Nucleic Acids Research, 2000, Vol. 28, No. 4 853–861

ATP-hydrolysis-dependent conformational switch modulates the stability of MutS–mismatch complexes

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Received November 8, 1999; Revised and Accepted December 22, 1999

ABSTRACT

The mismatch repair pathway in *Escherichia coli* has been extensively studied *in vitro* as well as *in vivo*. The molecular mechanisms by which nucleotide cofactors regulate the whole process constitute an area of active debate. Here we demonstrate that nucleotide (ADP or ATP) binding to MutS mediates a switch in protein conformation. However, in MutS that is DNA bound, this switch ensues only with ATP and not with ADP and is similar, irrespective of whether it is bound to a homo- or a heteroduplex. The results envisage a minimal model of three conformational states of MutS as reflected in: (i) a specific and highly stable MutS–mismatch complex in the absence of a nucleotide; (ii) a specific but less stable complex in the presence of ATP hydrolysis; and (iii) an irreversibly dissociated complex in the presence of ATP binding (ATPγS). Such transitions are of relevance to the protein's function *in vivo* where it has to first recognize a mismatch, followed by a search for hemimethylated sites.

INTRODUCTION

The mismatch repair system, which is evolutionarily conserved across prokaryotes and eukaryotes, plays a crucial role in maintaining the genomic integrity of the cell. The system safeguards and ensures high fidelity during key processes of replication and recombination. This repair pathway corrects mispaired or unpaired bases that arise as replication and recombination errors (1–4). Mismatch repair in prokaryotes has been well studied in the prokaryotic colon bacillus, *Escherichia coli*. The *E.coli* MutS can recognize and bind to DNA containing single base mismatches and small insertion–deletion loops of up to 4 nt (5–8). Mismatch bound MutS forms a complex with MutL in the presence of ATP, which then activates MutH, an endonuclease that incises the DNA at an upstream hemimethylated d(GATC) site. This directs excision and repair synthesis to the newly synthesized strand (3,8–11). Sequence information shows that MutS possesses a highly conserved Walker type-A nucleotide-binding motif which is involved in ATP binding and hydrolysis (12). MutS–ATP interactions have long since been shown to be important in modulating mismatch repair (12,13). Experiments done with MutS as well as with its eukaryotic counterparts, showed that addition of ATP to the mismatch bound protein can cause it to dissociate from the mismatch (9,14–20). It was believed that this dissociation is brought about as a result of hydrolysis of the ATP, the energy of which is channeled into translocating the mismatch repair complex over the flanking homoduplex DNA tracts in search of the hemimethylated sites (16). A contrasting model was put forth with the human mismatch repair proteins, where it has been shown that ATP binding alone, and not hydrolysis, can bring about a destabilization of the protein from the mismatch (17–19). In this model, a 'molecular-switch' role was ascribed to hMSH2–hMSH6, where the protein cycles between an ADP-bound 'ON' state (mismatch recognizing) and an ATP-bound 'OFF' state. The protein assumes a 'clamp' form in the OFF state that can freely slide on the helix in a hydrolysis-independent manner and now serves as a signal for the recruitment of further components of the repair pathway. This interesting switch of modes by nucleotide co-factors prompted us to look at the role of ATP in such a dynamic process.

The present study aims at understanding the events that transform the protein from a mismatch recognition mode to the so-called 'tracking' mode and the controversial role that ATP plays in such a process. We show that the addition of ATP facilitates the transition of a stable complex to one that is less stable but which still shows a high steady-state association of MutS with a mismatch. However, the complex dissociates when the hydrolysis of ATP is blocked, thereby abolishing MutS function. The results suggest that this dissociation is related to an ATP-dependent conformational switch in MutS that leads to a reduction in its affinity towards the mismatch. Moreover, initial rates of hydrolysis suggest that the effects of ATP are mediated through the mismatch bound MutS because of its higher affinity for ATP than for the free protein. Our experiments also show that ADP is not required for mismatch recognition, nor does it show any detectable effect on a preformed MutS–mismatch-DNA complex. Thus, we propose the existence of three plausible states of MutS: one which specifically binds to a mismatch in the absence of any nucleotide; the second, which irreversibly dissociates from the mismatch when ATP hydrolysis is prevented (in ATPγS conditions); and the third, which shows reduced stability but high level of steady-state association of MutS with the heteroduplex during ATP hydrolysis.

