Atomic force microscopy captures MutS tetramers initiating DNA mismatch repair

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In spite of extensive research, the mechanism by which MutS initiates DNA mismatch repair (MMR) remains controversial. We use atomic force microscopy (AFM) to capture how MutS orchestrates the first step of E. coli MMR. AFM images captured two types of MutS/DNA complexes: single-site binding and loop binding. In most of the DNA loops imaged, two closely associated MutS dimers formed a tetrameric complex in which one of the MutS dimers was located at or near the mismatch. Surprisingly, in the presence of ATP, one MutS dimer remained at or near the mismatch site and the other, while maintaining contact with the first dimer, relocated on the DNA by reeling in DNA, thereby producing expanding DNA loops. Our results indicate that MutS tetramers composed of two non-equivalent MutS dimers drive E. coli MMR, and these new observations now reconcile the apparent contradictions of previous ‘sliding’ and ‘bending/looping’ models of interaction between mismatch and strand signal.

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Introduction

DNA mismatch repair (MMR) is a conserved pathway targeting mismatched base pairs that arise through DNA replication errors and during genetic recombination (Modrich, 1987; Modrich and Lahue, 1996; Kunkel and Erie, 2005; Iyer et al., 2006). In E. coli, the strand specificity necessary for removal of DNA biosynthetic errors from the daughter strand is based on the transient absence of d(GATC) methylation in newly synthesized DNA (Pukkila et al., 1983). Genetic and biochemical experiments have implicated 11 gene products in the methyl-directed reaction (Iyer et al., 2006) and repair in this system has been reconstituted using purified proteins (Lahee et al., 1989; Cooper et al., 1993; Burdett et al., 2001; Viswanathan et al., 2001). Repair is initiated by the binding of MutS to a mismatch or to a small insertion–deletion loop (Allen et al., 1997). Subsequently, ATP-, MutS-, and MutL-dependent activation of MutH endonuclease results in an incision of the unmethylated strand at a d(GATC) site that can reside at a distance of 1000 bp or more from the mismatch, producing a strand break either 3’ or 5’ to the mismatch. An excision system that includes DNA helicase II and an appropriate single-strand exonuclease is loaded at the strand break in a MutS- and MutL-dependent manner. Excision directed by a 3’-strand break depends on the 3’-5’ hydrolytic activity of exonuclease I, exonuclease VII, or exonuclease X, while the 5’-3’ activity of either RecJ exonuclease or exonuclease VII can support hydrolysis directed by a 5’-strand break. Following excision of the unmethylated strand to a point beyond the mismatch, the excised DNA segment is resynthesized and covalent continuity is restored to the repaired strand by the action of DNA ligase.

A key unanswered question with respect to MMR in E. coli is how MutS directs MutH to incise at a site, which can be separated by a considerable distance (1000 bp or more) from the mismatch. And after the incision, how the orientation-dependent loading of the excision system occurs? Three different models have been proposed to explain the interactions of the mismatch and incision sites. One postulates ATP-dependent diffusion (Gradia et al., 1999; Acharya et al., 2003) and hydrolysis-dependent unidirectional translocation (Allen et al., 1997; Blackwell et al., 2001b) of MutS or of a MutS/MutL complex along the helix contour between the mismatch and the strand signal. Another model suggests that mismatch recognition by MutS triggers polymerization of a second protein along the helix between the two DNA sites (Modrich, 1987; Hall et al., 2001). The third model proposes that the two sites interact by a DNA bending mechanism, with MutS remaining at or near the mismatch; this model does not require signalling along the helix contour. And a variant of this model includes the supposition that MutS bound to a mismatch site in trans can activate MutH (Junop et al., 2001; Schofield et al., 2001; Wang and Hays, 2004).

MutS-mediated loops in DNA have been observed by electron microscopy and atomic force microscopy (AFM; Allen et al., 1997; Iia et al., 2008); however, it is not clear whether the MutS oligomer involved in loop formation is a dimer, tetramer, or some other oligomeric structure. This point, namely, the oligomeric state of MutS in MMR, is another aspect of the MMR mechanism that remains controversial. The existence of tetramers of MutS has been demonstrated by analytical ultracentrifugation and inferred from gel electrophoresis assays (Bjornson et al., 2003; Mendillo et al., 2007). However, studies with MutS mutants defective in tetramer formation have yielded conflicting results, with some studies indicating severe defects in key MMR functions (Bjornson et al., 2003; Calmann et al., 2005;
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Manelyte et al, 2006; Huang and Crothers, 2008), while other researchers observed smaller MMR defects (Mendillo et al., 2007). Therefore, some researchers propose that MutS dimers have the key role in E. coli MMR (Mendillo et al., 2007), while others suggest that the functional unit is a tetramer (Huang and Crothers, 2008).

AFM allows direct imaging of DNA, proteins, and their complexes at the single molecule level (Hansma et al., 2004) and is ideal for visualizing various DNA transactions (Mikhelkin et al., 2006; Shlyakhtenko et al., 2007). So far, however, AFM has only been rarely employed in the investigations of MMR, whose primary focus was on unravelling the mechanism by which MutS detects mismatches (Wang et al., 2003; Jia et al., 2008; Tessmer et al., 2008).

