Distinct MutS DNA-binding Modes That Are Differentially Modulated by ATP Binding and Hydrolysis*

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The role of MutS ATPase in mismatch repair is controversial. To clarify further the function of this activity, we have examined adenine nucleotide effects on interactions of Escherichia coli MutS with homoduplex and heteroduplex DNAs. In contrast to previous results with human MutSα, we find that a physical block at one end of a linear heteroduplex is sufficient to support stable MutS complex formation in the presence of ATP-Mg2+. Surface plasmon resonance analysis at low ionic strength indicates that the lifetime of MutS complexes with heteroduplex DNA depends on the nature of the nucleotide present when MutS binds. Whereas complexes prepared in the absence of nucleotide or in the presence of ADP undergo rapid dissociation upon challenge with ATP-Mg2+, complexes produced in the presence of ATP-Mg2+, adenosine 5’-(β,γ-imino)triphosphate (AMPPNP)-Mg2+, or ATP (no Mg2+) are resistant to dissociation upon ATP challenge. AMPPNP-Mg2+ and ATP (no Mg2+) reduce MutS affinity for heteroduplex but have little effect on homoduplex affinity, resulting in abolition of specificity for mispaired DNA at physiological salt concentrations. Conversely, the highest mismatch specificity is observed in the absence of nucleotide or in the presence of ADP. ADP has only a limited effect on heteroduplex affinity but reduces MutS affinity for homoduplex DNA.

DNA biosynthetic errors that escape the proofreading function of DNA polymerase are corrected by mismatch repair. Although mismatch repair was initially characterized in bacteria (1–4), analogous systems have been identified in eukaryotes, and defects in the mammalian pathway have been implicated in tumor development (5–8).

Rectification of DNA biosynthetic errors by these systems relies on secondary signals within the helix that serve to distinguish newly synthesized DNA from the template strand. In Escherichia coli these signals are based on patterns of adenine methylation of d(GATC) sequences, which are hemimethylated in newly replicated DNA (2). Repair is initiated via mismatch recognition by MutS, with assembly of a MutL-MutS heteroduplex complex serving to activate the MutH d(GATC) endonuclease in an ATP-dependent reaction (9–11). Activated MutH cleaves the unmethylated strand of a hemimodified d(GATC) sequence that may be located to either side of the mismatch. Assembly of MutL-MutSheteroduplex is also sufficient to activate unwinding by DNA helicase II, which enters the helix at the MutH-produced strand break with an orientation bias such that it tracks back toward the mismatch (12). This orientation-dependent activation of the excision system at the strand break implies signaling between the two DNA sites along the helix contour. The fact that MutS and MutL are sufficient for this effect suggests that one or both of these activities function in signal transmission between the two DNA sites.

Several models have been proposed to account for interaction of the two DNA sites involved in the methyl-directed reaction. The recent demonstration that a mismatch within one oligonucleotide duplex can activate MutS- and MutL-dependent MutH cleavage of a d(GATC) sequence on a second oligonucleotide duplex has led to the suggestion that the two sites are brought into proximity by DNA bending with the presence of MutS at the mispair signaling activation of downstream activities at the strand signal via a MutL interface (13). However, the efficiency MutH activation reported in these trans-activation experiments is several hundred times lower than that observed for d(GATC) cleavage activated in cis by a mismatch located 1,000 base pairs distant (11). Furthermore, whereas a DNA bending mechanism might account for activation of MutH cleavage at a d(GATC) sequence, it does not explain the MutS-, MutL-, and orientation-dependent activation of the excision system at the ensuing strand break.

Two alternate models, which can account for orientation-dependent signaling between the two DNA sites, invoke ATP-dependent movement of MutS and its eukaryotic homologs along the helix contour (14–16). The presence of ATP reduces the steady-state affinity of bacterial MutS and eukaryotic MutSα for a mismatch (10, 17–21), and ATP challenge of preformed MutS-DNA or hMutSα-DNA complexes can lead to release of the protein from a mismatch (14–16). Since ATPγS also results in release of a MutS homolog from a mispair (14, 16, 21), this effect may be due at least in part to binding of the triphosphate, although ATP hydrolysis by the heteroduplex-bound protein has been invoked by several laboratories (14, 15, 22). Electron microscopic analysis of the kinetics of evolution of MutSheteroduplex complexes has indicated that the presence of ATP results in the nucleotide-dependent formation of DNA loops, which grow with time and are stabilized by bound MutS at the base (14). However, loop formation was not supported by ATPγS,1 and ongoing ATP-dependent DNA loop growth was blocked upon addition of excess AMPPNP. Since the mismatch

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1 The abbreviations used are: ATPγS, adenosine 5’-O-(thiotriphosphate); AMPPNP, adenosine 5’-(β,γ-imino)triphosphate; bp, base pair; SPRS, surface plasmon resonance spectroscopy.
was found within the DNA loop with most heteroduplexes, these effects were attributed to ATP-dependent tracking of MutS from the mispair in a bidirectional fashion.

Analysis of ATP effects on the interaction of human MutSα with linear heteroduplexes that have biotin-avidin end-blocks or with circular DNAs has indicated that the MSH2-hMSH6 heterodimer also leaves a mispair in the presence of ATP by movement along the helix (15, 16, 22). This has led to the suggestion that in the presence of ATP, MutS homologs form a sliding clamp about the helix (15, 16). However, two distinct mechanisms for movement of this clamp have been proposed. One model invokes ATP binding and hydrolysis by DNA-bound hMutSα to modulate protein conformers leading to directional movement of a sliding clamp along the helix (15). The other posits mismatch recognition by the hMutSα-ADP complex, with the mispair acting as a nucleotide exchange factor that promotes formation of the hMutSα-ATP complex that is capable of free diffusion along the helix (16). In this mechanism ATP hydrolysis occurs after release from DNA and serves to regenerate the mismatch-binding form of the protein. These models differ with respect to an ADP requirement for mismatch recognition, whether movement on DNA is directional and whether ATP hydrolysis is supported by the DNA-bound form of the protein.

