when the protein is free and while it scans homoduplex DNA. Mismatch recognition restricts MutS conformation to a single state. Steady-state measurements in the presence of nucleotides suggest that both ATP and ADP must be bound to MutS during its conversion to a sliding clamp form that signals repair. The transition from mismatch recognition to the sliding clamp occurs via two sequential conformational changes. These intermediate conformations of the MutS:DNA complex persist for seconds, providing ample opportunity for interaction with downstream proteins required for repair.

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Introduction

Genomic stability depends on a system of mismatch repair (MMR) proteins that identifies post-replicative base mismatches or short insertion errors in DNA and signals their repair (Kunkel and Erie, 2005; Iyer *et al*, 2006; Modrich, 2006; Li, 2008). MMR proteins also activate apoptosis on encountering a variety of damage lesions in DNA. Given

their critical functions, it is not surprising that mutations in MMR proteins are associated with many cancers; for example, >85% of hereditary non-polyposis colorectal cancers (Fishel, 1999; Li, 2003; Hsieh and Yamane, 2008).

MMR is initiated by MutS homologues, which contain DNA-binding and ATPase activities that are essential for MMR *in vivo* (Kunkel and Erie, 2005; Iyer *et al*, 2006; Modrich, 2006; Li, 2008). MutS homologues are homodimers in prokaryotes and heterodimers in eukaryotes, but their functionality and mechanism of action appear to be conserved. In search of their targets, MutS proteins diffusively slide along correctly matched regions of DNA (Gorman *et al*, 2007; Jeong *et al*, 2011). Upon encountering a DNA defect, specific interactions stabilize MutS at the mismatch. After recognition of a target site, MutS undergoes an ATP-induced activation to a sliding clamp state (Gradia *et al*, 1999), which is believed to be essential for signalling initiation of MMR.

X-ray crystal structures of MutS proteins bound to various DNA defects have been solved (Lamers *et al*, 2000; Obmolova *et al*, 2000; Junop *et al*, 2001; Natrajan *et al*, 2003; Warren *et al*, 2007). In all structures, the DNA is kinked sharply at the mismatch and traverses one of two channels formed between the subunits of the MutS dimer (Figure 1A). Single-molecule AFM and fluorescence studies revealed that MutS-mismatch complexes adopt multiple conformations with different extents of DNA bending that are in dynamic equilibrium (Wang *et al*, 2003; Tessmer *et al*, 2008; DeRocco *et al*, 2010; Sass *et al*, 2010). Another key feature of the MutS crystal structures is an asymmetric interaction between MutS and the mismatch in which only one subunit of the MutS dimer makes specific contacts with the mismatched base.

This asymmetric DNA–MutS interaction is mirrored in the asymmetry of the nucleotide-binding and hydrolysis activity of the two ATPase sites of bacterial MutS (Bjornson and Modrich, 2003; Lamers *et al*, 2003; Antony and Hingorani, 2004) and eukaryotic MutS homologues (MSH proteins) (Iaccarino *et al*, 1998; Studamire *et al*, 1998; Drotschmann *et al*, 2002; Antony and Hingorani, 2003; Martik *et al*, 2004; Antony *et al*, 2006; Mazur *et al*, 2006). The distinct ATPase rates of each subunit are altered upon binding mismatched DNA. Coordination of nucleotide processing at the two non-equivalent ATPase sites plays a role in MutS progressing from recognition of a mismatch to signalling for repair (Bjornson *et al*, 2000; Drotschmann *et al*, 2002; Bjornson and Modrich, 2003; Antony and Hingorani, 2004; Antony *et al*, 2006; Lebbink *et al*, 2010; Heinen *et al*, 2011; Monti *et al*, 2011). Studies of MSH mutants in which nucleotide processing is selectively disrupted on each subunit of the heterodimer suggest that the Msh2 subunit controls all aspects of the ADP/ATP cycle (Drotschmann *et al*, 2002; Hargreaves *et al*, 2010; Heinen *et al*, 2011).

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Figure 1 Dye-labelling sites and molecular dynamics simulations of MutS. (A, B) Crystal structure of Taq MutS dimer in the presence of mismatched DNA (PDB-1EWQ). One subunit of the dimer is shaded light and the other is dark. Domains I are green. (B) Red dots show the location of the average of the centre of mass of fluorescent dyes attached to cysteine mutations introduced at Met88 derived from molecular dynamics simulations. DNA is not shown. The average separation from these simulations is 22 Å. (C) Crystal structure of Taq MutS dimer in the absence of mismatched DNA (PDB-1EWR).

Several biochemical, biophysical and computational studies suggest that MutS undergoes nucleotide-dependent conformational changes during MMR (Gradia *et al*, 1999; Biswas and Vijayvargia, 2000; Joshi *et al*, 2000; Junop *et al*, 2001; Kato *et al*, 2001; Alani *et al*, 2003; Drotschmann *et al*, 2004; Lamers *et al*, 2004; Mukherjee and Feig, 2009; Salsbury, 2009; Hargreaves *et al*, 2010; Mendillo *et al*, 2010; Winkler *et al*, 2011). Nonetheless, a detailed temporal view of specific conformational states of MutS working on DNA remains elusive. Knowledge of key conformations adopted by MutS as it forms an activated mismatch recognition complex is essential for understanding its subsequent interactions with downstream MMR proteins that carry out DNA repair.

Here, we use single-molecule fluorescence resonance energy transfer (smFRET) to characterize conformational changes in *Thermus aquaticus* (Taq) MutS throughout the process of scanning homoduplex DNA, mismatch recognition, and finally, mismatch- and ATP-dependent activation of the MutS sliding clamp. A highlight of our findings is that

after mismatch recognition, ATP binding induces a large movement of the MutS DNA-binding domains I as activated MutS starts to slide away from the mismatch. Our study further reveals that MutS converts into a sliding clamp via two sequential conformational transitions, and that this activation process requires an ATP:ADP-liganded MutS intermediate.

Results

To perform single-molecule FRET measurements of MutS conformations as it binds DNA, recognizes mismatches and forms a sliding clamp, we generated a single cysteine mutant of Taq MutS (C42A/M88C) that allowed donor and acceptor fluorophore attachment in domains I of the homodimer (Figure 1; domain nomenclature follows the structure PDB-1EWQ (Obmolova *et al*, 2000)). The ATPase and DNA-binding activities of the fluorescently labelled mutant MutS protein are within error of those of wild-type MutS (see Materials and methods and Supplementary Methods). Simulations suggest the average fluorophore separation (red/magenta dots in Figure 1B) is about 20 Å in the crystal structure conformation, which predicts FRET efficiency near 1. For smFRET measurements, individual DNA-free proteins were encapsulated inside surface-immobilized biotinylated liposomes (Boukobza *et al*, 2001), or MutS was incubated with 5'-end-biotinylated DNA substrates bound to widely spaced streptavidin islands on lipid bilayer-passivated surfaces (Graneli *et al*, 2006). Most experiments used 500 base pair (bp) duplexes with or without a mismatch (GT, T-bulge) located at the midpoint. Digoxigenin modification at the non-biotinylated end allowed blocking of the free end of immobilized DNA with anti-digoxigenin antibodies. Additional assay details are available in Materials and methods and Supplementary data.

