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α-Shaped DNA loops induced by MutS

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ABSTRACT

DNA mismatch repair (MMR) is critical for the maintenance of genomic stability. MMR is initiated by recognition of DNA mismatches by the protein, MutS, which subsequently recruits downstream repair factors. To better understand the mechanism by which MutS identifies and specifically binds mismatched basepairs embedded in random DNA sequences, we monitored the interaction between MutS and DNA substrates using atomic force microscopy (AFM). An α-shaped DNA loop formed by the interaction between MutS and DNA, which was independent of whether or not a mismatch was present in the DNA substrate. These data indicate that MutS associates with DNA non-specifically and forms an α-loop interaction with the DNA substrate. In this conformation, MutS is able to scan two arms of DNA simultaneously for each MutS dimer formed.

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The DNA mismatch repair (MMR) system corrects DNA replication errors and prevents recombination between divergent DNA sequences. The MMR system also mediates DNA lesion responses [1–3]. Dysfunctions in DNA MMR greatly increases spontaneous mutation rates, and in humans, can lead to hereditary forms of colon cancer and other sporadic tumors [3–6]. Therefore, the DNA MMR system is essential for maintaining genomic stability.

The methyl-directed mismatch repair system of *Escherichia coli* represents the most well-characterized MMR system studied to date. The process involves 11 proteins. MutS, MutL, and MutH are responsible for the initiation of MMR. The first step in the initiation of MMR, mismatch recognition, is performed by the protein, MutS, which can efficiently recognize and bind seven out of eight types of mispaired or 1–4 unpaired bases (except for C:C) [7]. However, considering that the rate of mismatch during DNA replication is approximately 1 out of 10^6–10^8 base pairs [8,9], how does MutS specifically bind to mismatches embedded in such a large excess of correctly paired sequence?

Since MutS specifically binds mismatches, it represents a site-specific DNA-binding protein. For these types of proteins, two models have been suggested for target recognition: (1) direct binding by three-dimensional diffusion of both protein and DNA; and (2) initial non-specific binding with subsequent one-dimensional diffusion of protein along DNA to a specific site [10,11]. It is unknown whether MutS binds a mismatched DNA region directly without any initial searching, or binds to DNA non-specifically at first, then searches for mismatches through mechanisms such as sliding [10,12–14]. The mechanism by which MutS specifically recognizes a mismatch is a critical aspect of its function since the information acquired in this step sets up the MMR process. To gain insight into these mechanistic hypotheses, we have examined the interaction between MutS and DNA using atomic force microscopy (AFM).

Previously, a translocation model was proposed by Allen et al. [15] in which MutS specifically binds to a mismatch, then MutL associates with the MutS-mismatch complex to activate the endonuclease activity of MutH through an α-loop mechanism. Similarly, our data indicate the formation of α-shaped DNA loops, although the appearance of an α-loop was not necessarily associated with mismatched DNA. Furthermore, we found that MutS associates with mismatched DNA regions when provided with extended incubation times, which corresponded with a decrease in α-loop frequency. Our novel observations provide further insight into the long-standing question of how MutS searches for mismatches embedded in long stretches of random DNA sequence.

Materials and methods

Materials. All chemicals were purchased from Sigma. Restriction enzymes, DNA polymerase (Pyrobest), and T4 DNA ligase were from Fermentas and Promega. Wizard® SV Gel and PCR Clean-Up System was from Promega. ALON™ metal affinity resin

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was from Novagen and Millipore Milli-Q water was used in all assays.

Mutagenesis and protein purification. Recombinant strain M15/pQE30-*mutS* was available from our lab. *E. coli* MutS mutants R194A/R198A/R275A and D835R were constructed by overlap PCR using pQE30-*mutS* as a template. The mutant genes were cloned into pQE30 and confirmed by nucleotide sequencing. His_6-tagged MutS and variants were expressed in *E. coli* M15. Cells were grown at 37 °C and when the absorbance (λ = 600 nm) of the cultures reached 0.6, cells were induced with 0.4 mM IPTG for 4 h. Proteins were purified using nickel ion affinity resin columns and eluted with 250 mM imidazole. Protein purity was determined to be > 95% by SDS–PAGE analysis.

