Nucleotide-promoted Release of hMutSα from Heteroduplex DNA Is Consistent with an ATP-dependent Translocation Mechanism*

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ATP hydrolysis by bacterial and eukaryotic MutS activities is required for their function in mismatch correction, and two different models for the role of ATP in MutS function have been proposed. In the translocation model, based on study of bacterial MutS, ATP binding reduces affinity of the protein for a mismatch and activates secondary DNA binding sites that are subsequently used for movement of the protein along the helix contour in a reaction dependent on nucleotide hydrolysis (Allen, D. J., Makarov, A., Grilley, M., Taylor, J., Thresher, R., Modrich, P., and Griffith, J. D. (1997) EMBO J. 16, 4467–4476). The molecular switch model, based on study of human MutSα, invokes mismatch recognition by the MutSα-ADP complex. After recruitment of downstream repair activities to the MutSα-mismatch complex, ATP binding results in release of MutSα from the heteroduplex (Gradia, S., Acharya, S., and Fishel, R. (1987) Cell 91, 995–1005). To further clarify the function of ATP binding and hydrolysis in human MutSα action, we evaluated the effects of ATP, ADP, and non-hydrolyzable ATP analogs on the lifetime of protein-DNA complexes. All of these nucleotides were found to increase the rate of dissociation of MutSα from oligonucleotide heteroduplexes. These experiments also showed that ADP is not required for mismatch recognition by MutSα, but that the nucleotide alters the dynamics of formation and dissociation of specific complexes. Analysis of the mechanism of ATP-promoted dissociation of MutSα from a 200-base pair heteroduplex demonstrated that dissociation occurs at DNA ends in a reaction dependent on ATP hydrolysis, implying that release from this molecule involves movement of the protein along the helix contour as predicted for a translocation mechanism. In order to reconcile the relatively large rate of movement of MutS homologs along the helix with their modest rate of ATP hydrolysis, we propose a novel mechanism for protein translocation along DNA that supports directional movement over long distances with minimal energy input.

In addition to their mismatch recognition activities, bacterial and eukaryotic MutS activities have an associated ATPase activity that is required for function of the proteins in mismatch repair (1–5). Two distinct functions have been proposed for nucleotide binding and hydrolysis by MutS homologs, both of which are based on the effects of ATP on MutS-heteroduplex interaction. The presence of ATP greatly reduces the efficiency of specific complex formation between bacterial MutS or eukaryotic MutSα and heteroduplex DNA (5–10), and ATP challenge of preformed MutS-heteroduplex complexes has been shown to result in departure of the protein from the mismatch (11). Available information indicates that some of these effects are attributable to ATP binding. Thus, ATPγS has been shown to promote departure of MutS from the mismatch in heteroduplex DNA (11), while ATPγS or ATP (in the absence of a divalent cation) reduce the binding efficiency human MutSα (hMutSα) to synthetic heteroduplexes (5, 10).

Electron microscopy of complexes between bacterial MutS and large heteroduplexes prepared from natural DNAs has demonstrated that ATP-promoted release of MutS from a mismatch is associated with efficient conversion of protein-DNA complexes to Ω-shaped loop structures stabilized by MutS at the base (11). Loop formation requires a mismatch, loop size increases linearly with time, loop growth depends on continued ATP hydrolysis, and the mismatch usually ends up in the loop. These observations have been interpreted in terms of a mechanism in which ATP binding reduces affinity of the protein for a mismatch and activates secondary DNA binding sites that are subsequently used for movement of the protein along the helix contour in a reaction dependent on nucleotide hydrolysis (11). MutS movement in this manner has been postulated to be important for the coupling of mismatch recognition to loading of the excision system at the strand break that directs repair (12, 13), a site that can be located hundreds of base pairs from this mismatch.

The finding that ATP binding reduces the efficiency of specific complex formation between hMutSα and oligonucleotide heteroduplexes has led to proposal of a molecular switch model for action of MutS activities. Like a G-protein, hMutSα is postulated to exist in two states, an ADP-bound form that binds with near irreversible affinity to a mismatch and an ATP-bound form that does not (10). In this proposal hMutSα-ADP binds to a mismatch and recruits downstream activities to this site. After assembly of the excision system, ATP binding results in dissociation of hMutSα from the heteroduplex so that repair may proceed (10).

To further clarify the role(s) of ATP binding and hydrolysis in hMutSα action, we have evaluated the effects of ATP, ADP, and nonhydrolyzable ATP analogs on the lifetime of hMutSα-DNA complexes and have examined the effect of DNA topology on ATP-promoted dissociation of hMutSα complexes with small heteroduplexes. We demonstrate that ADP is not required for mismatch recognition by hMutSα, but that the nucleotide alters the dynamics of formation and dissociation of specific hMutSα-mismatch complexes. We also show that ATP-pro-
moted dissociation of hMutSα from small heteroduplexes is blocked by physical barriers placed at the ends of the DNA. This implies that ATP-promoted dissociation of hMutSα from small heteroduplexes involves movement along the helix contour, as predicted for a translocation mechanism.