Nucleotides modulate the conformational dynamics of free MutS

We first determined the effect of nucleotides on MutS conformations in the absence of DNA. We measured smFRET from domain I dye-labelled single MutS proteins encapsulated in liposomes with and without nucleotides (Figure 2A). In the absence of nucleotides, the observed FRET efficiencies spanned the entire range from 0 to 1 (Figure 2B), and the time trajectories of FRET from each molecule showed several behaviours (Supplementary Figure 1). About two-thirds of the molecules exhibited stable FRET levels for the entire measurement before photobleaching (typically tens of seconds). The FRET level itself was variable among these molecules with about half at very high FRET (0.7–1.0), a quarter at mid fret (0.3–0.7) and a quarter at low fret (0–0.3). The remaining third of all molecules exhibited variable switching between multiple FRET levels with many time-scales (Figure 2C; Supplementary Figure 1; photobleaching prevents us from completely quantifying the switching rates). The low FRET values correspond to a MutS population in which the fluorophores on domains I are ~70 Å apart, as compared with 20–30 Å apart in the high FRET population. The MutS conformation in the high FRET species is likely similar to that in the MutS–DNA (T-bulge) structure with domains I close to each other (Figure 1) (Obmolova *et al*, 2000). Large conformational changes in domains I are

Figure 2 smFRET measurements between domains I in DNA-free MutS. **(A)** Schematic of surface immobilization using liposome encapsulation (not to scale). **(B)** smFRET histogram assembled for MutS in the absence of DNA without added nucleotides. **(C)** Donor and acceptor intensity time courses for a MutS molecule selected from experiments with DNA-free MutS with no added nucleotide that switches among various FRET states, demonstrating conformational variability. Illumination colour is indicated along the top edge. **(D)** smFRET histograms for MutS where nucleotides were added before liposome encapsulation (as indicated). Added nucleotides stabilized low and high FRET states. FRET values calculated at every time point during active donor/acceptor emissions from each MutS dimer are accumulated from many different molecules to compile the histograms.

required to account for the low FRET species (Supplementary Movie). The variability in FRET emissions from MutS in the absence of DNA is consistent with the DNA-free crystal structure in which the domains I are unresolved (Obmolova *et al*, 2000) (Figure 1C), with deuterium exchange studies on *Escherichia coli* MutS and *Saccharomyces cerevisiae* MutS α (Msh2–Msh6) (Mendillo *et al*, 2010), and with MD analysis of human MutS α (Mukherjee and Feig, 2009).

Addition of ADP or ATP shifts the mid FRET population to low FRET. FRET histograms have a small high FRET peak at 0.88 and a larger peak at low FRET at 0.17 for both nucleotides (Figure 2D). Approximately, 20% of the molecules undergo transitions between the high and low FRET populations. MutS hydrolyses ATP rapidly in the absence of mismatched DNA and exists predominantly in ADP-bound form (Antony and Hingorani, 2004; Monti *et al*, 2011), which accounts for the similarity of our ADP and ATP experiments. In contrast, ATP γ S stabilizes MutS in a high FRET state (Figure 2D, cyan curve), which suggests a configuration with the DNA-binding domains in a closed position similar to the mismatched DNA-bound arrangement captured in crystal structures (Figure 1A). Interestingly, addition of a mixture of ADP and ATP γ S yields nearly equal high and low FRET populations (Figure 2D).

Conformations of MutS bound to homoduplex DNA

MutS has been observed to move on homoduplex DNA by one-dimensional diffusional sliding (Gorman *et al*, 2007; Jeong *et al*, 2011), presumably in search of mispaired bases. We used a surface-immobilized end-blocked 500 bp matched DNA substrate to monitor the conformations of domain I dye-labelled MutS during this search phase (Figure 3A). We observed FRET distributions with two populations: one at low FRET and one at high FRET (Figure 3B; Supplementary Figure 2). The low FRET peak is similar in the absence and presence of ADP or ATP. In contrast, the high FRET peak is at a slightly higher FRET efficiency in the absence of nucleotide (FRET 0.91; $\sigma = 0.16$) as compared with in the presence of ADP (FRET 0.82; $\sigma = 0.17$) or ATP (FRET 0.81; $\sigma = 0.24$). Transitions between high and low FRET states occur in all nucleotide conditions with the high-to-low transition being more frequent (76.2% without nucleotide, 77.3% with ADP, 84.2% with ATP). In all, 93.5% of MutS bound initially into high FRET state and then transitioned to low FRET at a rate of 0.4 s^{-1} . The low FRET state lasted longer than the observation times, which were limited by photobleaching to about 15 s. Unblocking the end of homoduplex DNA (Figure 3C and D) reduced the population in the low FRET peak (fraction with FRET < 0.5 when blocked is 41% (no nucleotide), 46% (ADP), 39% (ATP) and when unblocked is 15% (no nucleotide), 16% (ADP), 12% (ATP)). This result indicates that the low FRET state has lower stability on unblocked homoduplex DNA than the high FRET state. Photobleaching of the Alexa fluorophores on MutS prevented quantification of the long lifetimes of these states on homoduplex DNA.

Mismatch recognition stabilizes the high FRET MutS conformation

To characterize the conformational transitions of MutS associated with mismatch recognition, we monitored FRET from domain I dye-labelled MutS on unblocked and end-blocked surface-immobilized 500 bp DNA containing a GT mismatch, with and without nucleotides (Figure 3E–H). On both DNAs (blocked and unblocked), we observe a narrow high FRET peak at 0.94 ($\sigma = 0.12$) in the absence of nucleotide and 0.88 ($\sigma = 0.12$ – 0.13) in the presence of ADP or ATP. Similar results were obtained with a 500-bp T-bulge substrate and with a 50-bp T-bulge substrate surface-immobilized or co-encapsulated with MutS in liposomes (Supplementary Figure 3). The decreased low FRET population on mismatched DNA

Figure 3 smFRET measurements between domains I in MutS bound to DNA. (A, B) smFRET measurements of donor/acceptor labelled MutS bound to immobilized 500 bp homoduplex, end-blocked DNA substrates with various nucleotides in solution. Fluorescein conjugated to the anti-dig block was used to locate the DNA. (C, D) smFRET measurements of donor/acceptor labelled MutS bound to immobilized 500 bp homoduplex, DNA substrates that are not end blocked with various nucleotides in solution. Alexa 488 at the free end was used to locate the DNA. These data were acquired at 500 ms per frame, unlike the rest of the experiments in this figure as well as all the other data in this paper, which were at 100 ms per frame. The increased number of photons in each 500 ms frame decreases the shot noise and results in narrower distributions of FRET for a single population. (E, F) smFRET measurements of donor/acceptor labelled MutS bound to 500 bp GT mismatched DNA that is not end blocked under several nucleotide conditions. (G, H) Measurements of donor/acceptor labelled MutS bound to end-blocked DNA containing a GT mismatch under several nucleotide conditions. For all, measurements are made in steady state with 10 nM MutS and indicated nucleotides in solution. Instances of multiple MutS loading on individual DNAs are excluded from this analysis. FRET values calculated at every time point during active donor/acceptor emissions from each MutS dimer are accumulated from many different molecules to compile the histograms. Schematics not to scale.

compared with homoduplex DNA when using blocked substrates in ADP or no nucleotide (green and red traces in Figure 3B compared with 3H) demonstrates that mismatch binding stabilizes the high FRET state. The high FRET values are consistent with MutS adopting a mismatch-bound

conformation as observed in crystal structures (Obmolova *et al*, 2000; Junop *et al*, 2001). The small decrease in FRET efficiency in the presence of ADP relative to no nucleotide is similar to that observed for MutS bound to homoduplex DNA (Figure 3B and D). These results indicate that nucleotide

binding induces changes in the relative positions of domains I of subunits A and B when MutS is bound to DNA. Consistent with this observation, the crystal structures of Taq MutS in the absence and presence of ADP reveal an altered position of subunit B domain I and increased mobility of subunit A domain I (Junop *et al*, 2001).

Large movements of the DNA-binding domains accompany ATP-triggered conversion of MutS to a sliding clamp

On unblocked mismatched DNA, the FRET distributions for MutS in the presence of ATP or ADP are essentially identical, with a single peak at 0.89 (Figure 3F). In contrast, on end-blocked DNA, in the presence of ATP, but not ADP, an additional low FRET peak at 0.2 is observed (Figure 3H). The lack of an ATP-induced low FRET peak on unblocked DNA can be explained if this low FRET MutS species slides on DNA as a clamp and falls off the free end, but the sliding clamp is trapped on end-blocked DNA. We also tested the effect of ATP γ S (100 μ M) on MutS in this experiment, and consistent with the ATP γ S-bound dimer adopting a closed conformation, no binding to DNA was detected within 10 min. With a mixture of ADP and ATP γ S, however, MutS exhibits a FRET distribution on end-blocked DNA similar to that in the presence of ATP (Figure 3H), suggesting that ATP hydrolysis is not necessary for sliding clamp formation. The large change in FRET from 0.89 to 0.2 indicates substantial movement (~ 30 Å) of the mismatch-binding domains I upon sliding clamp formation.