As noted above, the substantial evidence supporting ATP-dependent movement of MutS homologs along the helix has been interpreted in terms of a mechanism for signaling between the mismatch and the strand signal that directs repair. However, it is important to note that other potential functional roles for movement of the protein along the helix have not been excluded, for example, involvement in the kinetic path by which mispairs are located.

A distinct function for ATP binding and hydrolysis by MutS homologs has been suggested in the context of the DNA bending mode for interaction of the mismatch and strand signal sites that was described above. This proposal stipulates that once mismatch recognition occurs, MutS remains bound to the mispair during the course of the repair reaction. In this scheme, ATP binding and hydrolysis by DNA-bound MutS functions in a kinetic proofreading mechanism that is used to verify mismatch recognition prior to initiation of repair (13). ATP binding by DNA-bound MutS is envisioned to reduce the affinity of the protein for both mismatches and homoduplex sites. The ATP-induced reduction in affinity is postulated to be greater for the MutS-homoduplex complex than for the MutS-mismatch complex, resulting in preferential dissociation of MutS from correctly paired sequences.

This paper addresses the effects of adenine nucleotides on the interaction of MutS with heteroduplex and homoduplex DNAs. We show that as in the case of human MutSα, ATP-promoted dissociation of a MutS heteroduplex complex requires free DNA ends. Interestingly, a single avidin-biotin end-block is sufficient to prevent dissociation, implying that this protein does not form a simple sliding clamp in the presence of ATP that is capable of free diffusion along a heteroduplex. By using surface plasmon resonance spectroscopy, we also show that MutS is capable of specific mismatch recognition in the presence of ADP, ATP-Mg$^{2+}$, or in the absence of nucleotide, although mismatch specificity is not detectable at physiological ionic strength in the presence of AMPNP-Mg$^{2+}$ or in the presence of ATP in the absence of a divalent cation. These observations indicate that adenine nucleotides differentially modulate mismatch and homoduplex interactions and indicate that the protein has two distinct DNA-binding modes.

**EXPERIMENTAL PROCEDURES**

**Proteins and DNAs—**MutS was isolated by a modification of the procedure described previously (9). Frozen cell paste (50 g) was resuspended in 20 mM KPO₄, pH 7.4, 1 mM EDTA, 10 mM 2-mercaptoethanol (2 ml/g of cell paste), and cells were disrupted by sonication. The extract was clarified by centrifugation (15,000 × g, 45 min), and the resultant supernatant was treated with 0.25 volume of 25% (v/v) streptomycin sulfate. After stirring on ice for 30 min, the precipitate was removed by centrifugation (15,000 × g, 30 min), and the supernatant was treated with ammonium sulfate (0.18 g/ml) added over a period of 30 min. After stirring on ice for 45 min, the precipitate was collected by centrifugation (15,000 × g, 45 min) and resuspended in 60 ml of Buffer A (25 mM Tris-HCl, pH 7.4, 10 mM 2-mercaptoethanol) containing 50 mM KCl and 1 mM EDTA. Aliquots of resuspended protein were diluted with Buffer A containing 50 mM KPO₄, pH 7.4, 10 mM 2-mercaptoethanol) containing 50 mM KCl and 1 mM EDTA. MutS fractions were pooled and dialyzed against Buffer A containing 100 mM KCl in a 10-mL DE52 column equilibrated with Buffer A containing 100 mM KCl and 1 mM EDTA at a flow rate of 30 mL/h. After washing with 10 column volumes of this buffer, the column was eluted with a 900-mL linear gradient of KPO₄, pH 7.4 (200–200 mM) containing 100 mM KCl and 1 mM EDTA. MutS containing fractions were pooled and loaded onto a 90-ml DE52 column equilibrated with Buffer A containing 100 mM KCl and 1 mM EDTA at a flow rate of 90 mL/h. After washing with 10 column volumes of this buffer, the column was eluted with an 800-mL linear gradient of KPO₄, pH 7.4 (0.1–200 mM) containing 100 mM KCl and 1 mM EDTA. MutS containing fractions were pooled and dialyzed against Buffer B containing 150 mM KCl and 50% (v/v) glycerol. Protein was stored at −20 °C. MutS concentrations were determined from the absorbance at 280 nm using an extinction coefficient of 69420 M⁻¹ cm⁻¹ calculated from the primary amino acid sequence.

Oligodeoxyribonucleotides were purchased from Oligos Etc. (Wilsonville, OR) and, when indicated, were radiolabeled at the 5' end with T4 polynucleotide kinase and [γ-32P]ATP (3,000 Ci/nmol, PerkinElmer Life Sciences) to a specific activity of 1 × 10⁷ cpm/μl. A 41-base pair (bp) G-T heteroduplex that contained a terminal biotin at one or both ends of the duplex was prepared by combining 180 μM 5'-32P-labeled d(CGCCGAATTCTGCAAGCTTAAATTTTGGTTTCGAGGT) and 320 μM of unlabeled d(AAGCGGAATTCTAGCTGATAGTTGCTGACATCCTAAATTTCGGCG) that was synthesized with or without a 3'-terminal biotin. DNA samples (100 μl) were annealed in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl by heating at 99 °C in a PerkinElmer Life Sciences Gene Amp 9600 thermocycler for 2 min and cooling to 25 °C over a period of 90 min. Otherwise identical A-T homoduplex and A-T homoduplex substrates were prepared by annealing d(AAGCGGAATTCTAGCTGATAGTTGCTGACATCCTAAATTTCGGCG) and 320 μM of unlabeled d(CGCCGAATTCTGCAAGCTTAAATTTTGGTTTCGAGGT) with 2 μM 5'-32P-labeled d(AAGCGGAATTCTAGCTGATAGTTGCTGACATCCTAAATTTCGGCG) for 41-bp G-T heteroduplex and A-T homoduplex, respectively. Oligodeoxyribonucleotides (100 μl) were annealed as described above and diluted 10-fold prior to conjugation to the streptavidin sensor chip as described below.