**EXPERIMENTAL PROCEDURES**

**ATP and ADP Content of hMutSα Preparations and Nucleotide Depletion**—Human MutSα was purified as described previously to a purity in excess of 95% (7). ADP and ATP levels in purified hMutSα were determined using an ATP bioluminescence assay kit (Sigma). For background determination, 50 μl of hMutSα (108 ng in 5 μl Tris-HCl, pH 8.0, and 1 mM EDTA) was heat-denatured at 90 °C for 5 min, followed by addition of 50 μl of 120 mM Tris acetate, pH 7.8, 20 mM MgCl2, 2 mM KCl, 3 mM EDTA, 5 mM 2-mercaptoethanol. The diluted solution was mixed with 100 μl of a 25-fold dilution of the luminescent assay mix (Sigma), placed in a scintillation vial, and photon emission determined in the tritium channel of a Beckman LS6500 scintillation counter. ATP standards prepared in a similar manner yielded a linear response throughout the range tested (0.4–12.5 nm). ADP was determined by the same procedure after conversion to ATP by pyruvate kinase (14, 15).

Preparations of MutSα were depleted of nucleotide using Sephadex G50 spin column chromatography. Protein samples were either kept on ice or spun through a 1-ml G50 Sephadex column equilibrated in 20 mM TRIS-HCl, pH 8.0, 1 mM EDTA and heating to 99 °C in a Perkin-Elmer Gene Amp 9600 thermocycler, followed by cooling to 25 °C over a 30-min period. The solution of annealed duplex (50 μl) was adjusted to 1 mM NaCl and mixed with 5–10 μl of benzoylated naphthoylated DEAE-cellulose suspension (50% settled volume) in batch to remove single-stranded DNA (16). The oligonucleotide/ benzoylated naphthoylated DEAE-cellulose mixture was heated to the top of a Sephadex G50 spin column equilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and dithiothreitol, 5 mM MgCl2, 2 mM Mg/ml bovine serum albumin were loaded onto spin columns, which were centrifuged briefly in a clinical centrifuge. Presence of nucleotide in gel-filtered samples was determined as described above.

**Oligonucleotides and DNA—**Oligonucleotides were purchased from Oligos Etc. (Wilsonville, OR) and, when indicated, were 5′-end labeled with T4 polynucleotide kinase (U. S. Biochemical Corp.) and [γ-32P]ATP (3000 Ci/mmol, NEN Life Science Products) to a specific activity of 1 × 106 to 3 × 106 cpm/μmol. Duplexes were prepared by mixing molar equivalents of an unlabeled or 5′-32P-labeled oligonucleotide with an appropriate unlabeled complementary sequence in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and heating to 99 °C in a Perkin-Elmer Gene Amp 9600 thermocycler. After cooling to 25 °C over a 30-min period. The solution of annealed duplex (50 μl) was adjusted to 1 mM NaCl and mixed with 5–10 μl of benzoylated naphthoylated DEAE-cellulose suspension (50% settled volume) in batch to remove single-stranded DNA (16). The oligonucleotide/ benzoylated naphthoylated DEAE-cellulose mixture was heated to the top of a Sephadex G50 spin column equilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and dithiothreitol, 5 mM MgCl2, 2 mM Mg/ml bovine serum albumin were loaded onto spin columns, which were centrifuged briefly in a clinical centrifuge. Presence of nucleotide in gel-filtered samples was determined as described above.

**Binding of bacterial MutS to f1MR heteroduplex DNA**—Binding of bacterial MutS to f1MR heteroduplex DNA was assayed using a scintillation proximity assay (SPA) method as described previously (11). Briefly, a 5′-end-labeled 600-bp DNA fragment containing the f1MR gene was immobilized on a nylon membrane using Biochip Technology (24). The NYGeneChip was washed at 37 °C for 5 min with radiolabeled dCTP, followed by another 10 min incubation after addition of the unlabeled triphosphates. Reactions were terminated by addition of EDTA to 24 mM, and free nucleotides were removed by G50 spin column chromatography. DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 100 μl of 10 mM Tris-HCl, pH 8.0, 1 μM EDTA.

A 32P-labeled control 201-bp DNA that was biotin-tagged at only one end was prepared by a similar procedure, except that only one PCR primer was 5′-biotin-tagged.

**Nhel Protection Assay for hMutSα Mismatch Complexes—**Binding of bacterial MutS to f1MR heteroduplex DNA was assayed using a scintillation proximity assay (SPA) method as described previously (11). Briefly, a 5′-end-labeled 600-bp DNA fragment containing the f1MR gene was immobilized on a nylon membrane using Biochip Technology (24). The NYGeneChip was washed at 37 °C for 5 min with radiolabeled dCTP, followed by another 10 min incubation after addition of the unlabeled triphosphates. Reactions were terminated by addition of EDTA to 24 mM, and free nucleotides were removed by G50 spin column chromatography. DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 100 μl of 10 mM Tris-HCl, pH 8.0, 1 μM EDTA.