We observed loading of multiple MutS molecules onto individual DNAs when the ends were blocked, which is consistent with sliding clamp formation. For the analysis in Figure 3, we included only DNAs with a single-bound MutS. Loading of multiple MutS proteins was avoided by prebinding MutS to blocked mismatched DNA in the presence of ADP, followed by exchange to buffer containing ATP or ATP γ S but no additional MutS (Supplementary Figure 4). As expected, this approach yields a higher fraction of MutS in low FRET state for both ATP and ATP γ S, confirming that ATP γ S can induce sliding clamp formation on both GT and T-bulge containing DNAs (Supplementary Figure 4).

FRET between MutS and DNA confirms movement of the sliding clamp away from the mismatch

To test the hypothesis that the ATP-induced low FRET MutS species on mismatched DNA is a sliding clamp, we performed additional FRET experiments to monitor the interaction of MutS with the mismatch. We used Alexa Fluor 555 donor-only labelled MutS and a surface-immobilized 500 bp end-blocked DNA labelled with a Cy5 acceptor positioned 9 bases from a T-bulge (Figure 4A). Control experiments found minimal interactions between MutS and the fluorescent dye on the DNA (see Materials and methods and Supplementary Methods). Although the MutS dimer interacts asymmetrically with a mismatch, the distances between the FRET acceptor on DNA and the two possible locations of the donor on MutS differ by only ~ 3.5 Å (Obmolova *et al*, 2000); therefore, our measurements should not be significantly affected by the asymmetry. In the presence and absence of ADP, MutS binding to DNA results in two FRET populations: a small peak at 0 and a larger peak at 0.65 (Figure 4B and E). The 0

FRET peak likely represents MutS bound non-specifically 'far' from the T-bulge site, because this population is dramatically reduced on shorter DNAs. On unblocked T-bulge DNA, the 0 FRET peak included 35% of the population on 500 bp strands and 5% on 50 bp strands in the absence of nucleotides; with 2 mM ADP, the 0 FRET peaks are 13% for 500 bp and 3% for 50 bp. The reduction of the 0 FRET species in the presence of ADP relative to no nucleotides is consistent with the observation that ADP increases the specificity of *E. coli* MutS for mismatches (Blackwell *et al*, 2001). The FRET efficiency of 0.65 is consistent with the distance between the donor dye on domain I of MutS bound to the T-bulge and the acceptor dye 9 bases away. This 0.65 FRET state converts to 0 FRET following buffer exchange from ADP to ATP γ S (Figure 4C) or ATP (Figure 4D). Donor emission in the 0 FRET state verifies MutS presence on the end-blocked DNA, while the disappearance of acceptor emission indicates movement of MutS away from the T-bulge; thus confirming that ATP γ S or ATP can both induce ADP-bound MutS to become a sliding clamp that leaves the mismatch. The complete absence of the 0.65 FRET state confirms that once MutS is converted to a sliding clamp it no longer recognizes the mismatch although it remains bound to the DNA for a long time (Jeong *et al*, 2011).

Notably, we found that MutS bound to the mismatch in the absence of nucleotides does not convert into a sliding clamp and move away from the mismatch upon addition of ATP γ S (compare Figure 4C with F). In contrast, addition of ATP does convert MutS to the sliding clamp under the same conditions (Figure 4G). The latter result rules out the possibility that the conformation adopted by MutS on binding a mismatch in the absence of nucleotides is unable to convert to the sliding clamp. Taken together, these experiments suggest that both ADP and ATP must be bound to MutS for it to be converted into a post-mismatch recognition sliding clamp. These observations also suggest MutS can hydrolyse ATP while bound to a mismatch. Our determination of a heterogeneous nucleotide-bound state during transition to the sliding clamp is consistent with other studies of MutS homologues (Antony and Hingorani, 2004; Mazur *et al*, 2006; Monti *et al*, 2011), but contrasts with conclusions based on ATP hydrolysis mutants of human MSH2-MSH6 (Heinen *et al*, 2011), which suggest the ADP/ATP-liganded state may be a dead-end complex.

Resolving the steps leading to MutS sliding clamp formation

Measuring FRET between MutS and the DNA substrate also enabled us to follow MutS binding to and releasing from the mismatch site and the DNA substrate over time, and thus develop a temporal sequence of MutS actions as it recognizes a mismatch and signals repair. With donor-labelled MutS in solution and surface-immobilized 500 bp unblocked T-bulge DNA with an acceptor 9 bases away, we observed short bursts of FRET (Figure 5A) in the presence of 2 mM ADP, which should occupy both ATPase sites of MutS (Antony and Hingorani, 2004) (see Supplementary Figure 5 for lower ADP concentration measurements). The FRET efficiencies fit to a peak centred at 0.7 (Figure 5B), reporting MutS binding to the mismatch site. The FRET peak width is consistent with a single static state (Gopich and Szabo, 2005). The distribution of dwell times for these bursts fits well to a

Figure 4 smFRET between Cy5-labelled T-bulge DNA and Alexa 555-labelled MutS reveals the position of MutS on DNA. (A) Experiment schematic (not to scale). (B–D) MutS was incubated with the immobilized end-blocked mismatched DNA in buffer containing 10 μ M ADP resulting in the FRET histogram in (B) acquired with MutS in solution. Cy5 was 9 bases from the mismatch, resulting in FRET $\sim$ 0.6 when MutS bound at the mismatch. Exchanging the buffer to one containing no MutS and 100 μ M ATP γ S (C) or 100 μ M ATP (D) caused the disappearance of the 0.6 FRET state. (E–G) MutS was incubated with the immobilized end-blocked mismatched DNA in buffer containing no added nucleotides resulting in the FRET histogram in (E) acquired with MutS in solution, and further exchange to MutS-free buffer containing 100 μ M ATP γ S (F) did not change the FRET distribution. In contrast, exchange to MutS-free buffer with 100 μ M ATP (G) caused the disappearance of the 0.6 FRET state. In all, the population at FRET zero only includes donor-labelled MutS that co-localize with active acceptor dyes on DNA that were verified with direct excitation at the beginning and end of every observation. A single average FRET value is calculated from each co-localized donor/acceptor pair, and those values are accumulated from many different molecules to compile the histograms. Note, observations in (C, D, F, G) began 1–2 min after buffer exchange.

single exponential with a lifetime of 2.3 s (Figure 5C). This lifetime is longer than the 0.6 s value determined from ensemble kinetics of MutS releasing a 2-aminopurine-labelled mismatch site, but this difference can be attributed to the higher temperature of the ensemble experiments (40 versus 21°C) (Jacobs-Palmer and Hingorani, 2007). When the same experiment was performed with ATP, 78% of the binding events resulted in bursts of FRET (Figure 5D) with a single FRET efficiency where distributions and lifetime (Figure 5E and F) are almost identical to those with ADP. These events likely report the same ADP-bound state of MutS given ongoing ATP hydrolysis in the reaction. (Note: given the temperature dependence of ATP hydrolysis, the fraction of MutS doubly liganded with ADP may vary with temperature, which could impact repair efficiency (Lebbink *et al*, 2006).)

The other 22% of the binding events in the presence of 2 mM ATP exhibited discrete transitions in FRET levels before MutS dissociated from DNA (Figure 5G). Analysis of this population revealed a two-step process involving three distinct FRET efficiencies prior to MutS dissociation: 0.7, 0.5 and 0 (Figure 5G–I). The lifetimes for the 0.7, 0.5 and 0 FRET states are 4.2, 1.5 and 2.2 s, respectively. These lifetimes are all at least five times shorter than the photobleaching

lifetimes of the fluorophores in our experiments (similar behaviour was observed with 200 μ M ATP; Supplementary Figure 5).