DNA substrates for AFM. DNA substrates for the AFM experiments were created by ligating three DNA fragments: an EcoRV-digested *mutL* gene (1717 bp), a BglII-digested *mutH* gene (528 bp), and a synthesized oligonucleotide fragment with or without a G/T base pair mismatch (34 bp) with EcoRV and BglII ends. The G/T mismatch in the synthesized oligonucleotide fragment is at the 17 bp position. The assembly of the G/T mismatched DNA substrate is illustrated in Fig. 1. The product is a 2279 bp DNA fragment with a G/T mismatch at the 545 bp position (which is about 1/4 the contour length away from one end of the DNA substrate).

AFM experiment. Protein–DNA complexes were formed by incubating 1 ng/μl MutS, or variant protein, with 0.5 ng/μl mismatched or perfectly paired DNA substrate at room temperature in a buffer solution [16] containing 10 mM Tris–HCl (pH 7.5), 90 mM NaCl, and 6 mM $MgCl_2$ in a total volume of 60 μl. When the effect of ATP was examined, 10 μM Na_2-ATP was added into the reaction mixture while the other components were kept constant. After the binding reaction had proceeded for 2, 4, 6, 8, 10, 30, 60, and 90 min, a 10-μl droplet of each reaction mixture was deposited onto freshly cleaved mica for 1 min, then rinsed with Millipore Milli-Q water and dried under a stream of nitrogen individually. AFM analysis was carried out with a Nanoscope IIIa, MultiMode system (Digital Instruments, Veeco, Santa Barbara, CA) in tapping mode in air. Ultrasharp NSC11/AIBS silicon cantilevers (MikroMasch, Tallinn, Estonia) with spring constants of ~48 N/m and resonant frequencies of ~330 kHz were used for imaging. Images were collected at a speed of 1–1.5 Hz with a resolution of 512 × 512 pixels.

Image analysis. The binding frequency of MutS and DNA was calculated relative to the number of DNA molecules present. The formation of the MutS–DNA complex was counted as one binding event, independent of whether multiple proteins were present on the same single DNA fragment. α-loop frequency was calculated in the same manner. The distance from a protein in the MutS–DNA complex to its closest DNA end, and the contour length of the DNA, were assayed using software Image J (NIH, Bethesda, Maryland, USA). The specific binding frequency of G/T mismatched DNA by MutS was calculated by subtracting the 1/4-site binding frequency of homoduplex control from that of heteroduplex DNA substrates. Images from at least three independent experiments were analyzed and counted.

Results

MutS binds both G/T mismatched and perfectly paired DNA

AFM images showed that both G/T mismatched and perfectly paired DNA could form a complex with MutS (Fig. 2). Although both types of DNA substrates were bound by MutS, the binding frequencies were observed to be different. As shown in Table 1, the binding frequency of the G/T mismatched DNA substrate with MutS (85.1%) was higher than the binding frequency of a perfectly paired DNA substrate with MutS (47.7%). The ability of MutS to bind DNA independent of mismatched base pairs seems to indicate a non-specific association of MutS with DNA.

MutS mediates formation of an α-loop structure

Allen et al. [15] previously proposed a translocation model for the interaction of MutS with DNA in which α-shaped DNA loops formed where MutS formed a junction point with the DNA. In our assays, α-shaped DNA loops were observed, too (Fig. 2). Linear DNA was bent to a form just like the letter α, with a visible protein at the intersection of the two DNA arms. We can see that both G/T mismatched and perfectly paired DNA substrates could form α-shaped DNA loops in the presence of MutS. The frequency of α-loops formed by MutS with G/T mismatched DNA was 22.6%, while that of α-loops formed by MutS with a perfectly paired DNA substrate was 8.2% (Table 1).

ATP has no obvious effect on the binding frequency of DNA with MutS or on the frequency of α-loop

MutS and MutS homologs are known to have ATPase domains which bind and/or hydrolyze ATP to modulate MMR activities [17–21]. Crystal structures of MutS indicate that an allosteric relationship exists between ATP- and DNA-binding sites of MutS