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MATERIALS AND METHODS

Materials

T4 polynucleotide kinase, IPTG, ATP, ADP, dithiothrietol (DTT) and DNase I were purchased from Amersham Life Science. Phosphocreatine, creatine phosphokinase, nuclease-free bovine serum albumin (BSA), PMSF and diaminobenzidine were from Sigma. ATPγS was from Boehringer Mannheim. Ni-NTA agarose resin was from Qiagen. Oligonucleotides were synthesized at DNA Technology (Denmark).

DNA substrates

The DNA substrates used in all assays were a single GT-mismatched duplex (heteroduplex) and its corresponding GC-matched duplex (homoduplex). The sequences are:
Heteroduplex (substrate A, 33 bp): top strand, 3′-ATT TAA CAC AGC TTT AGG CGC T**G**G ACG AGG TAC-5′; bottom strand, 5′-TAA ATT GTG TCG AAA TCC GCG A**T**C TGC TCC ATG-3′.
Homoduplex: 3′-ATT TAA CAC AGC TTT AGG CGC T**G**G ACG AGG TAC-5′ and 5′-TAA ATT GTG TCG AAA TCC GCG A**C**C TGC TCC ATG-3′.
Heteroduplex (substrate B, 61 bp): 3′-AGC GGA CTA TTT AAC ACA GCT TTA GGC GCT **G**GA CGA GGT ACA ATG AAT CGG CCT TGC TCC G-5′ and 5′-TCG CCT GAT AAA TTG TGT CGA AAT CCG CGA **T**CT GCT CCA TGT TAC TTA GCC GGA ACG AGG C-3′.

The oligonucleotides were purified by electrophoresis on a 10% denaturing polyacrylamide gel. The full-length oligonucleotide was excised from the gel and eluted into 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, by diffusion, followed by desalting through a Sep-pak C18 cartridge (21). Final purity was determined by 5′ end labeling using [γ-^{32}P]ATP and analysis on a 12% denaturing polyacrylamide gel. DNA concentrations expressed refer to oligonucleotide molecules unless mentioned otherwise.

DNA labeling and annealing

The standard protocol was used to label 5′ ends of the oligonucleotides (100 μM as nucleotides) using [γ-^{32}P]ATP (10 μCi) in 5 μl reactions with 3 U of T4 polynucleotide kinase. Samples were heated at 90°C for 5 min to inactivate the kinase. Annealing was done in 20 mM Tris–HCl (pH 7.6) and 5 mM $MgCl_2$ in a total volume of 10 μl. The sample was heated to 90°C for 4 min followed by slow cooling to room temperature.

Protein purification

The MutS clone was obtained from Dr Leroy Worth, NIEHS, USA. The MutS gene is in a His-tag expression vector pQE30. The protocol followed to purify MutS is as described (22). The His-tag was not cleaved from the protein as it does not seem to alter the biochemical properties of MutS (22).

MutS binding to heteroduplexes

Duplexes [0.03 μM, substrate A or B (see figure legends); formed by annealing 1:1 molar ratios of complementary oligonucleotides at a stock concentration of 10 μM in terms of nucleotides] were incubated with 2.5 μM MutS in a binding buffer containing 20 mM Tris (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 50 μg/ml nuclease-free BSA and 100 μM (nucleotide concentration) oligo T (30mer) (as carrier DNA) in a final volume of 10 μl at 0°C for 30 min (for gel shift assays) or at 37°C for 15 min (DNase I footprinting assays). In reactions involving ATP, a regeneration system that comprises 8 mM phosphocreatine and 10 U/ml creatine phosphokinase was incorporated. Specified concentration of a given nucleotide (ATP, ATPγS or ADP; see figure legends) was present in the binding buffer where required.

Gel shift assays

Binding was carried out in conditions described above except that KCl was excluded from the buffer. After carrying out the binding reaction as described above, the samples were mixed with 3 μl 50% (w/v) sucrose and electrophoresed on a 6% native polyacrylamide gel at 4°C at 100 V using Tris–borate buffer (89 mM, pH 8.0) and EDTA (2 mM). Gels were dried prior to exposure and quantified using a Bio-Rad PhosphorImager.