As shown by the transition density plot (Figure 5J), the three FRET states overwhelmingly occurred in a specific sequence. The lines with arrows in Figure 5J define the major transition pathway, in which MutS binds the mismatch with FRET 0.7, transitions to FRET 0.5 and then transitions again to FRET 0. Finally, disappearance of the donor signal reports dissociation of MutS from DNA, most likely by sliding off the unblocked end. We verify acceptor dye emission on DNA at the end of the observation period to confirm the 0 FRET state definitively reports that MutS is on DNA but has moved away from the mismatch site. Experiments with a shorter acceptor-labelled DNA substrate (50 bp) containing a T-bulge 9 bp from an unblocked end confirmed these results (Supplementary Figure 6). Note that sliding clamps fall off the unblocked end of the 50-bp DNA faster than off the 500-bp DNA, so the 0 FRET state observed for the longer substrate is only rarely observed on the 50-bp substrate (Supplementary Figure 6, but see also rare exceptions illustrated in Figure 6E). Analysis of rare events where FRET 0 was observed before the FRET 0.7 emissions (Figure 6) and the

Figure 5 Dynamics of smFRET between Cy5 labelled, unblocked T-bulge DNA and Alexa 555-labelled MutS demonstrate a two-step process for ATP-dependent conversion of the mismatch recognition complex to a sliding clamp. (A–C) Experiments using 10 nM MutS and 2 mM ADP result in short binding events with a single FRET value: (A) typical intensity trace under 532 nm illumination of a single MutS-binding event in ADP buffer; (B) histogram of FRET from many such events; (C) distribution of dwell times for these events with exponential fit. No transitioning events were observed with ADP buffers. (D–J) Experiments using 10 nM MutS and 2 mM ATP in solution generate events that divided to those with no transitions (D–F) and those with transitions in FRET levels (G–J). In the subpopulation of ATP events without transitions, the intensity traces (D), FRET histogram (E) and dwell time distributions (F) were similar to ADP experiments. For ATP events with FRET transitions (G), three FRET levels were found (H) with three distinct lifetimes (I). The transitions between the FRET levels nearly always occur in the sequence high-to-medium-to-low as indicated by the lines with arrows in the transition density plot (J). Note some backward transitions. A single average FRET value is calculated from each FRET event between transitions, and those values are accumulated from many different molecules to compile the histograms. For all, measurements acquired under 532 nm illumination.

2.15-s lifetime of the 0 FRET state following sliding clamp activation on the 500-bp DNA (Figure 5I) allows estimation of MutS diffusivity in both the homoduplex scanning and mis-

match-activated sliding clamp states (Supplementary Methods). This analysis yields a lower diffusivity for the sliding clamp relative to MutS searching on homoduplex DNA.

Figure 6 Diffusion revealed by rare events with particular FRET signals. (A–C) Schematic for and examples of rare binding events in 2 mM ATP buffer of donor-labelled MutS on Cy5-labelled 500 bp T-bulge DNA (as in Figure 5D–J) where a brief FRET 0 state is observed before the FRET 0.7 state is achieved that can be used to estimate diffusivity of MutS in the searching phase. (D, E) A schematic for and an example of a rare event in 2 mM ATP buffer on 50 bp unblocked T-bulge DNA (similar to Supplementary Figure 6) where a brief burst of FRET 0 is seen following the mid FRET state reporting binding at the mismatch before the donor signal disappears due to MutS dissociation from the DNA constrain the diffusivity of the activated post-mismatch recognition sliding clamp. For all, measurements acquired under 532 nm illumination. Schematics not to scale.

In a recent study that measured FRET between DNA labelled near the mismatch and MutS labelled on domain IV, sliding clamp formation appeared to occur in a single step, and the authors concluded that an intermediate state was unlikely during sliding clamp activation (Jeong *et al*, 2011). Our ability to detect an intermediate is likely due to significant experimental differences. We labelled domain I instead of domain IV (M88C versus T469C in domain IV). Domain IV may move differently during sliding clamp activation such that the intermediate state is not distinguishable by FRET. In addition, we used longer DNA substrates (500 bp versus 74 or 100 bp), which yield a wider range of FRET values during recognition and sliding clamp formation (from 0.7 to 0 here versus from 0.8 to 0.5 in the previous study). Future work using other dye attachment sites will be useful.

To complete our analysis of MutS conformational dynamics on DNA, we monitored FRET within MutS using molecules labelled with one donor and one acceptor on domains I as the protein transitioned through intermediate states on surface-immobilized unlabelled, unblocked 500 bp DNA substrates containing a T-bulge or GT mismatch. We found that MutS binding to the mismatch site was accompanied by high FRET, indicating closure of the mismatch-binding domains. In most cases, the FRET value changed from high-to-low or high-to-

mid-to-low before the signal vanished due to MutS dissociation or photobleaching of the dyes (Supplementary Figure 7). The dwell times for each state of those events that showed two-step transitions from high-to-mid-to-low FRET efficiency between labels in domains I (Figure 7) matched those determined from measurements of FRET between MutS and DNA (Figure 5). The similarity of the three dwell times measured by the two distinct types of FRET experiments (Figures 5 and 7) demonstrates that the dye labelling of the DNA substrate is not affecting the intermediate configuration. From these observations we conclude that MutS forms the initial recognition complex with domains I closed, then converts to an intermediate state with domains I partially open with a rate of 0.24 s^{-1} , and then finally to the sliding clamp state with domains I completely open with a rate of 0.66 s^{-1} .

Discussion

To initiate MMR, MutS must first locate and recognize a mismatch. After recognition, ATP induces MutS to change conformation to a sliding clamp form that can interact with MutL and trigger the cascade of events that leads to DNA repair. Although this process has been studied extensively (Kunkel and Erie, 2005; Iyer *et al*, 2006; Modrich, 2006; Li, 2008), limited information is available about the associated conformational changes within MutS as it works on DNA (Hargreaves *et al*, 2010; Mendillo *et al*, 2010). In this study, we used smFRET between MutS and DNA to follow MutS binding to DNA, mismatch recognition, sliding clamp formation, and release from the DNA, as well as smFRET between domains I of subunits A and B to monitor conformational changes in MutS.

Mismatch searching, recognition and sliding clamp formation

In the absence of DNA and nucleotides, labelled MutS exhibits a range of FRET values from near 0 to near 1, indicating that the distance between the dyes attached in domains I varies from under $30 \text{ \AA}$ to over $70 \text{ \AA}$ (Figure 2B). Adenine nucleotides greatly reduce both the population of intermediate states and the number of fluctuations between states, suggesting that in the presence of nucleotides, MutS exists primarily in two states: a high FRET state in which DNA-binding domains I are in close proximity, perhaps forming a bridge across the central cavity of the dimer as seen in MutS–mismatched DNA crystal structures (Lamers *et al*, 2000; Obmolova *et al*, 2000; Warren *et al*, 2007), and a low FRET state, in which domains I have moved far from one another. The lifetimes of these states, which extend for several seconds, indicate that they are relatively stable (note: quantitative characterization of the lifetimes is prohibited because acceptor dye photobleaching limits observations to $\sim 10 \text{ s}$). Our data, together with ensemble fluorescence data showing that the lower DNA-binding domains IV are far apart in the absence of DNA and nucleotides (Cho *et al*, 2007), suggest that the entire DNA-binding site is open in the low FRET state. Notably, ATP γ S shifts the population from dominant open state (low FRET) to almost entirely closed state (high FRET). This result provides an explanation for the observation that MutS readily binds DNA under all conditions except in the presence of ATP γ S, which likely

Figure 7 smFRET between Alexa 555 and Alexa 647 attached to domains I in MutS demonstrate a two-step MutS conformational change during sliding clamp activation. (A) Selected intensity time trace of donor and acceptor emission to show a two-step transition with 10 nM MutS and 2 mM ATP in solution. The FRET histograms (B) showed three distinct non-zero states, with dwell time distributions (C) for the distinct states that were similar to the experiments with the acceptor attached to the DNA (Figure 5I). A single average FRET value is calculated from each FRET event between transitions, and those values are accumulated from many different molecules in the histograms. For all, measurements acquired under 532 nm illumination but the laser intensity was decreased from that in Figure 5 to reduce photobleaching rates. In all, 200 ms camera integration was used to compensate for the lower excitation.

maintains both domains I and IV in a closed state that precludes DNA binding.