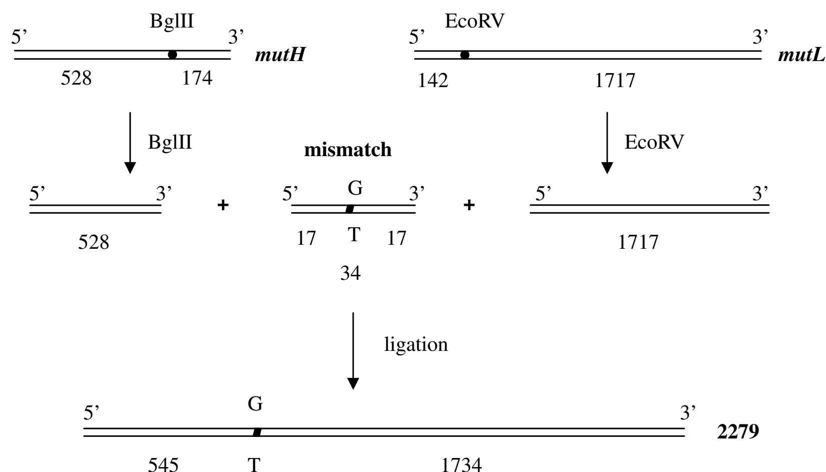

Fig. 1. Strategy for the construction of the 2279 bp G/T mismatched DNA substrate.

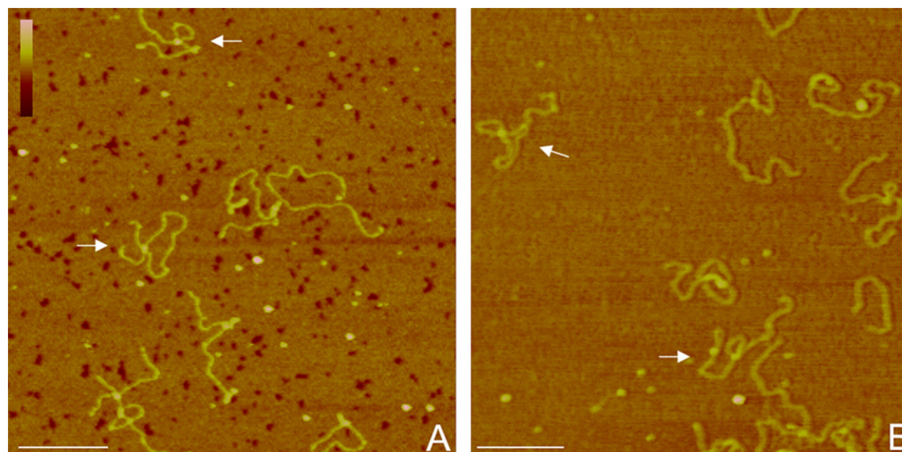

Fig. 2. AFM images of complexes formed between MutS and DNA. (A) Complexes formed between MutS and G/T mismatched DNA. (B) Complexes formed between MutS and perfectly paired DNA. Scale bars represent 250 nm. Color bar (height) represents 0–3 nm. Arrows indicate α-loop structures present. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 1
Frequencies of binding and α-loop formed by MutS and DNA with or without addition of Na_2-ATP during the first 10 min of a reaction assay[a]

Na_2-ATP (μM)	% Binding[b]		% α-Loop[c]	
	Heteroduplex	Homoduplex	Heteroduplex	Homoduplex
0	85.1 (978)	47.7 (1022)	22.6 (978)	8.2 (1022)
10	74.6 (1268)	43.6 (1266)	18.1 (1268)	5.9 (1266)

[a] Data in parentheses represent the numbers of DNA molecules counted. Without Na_2-ATP, frequencies were calculated from data obtained at 4, 6, 8, and 10 min; with 10 μM Na_2-ATP, frequencies were calculated from data obtained at 2, 6, 8, and 10 min. In both cases, similar amounts of perfectly paired and G/T mismatched DNA substrates were sampled at each time point.

[b] Percentage of DNA molecules bound by one or more MutS molecules relative to the total number of DNA molecules counted.

[c] Percentage of DNA molecules with one or more α-loops relative to the total number of DNA molecules sampled.

[22,23]. When ATP is bound to MutS, the affinity of MutS for DNA is decreased [21,24,25]. To determine if ATP affects the frequency of MutS–DNA interactions and α-loop formation, Na_2-ATP was incorporated into MutS–DNA-binding assays. In the presence of 10 μM ATP, the binding frequency of the G/T mismatched DNA substrate with MutS was only slightly lower than in the absence of exogenous ATP (74.6% vs. 85.1%). The binding frequency of the perfectly paired DNA substrate with MutS was nearly unchanged (43.6% vs. 47.7%) (Table 1). These results are consistent with the data from surface plasmon resonance (SPR) assays in which the response units of both G/T mismatched and perfectly paired DNA with MutS did not change upon addition of ATP (data not shown). The frequencies of α-loop formed in the presence of G/T mismatched DNA substrates and perfectly paired DNA substrates with and without ATP were 18.1% vs. 22.6%, and 5.9% vs. 8.2%, respectively (Table 1). These data suggest that ATP has no significant effect on the binding frequency of DNA with MutS, or on the frequency of α-loop.