In this study, we rechecked the recognition mechanisms of MutS detecting mismatch in detail by AFM. We captured free MutS oligomeric assemblies and characterized their interactions with homoduplex and heteroduplex DNA molecules in the presence of various nucleotides, as well as in the absence or presence of protein ‘end blocks’ or ‘roadblocks’. Our results show that MutS binds to mismatched DNA in several configurations, including one that results in the formation of a DNA ‘loop’ with two closely associated MutS dimers at its base. DNA loops occurred 5–18 times more frequently on mismatch-containing DNA than on homoduplex DNA, and increased in size when incubated in the presence of ATP. Importantly, our results show that in the presence of ATP, one MutS dimer remained at or near the mismatch site, while the other relocated on the DNA while maintaining close contact with the first dimer. Our observations provide evidence for the previously hypothesized non-equivalence of two MutS dimers within the tetramer (Obmolova et al., 2000; Bjorson and Modrich, 2003; Natrajan et al., 2003). These findings provide new mechanistic insights into how the two critical MMR DNA sites interact. This interaction is likely mediated not by one but by two MutS dimers and a DNA looping mechanism in which one MutS dimer moves along the helix in an ATP enhanced process.

Results

We used volumetric analysis (Ratcliff and Erie, 2001; Horcas et al., 2007) of AFM images to determine the oligomeric states of free MutS at near-physiological ionic strength and MutS concentration. Figure 1A shows a typical AFM image of MutS on fresh mica in the absence of any nucleotide; Figure 1B is a treated image using the flooding method (Horcas et al., 2007) showing magnified oligomeric structures of MutS in the boxed area of Figure 1A. The distribution of the measured volumes of MutS complexes in a large number of images is shown in the distribution histogram in Figure 1C. At the same time, the inset figure in Figure 1C shows the distribution histogram of the MutS at the experimental condition except ATP was added as comparison. Fitting Gaussian distributions to three independent volume distributions reveals that the measured MutS volumes fall into three independent distributions with peak values of 63 ± 16, 187 ± 37, and 414 ± 57 nm³ (mean ± s.d.).

By measuring AFM volumes of a set of reference proteins (see Supplementary Figure S1), we obtained a linear relationship between the measured volumes and their molecular weights (Ratcliff and Erie, 2001). Using this relationship as a standard, we determined the molecular weights of MutS complexes in images similar to those shown in Figure 1. The molecular weights determined by this method for the MutS complexes observed (92 ± 10, 165 ± 22, and 300 ± 34 kDa) correspond closely to the values of 95, 190, and 380 kDa expected for MutS monomer, dimer, and tetramer, respectively. This result strongly suggests that the MutS oligomeric assemblies represented by the three peak volume distributions observed in our AFM images are MutS monomers, dimers, and tetramers. These assemblies could be cleary identified by visual inspection of AFM images of MutS (see spots labelled M as monomer, D as dimer, and T as tetramer in Figure 1B). MutS tetramers appeared in two different forms: one appeared as a simple association of two dimers (marked T1 in Figure 1B); the other resembled an amalgamation of two dimers into a larger particle (T2 in Figure 1B). These two apparent forms (T1 and T2) have similar volumes as measured by AFM (data not shown) and their different appearance could be a consequence of different orientations of tetramers on the mica surface.

The number of MutS molecules in each peak in the volume distribution was integrated, and the integrated values were multiplied by the degree of oligomerization to obtain the weight percentages of MutS in each of the oligomeric structures observed (Figure 1D). In the absence of nucleotide, 41 ± 2% of the MutS particles had volumes similar to that expected for a dimer; substantial amounts of monomer- and tetramer-sized MutS (28 ± 3 and 31 ± 4%, respectively) were also seen in Figure 1D. The inclusion of all adenine nucleotides (ADP, ATP, AMPPnP, or a mixture of equal parts ADP and AMPPnP) in the incubation solution containing MutS decreased the amount of monomer observed from 28 to ~5% and increased the relative amounts of dimer and tetramer to ~53 and ~42% shown in Figure 1D. Although all nucleotides had a significant effect on the proportion of monomer present, there were only small differences in the proportions of monomer, dimer, and tetramer depending on which nucleotide was present.