A 500-bp G-T heteroduplex and A-T homoduplex DNAs were prepared by the polymerase chain reaction method described previously (15). Briefly, homoduplex and heteroduplex molecules were prepared by hybridizing single strands isolated by denaturing high pressure liquid chromatography from duplex fragments produced by the amplification of base pairs 5531–5732 of bacteriophages f1MR1 and f1MR3 (24). A biotinylated f1MR1 fragment was produced using 5'-biotin-d(TACCGCGAGCTCCTGAAAAAATATTCTTGGGTTGCGAGGAG) as a forward primer and d(TACCGCGAGCTCCTGAAAAAATATTCTTGGGTTGCGAGGAG) for a 41-bp G-T heteroduplex and A-T homoduplex, respectively. Oligodeoxyribonucleotides (100 μl) were annealed as described above and diluted 10-fold prior to conjugation to the streptavidin sensor chip as described below.

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**Surface plasmon resonance measurements—**Surface plasmon resonance measurements were performed using the Biacore 2000 instrument (Biacore AB, Uppsala, Sweden) equipped with a BSA-Spectraflow sensor chip (Flow Associates, Inc., Beverly, MA) to monitor changes in the surface plasmon resonance signal (25). The system was equipped with a 15-μl flow cell that was precoated with biotinylated-f1MR1 fragment and the avidin sensor chip. A 5'-biotinylated viral strand from f1MR1 (0.3 μM), complementary to the biotinylated viral strand from f1MR1 (0.3 μM), was combined in a 100-μl volume with 0.6 μM of the complementary sequence prepared from f1MR1 or f1MR3 to form a homoduplex or heteroduplex, respectively. Duplexes were annealed as described above.

**Gel Shift Analysis—**DNA-binding reactions (20 μl) contained 20 μg of DNA and 10 μM ATP in Buffer A.
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Tris-HCl, pH 7.6, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 5 mM MgCl₂, 50 mM KCl. 41-bp 32P-homoduplex or 32P-heteroduplex, 25 μg/ml of a BstEII digest of bacteriophage λ DNA (New England Biolabs) as nonspecific competitor, streptavidin, and ATP were present as indicated. Solutions were preincubated at 25 or 37 °C as noted for 10 min, the reaction initiated by addition of MutS, and incubation continued for 10 min. Reactions were terminated by the addition of 2 μl of 50% (v/v) glycerol, 0.05% xylene cyanol, 0.05% bromphenol blue, and 20 mM EDTA, placed on ice, and loaded onto 4 or 5% native polyacrylamide gels (acylamide/ bisacrylamide, 37.5:1) in 6.7 mM Tris acetate, pH 7.5, and 1 mM EDTA. Gels were electrophoresed at room temperature at 11.4 V/cm in this buffer.

32P-Labeled complexes were visualized by autoradiography after drying and quantitated using a Molecular Dynamics PhosphorImager.

Surface Plasmon Resonance Spectroscopy—Surface plasmon resonance measurements were performed on a BiACore 2000. A streptavidin sensor chip was derivatized with 32P-Labeled 41-bp homoduplex (305 response units) or G-T heteroduplex (306 response units) DNAs described above in which one strand was derivatized with a 5′-biotin. Additional sensor chips were derivatized with 299 response units and 298 response units of a 5′-biotin-tagged 200-bp homoduplex or G-T heteroduplex, respectively. Solutions (100 μl) of MutS in 20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 0.005% surfactant P-20, 5 mM MgCl₂ containing KCl and nucleotide concentrations as indicated, were flowed across the SA chip at a rate of 20 μl/min. After MutS association, the chip was washed at 20 μl/min for 20 min with 20 mM Tris, pH 7.6, 1 mM dithiothreitol, 0.005% surfactant P-20, and KCl as indicated, followed by 60 μl of the same buffer/KCl solution containing 5 mM MgCl₂ and 1 mM ATP. Measurements were performed at 25 °C, and samples were maintained at 4 °C prior to injection. The SA chip was regenerated by a 20-μl injection of 0.5% sodium dodecyl sulfate.

ATPase Assays—ATP hydrolysis by MutS was measured in 20-μl reactions at 37 °C in 20 mM Tris-HCl, pH 7.6, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 200 μM (α32P)-ATP (2500 μCi/mmol), 0.5 mg/ml streptavidin, and DNA substrates as indicated. These components, except for ATP, were assembled on ice and incubated for 10 min at room temperature to allow the conjugation of streptavidin to biotin-tagged DNAs. After supplementation with MutS (300 μM, monomer equivalent) and incubation at 37 °C for 5 min, reactions were initiated by addition of the (α32P)-ATP. Rate determinations were based on removal of 2-μl samples, which were quenched in 50 μl of 0.5 M EDTA, pH 8.0. ATP hydrolysis was determined by chromatography of 1 μl of quenched sample on PEI cellulose plates (EM Science, Gibbstown, NJ) developed in 0.3 M KPO₄, pH 7.0. Dried plates were quantitated on a Molecular Dynamics PhosphorImager after overnight exposure. Initial steady-state rates of ATP hydrolysis were determined by least squares analysis of the linear portion of the progress curve.