**Gel Shift Assay—**Binding reactions (20 μl) contained 20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 5 mM MgCl2, and 50 mM KCl, and [32P]G-T heteroduplex and hMutSα as indicated. After incubation at 37 °C for 15 min, reactions were stopped by addition of 2.2 μl of 50% (w/v) urea in formamide containing 0.05% xylene cyanol and 0.05% bromphenol blue. DNA fragments were separated by electrophoresis through a 10% denaturing polyacrylamide gel in 89 mM Tris, 89 mM boric acid, 2 mM EDTA, 7 μl urea at 40 mM. DNA samples were quantitated using a Molecular Dynamics PhosphorImager.

**Surface Plasmon Resonance Spectroscopy—**Surface plasmon resonance measurements used a BLAcore 2000. Streptavidin SA sensor chips (Pharmacia Biosensor) were preconditioned according to the manufacturer’s protocol and then derivatized with about 100 response units of a biotinylated 31-bp G-T heteroduplex that has been described previously (11). Human MutSα was bound to the derivatized chip by injecting a 50 nM solution of the heteroduplex at a flow rate of 15 μl/min in HBS buffer (10 mM HEPES-KOH, pH 7.4, 0.15 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) containing 2 mg/ml bovine serum albumin, and 32P-labeled substrate, and unlabeled competitor DNAs present as indicated. After incubation at 37 °C for 15 min, reactions were stopped by addition of 2.2 μl of 50% (w/v) glycerol, 0.05% xylene cyanol, 0.05% bromphenol blue, placed on ice, and loaded immediately onto a 5% native polyacrylamide gel (acrylamide-bisacrylamide 37.5:1). Gels were electrophoresed at 11.4 V/cm in 6.7 mM Tris acetate, pH 7.5, 1 mM EDTA, and 32P-labeled protein-DNA complexes visualized by autoradiography after drying.

**Surface Plasmon Resonance Spectroscopy—**Surface plasmon resonance measurements used a BLAcore 2000. Streptavidin SA sensor chips (Pharmacia Biosensor) were preconditioned according to the manufacturer’s protocol and then derivatized with about 100 response units of a biotinylated 31-bp G-T heteroduplex that has been described previously (11). Human MutSα was bound to the derivatized chip by injecting a 50 nM solution of the heteroduplex at a flow rate of 15 μl/min in HBS buffer (10 mM HEPES-KOH, pH 7.4, 0.15 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) containing 2 mg/ml bovine serum albumin, and 32P-labeled substrate, and unlabeled competitor DNAs present as indicated. After incubation at 37 °C for 15 min, reactions were stopped by addition of 2.2 μl of 50% (w/v) glycerol, 0.05% xylene cyanol, 0.05% bromphenol blue, placed on ice, and loaded immediately onto a 5% native polyacrylamide gel (acrylamide-bisacrylamide 37.5:1). Gels were electrophoresed at 11.4 V/cm in 6.7 mM Tris acetate, pH 7.5, 1 mM EDTA, and 32P-labeled protein-DNA complexes visualized by autoradiography after drying.
This possibility, we have tested near homogeneous hMutS MutS activities may purify as the ADP complex. To address protein for heteroduplex DNA to a significant degree (10). ADP does not alter the specific equilibrium affinity of the ADP complex as the mismatch binding and hydrolysis cycles the mismatch recognition activity between several conformational states that are involved in departure of the protein from the mismatch and movement along the helix contour (11). By contrast, the molecular switch model invokes the MutS dependence promoting release of the protein from the heteroduplex so that excision repair may occur (10).

One potential problem with the molecular switch model is that bacterial, mammalian, and yeast MutS activities bind with high specificity to mismatched base pairs in the absence of exogenous nucleotide (7–9, 17–19). Furthermore, ADP has no significant effect on the apparent affinity of human or yeast MutS for a mispair (10, 20). However, it is possible that these MutS activities may purify as the ADP complex. To address this possibility, we have tested near homogeneous hMutS isolated from HeLa cells for presence of adenine nucleotides. Analysis of several independent hMutS preparations using a bioluminescent assay (see “Experimental Procedures”) indicated presence of 0.003 mol of ATP and 0.2 mol of ADP per mol of the MSH2-MSH6 heterodimer. The presence of nucleotide in hMutS preparations was expected since the protein is purified by a two-step procedure, the first of which involves elution from DNA cellulose by ATP and Mg$^{2+}$ (7). In fact, ATP and ADP present in hMutS preparations were reduced by more than 90% by gel filtration spin column chromatography (see “Experimental Procedures”). As shown in Fig. 1, nucleotide depletion had no effect on the ability of hMutS to bind specifically to G-T heteroduplex DNA, as judged by restriction endonuclease protection assay. Therefore, ADP is not required for hMutS mismatch binding.