DNase I footprinting assays

MutS binding to heteroduplexes was carried out in standard binding buffer (see above) at 37°C for 15 min. The samples (12 μl) were equilibrated at room temperature for 5 min following which 2.4 μl DNase I (15 ng, 37.5×10^{-3} U) was added and incubated for 2 min at room temperature. The reaction was quenched by adding an equal volume (14.4 μl) of 90% formamide containing bromophenol blue and 10 mM EDTA. Subsequently, the samples were analyzed on a 12% denaturing polyacrylamide gel. Gels were dried prior to exposure and quantified using a Bio-Rad PhosphorImager.

ATPase assays

MutS (0.5 μM) was incubated with the duplex (substrate A, 1 μM) at 37°C in a standard binding buffer containing 0.1 mM ATP and 4 nM [γ-^{32}P]ATP. Aliquots (10 μl) were withdrawn at the indicated time-points and quenched by adding 2 μl of 2% SDS following which 100 μl of 1% activated charcoal suspension was added to each sample. The samples were vortexed for 30 s followed by incubation on ice for 10 min. The samples were centrifuged to pellet the charcoal and, subsequently, 25 μl aliquots of the supernatant were counted in duplicates.

RESULTS

Mismatch recognition by MutS

Three different approaches, namely gel shift, filter binding and footprinting assays were used to study the modulation of MutS binding to a 33 bp mismatch duplex (heteroduplex, substrate A). Our aim was to assess how the nucleotide cofactor ATP influences interactions between MutS and a mismatch. In the current study we confirmed that under the experimental conditions used, MutS binds to a heteroduplex in a highly specific manner. Binding of MutS to a duplex containing a single GT mismatch yielded a specific footprint. The footprint was about 19 bases in size encompassing both strands of the duplex (Fig. 1). Importantly, negative controls, i.e. heteroduplex minus MutS (Fig. 1) and homoduplex plus MutS (data not shown), showed no such footprint indicating the absence of a specific complex. We studied the modulation of MutS–mismatch-DNA interactions in three different conditions, i.e. with ATP under regeneration, ATPγS and ADP.

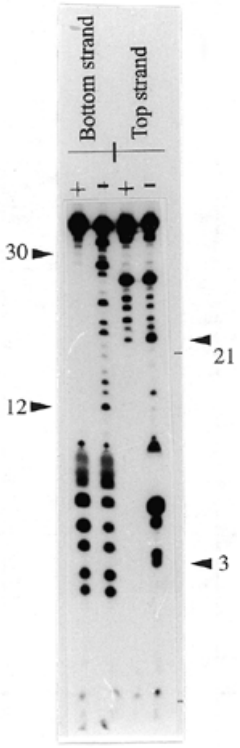

Figure 1. DNase I footprinting of MutS–mismatch-DNA complexes. DNase I footprinting was carried out on a 33 bp heteroduplex (substrate A in Materials and Methods; top and bottom strands are as described). The footprints are located between the arrowheads and the numbers indicate corresponding nucleotide positions. + and – indicate the presence and absence of MutS respectively.

A study of the stability of MutS–mismatch-DNA interactions: gel shift assay

Previous studies have shown that MutS and its homologs in other systems dissociate from the mismatch in the presence of ATP (9,14–20,22). We wanted to address this issue in detail. A regeneration system was included to ensure that ATP concentrations are stably maintained in spite of continual ATP hydrolysis. Yield of specific MutS–mismatch-DNA complexes was quantified as a function of increasing ATP concentrations. Since gel shift and filter binding assays yielded very similar quantitative data, here we present the data obtained only from the gel shift assays. Most of the gel-shifted complexes were destabilized by 0.25–0.4 mM ATP with no further loss at higher concentrations (Fig. 2A and B). In order to understand whether destabilization of MutS–mismatch-DNA complexes was due to mere binding of ATP as against its hydrolysis, we repeated the same experiment with ATPγS, a poorly hydrolyzable analog of ATP (23). The effect of ATPγS on these complexes was more severe. The destabilization was essentially complete at as low as 25 μM ATPγS. Moreover, in neither of these conditions (with or without ATP/ATPγS) were such gel-shifted complexes detectable in homoduplex controls revealing the specific nature of MutS–heteroduplex complexes. One must note that the smear that is evident in –ATP/ATP gel shifts might stem from the slow dissociation of specific complexes during electrophoresis. However, a similar trend was lacking in ATPγS, perhaps