Estimation of MutS-DNA Dissociation Constants—Values for the dissociation constant (Kₛ) for MutS DNA complexes were determined by surface plasmon resonance spectroscopy by titration of chip-bound DNA with increasing concentrations of MutS. Maximum binding values obtained in this manner were plotted as a function of the protein concentration and data fit to a square hyperbola, from which the Kₛ value was extracted.

At KCl concentrations above 100 mM in the presence or absence of nucleotide, MutS binding to 41-bp homoduplex DNA was not saturable as monitored by surface plasmon resonance spectroscopy, even at the highest concentration tested (800 mM). Binding to heteroduplex also was not saturable at these salt concentrations in the presence of ATP, AMPPPNP, or ATP in the absence of Mg²⁺. In these cases Kₛ values were estimated for purposes of comparison with dissociation constants obtained under conditions where saturable behavior was observed. To this end, binding isotherms were formulated by fitting binding data to a hyperbola that was constrained to the saturation value determined with heteroduplex DNA under conditions of high affinity binding (in the absence of nucleotide or in the presence of ADP).

RESULTS

One or Two Streptavidin End-blocks Interfere with ATP-promoted Release of MutS from Linear Heteroduplex DNA—Terminal streptavidin-biotin complexes have been used to study the mechanism of nucleotide-promoted release of human MutSα from linear heteroduplex DNA (15, 16, 22). We have utilized this method to explore further the nature of bacterial MutS movement along the helix in the presence of ATP. These experiments used a 41-base pair G-T heteroduplex with a centrally located mispair or an otherwise identical A-T homoduplex DNA (see “Experimental Procedures”) possessing either two free termini or one or two biotin-streptavidin terminal end-blocks. The ability of MutS to form stable DNA complexes with these DNAs in the absence or presence of ATP was tested by gel shift analysis.

Due to the presence of unlabeled homoduplex competitor in all reactions, no binding was observed with the A-T homoduplex control that contained one or two streptavidin-biotin end-blocks (Fig. 1, upper panel, lanes 10–13), indicating that MutS does not interact nonspecifically with the streptavidin component of such substrates. As observed previously (14), the presence of ATP and Mg²⁺ reduced the yield of MutS complexes with the streptavidin-free G-T heteroduplex by about 90% (compare lanes 1–3). However, the yield of specific complexes with the G-T DNA containing a bi-terminal streptavidin end-block was unaffected by the presence of the nucleotide (lanes 7–9), a finding similar to those obtained with human MutSα (15, 16). Furthermore, a single streptavidin block was largely sufficient to stabilize specific complexes in the presence of ATP (lanes 4–6). The latter finding was independent of placement of the streptavidin complex at the 3′-end of one strand or the other, or 3′ or 5′ derivatization of a particular duplex terminus (not shown), ruling out strand polarity or position effects with respect to placement of the single end-block. Quantitation of the results shown in Fig. 1 (upper panel) demonstrated that the yield of specific MutS-heteroduplex complexes in the presence of ATP, as compared with that observed in the absence of nucleotide, was 10% for the heteroduplex with free ends, 100% for the substrate with the bi-terminal block, and 80% for G-T DNA with a streptavidin-biotin complex at only one end. It is noteworthy that the complexes observed with end-blocked heteroduplexes were obtained in reactions that were initiated by addition of MutS to solutions that contained both DNA and nucleotide (“Experimental Procedures”), i.e. the formation of such complexes did not require pre-binding of MutS to DNA in the absence of nucleotide.

Since these experiments were performed under conditions where MutS is catalyzing ATP hydrolysis, the enhanced yields of specific MutS-heteroduplex complexes observed with end-blocked DNAs is indicative of increased steady-state levels of these protein-DNA complexes. This effect is due at least in part to an increased lifetime of such complexes as judged by substrate challenge experiments. As shown in Fig. 1 (lower panel), MutS complexes with G-T heteroduplex lacking a terminal end-block dissociated rapidly and completely upon addition of a 600-fold excess of unlabeled G-T heteroduplex. By contrast, complexes with DNA containing a bi-terminal end-block did not dissociate significantly over a 30-min period under these conditions. MutS complexes with heteroduplex DNA blocked at one terminus behaved in an intermediate fashion. About half of the complexes dissociated upon challenge with unlabeled heteroduplex, but the remainder was stable over a 30-min period.

The results obtained with MutS and the heteroduplex containing a bi-terminal block are similar to those previously obtained with human MutSα, where stabilization of specific complexes in the presence of ATP was also observed (15, 16). However, results with the bacterial protein differ from those obtained with hMutSα for substrates with a single end-block. In contrast to the substantial stabilization afforded specific complexes of the bacterial protein in the presence of ATP, a single end-block is not sufficient to stabilize hMutSα-heteroduplex complexes in the presence of this nucleotide (15, 16). Our findings suggest that as in the case of hMutSα, DNA termini are required for MutS dissociation in the absence of other repair activities, but they also imply that a single free
terminus does not support efficient release of the bacterial protein.

Surface Plasmon Resonance Spectroscopy of MutS-DNA Complexes and the Effect of Adenine Nucleotides on Association and Dissociation—The entrapment of MutS on end-blocked heteroduplexes suggested that MutS might retain the ability to recognize a mismatch in the presence of ATP and Mg$^{2+}$. We further examined the effects of adenine nucleotides on the dynamics of formation and dissociation of MutS-heteroduplex complexes using the real time technique of surface plasmon resonance spectroscopy (SPRS). Since analysis of protein-DNA interactions by SPRS is based on linkage of DNA to a streptavidin-derivated sensor chip via a single terminal biotin (see “Experimental Procedures”), this experimental system is similar to that described above using a heteroduplex with a single end-block.