Upon binding homoduplex DNA, MutS predominantly adopts a high FRET state in which domains I are closed and then converts to a low FRET state in which domains I are open (at 0.4 s^{-1}), while still on DNA, in both the presence and absence of ADP and ATP. In this case, even with domains I in open state, domains IV presumably remain closed allowing MutS to remain bound to DNA. The state with domains I open is less stable on unblocked homoduplex DNA than the closed domains I state as the low FRET state is significantly reduced without the block (Figure 3D). Further, it appears MutS can diffuse one-dimensionally along the homoduplex DNA in the pre-mismatch recognition state at an average rate of $\sim 0.036 \mu\text{m}^2 \text{ s}^{-1}$ (Jeong *et al*, 2011). As expected, mismatch recognition shifts MutS into a stable high FRET state in which domains I are closed (Figure 3F), consistent with crystal structures of MutS–mismatched DNA complexes (Lamers *et al*, 2000; Obmolova *et al*, 2000; Junop *et al*, 2001; Natrajan *et al*, 2003; Warren *et al*, 2007).

Although MutS can recognize mismatches in a multitude of nucleotide-bound and -free states, the fate of MutS after mismatch recognition is dependent on the nucleotide occupancy of both ATPase sites (Iaccarino *et al*, 1998; Mazur *et al*, 2006; Lebbink *et al*, 2010). As discussed below, our data strongly suggest that under nucleotide conditions expected *in vivo* mismatch-bound MutS with doubly liganded ADP dissociates directly from the mismatch instead of undergoing ADP→ATP exchange to form a sliding clamp; whereas, MutS containing both ADP and ATP can undergo conformational

changes leading to the post-recognition sliding clamp state. After mismatch recognition, MutS proteins competent to form sliding clamps convert from the recognition state (domains I closed) to an intermediate state (domains I partially open) and finally to the clamp state (domains I completely open), which can slide away from the mismatch. In this open state, the specific contacts between MutS and the mismatched base would be lost and the two channels made by domains I bridging the central cavity of the MutS dimer would presumably become one. These conformational changes would allow MutS to slide away from the mismatch site with domains IV remaining closed to keep MutS topologically linked to DNA. Comparing experiments that observe MutS move away from the mismatch as a clamp (Figure 4D) to experiments that monitor domains I conformation under identical conditions at times well after sliding clamp activation (Supplementary Figure 4i) suggests that eventually domains I may be open or closed in the sliding clamp state. Large-scale movement of domains I has been proposed recently based on protein cross-linking studies of *E. coli* MutS (Winkler *et al*, 2011).

Notably the rate of diffusivity of the post-mismatch recognition MutS is lower than that during the search for a mismatch (Supplementary Methods). This change in the diffusivity implies that interactions between MutS and homoduplex DNA are different when MutS is in a post-recognition sliding clamp conformation relative to its conformation in the initial search phase. Jeong *et al* (2011) also reported differences in pre- and post-mismatch recognition MutS sliding clamps.

Both ADP and ATP must be bound to MutS for sliding clamp formation

A striking finding from our smFRET study is that, even at high ATP concentration (2 mM), ~80% of MutS mismatch-binding events do not result in formation of a sliding clamp, but result in direct dissociation from the mismatch (Figure 5D). The kinetic properties of this population are essentially identical to those of MutS in the presence of 2 mM ADP, suggesting that their nucleotide ligation states are the same. At 2 mM ADP, both sites on the MutS dimer are occupied ($K_{d1}=0.5\ \mu\text{M}$, $K_{d2}=30\ \mu\text{M}$ for Taq MutS (Antony and Hingorani, 2004; Monti *et al*, 2011), which leads us to conclude that before binding DNA a majority of MutS is doubly ADP liganded, even in high concentrations of ATP. This result is consistent with kinetic studies indicating that ATP hydrolysis and phosphate release occur faster than ADP release in the absence of DNA (Gradia *et al*, 1997; Antony and Hingorani, 2004), and with a recent mass spectrometry study, which revealed that ~60% of *E. coli* MutS contains two ADPs in the presence of 16 μM ATP (Monti *et al*, 2011). Taken together, these results suggest that the doubly liganded ADP MutS is not converted to a sliding clamp in the presence of ATP. A widely accepted model of MutS function proposes that mismatch recognition by ADP-bound MutS leads to rapid ADP→ATP exchange and sliding clamp formation (Acharya *et al*, 2003). Our results imply that this transition does not occur when MutS is doubly liganded with ADP. Notably, although mismatched DNA stimulates release of ADP, the rate of ADP release ($\sim 0.1\ \text{s}^{-1}$) is similar to or slightly slower than the rate of ATP-induced release of MutS from unblocked DNA (Acharya *et al*, 2003; Antony and Hingorani, 2003; 2004; Antony *et al*, 2006; Monti *et al*, 2011), and it is four times slower than the rate of release of doubly ADP-liganded MutS from the mismatch observed here (Figure 5), which supports the possibility that doubly ADP-liganded MutS dissociates from DNA rather than converting to sliding clamp form.

Given the high concentration of ATP (2 mM), the remaining fraction (~20%) of MutS that can be activated to form

sliding clamps likely has 1 ADP and 1 ATP bound, because one subunit binds ADP with higher affinity than the other, and ATP binds rapidly once ADP dissociates (Antony and Hingorani, 2004; Mazur *et al*, 2006). This population forms sliding clamps in a two-step process in which the first step is reversible followed by irreversible sliding clamp formation, with an overall rate of ($0.16\ \text{s}^{-1}$). One possibility is that mismatch-stimulated ADP release occurs from the mixed ADP:ATP-liganded state during the irreversible step of sliding clamp formation. It has also been suggested that formation of the sliding clamp requires both ATPase sites to be filled with ATP, and high concentrations of ATP γ S or AMPPNP appear to be able to activate yeast MutS α or *E. coli* MutS to form a sliding clamp in the absence of ADP (Mazur *et al*, 2006; Monti *et al*, 2011). ADP carried through from MutS purification (Monti *et al*, 2011) or hydrolysis of ATP γ S during extended incubations could account for the apparent lack of ADP requirement in these previous studies. In our study, we found that 0.1 mM ATP γ S cannot convert mismatch-bound MutS to a sliding clamp state in the absence of ADP (Figure 4F), which strongly suggests that the only recognition state that can be converted to a sliding clamp is one in which both ADP and ATP are bound (Bjornson and Modrich, 2003; Antony and Hingorani, 2004). Our observations do not exclude the possibility that an ADP:empty state could be a precursor to a sliding clamp when mismatch-bound MutS is exposed to unphysiologically low nucleotide concentrations.