Figure 2 shows histograms representing the percentage of MutS molecules found in each of the three oligomeric states after incubation of MutS with linear homoduplex or G-T heteroduplex DNA (41 or 201 bp in length). The presence of 41-bp DNA (homoduplex or G-T mismatch) either with or without added nucleotides had minimal effect on the distribution of MutS aggregation states (Figure 2A). However, inclusion of 201-bp DNA significantly increased the percentage of MutS appearing as tetramer, particularly in the presence of adenine nucleotide (Figure 2B). In the presence of both 201-bp DNA and 0.5 mM ADP, ATP, AMPPnP, or ADP + AMPPnP, the proportion of MutS existing as apparent tetramers increased from ~40% to ~70%. Thus, at a MutS concentration of 100 nM, close to its estimated in vivo concentration (Feng et al., 1996), tetramers were the main form observed for MutS bound to DNA. The results of the control experiments (data not shown) confirmed that, when the concentrations of MutS and 41-bp DNA or 201-bp DNA were changed, the change in the ratio of dimer to tetramer was minimal. This observation supports the conclusion that the percentages of MutS dimers and tetramers were not affected by the concentration of DNA, but rather by the length of the DNA.
In order to address the interaction of MutS with DNA in further detail, images were prepared after incubation of MutS with linear 1120-bp heteroduplex DNA containing a single G-T mismatch (for a detailed description of this DNA, see Supplementary Figure S2). Two types of MutS/DNA complex were observed in these experiments. The first involved MutS binding at a single location (single-site binding). MutS proteins involved in single-site binding might be dimers or tetramers (see Table I), and most were located close to the mismatch site. Figure 3A and C show examples of single-site binding of MutS in the absence of any nucleotide and in the presence of ATP, respectively, to a G-T mismatch-containing DNA with two biotinylated ends which had been previously bound with monofunctional streptavidin (Howarth et al., 2006) before incubation with MutS. Experiments were also performed using single-end-biotinylated DNA that had been previously incubated with streptavidin, and with DNA that had not been incubated with streptavidin. Supplementary Figure S3 shows a detailed analysis of the distribution of single-site-bound MutS on streptavidin-bound biotinylated DNAs. Single-site-bound MutS can be divided into two groups: specific bound and non-specific bound. The specific-bound MutS located at the relative position of 50 ± 6 and 50 ± 5% of the relative DNA length before and after ATP was added (Supplementary Figure S3C and D).

The second type of MutS/DNA complex that was observed frequently promoted a segment of DNA to form a loop, at the base of which were situated two closely associated MutS dimers or a MutS tetramer. Figure 3B illustrates MutS/DNA loop binding in the absence of nucleotide. Under these conditions, loops formed most frequently by two easily distinguishable, closely associated MutS dimers. When ATP was present, loop formation was most often mediated by a MutS tetramer that covered the DNA strands at the base of the loop (Figure 3D).

Figure 1 Volume analysis of MutS. MutS (100 nM) was incubated in the absence of a nucleotide in 20 mM Tris–HCl (pH 7.5), 100 mM K+ glutamate, 5 mM MgCl2, and 0.4 mM DTT for 10 min before deposition on mica, washing, and air-drying as described in Materials and methods. (A) A typical AFM image showing MutS on mica; the scan size of this image is 2 × 2 μm2. (B) An enlargement of the area shown in the blue box of image (A). The mica surface was flooded by a blue colour to make MutS more apparent. The size of this image is 0.5 × 0.5 μm2. Protein particles labelled ‘M’, ‘D’, and ‘T’ were identified as a MutS monomer, dimer, and tetramer, respectively, by further comparison with the volumes of proteins of known molecular weights (Supplementary Figure S1). (C) The histogram showing the volume distribution of MutS particles measured in images similar to those shown in (A). The inset figure shows the volume distribution of MutS particles in the presence of ATP for comparison. Three individual peaks were verified as monomer, dimer, and tetramer, respectively, according to their volumes. (D) Percentage histogram showing the percentage of MutS found in each oligomeric state in the presence of various nucleotides (0.5 mM). The bars, from left to right, indicate no nucleotide (red), ADP (green), AMP/PnPP (purple), half ADP and half AMP/PnPP (yellow), and ATP (blue). Error bars indicate ± s.d. The bar graphs are percentages of MutS monomer presented as each species.
The oligomeric forms of MutS bound to 1120-bp DNA in single-site and loop binding were verified by AFM volume measurement (Table I). In the absence of ATP, the statistical distribution of the volumes of single-site-bound MutS yielded two peaks, one corresponding closely to the expected molecular weight of a MutS dimer (181 ± 27 kDa) and the other to the expected molecular weight of a MutS tetramer (361 ± 29 kDa). The calculated molecular weight of loop-bound MutS almost always corresponded to that of a tetramer (365 ± 42 kDa). In the presence of ATP, MutS dimers and tetramers appeared larger than in its absence (Table I). Single-site-bound MutS exhibited an apparent increase of 28–45% in volume, while free MutS appeared 17–20% larger (Table I). Furthermore, while in the absence of ATP loops appeared to contain two associated MutS dimers, the outline of each of which was separately distinguishable (example shown in Figure 3B), in the presence of ATP the two MutS dimers at the base of loops appeared to merge into a more unified structure (Figure 3D), with an average volume ~28% larger than that of the loop-bound tetramers seen in the absence of ATP. These phenomena may indicate a conformational change in MutS that depends on the presence of both ATP and DNA.