Representative sensorgrams performed in the presence of 50 mM KCl display three elements (Fig. 2) as follows: a binding phase during protein flow across the chip, a dissociation phase upon buffer wash, and second dissociation phase upon challenge with ATP-Mg$^{2+}$. MutS readily associated with chip-bound 41-base pair G-T heteroduplex in the presence of ADP-Mg$^{2+}$ (A), ATP-Mg$^{2+}$ (B), AMPPNP-Mg$^{2+}$ (C), or in the presence of ATP but in the absence of MgCl$_2$ (D). In each case a major component of the binding observed was mismatch-dependent, as demonstrated by parallel analyses with an otherwise identical homoduplex control (the effects of adenine nucleotides on heteroduplex and homoduplex affinities are considered in detail below). The demonstration of stable, specific complexes in the presence of ATP-Mg$^{2+}$ (B) confirms the results obtained from the gel shift experiments of Fig. 1. Stable, specific MutS-heteroduplex complexes were also formed in the presence of nonhydrolyzable AMPPNP or in the presence of ATP but in the absence of divalent cation (C and D), suggesting that formation of these complexes does not require ATP hydrolysis. Furthermore, the resistance of MutS-DNA complexes to dissociation upon subsequent ATP-Mg$^{2+}$ challenge was dependent upon the nature of the nucleotide present during the binding phase. Complexes prepared with heteroduplex or homoduplex DNA in the presence of ADP dissociated rapidly and completely upon subsequent challenge with ATP-Mg$^{2+}$ (A). Similar results were obtained if MutS was allowed to bind in the absence of nucleotide (not shown). However, MutS-DNA complexes prepared in the presence of ATP-Mg$^{2+}$, AMPPNP-Mg$^{2+}$, or ATP (no Mg$^{2+}$) were largely refractory to dissociation upon ATP challenge (B–D).

MutS Affinities for Homoduplex and Heteroduplex DNA Are Differentially Modulated by Adenine Nucleotides and Ionic Strength—Dissociation constants ($K_d$s) were estimated by SPRS by evaluating the extent of MutS-DNA complex formation as a function of MutS concentration in the absence or presence of adenine nucleotides (see “Experimental Procedures” and Fig. 3). In order to address the generality of the relative binding affinities obtained, these analyses were performed at several KCl concentrations, and the results are summarized in Table I and Fig. 4.

Comparison of adenine nucleotide effects as a function of KCl concentration (Table I and Fig. 4) indicates that MutS affinities for homoduplex and G-T heteroduplex DNAs are differentially modulated by the nature of the adenine nucleotide present. Whereas AMPPNP-Mg$^{2+}$ and ATP (no Mg$^{2+}$) have only a modest effect on homoduplex affinity as KCl concentration is increased (Table I and Fig. 4, upper panel), the presence of ADP results in a substantial affinity reduction that is particularly evident at salt concentrations in the physiological range. A similar KCl-dependent reduction in MutS homoduplex affinity
MutS (400 nM) was present during the binding phase. The chip was derivatized with 305 response units of 41-bp A\(^+\)/H18528T homoduplex DNA. -terminal biotin. A second flow cell using the same chip with a single 5 was derivatized with 306 response units (RUs) (dashed line). A, homoduplex DNA is denoted by a " under

ADP Mg\(^2+\) were observed in the absence of nucleotide or in the presence of ADP or ATP, but only those complexes formed with ADP dissociate rapidly upon ATP challenge. The observation that MutS affinities for both homoduplex and heteroduplex are reduced to a similar degree in the presence of ATP-Mg\(^2+\) and AMPPNP-Mg\(^2+\) on affinities for the two types of DNA. However, these findings are consistent with results of pre-steady-state studies, which have shown that the rate-limiting step for ATP turnover by MutS depends on the nature of the DNA cofactor present (25).

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The observation that MutS affinities for both homoduplex and heteroduplex are reduced to a similar degree in the presence of ATP-Mg\(^2+\) and AMPPNP-Mg\(^2+\) is consistent with the results of pre-steady-state studies, which have shown that the rate-limiting step for ATP turnover by MutS depends on the nature of the DNA cofactor present (25). In the presence of a homoduplex DNA, the rate-limiting step occurs subsequent to hydrolysis, implying significant binding site occupancy by ADP, but in the presence of heteroduplex DNA, this step occurs at or prior to covalent chemistry implying occupancy by ATP.

These results suggest that MutS has at least two distinct DNA-binding modes and that these can be modulated by the phosphorylation state(s) of bound adenine nucleotides. The differential effects of the di- and triphosphate on homoduplex

increased KCl concentration (Table I and Fig. 4, lower panel). By contrast, heteroduplex affinity is reduced dramatically, particularly at salt concentrations in the physiological range, in the presence of AMPPNP-Mg\(^2+\), ATP (no Mg\(^2+\)), or under hydrolytic conditions in the presence of ATP-Mg\(^2+\).

FIG. 2. MutS associates with heteroduplex DNA in the presence of ADP or ATP, but only those complexes formed with ADP dissociate rapidly upon ATP challenge. Surface plasmon resonance spectroscopy was performed in the presence of 50 mM KCl as described under “Experimental Procedures” using a streptavidin sensor chip derivatized with 306 response units of 41-bp A/T homoduplex DNA. MutS (400 nM) was present during the binding phase. A second flow cell using the same chip was derivatized with 305 response units of 41-bp A/T homoduplex DNA. MutS was observed in the presence of ATP-Mg\(^2+\) where ATP hydrolysis is ongoing. Conversely, the highest heteroduplex affinities were observed in the absence of nucleotide or in the presence of ADP-Mg\(^2+\), and these affinities are relatively insensitive to

Binding Buffer ATP-Mg\(^2+\)

FIG. 3. Determination of the binding affinity of MutS to oligo-nucleotide duplexes. SPRS sensograms of binding to a 41-bp G-T heteroduplex were obtained as a function of MutS concentration as described under “Experimental Procedures” in the presence of 1 mM ATP and 50 mM KCl (inset). The plateau value obtained from each trace was plotted as a function of the MutS concentration and fit to a square hyperbola using non-linear least squares analysis to yield a \( K_d \) value of 0.12 \( \pm \) 0.02 \( \mu \)M.