**ATP and ADP Greatly Increase the Rate of hMutS Dissociation from a Small G-T Heteroduplex**—In contrast to ATP, which reduces the affinity of hMutS for a mismatch (7, 8), ADP does not alter the specific equilibrium affinity of the protein for heteroduplex DNA to a significant degree (10).

**RESULTS**

**Nucleotide-free hMutS Binds with High Specific Affinity to a Mismatch**—The translocation and molecular switch models postulate different roles for ATP binding and hydrolysis in the function of MutS homologs. In the translocation model, ATP binding and hydrolysis cycles the mismatch recognition activity between several conformational states that are involved in departure of the protein from the mismatch and movement along the helix contour (11).

**Like ATP, ADP promotes dissociation of hMutS from a G-T heteroduplex**—The binding of hMutS to a 31-bp G-T heteroduplex was monitored by surface plasmon resonance spectroscopy (see “Experimental Procedures”). Upper panel, after maximum binding was achieved ($t = 0$), the chip was washed with buffer alone or with buffer containing 1 mM ATP, ADP, or AMPPNP as indicated. For the sample designated ATP($-$Mg$^{2+}$), MgCl$_2$ was omitted from the post-binding wash buffer (see “Experimental Procedures”). Dissociation, which was biphasic with a major and minor species, was fit to a sum of two exponentials ($A(t) = A_1 (1 - e^{-k_1 t}) + A_2 (1 - e^{-k_2 t})$). As summarized in Table I, the major species comprised 72–97% of the material. Lower panel, the rate constant for dissociation of the major species was determined as described above as a function of ATP concentration. Asymptotic standard errors returned by the fitting routine (27) for these rate constants were 2% or less in all cases and are not shown. The line shown was determined by non-linear least squares fit (27) to the hyperbolic function $k_{obs} = k_0 + k_{max} [ATP]/(K_m + [ATP])$, where $k_0$ corresponds to the rate constant for dissociation in the absence of ATP. The fit yielded the following values: $k_0 = 0.0028$ s$^{-1}$, $k_{max} = 1.26$ s$^{-1}$, and $K_m = 37$ μM.

However, ADP does alter the dynamics of hMutS- heteroduplex interaction as judged by several criteria. Fig. 2 (upper panel) shows the dissociation kinetics of specific hMutS complexes with a 31-bp G-T heteroduplex as visualized by surface plasmon resonance spectroscopy. Dissociation kinetics monitored by this method were biphasic, and results were fit to a sum of two exponentials. Dissociation rate constants corresponding to the major amplitude, which represented 72–97% of the protein-DNA complexes, are summarized in Table I. In the presence of Mg$^{2+}$ but in the absence of nucleotide, the major species dissociated with a half-life of about 5 min. Inclusion of 1 mM ATP increased the off-rate about 200-fold. This ATP effect is independent of hydrolysis since 1 mM AMPPNP or 1 mM ATP in the absence of Mg$^{2+}$ enhanced the dissociation rate to a similar degree, results consistent with previous findings suggesting that ATP binding is sufficient for mismatch dissociation in the case of bacterial MutS (11) and hMutS (5, 10).
presence of ADP also resulted in a large increase in the rate of dissociation of hMutS\(a\)-heteroduplex complexes: 1 m\(M\) ADP decreased the half-life of specific complexes 25-fold relative to that observed with buffer alone (Table I).

Surface plasmon resonance analysis demonstrated that the increased rate of hMutS\(a\)-heteroduplex dissociation is a saturable function of ATP concentration. As shown in Fig. 2, the rate of complex dissociation increases hyperbolically with ATP concentration, with a \(K_m\) of 37 \(\mu M\), a value very similar to the \(K_m\) for the nucleotide in the DNA-stimulated ATPase reaction (10, 21).

\(Nhe\) restriction endonuclease protection assay confirmed saturability of ATP-promoted dissociation and also indicated saturability for dissociation promoted by AMPPNP and ADP (Fig. 3). In this assay, preformed hMutS\(a\)-heteroduplex complexes were challenged with \(Nhe\)I and nucleotide, and incubation continued for 5 min (see “Experimental Procedures”). Although individual \(K_{1/2}\) values obtained by this method cannot be interpreted analytically due to the fact that dissociation occurs during the \(Nhe\)I assay period, comparison of \(K_{1/2}\) values provides an estimate of the relative efficacy of the different nucleotides for promoting dissociation of the hMutS\(a\)-heteroduplex complex. The relative \(K_{1/2}\) values for ADP and AMPPNP determined in this way are about 5 and 10 times greater than that for ATP, respectively (Fig. 3). The low relative affinity of the hMutS\(a\)-heteroduplex complex for AMPPNP is in accord with previous findings, indicating that this nucleotide is less effective than ATP in suppressing the formation of specific complexes with oligonucleotide heteroduplexes (5, 7, 10).