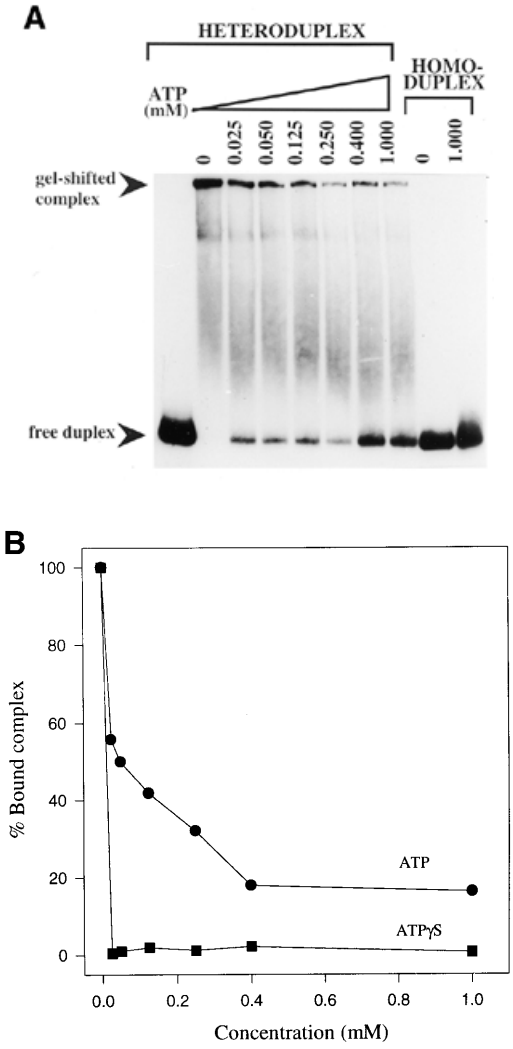

Figure 2. ATP and ATPγS destabilize the MutS–mismatch-DNA complexes. (**A**) Gel shift assay was performed with substrate A to monitor the levels of MutS–mismatch-DNA complexes in the presence of increasing concentrations of ATP. Positions of the MutS-bound duplex and free duplex are as indicated. (**B**) PhosphorImager quantitation of gel-shifted complexes. MutS–mismatch-DNA complexes were quantified in a gel shift assay (A) and expressed as percentage of radioactivity associated with the complex plus the free duplex in each lane. Values of percent bound complexes were calculated after normalizing with that obtained in the absence of a nucleotide, which is taken as 100%, and plotted. Squares, ATPγS; circles, ATP.

suggesting dissociation of the complexes very early on in the assay. Secondly, we also did an order-of-addition experiment wherein the ATP was added to preformed MutS–mismatch-DNA complexes. Destabilization levels were the same as shown in Figure 2 (data not shown). Therefore, one can conclude that the order of addition of ATP is not crucial in bringing about the complete dissociation of the complexes and that ATP binding itself, as against its hydrolysis, is responsible for this effect.

MutS footprint on the heteroduplex DNA is altered by ATP

Release of MutS from the mismatch in the presence of ATP could either be due to a direct dissociation of the protein from the duplex or a switch from a tightly bound form to a loosely