Determination of the binding affinity of MutS to oligo-base pair G-T heteroduplex and A\(^+\)T homoduplex DNAs

Dissociation constants (\( K_d \)) were determined for MutS complexes with 41-bp G-T heteroduplex or A\(^+\)T homoduplex substrates by surface plasmon resonance spectroscopy (see “Experimental Procedures”) in 50 or 150 mM KCl in the absence or presence of 1 mM adenine nucleotide as indicated. The binding of MutS at 150 mM KCl was not saturable with homoduplex DNA or with heteroduplex DNA in the presence of ATP, ATP (no magnesium), and AMPPNP. In these cases \( K_d \) values were estimated as described under “Experimental Procedures.” Specificity values correspond to the quotient \( K_d \) (A\(^+\)T)/\( K_d \) (G-T).

**TABLE I**

| Nucleotide | \( K_{d(G\_T)} \) | \( K_{d(A\_T)} \) | Specificity |
|------------|-----------------|-----------------|------------|
| None       | 0.021 \( \pm \) 0.001 | 0.074 \( \pm \) 0.007 | 3.5        |
| ATP        | 0.12 \( \pm \) 0.02 | 0.59 \( \pm \) 0.04 | 4.9        |
| ATP (\(-Mg^{2+}\)) | 0.17 \( \pm \) 0.01 | 0.56 \( \pm \) 0.04 | 3.3        |
| AMPPNP     | 0.078 \( \pm \) 0.01 | 0.68 \( \pm \) 0.14 | 8.7        |
| ADP        | 0.015 \( \pm \) 0.003 | 0.15 \( \pm \) 0.01 | 10         |
| None       | 0.030 \( \pm \) 0.003 | 1.4 \( \pm \) 0.05 | 47         |
| ATP        | 1.3 \( \pm \) 0.04 | 12.6 \( \pm \) 0.65 | 9.2        |
| ATP (\(-Mg^{2+}\)) | 1.2 \( \pm \) 0.13 | 12.6 \( \pm \) 0.24 | 1.0        |
| AMPPNP     | 0.95 \( \pm \) 0.09 | 0.76 \( \pm \) 0.21 | 0.80       |
| ADP        | 0.090 \( \pm \) 0.004 | 11 \( \pm \) 0.39 | 120        |
The presence of 1 mM ATP (circled). Values were determined in the absence of nucleotide (no Mg2+), or in the presence of 1 mM ATP, AMPPNP, ADP, or ATP (MgCl2 omitted). Upper panel, A-T homoduplex; lower, G-T heteroduplex. ADP and heteroduplex affinities have implications for the specificity of MutS-DNA interaction (Table I). At low ionic strength (50 mM KCl), MutS displays significant specificity for a 41-bp G-T heteroduplex under all conditions tested. The demonstration of specificity in the presence of the triphosphate implies that MutS binding to 41-bp A-T homoduplex, and G-T heteroduplex substrates were obtained from SPRS measurements (“Experimental Procedures” and Fig. 3) in the presence of 5 mM MgCl2 and KCl as indicated. Values were determined in the absence of nucleotide (○), or in the presence of 1 mM ATP (●), AMPPNP (▲), ADP (▲), or ATP (MgCl2 omitted, ■). Upper panel, A-T homoduplex; lower, G-T heteroduplex. ADP, or AMPPNP were estimated as described under “Experimental Procedures.”

and heteroduplex affinities have implications for the specificity of MutS-DNA interaction (Table I). At low ionic strength (50 mM KCl), MutS displays significant specificity for a 41-bp G-T heteroduplex under all conditions tested. The demonstration of specificity in the presence of the triphosphate implies that MutS is capable of mismatch recognition under these conditions. Interestingly, at near-physiological salt concentration (150 mM KCl), heteroduplex specificity was abolished by the presence of AMPPNP-Mg2+ or ATP (no Mg2+). Inspection of Table I and Fig. 4 shows that this effect is largely due to a dramatic reduction in heteroduplex affinity, consistent with previous work (14, 16, 21) demonstrating that nonhydrolyzable ATP analogs can promote release of a MutS homolog from a mismatch.

ADP Increases Mismatch Specificity Primarily by Reducing Homoduplex Interaction.—The SPRS experiments above, which utilized 41-base pair DNA substrates, demonstrated a large enhancement of MutS heteroduplex specificity with increased KCl concentration provided that the experiments were done in the absence of nucleotide or in the presence of ADP. Further ADP-dependent specificity enhancement, beyond that observed in the absence of nucleotide, was demonstrable with larger DNAs capable of accommodating multiple MutS molecules. SPRS analysis of MutS binding at 150 mM KCl to a 200-bp G-T heteroduplex with a centrally located mismatch provided a clear example of two MutS-binding modes to a single DNA substrate (Fig. 5). Two distinct phases of MutS binding were resolved under these conditions: a high affinity phase with an apparent Kd of 9 nM and a low affinity phase that did not saturate even at the highest MutS concentration tested (800 nM). Since binding to an otherwise identical A-T homoduplex indicated that the low affinity phase is due to nonspecific interactions (Fig. 5), the high affinity binding phase therefore corresponds to MutS-mismatch complex formation. The inclusion of ADP significantly reduced the nonspecific interaction of MutS with perfectly paired sequences as judged by reduced binding to control A-T homoduplex DNA, as well as by reduction of the low affinity binding phase to the G-T heteroduplex. By contrast, the nucleotide had little effect on mismatch recognition as the Kd values for the specific phase of the two binding curves were similar (9.1 ± 1.5 and 10 ± 1.9 nM without and with 1 mM ADP, respectively). Corresponding Kd values for homoduplex binding were 660 ± 98 and 490 ± 490 nM.