In order to determine the effect of ATP on loop size, we incubated MutS with 1120-bp G-T DNA for 10 min in the absence and presence of ATP (Figures 3 and 4). We then measured the resulting contour lengths of the DNA segments representing the ‘short leg’, ‘loop contour’, and ‘long leg’ of the DNA. Supplementary Figure S2F–G provides details of how the length of loop contour, short leg, and long leg were defined and measured. Figure 4A and E show the relative length distribution of the short legs, loop contours, and long legs of double-end-biotinylated DNA previously bound with streptavidin after incubation with MutS for 10 min in the absence or presence of ATP. The lengths of short legs, loop contours, and long legs were measured and fitted with Gaussian distributions (Figure 4B–D and F–H). In the absence of ATP, the lengths of the long legs fall into a Gaussian distribution centered at 46 ± 5% of the DNA length (Figure 4D). When ATP was included, the lengths of long leg remained centered at 47 ± 9% but the distribution broadened (Figure 4H). A comparison of Figure 4D and H shows that in the absence of nucleotide, the standard deviation is 5, while in the presence of ATP, the standard deviation increased to 9. However, the most important finding is that the mean of the Gaussian distribution of the loop contour length increased by about 15% (22 ± 5% for no nucleotide in Figure 4C and 37 ± 11% for adding ATP in Figure 4G) of the total DNA.

### Table 1

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Values represent mean ± s.d.; N indicates the number of proteins measured; P-values were obtained using Student’s t-test. *This type of MutS is rare. For a free MutS sample, MutS (100 nM) was incubated in a buffer containing 20 mM Tris–HCl (pH 7.5), 100 mM K⁺ glutamate, 5 mM MgCl₂, and 0.4 mM DTT for 5 min before deposition on mica, and the washing and air-drying was as described in Materials and methods. For MutS bound on DNA, MutS (50 nM) was incubated with 1120-bp G-T heteroduplex DNA (10 nM) in buffer containing 20 mM Tris–HCl (pH 7.5), 100 mM K⁺ glutamate, 5 mM MgCl₂, 0.4 mM DTT with or without 0.5 mM ATP for 5 min. The average volumes of MutS images in AFM and the corresponding molecular weights shown in the table were determined as described in the text and in Materials and methods.
length. And consequently, the length of short leg decreased from $32 \pm 4\%$ (Figure 4B) to $18 \pm 7\%$ (Figure 4F).

Because the end of the long leg remained at almost the same position ($47$–$53\%$) in the presence or absence of ATP, the MutS dimer associated with the long leg remained at the mismatch site. However, when ATP was added, the length of the short leg became shorter and the size of the DNA loop increased.

A series of experiments were performed in which biotinylated DNA that was previously bound with streptavidin was incubated with MutS in the presence of various nucleotides. The lengths of the short legs, loop contours, and long legs of the resulting loops were measured and compared, by fitting a Gaussian distribution to histograms representing the distribution of the percentage of the DNA length represented by each segment. The results of these experiments (Table II) show that time, temperature, and specific nucleotide all affect the growth of the loops. When MutS and DNA were incubated in the presence of 0.5 mM ADP, average loop size was slightly smaller than that in the absence of nucleotide, and the loops were somewhat more homogeneous in size. Loop size after adding AMPPnP for 10 min was bigger (average increase 107 bp) than the loop generated at 'no nucleotide' condition and was similar to the loop size after incubation with ATP. Loops that formed on homoduplex DNA showed a bigger size and broader distribution than loops on G-T mismatch substrates.

For DNA that contained biotin at both ends, it is impossible to identify one end of the DNA from another. The mismatch position could be either 47 or 53\% of the DNA length from the end in the looped complexes. Because of this uncertainty, the distribution for specific bindings that we determined was relatively broad. In order to eliminate this uncertainty, experiments were also performed using single-end-biotinylated DNA that had been previously bound with monovalent streptavidin on the single end. In this experiment, the mismatch position can be easily identified; it is located 53\% of the DNA length from its streptavidin-labelled end. Supplementary Figure S2B provides detailed structure of this DNA.

The results are shown in Figures 5 (no ATP) and 6 (with ATP). Importantly, the DNA loops originated at or very close to the position of the mismatch. Some loops were formed between the mismatch and the biotinylated end while some were formed between the mismatch and the free DNA end. We analysed these cases separately (Figure 5A). For DNA molecules carrying the loops between the mismatch and the free DNA end (lower part of Figure 5A), the lengths of the long legs fall into a narrow Gaussian distribution centered exactly at $53 \pm 3\%$ of the DNA length (Figure 5D) coinciding with the location of the mismatch. For DNA molecules carrying a loop between the mismatch and the biotinylated end (upper part of Figure 5A), the length of the long legs is $46 \pm 5\%$ (Figure 5G) again, consistent with the location of