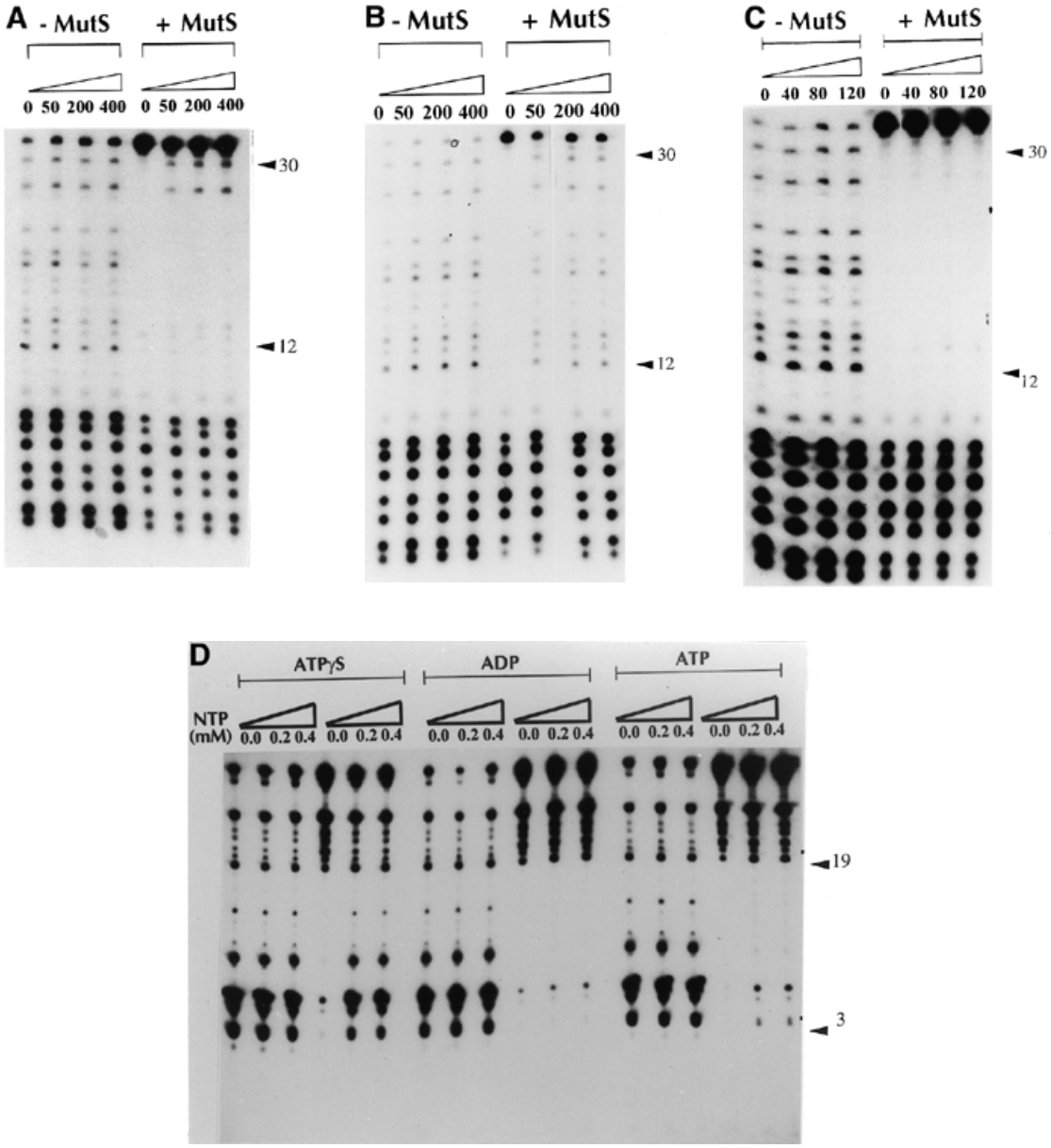

Figure 3. Modulation of MutS–mismatch-DNA complexes by nucleotide cofactors. An analysis by DNase I footprinting assay using substrate A. Footprinting was done following the formation of MutS–mismatch-DNA complexes in the presence of either ATP, ATPγS or ADP as described in Materials and Methods. Concentrations of nucleotides are expressed as μM. Footprint is located between the arrowheads, the nucleotide positions of which are numbered. Bottom strand labeled: (**A**) ATP, (**B**) ATPγS, (**C**) ADP. Top strand labeled: (**D**) (top and bottom strands are as described in Materials and Methods). Out of six lanes in each nucleotide set, the first three lanes are without MutS and the last three are with MutS.

associated state. Such a weakly bound complex is probably prone to dissociation during electrophoresis (in gel shift assay) and therefore may give rise to an apparent loss of bound complexes. We surmised that probing the complexes under equilibrium conditions by a comparatively mild assay would provide better insight about such protein–DNA interactions. Therefore, we resorted to a DNase I footprinting approach to study the effects of ATP on such seemingly unstable complexes. Since most of the MutS–mismatch-DNA complexes were destabilized by 0.4 mM ATP (Fig. 2), we used a concentration range up to 0.4 mM in the footprinting experiments.