The Coupled Equilibria between Nucleotide and DNA Binding Can Be Used to Estimate Nucleotide Kd Values.—The experiments described above demonstrate that heteroduplex affinity is reduced substantially in the presence of AMPPNP-Mg2+ or ATP (no Mg2+). The simplest interpretation of these observations is that binding of the triphosphate is responsible for the reduction in heteroduplex affinity. However, since the adenine nucleotide concentration in these experiments was 1 mM and since the AMPPNP and ATP preparations used were contaminated at the several percent level by ADP, it could be argued that this effect not only requires binding of triphosphate but is also dependent on binding of the contaminating diphosphate as well. To address this question...
yielding an apparent $K_d$ chip, an effect that was saturable and hyperbolic (Fig. 6, inset). AMPPNP resulted in a diminution of MutS binding to the DNA a 41-bp G-T heteroduplex. Increasing concentrations of traces were obtained at a constant protein concentration using a 41-bp G-T heteroduplex. Increasing concentrations of AMPPNP alone is sufficient to reduce heteroduplex affinity because the high affinity for AMPPNP implies that triphosphate binding to the MutS ADP complex, which is in the low nM range, well below the dissociation constant of the MutS-ADP complex, which is in the nM range.  

End-blocked DNA Substrates Stimulate the ATPase Activity of MutS—As discussed above, the several models for MutS action differentially invoke ATP hydrolysis by the DNA-bound ATPase activity relative to that of the free protein.

ATPase assay conditions were similar to those of the gel shift experiments of Fig. 1 and included streptavidin. As shown in Fig. 7, titration of MutS with each type of DNA substrate increased the rate of ATP hydrolysis in a manner similar to that observed previously (25). However, the degree of stimulation was reduced −2-fold for the heteroduplex with a bi-terminal end-block as compared with DNAs that lacked or contained only one biotin-streptavidin end-block (Fig. 7). If ATPase activity were dependent on the ability of MutS to dissociate from the DNA, MutS complexes trapped on end-blocked substrates would be expected to have reduced ATPase activity in the low nM range, well below the dissociation constant of the MutS-ADP complex, which is in the nM range.  

As discussed above, two classes of models have been proposed with respect to the role of the MutS ATPase in mismatch repair. One class invokes signaling between the mismatch and the strand signal via ATP-dependent movement of a MutS clamp-like structure along the helix, and two models of this type have been described. These differ in that the translocation model invokes directional tracking driven by ATP hydrolysis that occurs while MutS is DNA-bound (14, 15), whereas the molecular switch model postulates free diffusion of the MutS-ATP complex along the helix, with ATP hydrolysis occurring subsequent to dissociation from DNA (16).

The second class of model argues that MutS remains bound to the mismatch during the course of repair and that interaction of the two DNA sites occurs by a DNA binding mechanism that brings the distal DNA sites into proximity (13). This model posits that ATP binding and hydrolysis by MutS functions in a kinetic proofreading capacity to enhance mismatch specificity of the protein.

As in the case of hMutSα (15, 16, 22), the nature of the interaction of bacterial MutS with end-blocked heteroduplexes is consistent with formation of clamp-like structure that can move along the helix in the presence of ATP. However, results with the bacterial protein differ from those obtained with the eukaryotic protein in the case of heteroduplex DNA with a single end-block. In contrast to hMutSα where bi-terminal end-blocks are required to prevent ATP-promoted release of the protein from a heteroduplex (15, 16, 22), a single end-block is largely sufficient to prevent release in the case of the bacterial protein (Fig. 1). Although this sort of behavior can be viewed as consistent with a mechanism that supports directional movement, it is seemingly incompatible with a mechanism that invokes free diffusion of a protein clamp along the helix.

**Discussion**

As discussed above, the several models for MutS action differentially invoke ATP hydrolysis by the DNA-bound and free forms of the protein. To address the possibility that MutS-DNA complexes may support ATP hydrolysis, we examined the ATPase activity in the presence of DNA substrates with zero, one, or two streptavidin-biotin end-blocks (Fig. 7). If ATPase activity were dependent on the ability of MutS to dissociate from the DNA, MutS complexes trapped on end-blocked substrates would be expected to have reduced ATPase activity in the presence of terminal blocks inhibit ATPase activity. The stimulation of MutS ATPase activity by DNA with a bi-termi-

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2 K. P. Bjornson and P. Modrich, unpublished experiments.
The sliding clamp model also postulates that ATP hydrolysis occurs upon DNA release. By using oligonucleotide heteroduplexes with sterically blocked ends as an ATPase cofactor, we have observed stimulation of ATPase activity, not the diminution predicted by this model. The gel shift experiments (Fig. 1) and SPRS studies (Fig. 2) performed with substrates identical to those used for ATPase assay indicate that MutS remains DNA bound while undergoing multiple ATP hydrolytic turnovers. Together, these observations seem more consistent with an active role for ATP hydrolysis in the function of DNA-bound MutS as opposed to the passive ATP-binding role envisioned in the molecular switch model.