MutS dimer could mediate α-loop formation

There are two large channels in the MutS dimer structure. One is larger (~40 × 20 Å) than the other (30 × 20 Å), and the former is occupied by mismatched DNA [22,23]. However, for an α-loop to be formed, one DNA molecule must pass through a MutS protein twice. This leads to the question of whether there is more than one MutS dimer at the base of an α-loop, or is there more than one DNA-binding site in each MutS dimer so that DNA may pass through it twice? To probe the mechanism by which an α-loop forms, we constructed two mutants of MutS, D835R, and R194A/R198A/R275A, to investigate protein–DNA interactions. For both variants could complement the *mutS*-deficient strain *E. coli* KM75 (data not shown), they were functional *in vivo*. Therefore these variants are suitable for the investigation of protein–DNA interactions *in vitro*.

The D835R mutant has been reported to be a dimer in solution [26,27]. To assess the oligomeric status of MutS in an α-loop structure, we tested whether the tetramer-deficient MutS mutant D835R could mediate formation of an α-loop. The data indicate that the frequency of α-loop formed by the D835R variant was not lower than that of the wild type MutS (Table 2). Although it cannot be asserted that all the protein at the base of α-loops is arranged in dimers, it can be concluded that one MutS dimer is enough to mediate formation of an α-loop. However, according to crystallographic data, a DNA molecule only makes a single pass through the larger channel of a MutS dimer. Although, since the smaller channel in the MutS dimer is also accessible for binding DNA based on both size and electrostatic potential [23], there is the possibility that one DNA arm of the α-loop passes through the larger channel while the other arm passes through the smaller channel. To address this possibility, the R194A/R198A/R275A mutant was investigated. This MutS variant has three predicted DNA-binding residues in the smaller channel replaced by neutral alanines, thereby reducing the number of DNA-specific contacts within the smaller channel. While the frequency of α-loop mediated by the R194A/R198A/R275A variant was similar to that of α-loop formed by wild type MutS (Table 2). Therefore, it remains unclear where a second pass of DNA occurs.

Table 2
α-Loop frequency (%) by binding of MutS or MutS variants to G/T mismatched DNA without addition of Na_2-ATP

Reaction time (min)	Wild type	R194A/R198A/R275A	D835R
6	12.0 (267)	23.1 (182)	24.7 (320)
8	27.1 (350)	13.7 (315)	21.4 (365)
10	28.0 (175)	13.1 (160)	34.1 (179)

Data in parentheses represent the numbers of DNA molecules counted.

MutS tends to associate with mismatched DNA regions with extended incubation times, and α-loop frequency decreases

When binding assays with MutS and DNA substrates were incubated for extended amounts of time, a localization of MutS at specific regions in the mismatched DNA substrate was observed (Fig. 3). However, since the mismatched DNA substrate did not have its 5′ vs. 3′ end differentially labeled, there was uncertainty with respect to location of the mismatch in the AFM images (although the mismatch site is located at 1/4 of the contour length away from one end of the heteroduplex DNA). This problem was compensated for by subtracting the assay results obtained using a perfectly paired DNA control. At timepoints between 30 and 90 min, the specific binding frequency of the G/T mismatched DNA substrate by MutS increased from 38.1% to 51.8%. At the same timepoints, α-loop frequency was decreased (Table 3).

Discussion

AFM images taken of the interactions between MutS and DNA substrates in this study show that MutS can bind DNA non-specifically. The observation is consistent with other reports [28–30]. Our data also show that α-shaped DNA loops were formed in the presence of both G/T mismatched, and perfectly paired DNA substrates bound by MutS. These results are in contrast with a previously hypothesized translocation model for MutS:DNA interactions that postulates that MutS mediates α-loop formation only at a mismatched site [15].