It was observed that ATP, at concentrations that destabilized the complexes in gel shift assays, did not abolish the MutS footprint under equilibrium (footprinting) conditions (Fig. 3A). However, on addition of ATP, the footprint was partially uncovered by ~3–4 nt at the 3′ end of the labeled strand. The remaining portion of the footprint was still discernable and intact. In the same duplex, when the other strand (top strand) was labeled, the footprint was positioned towards the 5′ end and remained essentially unchanged upon the addition of ATP (Fig. 3D). Since the stretch encompassing first 3–4 nt at the very 5′ end of the strand (blank region below the footprint,

Fig. 3D) was intrinsically more resistant to DNase I digestion, we were unable to map this end of the duplex. Therefore, it was difficult to resolve the ambiguity whether ATP addition recesses the footprint at this end on the top strand, where it had done so on the bottom strand by shortening the footprint length by 3–4 nt (Fig. 3A). Hence, the effects of ATP addition were at best limited to the edges of footprints where the protein–DNA contacts were marginally reduced (see Discussion). We repeated the same experiment at an identical concentration range with ATPγS. The results obtained with ATPγS were distinctly different from that obtained with ATP. Under the same footprinting conditions, ATPγS virtually abolished the footprint on either strand (Fig. 3B and D). The same contrasting effects of ATP and ATPγS were observed when footprinting was analyzed on complexes that were preformed without ATP followed by the addition of either of the nucleotide cofactors (data not shown). The ATPγS control, which is artificial as far as *in vivo* correlates are concerned, only indicates the propensity of MutS to fall off the heteroduplex when bound by ATP in non-hydrolyzing conditions. Under hydrolyzing conditions, MutS does not dissociate appreciably but rather switches from one form to another as indicated by a marginal loss in the protein–DNA contacts at the edges of the footprint (Fig. 3A) (see Discussion). Why is the footprinting result different with ATP versus that with ATPγS? Is it the hydrolysis of ATP *per se* or the product of hydrolysis, namely ADP, which causes the retention of the footprint? We tested this possibility by checking the role of ADP in these reactions. The footprinting pattern remained unaffected by ADP (Fig. 3C and D). Under the conditions where ATPγS caused the dissociation of MutS–mismatch-DNA complex, ADP showed stable maintenance of the same. Moreover, in contrast with ATP, addition of ADP did not lead to any partial loss of the footprint at the edges (Fig. 3A and C). The entire footprint was intact at all ADP concentrations tested (Fig. 3C and D).

The results with ATP versus ATPγS show that although ATPγS brings about a complete dissociation of the protein from the heteroduplex, ATP does not do so. Retention of the footprint in the presence of ATP indicates a steady-state association of MutS with the mismatch. Is this due to the short size of the heteroduplex used where MutS fortuitously comes back onto the same mismatch? The duplex used here is marginally bigger than the site-size of MutS and therefore provides no other target for binding. In order to investigate this possibility, we repeated the footprinting experiment with a longer duplex (61 bp) (substrate B; see Materials and Methods). This duplex is identical to the former one (substrate A) in the middle, but has extra sequences on either side such that the mismatch is now centrally located. As with the shorter one, the longer duplex also showed a marginal loss of the footprint towards the 5′ half upon the addition of ATP (Fig. 4). However, the loss was not complete and the effect was only partial. So, the effect of ATP on the status of MutS–mismatch-DNA complex was qualitatively similar to that obtained with short duplex substrate. With the long substrate (as with the shorter one), it was observed that there was a retention of the footprint in the presence of ADP while a complete loss of the same ensued following the addition of ATPγS. All the results with long duplex which corroborated well with those obtained with the short duplex revealed that MutS, in the presence of ATP,

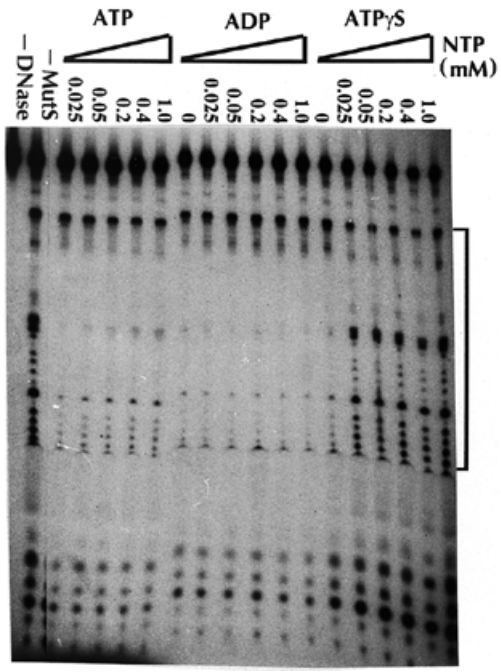

Figure 4. Footprinting assay to monitor the modulation of MutS by nucleotide cofactors on a longer substrate (substrate B). The assay was carried out in the presence of either ATP or ATPγS or ADP at indicated concentrations. Footprint position is indicated by a bracket.

retains the ability to interact with a mismatch in preference to homoduplex.