Adenine nucleotides differentially modulate MutS affinities for heteroduplex and homoduplex DNAs in a manner that can dramatically alter the specificity of the protein for a mismatched base pair. Conditions where nucleotide-binding site occupancy is restricted to the triphosphate (i.e. in the presence of AMPPNP-Mg\(^{2+}\) or ATP (no Mg\(^{2+}\))) result in a dramatic reduction in heteroduplex affinity but have only a modest effect on homoduplex affinity. As a consequence, specificity of MutS for a mismatch is abolished, or nearly so, at salt concentrations of 100 mM or above (Fig. 4, Table I). Conversely, restriction of nucleotide-binding site occupancy to ADP-Mg\(^{2+}\) has little effect on heteroduplex affinity but confers reduced homoduplex affinity and consequently high mismatch specificity. This effect was particularly evident with a 200-bp heteroduplex DNA capable of binding multiple MutS molecules in nonspecific manner (Fig. 5). It is important to note that a similar loss of heteroduplex specificity in the presence of ATP (no Mg\(^{2+}\)) has been observed with yeast MutSα, although reduction of homoduplex affinity by ADP has not been observed in this system (26).

Interestingly, in the presence of ATP-Mg\(^{2+}\) where hydrolysis is occurring, apparent homoduplex affinity is similar to that observed in the presence of ADP-Mg\(^{2+}\), whereas heteroduplex affinity is similar to that determined in the presence of the triphosphate under conditions where hydrolysis is blocked (i.e. in the presence of AMPPNP-Mg\(^{2+}\) or ATP (no Mg\(^{2+}\)), Fig. 4). At physiological salt concentration (150 mM KCl), this results in a specificity intermediate between those observed when binding site occupancy is restricted to the diphosphate or the triphosphate. There are several potential explanations for this interesting effect. As noted above, pre-steady-state studies have shown that the rate-limiting step for ATP hydrolysis in the presence of a homoduplex cofactor occurs subsequent to hydrolysis, implying occupancy of at least one binding site by ADP (25). However, in the presence of heteroduplex, the rate-limiting step occurs at or prior to covalent chemistry, indicating significant binding site occupancy by ATP. Thus, in the presence of heteroduplex and ATP-Mg\(^{2+}\), MutS might be expected to behave as the triphosphate-bound form, whereas in the presence of homoduplex and ATP-Mg\(^{2+}\) it would behave as the diphosphate form. This is consistent with what we observe, but the effects of ATP-Mg\(^{2+}\) on heteroduplex/homoduplex affinities could also be indicative of a MutS conformational state in which one class of nucleotide-binding site is occupied by ATP and a second by ADP.

These nucleotide effects on MutS specificity have implications for the postulated kinetic proofreading role of adenine nucleotides in the mismatch verification model (13). This model suggests that ATP binding by DNA-bound MutS preferentially reduces the affinity of the protein for homoduplex DNA sites, thereby resulting in enhanced mismatch specificity. We do not find this to be the case. With the exception of specificity values obtained at 50 mM KCl, which only varied over a 3-fold range in the absence or presence of various adenine nucleotides (Table I), heteroduplex specificity was abolished in the presence of AMPPNP-Mg\(^{2+}\) or ATP (no Mg\(^{2+}\)) at KCl concentrations of 100 mM or higher. In fact, at KCl concentrations of 100 mM or above, the highest specificity was observed in the absence of nucleotide or in the presence of ADP-Mg\(^{2+}\).

The dramatic and differential effects of adenine nucleotides on heteroduplex and homoduplex affinities imply that MutS is capable of several distinct modes of DNA interaction that are modulated by ATP binding and hydrolysis. This idea is also consistent with the finding that the kinetic stability of MutS-heteroduplex complexes upon ATP challenge depends on the nature of the nucleotide present during the DNA binding step (Fig. 2). Although the molecular bases of the nucleotide effects on mismatch and nonspecific affinities are unclear, the structure of the bacterial MutS-heteroduplex complex may bear on this point (27, 28). The amino-terminal 800 residues of MutS compose five domains (mismatch binding, connector, core-levers, DNA clamp, and ATPase) that dimerize into a Θ-like structure that possesses two channels (27, 28). One channel formed by the connector and DNA clamp is occupied by heteroduplex, and the second channel, which is unoccupied in the complex and of unknown function, could also accommodate a region of helix (27, 29, 30). A two DNA-binding site mechanism involving a sliding clamp and a static DNA-binding site has been postulated to account for MutS movement in the directional translocation model (15). An alternate possibility is based on the fact that the MutS ATPase is member of the ABC ATPase family which also includes Rad50. Although structural information on the MutS-ATP complex is not available, the Rad50-ATP complex has been solved (31). The structural homology of MutS and Rad50 ATPase domains has led to the suggestion that ATP binding by MutS could lead to repositioning of the mismatch binding domain to yield a simple sliding clamp structure (30) like that proposed by Fishel and colleagues (16).

Unfortunately, little is known concerning the possible states of occupancy of the MutS nucleotide-binding sites. This is an important question since the nucleotide binding center of each MutS monomer subunit can potentially exist in three possible states: empty, occupied by triphosphate, or occupied by diphosphate. Given that MutS exists as dimers and tetramers in solution (9, 32, 33) and the extensive evidence indicating interaction of nucleotide and DNA binding center(s), the number of potential conformational states available to the protein may be substantial (29). In fact, nucleotide occupancy has been established only in the case of the crystallographic ADP-MutS-heteroduplex complexes obtained with carboxyl-terminal truncated forms of the Taq and E. coli proteins. These results differ, however, in the Taq complex both nucleotide-binding sites are occupied by ADP (13), whereas in the E. coli structure one site is occupied and one is empty (28). Elucidation of the possible states of nucleotide occupancy in the absence and presence of DNA cofactors should further clarify the role of the MutS ATPase in mismatch repair.

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Distinct MutS DNA-binding Modes That Are Differentially Modulated by ATP Binding and Hydrolysis

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