Figure 3 AFM images showing single-site binding and loop binding of MutS to heteroduplex DNA. (A, B) Images of MutS deposited in the absence of nucleotide. Images in (A) show MutS single-site binding on DNA; those in (B) show MutS loop binding. (C, D) Images of MutS deposited in the presence of 0.5 mM ATP. Images in (C) shows MutS single-site binding on DNA, while (D) shows loop binding. In both cases, the ends of DNA were previously bound with streptavidin. The scale bar indicates 200 nm.
When ATP was included, as shown in Figure 6, the distribution of the long leg lengths broadened but remained centered at 54 ± 5% (Figure 6D) and 48 ± 8% (Figure 6G) of the DNA length. At the same time the mean of the loop contour length increased by additional 20% of the DNA length when the loops formed between the mismatch and the free DNA end (Figure 6C) and by additional 13% of the DNA length (Figure 6F) when the loops formed on the mismatch. When ATP was included, as shown in Figure 6, the distribution of the long leg lengths broadened but remained centered at 54 ± 5% (Figure 6D) and 48 ± 8% (Figure 6G) of the DNA length. At the same time the mean of the loop contour length increased by additional 20% of the DNA length when the loops formed between the mismatch and the free DNA end (Figure 6C) and by additional 13% of the DNA length (Figure 6F) when the loops formed on the...
other side of the mismatch. A detailed analysis of the distribution of single-site-bound MutS on single-end-biotinylated DNAs previously bound with streptavidin is shown in Supplementary Figure S4. These results show that the MutS molecules that bound on one end of the loops remained on the mismatch whether or not ATP was present. When ATP was present, the short leg became shorter and the DNA loop size increased, consistent with the results obtained on double-end-biotinylated DNA (Figure 4). Furthermore, we observed that DNA loops can form and grow on both sides of the mismatch, which suggests that there is no preferred direction for MutS tetramers to produce and expand loops.

To determine whether internal roadblocks on the DNA could prevent the increases in loop size, we used DNA previously bound with EcoRI. In the absence of ATP, the distribution of MutS showed no big difference whether or not EcoRI was added (Supplementary Figure S5). Also, the average size of loops formed in the presence of EcoRI and ATP was considerably larger than that measured in samples incubated with EcoRI and with ADP or with EcoRI but with no added nucleotide (Supplementary Figure S6; Table I). However, the loops formed in the presence of EcoRI blocks on MutS-bound DNA during the application of ATP for 10 min were on average smaller as compared with the loops formed on DNA under the same conditions but without EcoRI (a decrease from 37 to 31%; Table II; Supplementary Figure S6). This result suggests that the binding of EcoRI protein on DNA does prevent the increase in loop size. On DNA previously bound with EcoRI and subsequently incubated with MutS and ATP, loops which contained a protein (most likely EcoRI) within the loop contour were sometimes seen, but loops with proteins only at the loop base and no visible proteins within the loop contour were much more frequent (74%) (Figure 7).

The dissociation constant ($K_D$) and the occupancy of DNA by MutS under different conditions were determined from AFM images and the data are presented in Supplementary Figure S7 and Supplementary Table S1. Our results are consistent with previous reports (Yang et al., 2003; Huang and Crothers, 2008), when one considers various lengths of DNA substrates used in various studies.

### Discussion

MutS oligomeric states have been studied by means of ultracentrifugation, velocity sedimentation, and gel filtration. Previous studies have shown that MutS undergoes dimer-to-tetramer assembly, though the association constants for MutS dimers to tetramers are inconsistent (Bjornson et al., 2003; Lamers et al., 2004). In our current work, we evaluated the MutS dimer-to-tetramer conversion in the concentration range of 40–200 nM. Our results show that after deposition on mica from a solution containing 100 nM MutS (as monomer) in a buffer of comparable ionic strength to intracellular conditions (Feng et al., 1996), five-fold lower than the estimated in vivo MutS concentration (500 nM) in exponentially growing *E. coli* cells, approximately equal proportions of MutS exist in dimer and tetramer forms.

In the absence of any nucleotide, ~30% of the MutS monomers was identified, and the percentage dropped significantly to ~5% in the presence of all the nucleotides used. This result supports earlier observations about nucleotides’ role in MutS dimerization (Lamers et al., 2004). The association constant for the dimer-to-tetramer transition is about $1.3 \times 10^7$ M$^{-1}$, which is close to the value $(2.1 \times 10^7$ M$^{-1}$) obtained by previous biochemical studies (Bjornson et al., 2003).

The addition of the adenosine nucleotide at concentrations expected to saturate both high-affinity (ATP or ADP) and low-affinity (ATP or ADP) binding sites on the two monomers within a dimer of MutS (Bjornson and Modrich, 2003), or of a mixture of the two nucleotides at saturating concentration, did not significantly alter the relative proportions of dimer and tetramer observed. When 201-bp DNA in length was included in the incubation, tetramer was the main form of MutS observed, and when both nucleotide and 201-bp DNA were present, the equilibrium was markedly shifted in favour of tetramers. Based on the AFM measurement alone, we do not know how much of the MutS was bound to DNA because the 41- and 201-bp DNA are too small to be imaged by AFM if they are bound to MutS. But based on the concentrations of both MutS and DNA used in our experiments (100 nM of MutS as monomer and 200 nM of DNA) and the known $K_D$ of MutS/DNA complexes (21 nM) (Blackwell et al., 2001a), we can estimate that at this condition, ~90% of MutS (93.8%) was occupied by DNA. These results suggest that the interaction of MutS with 201-bp DNA promotes the formation of tetramers, and that tetramer formation of MutS in complex with DNA is influenced by the interaction of MutS with adenosine nucleotides. The reason that the association constant for the dimer-to-tetramer transition did not change after 41-bp DNA was included might be that 41-bp DNA is too short to support binding of two MutS dimers side-by-side. However, the 201-bp DNA (both homoduplex and G-T mismatched) is long enough to accommodate and stabilize two dimers forming a tetramer structure. The similar
effect of homoduplex and G-T DNA on the dimer-to-tetramer transition suggests that MutS may scan DNA for mismatches as a tetramer. However, mechanistic details of the tetramerization step in the presence of DNA are presently missing and warrant further studies. We observed MutS tetramers bound to DNA in a configuration in which one of the MutS dimers forming the tetramer appeared to be closely associated with the mismatch site, while the other occupied a non-specific site. In the absence of any nucleotides, the loops are quite small and contain...
~250 bp of DNA. The DNA seems to have propensity to bend in this region, and this may promote loop formation for MutS interacting with DNA at two sites, mismatch and a non-specific site, or even two non-specific sites. Future projects will test whether a different DNA sequence with a lesser propensity for DNA bending may affect the frequency at which loops are formed. MutS molecules in this configuration were positioned at the base of DNA loops, the size of which increased in the presence of ATP. We also saw apparent MutS tetramers that appeared to associate with only a single site on the DNA. We most frequently observed loops on DNAs that had two biotinylated ends and that had been incubated with both streptavidin and EcoRIE111Q before incubation with MutS. The MutS occupancy of DNAs which were end blocked or which were additionally blocked by EcoRIE111Q at sites flanking the mismatch was about 2-3 fold higher than that of DNA that have not been blocked by any proteins in the presence of ATP; this suggests that MutS was ‘trapped’ on these DNAs by the presence of the protein blocks.