Although MutS-mediated α-shaped DNA loops were observed both in the report by Allen et al. [15] and in the study presented here, the two kinds of α-loop seem different from each other. First, formation of α-loop described by Allen et al. depends on mismatch, but in the present research this is not the case, for both mismatched and perfectly paired DNA were found to form α-shaped DNA loops mediated by MutS. Second, the formation of α-loop reported by Allen et al. requires ATP, while that we observed does not. We have observed α-loops without addition of any ATP (Fig. 2). The third difference lies in the appearance of the two kinds of α-loop. Although both kinds of α-loop have MutS protein at the base, the oligomeric states of MutS seem different. The double-globe-shaped structure at the base of α-loop observed by Allen et al. could not be two MutS monomers, but be at least two dimers [23,24], because monomer is in the shape of a comma and dimer forms an oval disk [22,23], while tetramer is arranged head-to-head to form an extended conformation [26]. At the base of α-loop observed in this study, however, MutS protein seems to be a small globe (Fig. 2). For the shape and dimensions, it should be a dimer.

The fact that α-loop appeared during the process of mismatch recognition, independent of whether or not there was a mismatch in DNA, suggests that the significance of α-loop formation maybe is based on the ability of two arms of DNA to pass through MutS during the search for mismatched base pairs. Furthermore, the specific binding frequency of mismatched DNA by MutS increased with longer incubation times. This result, together with the observation that α-loops formed by mismatched DNA decreased at the same time, may suggest that for heteroduplex DNA, after an α-loop dis-

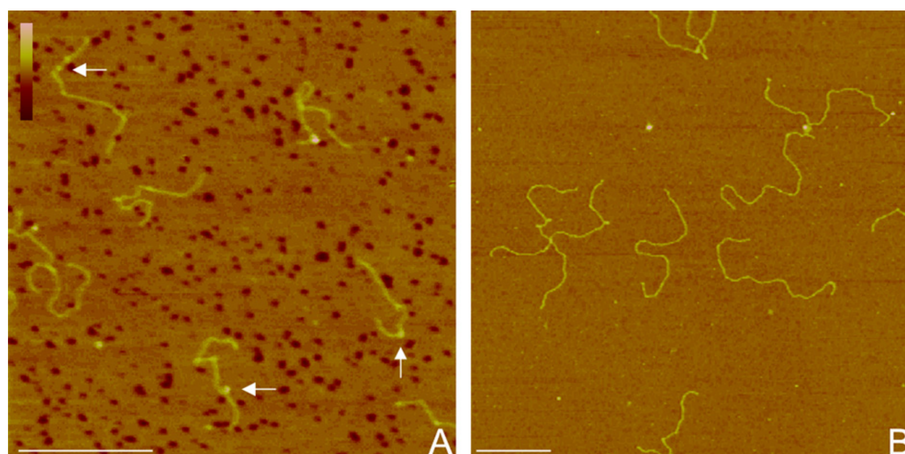

Fig. 3. AFM images of complexes formed between MutS and DNA after an extended incubation time (60 min), which represent the tendency of MutS to bind specific sites in the mismatched DNA substrate. (A) Complexes formed between MutS and G/T mismatched DNA substrates. Scale bar represents 500 nm. (B) Complexes formed between MutS and perfectly paired DNA substrates. Scale bar represents 250 nm. Color bar (height) represents 0–2 nm for (A) and 0–3 nm for (B). Arrows indicate the bound protein molecules which are approximately 1/4 contour length away from one end of the DNA substrate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 3
Frequencies of α-loop and specific binding of G/T mismatched DNA by MutS

Reaction time (min)	% 1/4-Site binding[b]		% Specific binding[c]	% α-Loop of heteroduplex[a,d]
	Heteroduplex[a]	Homoduplex[a]		
30	48.3 (263)	10.2 (283)	38.1	23.2 (263)
60	51.4 (325)	8.6 (326)	42.8	9.2 (325)
90	60.3 (199)	8.5 (199)	51.8	7.0 (199)

[a] Data in parentheses represent the numbers of DNA molecules counted.
[b] Percentage of DNA molecules bound by MutS at the binding site located 1/4 of the distance from either end relative to the total number of DNA molecules counted.
[c] Specific binding frequency of G/T mismatched DNA by MutS obtained by subtracting the 1/4-site binding frequency of homoduplex control from that of heteroduplex DNA.
[d] Percentage of G/T mismatched DNA molecules that have one or more α-loops relative to the total number of G/T mismatched DNA molecules sampled.

assembles, the MutS protein on it will bind to the mismatched site. These results lead us to propose that MutS searches for a mismatch in DNA through formation of α-shaped DNA loop. This proposal accounts for the non-specific interactions of MutS with DNA, for the presence of α-loop formation independent of a mismatch, and for the increased specific binding frequency despite a decrease in α-loop frequency. The formation of an α-loop allows two arms of DNA to be scanned simultaneously by a MutS dimer.

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