To further clarify the roles of ATP and ADP in the dynamics of hMutS\(a\)-DNA interaction, kinetic competition studies were performed. In these experiments, preformed complexes of hMutS\(a\) and a 50-bp \([\text{32}P]\)G-T heteroduplex were challenged with a 20-fold molar excess of unlabeled 50-bp heteroduplex or homoduplex DNA in the presence of ADP or ATP (Fig. 4). In the absence of nucleotide, only a modest reduction in preformed hMutS\(a\)-DNA complexes was observed (compare lanes 2 and 3 with lane 1), consistent with the kinetic stability of such complexes in the absence of added nucleotide (Table I). Addition of 100 \(\mu M\) ATP reduced the steady-state level of specific complexes about 3-fold (compare lane 4 with lane 1), but when the ATP challenge was performed in the presence of unlabeled competitor DNA, hMutS\(a\) complexes with the \([\text{32}P]\)heteroduplex were nearly abolished. Since heteroduplex and homoduplex were equally effective as competitor under these conditions (lanes 5 and 6), ATP binding (or hydrolysis) must greatly reduce the relative affinity of hMutS\(a\) for a mismatch as compared with that for nonspecific, canonically paired sequences. This result is in marked contrast to the limited effect of homoduplex competitor on mismatch recognition by hMutS\(a\) in the absence of ATP, conditions where specific complexes are readily formed in the presence of a several hundred-fold molar excess of homoduplex molecules (7).

| Nucleotide (1 \(\mu M\)) | Amplitude | \(k_{1/2}\) | \(t_{1/2}\) |
|--------------------------|-----------|-------------|-----------|
| Buffer only              | 0.93      | 0.002 ± 0.000001 | 350 ± 2   |
| ATP                      | 0.97      | 0.42 ± 0.01  | 1.7 ± 0.04 |
| ATP (\(-\text{Mg}^2+\)) | 0.88      | 0.45 ± 0.01  | 1.5 ± 0.03 |
| AMPPNP                   | 0.72      | 0.33 ± 0.01  | 2.1 ± 0.06 |
| ADP                      | 0.86      | 0.05 ± 0.001 | 14 ± 0.3  |

**TABLE I**

Kinetics of MutS\(a\)-heteroduplex dissociation promoted by nucleotide cofactors

- ATP and ADP promote dissociation of hMutS\(a\)-heteroduplex complexes.
- The low \(K_{1/2}\) values for ADP and AMPPNP compared to ATP indicate its lesser efficacy in promoting dissociation.
- The presence of ADP increases the rate of dissociation, whereas ATP promotes dissociation more effectively.

**Fig. 4.** DNA binding in the presence of ADP is specific for the mismatch, while that with ATP is nonspecific. hMutS\(a\) (38 \(\mu M\)) was preincubated for 10 min with 5 \(\mu M\) of 50-bp \([\text{32}P]\)G-T heteroduplex under gel shift assay conditions (see “Experimental Procedures”). Protein-DNA complexes were then challenged for 5 min with a 20-fold molar excess of unlabeled G-T heteroduplex or homoduplex DNA in the absence of nucleotide (lanes 1–3), or in the presence of 100 \(\mu M\) ATP (lanes 4–6) or 100 \(\mu M\) ADP (lanes 7–9).

Like ATP, ADP increases the rate of dissociation of hMutS\(a\)-heteroduplex complexes (Table I), but the effects of these two nucleotides on the dynamics of hMutS\(a\)-DNA complexes are distinct. Thus, addition of ADP to preformed hMutS\(a\)-heteroduplex complexes in the absence of competitor DNA did not reduce the steady-state level of specific complexes (Fig. 4, compare lanes 1 and 7). As expected, the yield of preformed complexes was greatly diminished when ADP challenge was performed in the presence of excess heteroduplex trap DNA due to the ADP-promoted increase in DNA dissociation rate (lanes 7 and 8). However, in contrast to results obtained in the presence of ATP, a 20-fold molar excess of homoduplex competitor was not an effective trap for capture of hMutS\(a\) released from preformed complexes upon challenge with ADP. These results imply that the hMutS\(a\)-ADP complex retains high specific affinity for a mismatch and are consistent with previous findings that ADP has little effect on the apparent affinity of human or yeast MutS\(a\) for a mispair (10, 20). In view of the increased rate of DNA dissociation promoted by ADP, retention of near normal mismatch affinity suggests that...
ADP also increases the rate of formation of hMutSα-mismatch complexed. Preliminary analysis of the kinetics of hMutSα heteroduplex complex formation by surface plasmon resonance is consistent with this view, with these experiments also indicating a modest decrease in specific affinity of the heterodimer for a mismatch in the presence of ADP as compared with that in its absence.2

Physical Barriers at DNA Termini Block ATP-promoted Dissociation of hMutSα from a Small Heteroduplex—As noted above, the two models for the role of ATP hydrolysis in the activity of MutS homologs are based on distinct results obtained with large versus small heteroduplexes. The mismatch- and ATP-dependent formation of α-shaped DNA loops with large heteroduplexes has led to the suggestion that ATP binding and hydrolysis cycles bacterial MutS through a set of conformational states that permit it to leave the mismatch and move along the helix contour (11). The finding that ATP binding greatly reduces the apparent affinity of hMutSα for small heteroduplexes has led to a molecular switch model that postulates ATP-promoted release of hMutSα from the DNA after repair complex assembly at the mismatch (10).