Initial attempts to evaluate ATP effects on MutS–DNA interaction were based in part on visualization of complexes of the E. coli protein with 6.4-kbp heteroduplex and homoduplex DNAs by electron microscopy (Allen et al., 1997). These experiments demonstrated the mismatch- and ATP-dependent formation of χ-shaped DNA loop structures up to several kbp in size, in which MutS were bound at the base. Loop size was found to increase with time, and in the majority of molecules, the mismatch was present in the loop. There are a number of differences between the loops observed in the present study and those observed by Allen et al. In the latter, formation of loops depended on the presence of ATP, while the loops we observed occurred in the absence of nucleotides as well as in the presence of ADP, ATP, and AMPPNP. The loops we observed were actually less frequent when ATP or an ATP analogue was included in the incubation than when no nucleotide or ADP was added; depending on the conditions, loops were up to 50% more frequent in the absence of ATP than in its presence. The only exception to the difference in the frequency of loop formation was where DNAs had been previously incubated with both streptavidin and EcoRIE111Q before binding to MutS. Second, complexes between MutS and homoduplex DNA were observed very infrequently by Allen et al (1997) and both loop binding and ‘no loop’ binding largely depended on the presence of a mismatch. In our study, loops were also seen on homoduplex DNA (2.4 ± 0.3%) with a specificity about 18.1 times less than that on heteroduplex DNA under the same conditions (Supplementary data; Supplementary Table S1).

In addition, the size of the loops on homoduplex DNA (40 ± 15%) was much bigger than that on heteroduplex DNA (22 ± 5%), and at the same time their size distribution was also three times broader as compared with the loops formed on heteroduplex DNA (Table II). Our interpretation of this result is that in the absence of a mismatch, neither of MutS dimers that participated in the loop formation had to remain near the center of homoduplex DNA. In the present study, the size of the loops increased when either ATP or the non-hydrolysable ATP analogue AMPPPNP was present, while in the study of Allen et al, non-hydrolysable ATP analogues failed to support large loop formation and ongoing loop growth was suppressed upon their addition to ATP-containing reactions. Another important difference between our results and those by Allen et al is that the DNA loops captured by AFM were on average significantly smaller than the loops captured by EM, which may be related to the smaller DNA substrate used in our study (1.12 versus 6.4 kbp).

Using AFM, Jia et al (2008) also reported similar χ-shaped loops formed by MutS. The loops they imaged were independent of whether or not a mismatch was present in the DNA substrate. Furthermore, they found MutS D835R (tetramer deficient) and R194A/R198A/R275A mutants of MutS formed χ-loops at a similar frequency as the wild-type MutS, suggesting that tetramer formation is not necessary for the formation of the loops in their system. However, that study did not provide evidence (e.g. by MutS volume measurements) that the MutS that formed χ-loops was in a dimer configuration.

Using fast-scanning AFM, Crampton et al (2007) visualized translocation and extruded looping by EcoP15I restriction enzyme, which is similar to MutS displays anomalously low rates of ATP hydrolysis. In order to efficiently cleave DNA, EcoP15I requires two recognition sites separated by up to 3.5 kbp. They observed that DNA loops were formed by contact between specific site-bound EcoP15I and a non-specific region of DNA. Further they showed that EcoP15I translocation must also occur because when it is blocked by a Lac repressor protein, DNA cleavage is inhibited. This observation provides a plausible mechanism by which an enzyme could communicate rapidly between two non-contiguous DNA sites through a combination of translocation-coupled and DNA looping mechanism.