Model and biological implications

The ability to parse heterogeneous and asynchronous populations by single-molecule FRET enabled us to monitor MutS dynamics under steady-state conditions and determine the fates of MutS at various stages of the reaction, including mismatch recognition and sliding clamp formation. The sequence of events emerging from our study yields a model of MutS actions (Figure 8). Given its ATPase kinetics and the high concentrations of ATP and ADP *in vivo*, most MutS likely

Figure 8 Model of MutS during MMR initiation. Schematic illustrating conformational changes and nucleotide ligation states associated with MutS binding DNA (far left), searching homoduplex DNA, identifying mismatches (centre) and becoming a mismatch recognition activated sliding clamp (far right). The suggested occupancy of the two nucleotide-binding sites is indicated above MutS (D = ADP and T = ATP). We expect both nucleotide-binding sites are occupied at the nucleotide concentrations in our experiments as well as those found *in vivo*. Therefore, we indicate the ADP:ATP ligation state of MutS as the most significant intermediate on the signalling pathway. It remains possible that an ADP:empty configuration, although not probable *in vivo*, could be competent to convert to the sliding clamp. Finally, the final nucleotide exchange during sliding clamp activation is unresolved in our experiment (grey shading).

exists in mixed ADP:ATP-liganded or doubly ADP-liganded states. These nucleotide-bound MutS states undergo dynamic open-closed conformational changes that facilitate loading of the protein onto DNA. Once loaded, MutS scans the DNA with domains IV closed and domains I either open or closed. When MutS encounters a mismatch, it adopts the conformation observed in crystal structures with the DNA kinked at the mismatch and domains I closed (Obmolova *et al*, 2000; Sass *et al*, 2010). The fate of this complex depends on the nucleotide ligation state of MutS. Doubly ADP-liganded MutS dissociates directly from DNA; whereas, mixed ADP:ATP-liganded (and possibly doubly ATP-liganded) MutS undergoes a subsequent reversible conformational change in which the mismatch-binding domains move apart (mid-range intra-molecular FRET between dyes on domains I), but the protein remains at or near the mismatch site. The FRET properties of this complex are consistent with previously observed MutS-mismatch complexes in which the DNA is unbent (Wang *et al*, 2003; Tessmer *et al*, 2008; Sass *et al*, 2010). Finally, this partially open state converts to a sliding clamp state that can slide away from the mismatch on DNA with a different mobility than the pre-recognition scanning state. We speculate that ADP→ATP exchange is associated with this final conversion step in the pathway.

Importantly, our smFRET observations reported here suggest that MutS remains at the mismatch for several seconds during sliding clamp formation, providing ample opportunity for downstream MMR proteins to interact with MutS at the mismatch site, possibly adding another layer of complexity into the nucleotide processing sequence during mismatch identification. Conformational changes in domains I associated with formation of both the intermediate and sliding clamp states expose new protein surfaces; thus, both states may provide binding sites for downstream proteins. Consistent with this proposal, there are several ATPase mutants of *S. cerevisiae* MutSα that cannot form sliding clamps but that can form ternary complexes with MutLα (Mlh1-Pms2). Interestingly, all of these mutants exhibit normal ADP binding to both subunits and normal ATP binding to Msh6, but impaired ATP binding to Msh2, suggesting that the doubly ATP-liganded state may be required for sliding clamp formation but not for ternary complex formation (Hargreaves *et al*, 2010). Taken together, these findings suggest that the ADP:ATP bound intermediate state of MutS may be recognized by MutL for initiation of MMR.

Materials and methods

Protein and DNA substrates

We generated a mutant of Taq MutS (C42A/M88C), in which the native cysteine is changed to alanine and Met88 in domain I is changed to cysteine. Taq MutS was expressed in *E. coli* and purified as described previously (Antony and Hingorani, 2004). We labelled M88C in both subunits A and B to generate donor/acceptor MutS by mixing an equimolar mixture of Alexa 555- and Alexa 647-maleimide or donor-only MutS by omitting the acceptor dye. Labelling efficiencies were typically about 75% per cysteine.

We performed simulations of dyes attached at M88C (Figure 1) using CNS and pdb files 1EWQ, 1EWR and 1FW6 as well as structures of Cy3 and Cy5 in lieu of the unpublished Alexa structures (Brunger, 2007; Choi *et al*, 2010; Brunger *et al*, 2011). The centres of mass of the averaged dye locations from the simulations are shown as red dots in Figure 1B (distribution in Supplementary Figure 8). The dyes are predicted to be ~20 Å apart in the crystal structure conformation (PDB-1EWQ (Obmolova *et al*, 2000)), which yields

FRET efficiency near 1 for the Alexa Fluor 555–647 pair (Förster radius: 50–60 Å).

Native-like mismatched DNA binding of MutS labelled at M88C with Alexa 555, Cy3 or IAANS dye was verified with ensemble assays (Supplementary Methods; Supplementary Figure 9). Single-molecule methods to directly measure the binding kinetics and equilibrium binding constants (Li *et al*, 2007) of Alexa 555-labelled MutS (C42A/M88C) to unblocked, 500-mer T-bulge DNA in both nucleotide-free buffer and 2 mM ADP containing buffer revealed native binding using both Cy5-labelled DNA and unlabelled DNA (Supplementary Figure 10). The single-molecule binding study confirmed the ensemble result that dye-labelling MutS does not affect binding affinity for unlabelled, mismatched DNA substrates. The consistency of binding kinetics determined with this single-molecule fluorescence approach using Cy5-labelled DNA (Supplementary Figure 10, left column) and unlabelled DNA (Supplementary Figure 10, right column) confirms that the Cy5 backbone modification 9 bases from the mismatch site has minimal effect on the interaction between MutS and the mismatch. The similarity in the ATP-activated intermediate state seen on a T-bulge using internal Cy5-labelled 500 bp DNA (Figure 5G–J) with the 50-bp DNA where the Cy5 is conjugated to a DNA end at a nick (Supplementary Figure 6g–j), suggests that the specific chemistry of dye attachment to the DNA does not affect MutS interactions with the mismatch.

MutS rarely bound directly to Cy5 as control experiments with Cy5-labelled, blocked, homoduplex 500 bp DNA found <6% of Alexa 555-labelled MutS-binding events generated FRET >0.3 (Supplementary Figure 11). The high affinity of MutS for a mismatch will decrease this fraction in experiments using mismatched DNA.

Near native-like ATPase activity of Alexa 555-labelled MutS was confirmed by measuring the pre-steady state phosphate release rate (Supplementary Methods; Supplementary Figure 12) using the previously established MDCC-PBP assay (Antony and Hingorani, 2004). The burst rate for the Alexa 555-labelled M88C mutant is within a factor of 2 of the wild-type protein in the absence of DNA. The steady-state mismatch-bound ATP hydrolysis rates are identical for the labelled mutant and wild-type MutS.

Our experiments focused on MutS dimers although MutS can also form tetramers (Biswas *et al*, 1999; Bjornson *et al*, 2003; Wang *et al*, 2003; Jiang and Marszalek, 2011; Monti *et al*, 2011). In the smFRET analysis, we only included events that met criteria consistent with MutS dimers: containing exactly one donor and one acceptor dye in donor/acceptor MutS experiments or only one or two donors in donor-only MutS experiments. Due to incomplete labelling of samples, some tetramers could also meet these criteria. We performed measurements with mixed samples of Alexa 555-only MutS and Alexa 647-only MutS to assess the degree of tetramerization (appearing as events with both dyes present). As discussed in detail in supporting methods, we determined that in our experimental conditions, <10% of included events could be incompletely labelled tetramers meeting dimer acceptance criteria. Furthermore, key experiments performed at a lower MutS concentration (with verified lower tetramer fraction) yielded the same results as higher MutS concentration, indicating little if any contribution of MutS tetramers to the results of this study (Supplementary Figure 13).

To characterize MutS conformations in the absence of DNA, individual proteins were encapsulated inside biotinylated liposomes (Boukobza *et al*, 2001), which were then tethered to a streptavidin-coated quartz slide. For measurements of MutS interacting with DNA, 5'-end-biotinylated DNA substrates were bound to widely spaced streptavidin islands on lipid bilayer-passivated quartz surfaces (Graneli *et al*, 2006). Non-specific binding of MutS to the bilayer was negligible, thus any MutS retained on the surface was bound to DNA. The primary DNA substrate is a 500-bp duplex with or without a mismatch (GT, T-bulge) located at midpoint, and key control experiments were performed with 50 bp DNA. The 500-bp biotinylated DNA was modified with digoxigenin at the free end, which could be blocked with digoxigenin antibodies. In control experiments, over 75% of Cy5-labelled DNA co-localized with fluorescein-labelled anti-digoxigenin (Supplementary Methods). For experiments with homoduplex DNA, a brief 473 nm illumination was used to excite either a terminal Alexa 488 (Figure 3C) or fluorescein-labelled anti-digoxigenin (Figure 3A) to locate tethered DNA substrates. Emission from the

single Alexa 488, detected through the optical filters optimized for Alexa 555 emission, was weak so we allowed the camera to integrate 500 ms in these experiments. This protocol was not required for the fluorescein-labelled anti-digoxigenin, which was labelled with 3–5 dyes per molecule. The longer camera integration time decreases shot noise and results in narrower Gaussian peaks for single states within a FRET efficiency histogram. Relationships between shot noise and histogram widths are described elsewhere (Choi *et al*, 2011).