In order to distinguish between these two models and in an attempt to reconcile the different results obtained with large and small substrates mentioned above, we have tested the possibility that dissociation of hMutSα from a small heteroduplex may occur by movement along the helix. If this were the case, then dissociation should be blocked by placing physical barriers at DNA termini where dissociation is expected to occur, as has been shown to be the case for the β sliding clamp of DNA polymerase III holoenzyme (22). To address this issue, a 201-bp heteroduplex with a central G-T mismatch and an otherwise identical A-T homoduplex were prepared, both of which were 5'-biotin-tagged at each end (“Experimental Procedures”). Gel shift analysis in the presence of streptavidin demonstrated formation of two streptavidin-DNA complexes in similar yield (Fig. 5, lanes 13 and 14). Two approaches were used to identify these species as streptavidin-DNA complexes with protein bound at one or both ends. Cleavage with HindIII, which yields a 96-bp 32P-labeled fragment and an unlabeled 105-bp product, converted the two types of avidin-DNA complex into single streptavidin-tagged, 32P-labeled species (not shown). Furthermore, only the faster migrating streptavidin-DNA complex was observed with a 201-bp control DNA that contained a biotin tag at just one end. Despite the multivalent binding potential of streptavidin, polymerization of biotin-labeled DNA fragments therefore did not occur under the conditions used for the experiments described here.

In the absence of streptavidin, hMutSα specifically recognized the G-T mismatch in the biotin-tagged 201-bp heteroduplex, forming a single shifted band whose intensity increased with increasing protein concentration (Fig. 5, lanes 1–3). However, this species was essentially abolished by ATP addition to preformed complexes (lanes 4–6), a finding similar to that obtained with smaller oligonucleotide substrates (lanes 7–10). It is important to note that the hMutSα-heteroduplex complexes shown in Fig. 5 were prepared in the presence of homoduplex competitor DNA. This accounts for the more dramatic ATP effect in this experiment as compared with that shown in lane 4 of Fig. 4.

Human MutSα was also efficiently bound by the 201-bp heteroduplex in the presence of streptavidin, but in this case four hMutSα-bound species were evident (lanes 7–9). The intensity of each of these species increased with hMutSα concentration, with a concomitant decrease in hMutSα-free DNA species with 0, 1, or 2 bound streptavidin molecules. As summarized quantitatively in the legend to Fig. 5, these observations indicate that the DNA terminal streptavidin complexes did not interfere with hMutSα binding, and the failure to observe hMutSα-shifted species with a streptavidin-tagged A-T homoduplex control (lane 15) demonstrated that complexes produced with heteroduplex DNA were mismatch-specific.

In contrast to the effect of ATP on hMutSα-heteroduplex complexes in the absence of streptavidin, ATP challenge of preformed complexes with streptavidin-tagged heteroduplex yielded a stable complex resistant to nucleotide-promoted dissociation (Fig. 5, compare lanes 10–12 with lanes 7–9). Of the four shifted bands observed in the presence of hMutSα, ATP promoted dissociation of the two more rapidly migrating species. Loss of these two species was accompanied by reappearance of free forms of the 201-bp heteroduplex with 0 and 1 molecule of bound streptavidin (compare lanes 7–9 and 10–12 with lane 13), and quantitation of the hMutSα-free DNA spe-

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2 D. Martik and P. Modrich, unpublished experiments.
cies (Table II) showed that dissociation of the protein from these two species was virtually complete. Inasmuch as the two ATP-resistant hMutSα-DNA complexes were species of the slowest mobility and since ATP challenge failed to restore original levels of the 201-bp heteroduplex with biterminal streptavidin complexes (Table II; and compare lanes 10–12 with lane 13 in Fig. 5), these complexes must have originated from this heteroduplex species. Inspection of the data in Table II under the “2-streptavidin” entry indicates that 75–78% of the complexes formed between hMutSα and the biterminal tagged heteroduplex were resistant to fairly prolonged incubation with ATP in the presence of Mg2+.