Our data show that in the absence of ATP, both end binding by streptavidin and roadblocking by EcoRI had no effect on binding and looping. But in the presence of ATP, binding by streptavidin and EcoRI increased the binding affinity and the fraction of loop binding (Supplementary data). On DNA previously bound with EcoRIE111Q and subsequently incubated with MutS and ATP, 74% of loops was seen which did not contain EcoRIE111Q within the loop contour. These loops were on average smaller than those loops formed in the presence of ATP on DNA without EcoRIE111Q. Therefore, roadblocking by EcoRI seems to prevent the increase in loop size. Considering the residency half-life of EcoRIE111Q at a d(GAATTCC) sequence is <40 min (Pluciennik and Modrich, 2007), one cannot exclude that part of EcoRI

Figure 5 Normalized length distributions for MutS bound in loop configuration to heteroduplex DNA containing biotin at a single DNA end in the absence of ATP. MutS (100 nM) and 1120-bp G-T mismatch DNA (20 nM) that contained biotin at the end further from the mismatch site and that had been previously bound with monovalent streptavidin (see Materials and methods) were incubated in 20 mM Tris–HCl (pH 7.5), 100 mM K glutamate, 5 mM MgCl₂, and 0.4 mM DTT for 10 min at room temperature in the absence of ATP. Panel (A) shows the contour lengths of each segment on individual DNA molecules. The segment of DNA between MutS and the biotinylated end of the DNA is represented in blue, the loop is represented in red, and the segment of DNA between MutS and the other DNA end is represented in green. The position of the mismatch is indicated by a vertical dashed line. Two sets of loops can be observed from panel (A). Set one: the loops are between MutS and biotinylated ends, and set two: the loops are between MutS and free ends. The histograms (B–D) and (E–G) show Gaussian fits of the contour length data of these two sets of loops, respectively.
Figure 6 Normalized length distributions for MutS bound in loop configuration to heteroduplex DNA containing biotin at a single DNA end in the presence of ATP. MutS (100 nM) and 1120-bp G-T mismatch DNA (20 nM) that contained biotin at the end further from the mismatch site and that had been previously bound with monovalent streptavidin (see Materials and Methods) were incubated in 20 mM Tris–HCl (pH 7.5), 100 mM K⁺ glutamate, 5 mM MgCl₂, and 0.4 mM DTT for 10 min at room temperature in the presence of ATP. Panel (A) shows the contour lengths of each segment on individual DNA molecules. The colours of these segments have the same means as Figure 5. Two sets of loops can be observed from panel (A). Set one: the loops are between MutS and biotinylated ends, and set two: the loops are between MutS and free ends. The histograms (B–D) and (E–G) show Gaussian fits of the contour length data of these two sets of loops, respectively.
may dissociate and rebind within 10 min incubation in our experiments. This effect would explain the observation that 26% of DNA loops contained EcoRI E111Q inside the loop contour (Figure 7). These observations support the idea that MutS moves along the DNA contour by means of a translocation (sliding) mechanism. In addition, our results strongly suggest that at least part of the enzyme, possibly one dimer of a tetramer, must remain at or near its target site (mismatch site). Moreover, DNA is being reeled from one side of the mismatch site, thereby producing expanding DNA loops. Adding ATP has the effect of making the size of the loop increase, and present observations support MutS translocation which depends on the binding of ATP. Looking ahead into the future, whether or not MutS translocation is ATP hydrolysis dependent needs to be clarified. Moreover, when AMPPnP was added (in Table II), the loop size can expand too. It might imply that ATP hydrolysis is not completely necessary for this unidirectional ‘translocation’.

In conclusion, the AFM data presented here support the notion that MutS tetramers are involved in the early stages of MMR. MutS tetramers can be observed bound to heteroduplex DNA in a configuration in which one of the MutS dimers is closely associated with the mismatch site, while the other occupies a non-specific site. Both dimers are found at the base of DNA loops, the size of which increases in the presence of ATP. This growth is partially inhibited by protein roadblocks. Taken together, our results provide new insights into the mechanisms of MMR initiation in E. coli by MutS, and also reconcile the main features of sliding and looping models for the interaction of DNA mismatch and GATC sites.

Figure 7  AFM images showing MutS/DNA loops with and without other proteins bound inside the loop. MutS (100 nM in Tris–K+ glutamate buffer) was incubated with G-T heteroduplex DNA which had been previously bound with 50 nM streptavidin and 100 nM EcoRI E111Q. ATP was added after MutS. Panel (A) shows loops without any EcoRI E111Q within the loop contour; panel (B) shows loops with EcoRI inside. The percentage histogram in panel (C) indicates the frequency of each type of loop.

Materials and methods

Proteins
MutS (Blackwell et al., 2001a) and EcoRI E111Q (Wright et al., 1989) were purified by published methods. Expression plasmids for ‘live’ (wt) and ‘dead’ (N23A/S45A/S27D) streptavidin were obtained from Alice Ting, Mass. Inst. of Technology. Monovalent streptavidin containing one active (biotin binding) subunit and three inactive subunits was prepared in accord to Howarth et al. (2006).