Single-molecule FRET measurements

smFRET measurements were made with a prism-type total internal reflection microscope described previously (Sass *et al*, 2010) with 10 Hz frame rate unless noted otherwise. During FRET measurement, an alternation laser illumination sequence (635 nm 0–1 s; 532 nm middle interval; 635 nm last 10 s) was used to locate single molecules and distinguish the number of acceptor and donor dyes on each molecule by single-step photobleaching. Only molecules with one donor dye and one acceptor dye were used for analysis.

After correcting donor and acceptor for crosstalk and backgrounds, FRET histograms for donor–acceptor labelled MutS were generated by calculating FRET efficiency in each frame of traces where both donor and acceptor were active using $\text{FRET} = 1/(1 + (I_D/I_A))$. Histograms were fit with Gaussian functions and the centre value and standard deviation parameter (σ) of the fitted function are used when peak or width is indicated (Supplementary Table). For the FRET between MutS and DNA, the maximum of the length of the FRET state or 35 frames were averaged to determine a FRET value for each state. Distances (d) were estimated using the relation $\text{FRET} = 1/(1 + (d/R_0)^6)$ and R_0 of 53 Å (Haugland, 2005). Effects of local environment on quantum yield and dye mobility that can change with dynamic protein conformations have the potential to alter R_0 and FRET efficiencies, so here we do not claim better than 10 Å precision in converting FRET to distance. We determined

a global γ -factor around 1.27, which gives <5% corrections to converted distances (McCann *et al*, 2010). Transitions were determined by examining traces by hand or using an edge finding algorithm previously described (Sass *et al*, 2010).

All experiments were performed with custom-built quartz cells at 21°C in 20 mM Tris:Acetic acid, 100 mM sodium acetate, 5 mM MgCl₂, 2% glucose (w/w), pH 7.8 with oxygen scavenging system (100 U ml^{−1} glucose oxidase, 1000 U ml^{−1} catalase, 0.05 mg ml^{−1} cyclooctatetraene and 143 mM 2-mercaptoethanol).

Additional experimental details are available as Supplementary Methods.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: RQ performed the measurements and analysed the data; VCD, CH and RQ created DNA substrates; AS and MMH provided protein and performed ensemble DNA-binding and ATPase assays; RQ, DAE and KRW designed the research; all authors contributed to interpretations and manuscript preparation.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Acharya S, Foster PL, Brooks P, Fishel R (2003) The coordinated functions of the E. coli MutS and MutL proteins in mismatch repair. *Mol Cell* **12**: 233–246
- Alani E, Lee JY, Schofield MJ, Kijas AW, Hsieh P, Yang W (2003) Crystal structure and biochemical analysis of the MutS:ADP:beryllium fluoride complex suggests a conserved mechanism for ATP interactions in mismatch repair. *J Biol Chem* **278**: 16088–16094
- Antony E, Hingorani MM (2003) Mismatch recognition-coupled stabilization of Msh2-Msh6 in an ATP-bound state at the initiation of DNA repair. *Biochemistry* **42**: 7682–7693
- Antony E, Hingorani MM (2004) Asymmetric ATP binding and hydrolysis activity of the Thermus aquaticus MutS dimer is key to modulation of its interactions with mismatched DNA. *Biochemistry* **43**: 13115–13128
- Antony E, Khubchandani S, Chen S, Hingorani MM (2006) Contribution of Msh2 and Msh6 subunits to the asymmetric ATPase and DNA mismatch binding activities of Saccharomyces cerevisiae Msh2-Msh6 mismatch repair protein. *DNA Repair (Amst)* **5**: 153–162
- Biswas I, Ban C, Fleming KG, Qin J, Lary JW, Yphantis DA, Yang W, Hsieh P (1999) Oligomerization of a MutS mismatch repair protein from Thermus aquaticus. *J Biol Chem* **274**: 23673–23678
- Biswas I, Vijayvargia R (2000) Heteroduplex DNA and ATP induced conformational changes of a MutS mismatch repair protein from Thermus aquaticus. *Biochem J* **347**(Part 3): 881–886
- Bjornson KP, Allen DJ, Modrich P (2000) Modulation of MutS ATP hydrolysis by DNA cofactors. *Biochemistry* **39**: 3176–3183
- Bjornson KP, Blackwell LJ, Sage H, Baitinger C, Allen D, Modrich P (2003) Assembly and molecular activities of the MutS tetramer. *J Biol Chem* **278**: 34667–34673
- Bjornson KP, Modrich P (2003) Differential and simultaneous adenosine di- and triphosphate binding by MutS. *J Biol Chem* **278**: 18557–18562
- Blackwell LJ, Bjornson KP, Allen DJ, Modrich P (2001) Distinct MutS DNA-binding modes that are differentially modulated by ATP binding and hydrolysis. *J Biol Chem* **276**: 34339–34347
- Boukobza E, Sonnenfeld A, Haran G (2001) Immobilization in surface-tethered lipid vesicles as a new tool for single biomolecule spectroscopy. *J Phys Chem B* **105**: 12165–12170
- Brunker AT (2007) Version 1.2 of the crystallography and NMR system. *Nat Protoc* **2**: 2728–2733
- Brunker AT, Strop P, Vrljic M, Chu S, Weninger KR (2011) Three-dimensional molecular modeling with single molecule FRET. *J Struct Biol* **173**: 497–505
- Cho M, Chung S, Heo SD, Ku J, Ban C (2007) A simple fluorescent method for detecting mismatched DNAs using a MutS-fluorophore conjugate. *Biosens Bioelectron* **22**: 1376–1381
- Choi UB, McCann JJ, Weninger KR, Bowen ME (2011) Beyond the random coil: stochastic conformational switching in intrinsically disordered proteins. *Structure* **19**: 566–576
- Choi UB, Strop P, Vrljic M, Chu S, Brunker AT, Weninger KR (2010) Single-molecule FRET-derived model of the synaptotagmin 1-SNARE fusion complex. *Nat Struct Mol Biol* **17**: 318–324
- DeRocco V, Anderson V, Piehler J, Erie DA, Weninger K (2010) Four-color single-molecule fluorescence with noncovalent dye labeling to monitor dynamic multimolecular complexes. *Biotechniques* **49**: 807–816
- Drotschmann K, Topping RP, Clodfelter JE, Salsbury FR (2004) Mutations in the nucleotide-binding domain of MutS homologs uncouple cell death from cell survival. *DNA Repair (Amst)* **3**: 729–742
- Drotschmann K, Yang W, Kunkel TA (2002) Evidence for sequential action of two ATPase active sites in yeast Msh2-Msh6. *DNA Repair (Amst)* **1**: 743–753
- Fishel R (1999) Signaling mismatch repair in cancer. *Nat Med* **5**: 1239–1241
- Gopich I, Szabo A (2005) Theory of photon statistics in single-molecule Forster resonance energy transfer. *J Chem Phys* **122**: 14707
- Gorman J, Chowdhury A, Surtees JA, Shimada J, Reichman DR, Alani E, Greene EC (2007) Dynamic basis for one-dimensional DNA scanning by the mismatch repair complex Msh2-Msh6. *Mol Cell* **28**: 359–370