The experiments in Fig. 5 demonstrating ATP-resistant hMutSα-heteroduplex complexes were performed under conditions where nucleotide hydrolysis was occurring (“Experimental Procedures”). To determine whether this result is dependent on ATP hydrolysis, similar experiments were performed with AMPPNP and ATPγS, as well as with ATP in the absence of a divalent cation (Fig. 6). Interestingly, under all conditions of hydrolysis block, nucleotide challenge resulted in a similar degree of hMutSα-heteroduplex dissociation without regard to the streptavidin status of the DNA (compare lanes 3–5 with lane 2), an effect that is evident not only from inspection of hMutSα-heteroduplex shifted species but also from the levels of free heteroduplex with a biterminal avidin block (arrow). These results imply that nucleotide hydrolysis is necessary for formation of the stable hMutSα-DNA complex that we have observed in the presence of ATP.

**DISCUSSION**

The experiments described here further clarify the role of nucleotides as modulators of hMutSα-heteroduplex interaction. As noted above, previous work has demonstrated that ATP greatly reduces the efficiency of specific complex formation between MutS homologs and small heteroduplexes (6–9), an effect that appears to be due largely to binding of the triphosphate (5, 10). We have extended these observations to show that ATP, AMPPNP, or ATP in the absence of Mg2+ increases the rate of dissociation of preformed complexes between hMutSα and small heteroduplexes by several orders of magnitude. This observation is consistent with findings in the bacterial system that addition of ATP or ATPγS to preformed complexes between MutSα and a large heteroduplex promotes departure of the protein from the mismatch, but not from the DNA molecule (11). Although previous experiments have indicated that the effects of AMPPNP on hMutSα-heteroduplex complex formation are much more dramatic than those of ATP or ATPγS (5, 7, 10), we have shown this to be due to a relatively low affinity of the complex for the former nucleotide. At high concentrations, AMPPNP is as effective as ATP in promoting dissociation of preformed specific complexes.

There is general experimental agreement in the literature with respect to the modulating effects of ATP and ATP analogs on complex formation between MutS homologs and heteroduplex DNA, but these effects have been interpreted in two different ways with respect to the role of these effects in mismatch repair. The translocation model, which is based on electron microscopy of complexes between bacterial MutS and large heteroduplexes, invokes ATP binding and hydrolysis in movement of the protein along the helix contour (11). This provides a physical mechanism by which mismatch recognition may be coupled to recognition of a strand break elsewhere on the helix that serves as the site for initiation of excision (12, 13). The molecular switch model, which is based on the demonstration that ATP binding reduces the efficiency of complex formation between hMutSα and small heteroduplexes, postulates mismatch recognition by the hMutSα-ADP complex, which binds to the mispair with nearly irreversible affinity (10). The resulting ADP-hMutSα-DNA complex serves as a flag for assembly of other repair activities of the mismatch, with subsequent ATP binding resulting in release of hMutSα from the DNA so that repair may ensue.

Examination of a possible ADP requirement has shown that formation of the hMutSα-ADP complex is not necessary for mismatch recognition and that the affinity of this complex for oligonucleotide heteroduplexes is no greater than that of the nucleotide-free form of the hMSH2-hMSH6 heterodimer. However, we have also found that binding of ADP by hMutSα enhances the dynamics of its interaction with heteroduplex DNA by increasing the rates of dissociation and formation of specific complexes.

We also examined the molecular pathway by which hMutSα dissociates from small heteroduplexes after challenge of preformed complexes with ATP. The aim of these experiments was to resolve an apparent discrepancy with respect to the effect of ATP on the fate of the mismatch-MutS homolog complex, e.g., movement from the mispair along the helix contour with large DNAs as opposed to rapid dissociation from the DNA in the case of small heteroduplexes. Since the translocation model predicts more rapid dissociation of MutS activities from small DNA.
versus large heteroduplexes due to a requirement for movement along the helix and release at DNA termini, we tested the involvement of DNA ends in ATP-promoted dissociation. The finding that biterminal physical barriers block ATP-promoted dissociation of hMutSα from a 200-bp heteroduplex indicates that DNA release involves movement of the hMSH2-hMSH6 heterodimer along the helix contour, as expected for a translocation mechanism.

The use of terminally blocked DNA has also shown that ATP-promoted dissociation from the small heteroduplex can occur via two distinct pathways. Under conditions where ATP hydrolysis was permitted, the biterminal physical barriers blocked dissociation of hMutSα from the DNA, but when nucleotide hydrolysis was prevented by omission of a divalent cation or by use of nonhydrolyzable ATP analogs, dissociation did occur within the 5-min incubation period after nucleotide addition to the terminally blocked complex (Fig. 6). While ATP binding is clearly sufficient to release hMutSα from the mismatch, the latter observation suggests that nucleotide hydrolysis by at least one subunit of the heterodimer is required for production of the dissociation-resistant protein-DNA complex. However, it is also possible that binding of ATP-Mg\(^{2+}\) is sufficient to drive formation of this complex, with the nonhydrolyzable analogs (or ATP in the absence of a divalent cation) being unable to do so, perhaps due to an inability to adopt an appropriate conformation upon binding to hMutSα. In any case, these results imply that dissociation of hMutSα from biterminal blocked heteroduplex under the latter conditions occurs via a side reaction, possibly dissociation of a metastable intermediate that otherwise decays to yield the dissociation-resistant complex that is observed in the presence of ATP and a divalent cation.