DNA substrates
Linear 41- and 201-bp DNAs containing a G-T mismatch or an A/T base pair at a specific site were prepared as described previously (Blackwell et al., 2001a). Linear 1120-bp G-T mismatch and A-T homoduplex DNAs were prepared following the same protocol used by Pluciennik and Modrich (2007), except that 5'-TTACGCTTT CAGTCAAGAACCTTTACTT-3' and 5'-TTCTAGCCGGAGAGGG TAGCTATTTTG-3' were used as primers, and phages f1MR65 and f1MR66 were used as templates. For both 1120-bp DNAs, two EcoRI binding sites are present, one 40% and one 81% of the DNA length from the end. In the 1120-bp G-T substrate, the G-T mismatch is located 53% of the distance from the same end (see Supplementary Figure S2A–C). PCRs were performed in which one or both primers were biotinylated at the 5’ ends, so that the final products contained biotin at one end (single-biotin DNAs) or both ends (double-biotin DNAs). Since the position of the mismatch on the DNA was known and a specific end of the DNA length from the end. In the 1120-bp G-T substrate, the G-T mismatch is located 53% of the distance from the same end (see Supplementary Figure S2A–C). PCRs were performed in which one or both primers were biotinylated at the 5’ ends, so that the final products contained biotin at one end (single-biotin DNAs) or both ends (double-biotin DNAs). Since the position of the mismatch on the DNA was known and a specific end of the DNA could be identified by streptavidin bound to single-biotin DNA, we were able to discriminate between MutS bound at a mismatch (specific complex) and MutS bound at a homoduplex site (non-specific complex) on single-biotin DNAs, by measuring the distance from MutS to the ends of the DNA. For both heteroduplex and homoduplex DNA, they were annealed using the same method.

Binding of EcoRI E111Q MutS, and streptavidin to 41-, 201-, and 1120-bp DNAs
MutS was diluted in dilution buffer daily for each experiment (20 mM KPO4 pH 7.4, 150 mM KCl, 1 mM EDTA, and 1 mM DTT)
to a concentration of 0.5–2 μM monomer and kept on ice until use. DNA binding reactions (10 μl) contained 20 mM Tris–HCl pH 7.5, 100 mM K+ glutamate, 5 mM MgCl2, 0.4 mM DTT, 100 nM MutS, and 200 nM linear heteroduplex or homoduplex DNA (for 41- and 201-bp substrates) or 40–200 nM MutS and 5–20 nM DNA (for 1120-bp substrates). For comparison, in some experiments, HEPEs reaction buffer (50 mM HEPES-KOH pH 8.0, 20 mM KCl, 5 mM MgCl2, and 1 mM DTT) or phosphate reaction buffer (20 mM KPO4 pH 7.4, 20 mM KCl, 5 mM MgCl2, and 1 mM DTT) was used. Also, in some experiments, ATP, ADP, or AMPPPn was added to a final concentration of 0–2 mM. For EcoRIE111Q binding, 100 nM EcoRIE111Q (as dimers) was mixed with 10 nM 1120-bp DNA in Tris–K+ glutamate reaction buffer in a total volume of 10 μl. The solution was incubated at room temperature for 10 min. The solution was then either directly used for AFM imaging, or components were added before AFM imaging was performed. For streptavidin which was previously bound, 50 nM streptavidin was mixed with 10 nM DNA (previously bound with 100 nM EcoRIE111Q or mock previously bound) and the solution was incubated at room temperature for 10 min. EcoRIE111Q and streptavidin binding were evaluated by AFM imaging. The statistical results show that about 61% of DNA had both ends blocked by EcoRIE111Q. For double-biotin labelled DNA, 78% of DNA had both ends blocked by streptavidin. For single-biotin labelled DNA, 89% of DNA had one end blocked by streptavidin. These observations were not critical, because when contour lengths or a K0 was determined, only the DNA molecules with both ends blocked by streptavidin were counted (for experiments with double-biotin labelled DNA). Control reactions were performed in the absence of EcoRIE111Q as well as the absence of streptavidin. For MutS binding to 1120-bp DNA, MutS was incubated with DNA previously bound with EcoRIE111Q and/or streptavidin in a total volume of 10 μl at room temperature for 10 min, followed by addition of nucleotide from 10 s to 10 min. Following incubation, the mixture was immediately deposited directly onto an APS-mica surface.

AFM sample preparation and imaging

1-(3-Aminopropyl)silatrane-functionalized mica (APS-mica) was used as a substrate for the binding of MutS and DNA molecules. APS-mica was prepared as described by Shlyakhtenko et al. (2003). A 2–3 μl droplet of MutS/DNA solution was deposited on the APS-mica surface at room temperature for 1–3 s. The sample was immediately rinsed with deionized water and air-dried before imaging. Images were taken using a Nanoscope IIIa Multimode Scanning Probe Microscope (Veeco Instruments Inc., Santa Barbara, CA) in tapping mode with an E scanner. RTESP probes (Veeco) were used for imaging in the air. The spring constant of AFM cantilevers used for imaging in the air. The spring constant of AFM cantilevers was previously bound with EcoRIE111Q and/or streptavidin in a total volume of 10 μl at room temperature for 10 min, followed by addition of streptavidin in a total volume of 10 μl at room temperature for 10 min, followed by addition of nucleotide from 10 s to 10 min. Following incubation, the mixture was immediately deposited directly onto an APS-mica surface.

References


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