- Gradia S, Acharya S, Fishel R (1997) The human mismatch recognition complex hMSH2-hMSH6 functions as a novel molecular switch. *Cell* **91**: 995–1005
- Gradia S, Subramanian D, Wilson T, Acharya S, Makhov A, Griffith J, Fishel R (1999) hMSH2-hMSH6 forms a hydrolysis-independent sliding clamp on mismatched DNA. *Mol Cell* **3**: 255–261
- Graneli A, Yeykal CC, Prasad TK, Greene EC (2006) Organized arrays of individual DIVA molecules tethered to supported lipid bilayers. *Langmuir* **22**: 292–299
- Hargreaves VV, Shell SS, Mazur DJ, Hess MT, Kolodner RD (2010) Interaction between the Msh2 and Msh6 nucleotide-binding sites in the *Saccharomyces cerevisiae* Msh2-Msh6 complex. *J Biol Chem* **285**: 9301–9310
- Haugland RP (2005) *The Handbook: A Guide to Fluorescent Probes and Labeling Technologies*. Carlsbad, California: Molecular Probes
- Heinen CD, Cyr JL, Cook C, Punja N, Sakato M, Forties RA, Lopez JM, Hingorani MM, Fishel R (2011) Human MSH2 (hMSH2) Protein Controls ATP Processing by hMSH2-hMSH6. *J Biol Chem* **286**: 40287–40295
- Hsieh P, Yamane K (2008) DNA mismatch repair: molecular mechanism, cancer, and ageing. *Mech Ageing Dev* **129**: 391–407
- Iaccarino I, Marra G, Palombo F, Jiricny J (1998) hMSH2 and hMSH6 play distinct roles in mismatch binding and contribute differently to the ATPase activity of hMutSalpha. *EMBO J* **17**: 2677–2686
- Iyer RR, Pluciennik A, Burdett V, Modrich PL (2006) DNA mismatch repair: functions and mechanisms. *Chem Rev* **106**: 302–323
- Jacobs-Palmer E, Hingorani MM (2007) The effects of nucleotides on MutS-DNA binding kinetics clarify the role of MutS ATPase activity in mismatch repair. *J Mol Biol* **366**: 1087–1098
- Jeong C, Cho WK, Song KM, Cook C, Yoon TY, Ban C, Fishel R, Lee JB (2011) MutS switches between two fundamentally distinct clamps during mismatch repair. *Nat Struct Mol Biol* **18**: 379–385
- Jiang Y, Marszalek PE (2011) Atomic force microscopy captures MutS tetramers initiating DNA mismatch repair. *EMBO J* **30**: 2881–2893
- Joshi A, Sen S, Rao BJ (2000) ATP-hydrolysis-dependent conformational switch modulates the stability of MutS-mismatch complexes. *Nucleic Acids Res* **28**: 853–861
- Junop MS, Obmolova G, Rausch K, Hsieh P, Yang W (2001) Composite active site of an ABC ATPase: MutS uses ATP to verify mismatch recognition and authorize DNA repair. *Mol Cell* **7**: 1–12
- Kato R, Kataoka M, Kamikubo H, Kuramitsu S (2001) Direct observation of three conformations of MutS protein regulated by adenine nucleotides. *J Mol Biol* **309**: 227–238
- Kunkel TA, Erie DA (2005) DNA mismatch repair. *Annu Rev Biochem* **74**: 681–710
- Lamers MH, Georgijevic D, Lebbink JH, Winterwerp HH, Agianian B, de Wind N, Sixma TK (2004) ATP increases the affinity between MutS ATPase domains. Implications for ATP hydrolysis and conformational changes. *J Biol Chem* **279**: 43879–43885
- Lamers MH, Perrakis A, Enzlin JH, Winterwerp HH, de Wind N, Sixma TK (2000) The crystal structure of DNA mismatch repair protein MutS binding to a G x T mismatch. *Nature* **407**: 711–717
- Lamers MH, Winterwerp HH, Sixma TK (2003) The alternating ATPase domains of MutS control DNA mismatch repair. *EMBO J* **22**: 746–756
- Lebbink JH, Fish A, Reumer A, Natrajan G, Winterwerp HH, Sixma TK (2010) Magnesium coordination controls the molecular switch function of DNA mismatch repair protein MutS. *J Biol Chem* **285**: 13131–13141
- Lebbink JH, Georgijevic D, Natrajan G, Fish A, Winterwerp HH, Sixma TK, de Wind N (2006) Dual role of MutS glutamate 38 in DNA mismatch discrimination and in the authorization of repair. *EMBO J* **25**: 409–419
- Li GM (2003) DNA mismatch repair and cancer. *Front Biosci* **8**: d997–d1017
- Li GM (2008) Mechanisms and functions of DNA mismatch repair. *Cell Res* **18**: 85–98
- Li Y, Augustine GJ, Weninger K (2007) Kinetics of complexin binding to the SNARE complex: correcting single molecule FRET measurements for hidden events. *Biophys J* **93**: 2178–2187
- Martik D, Baitinger C, Modrich P (2004) Differential specificities and simultaneous occupancy of human MutSalpha nucleotide binding sites. *J Biol Chem* **279**: 28402–28410
- Mazur DJ, Mendillo ML, Kolodner RD (2006) Inhibition of Msh6 ATPase activity by mispaired DNA induces a Msh2 (ATP)-Msh6 (ATP) state capable of hydrolysis-independent movement along DNA. *Mol Cell* **22**: 39–49
- McCann JJ, Choi UB, Zheng L, Weninger K, Bowen ME (2010) Optimizing methods to recover absolute FRET efficiency from immobilized single molecules. *Biophys J* **99**: 961–970
- Mendillo ML, Putnam CD, Mo AO, Jamison JW, Li S, Woods Jr VL, Kolodner RD (2010) Probing DNA- and ATP-mediated conformational changes in the MutS family of mispair recognition proteins using deuterium exchange mass spectrometry. *J Biol Chem* **285**: 13170–13182
- Modrich P (2006) Mechanisms in eukaryotic mismatch repair. *J Biol Chem* **281**: 30305–30309
- Monti MC, Cohen SX, Fish A, Winterwerp HH, Barendregt A, Friedhoff P, Perrakis A, Heck AJ, Sixma TK, van den Heuvel RH, Lebbink JH (2011) Native mass spectrometry provides direct evidence for DNA mismatch-induced regulation of asymmetric nucleotide binding in mismatch repair protein MutS. *Nucleic Acids Res* **39**: 8052–8064
- Mukherjee S, Feig M (2009) Conformational change in MSH2-MSH6 upon binding DNA coupled to ATPase activity. *Biophys J* **96**: L63–L65
- Natrajan G, Lamers MH, Enzlin JH, Winterwerp HH, Perrakis A, Sixma TK (2003) Structures of *Escherichia coli* DNA mismatch repair enzyme MutS in complex with different mismatches: a common recognition mode for diverse substrates. *Nucleic Acids Res* **31**: 4814–4821
- Obmolova G, Ban C, Hsieh P, Yang W (2000) Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. *Nature* **407**: 703–710
- Salsbury Jr. FR (2009) Effects of Cisplatin binding to DNA on the dynamics of the *E. Coli* MutS dimer. *Protein Pept Lett* **17**: 744–750
- Sass LE, Lanyi C, Weninger K, Erie DA (2010) Single-molecule FRET TACKLE reveals highly dynamic mismatched DNA-MutS complexes. *Biochemistry* **49**: 3174–3190
- Studamire B, Quach T, Alani E (1998) *Saccharomyces cerevisiae* Msh2p and Msh6p ATPase activities are both required during mismatch repair. *Mol Cell Biol* **18**: 7590–7601
- Tessmer I, Yang Y, Zhai J, Du C, Hsieh P, Hingorani MM, Erie DA (2008) Mechanism of MutS searching for DNA mismatches and signaling repair. *J Biol Chem* **283**: 36646–36654
- Wang H, Yang Y, Schofield MJ, Du C, Fridman Y, Lee SD, Larson ED, Drummond JT, Alani E, Hsieh P, Erie DA (2003) DNA bending and unbending by MutS govern mismatch recognition and specificity. *Proc Natl Acad Sci USA* **100**: 14822–14827
- Warren JJ, Pohlhaus TJ, Changela A, Iyer RR, Modrich PL, Beese LS (2007) Structure of the human MutSalpha DNA lesion recognition complex. *Mol Cell* **26**: 579–592
- Winkler I, Marx AD, Lariviere D, Heinze RJ, Cristovao M, Reumer A, Curth U, Sixma TK, Friedhoff P (2011) Chemical trapping of the dynamic MutS-MutL complex formed in DNA mismatch repair in *Escherichia coli*. *J Biol Chem* **286**: 17326–17337