Movement of bacterial MutS and, as shown here, hMutSα along the helix provides a mechanism by which mismatch recognition can be coupled to recognition of the strand break that directs repair to the new DNA strand. The rate of movement of hMutSα along the helix has not been determined, but bacterial MutS extrudes a loop from heteroduplex DNA at a rate of several hundred to several thousand bp per min depending on reaction conditions (11). These values compare with a \(k_{\text{cat}}\) value for DNA-stimulated ATP hydrolysis of about 5 min\(^{-1}\), similar to that for hMutSα.\(^3\) It is difficult to reconcile these numbers, which imply translocation over hundreds of bp per ATP hydrolytic event, with the action of a conventional molecular motor. Consequently, we have considered a novel mechanism for heteroduplex loop formation that involves subunit movement along the helix by a linear diffusion process that occurs against a reflecting barrier (Fig. 7).

This model postulates that each subunit contains two sites that function in the process of movement, a latch site (L) and a site (D) through which DNA may freely diffuse. The latter site is similar to the ring structures adopted by the E. coli \(\beta\) clamp or the eukaryotic proliferating cell nuclear antigen processivity factors that topologically constrain diffusion of these proteins along the helix (23). The L site on the other subunit, acts as a molecular latch that when closed, blocks physical movement of DNA through this site and serves as a reflecting barrier against which DNA diffuses through site D on the alternate subunit. As described in the text, this model invokes two sites on each MutS subunit that participate in the process of movement of the protein along the helix, a latch site L and a site D, through which DNA may freely diffuse. When closed, the latch site L blocks physical movement of DNA through this site and serves as a reflecting barrier against which DNA diffuses through site D on the alternate subunit. The latch, which is closed in two upper diagrams, opens in the lower in response to a change in phosphorylation state of the bound nucleotide. For simplicity, only half of each subunit is shown, e.g., the L site on the upper subunit and the D site on the lower subunit are not shown. Similar action of the sites that are not shown would result in bidirectional translocation, as has been observed for bacterial MutS (11).

\[
l = \frac{1}{\sqrt{\pi D t}} e^{-\frac{x^2}{4Dt}} dx = \frac{2}{\sqrt{\pi}} \sqrt{\frac{D t}{\pi}} \quad (\text{Eq. 1})
\]

\(l\) is time, and \(D\) is the diffusion coefficient governing movement of DNA through site D. Change in occupancy of the nucleotide binding site results in brief opening of the latch L, permitting the small DNA segment to join a growing DNA loop that is being extruded by the two subunits via repetition of these steps.

This model is consistent with observations on the growth of the heteroduplex DNA loops that are extruded by bacterial MutS. The small intermediate loops produced by DNA diffusion past site D against the barrier L grow as a function of \(t^{1/2}\) during the course of an ATP hydrolytic cycle. However, since the DNA segment in this small intermediate loop efficiently transferred into the primary loop upon opening of the latch L, the primary loop grows linearly with time on a scale that is long relative to the turnover time for ATP hydrolysis. This mechanism can thus account for linear rate of growth of heteroduplex loops extruded by bacterial MutS (11). Assuming that the small intermediate loops grow over a period of about 10 s, corresponding to the time for an ATP turnover event, their ultimate size

\(^3\) K. Bjornson, W. Bedale, and P. Modrich, unpublished experiments.
can be estimated from the macroscopic rate of loop growth (≈500–5000 bp min⁻¹; Ref. 11) to be in the range of 30–400 bp, corresponding to a diffusion constant \( D \) in the range of \( 8 \times 10^{-14} \) to \( 1 \times 10^{-11} \) cm² s⁻¹, considerably less than the \( 10^{-9} \) cm² s⁻¹ value estimated for lac repressor (24) or the \( 10^{-11} \) to \( 10^{-10} \) cm² s⁻¹ range for EcoRI endonuclease (25). The diffusion rates required by the model are therefore reasonable. Finally, the heteroduplex loops extruded by bacterial MutS appear to be free of superhelical turns (11). A diffusion site based on the sliding clamp structure of \( E. coli \) \( \beta \) protein and proliferating cell nuclear antigen, like that shown in Fig. 7, can account for this finding, but this effect can also be explained in other ways. This model is attractive in the sense that it permits directional movement over large distances at minimal energy cost and can account for experimental observations linking nucleotide binding and hydrolysis to movement of MutS homologs along the helix. However, its validity awaits further experimental test. Of potential interest in this regard is the fact that electron microscopy of bacterial MutS-heteroduplex loops revealed occasional molecules with a small secondary loop emerging from the protein complex (11, 26).

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