ATP reduces the stability of MutS–mismatch-DNA complexes

How does ATP affect the stability of MutS–mismatch-DNA complex? In order to assess this, we carried out a competition experiment. MutS–mismatch-DNA complexes were formed with labeled DNA followed by the addition of unlabeled mismatched duplex as a competitor. The competitor was the same duplex as the labeled substrate used for footprinting. Addition of molar excess of unlabeled competitor establishes a new binding equilibrium thereby displacing the protein out from the initial complex, which manifests as a partial loss in the footprint. We quantified the relative decay in the MutS–mismatch-DNA complex as a function of time by analyzing this loss in footprint in the presence and absence of ATP following the addition of large excess (15-fold molar excess) of competitor. A time-course analysis revealed that a new equilibrium was already established by the first time-point chosen as early as 30 s (data not shown). Subsequently, for the entire time-course studied (up to 5 min), there was no change in the degree of footprint loss. This was so for reactions containing ATP as well as those without (data not shown). This revealed that the footprinting assay (which itself involves a 2 min incubation) is too slow to score the rate at which the new equilibrium is reached. Since we could not capture the time-course of footprint loss, we quantified steady-state levels of the footprint at a single time-point, which scored the binding in its new equilibrium. The footprint losses for multiple reactions done in parallel were quantified and plotted (Fig. 5 and a representative panel

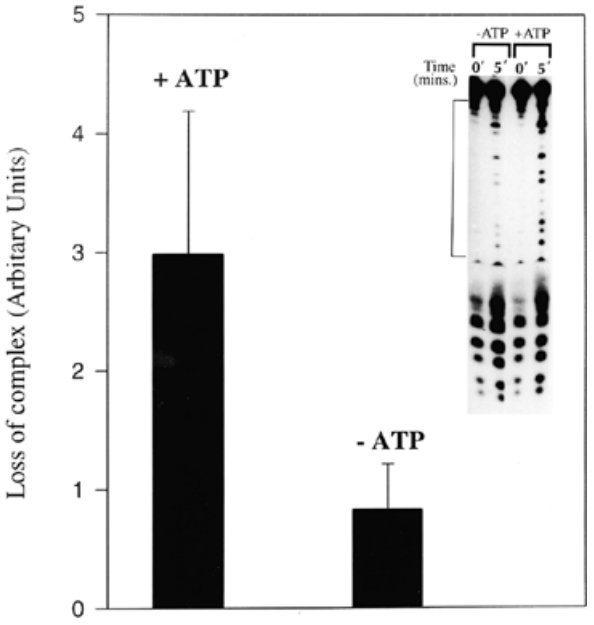

Figure 5. Histogram to compare the steady-state levels of MutS–mismatch-DNA complexes in the presence and absence of ATP following a 'chase' by excess cold competitor. MutS–mismatch-DNA complexes were formed using labeled DNA (substrate A, 0.03 μM) and MutS (1.2 μM) as described in Materials and Methods. Either ATP (500 μM) or an equal volume of 10 mM Tris–HCl (pH 7.6) (–ATP control) was added simultaneously with unlabeled competitor (0.45 μM) (15-fold molar excess relative to the labeled duplex), followed by incubation at 37°C for a further 5 min. Ten such competition experiments were done in parallel with or without ATP followed by DNase I footprinting assay. All footprinting samples were analyzed on a denaturing gel as described in Materials and Methods. In each competition lane, a set of four common bands, one in the footprint region (depicted as A) and the other three outside of the footprint (depicted as B, C and D), were quantified but beads A–D are not marked. Loss of the complex was calculated as A/A+B+C+D for each lane and expressed as a histogram. A representative autoradiogram is shown as the inset where the footprint position is indicated by a bracket.

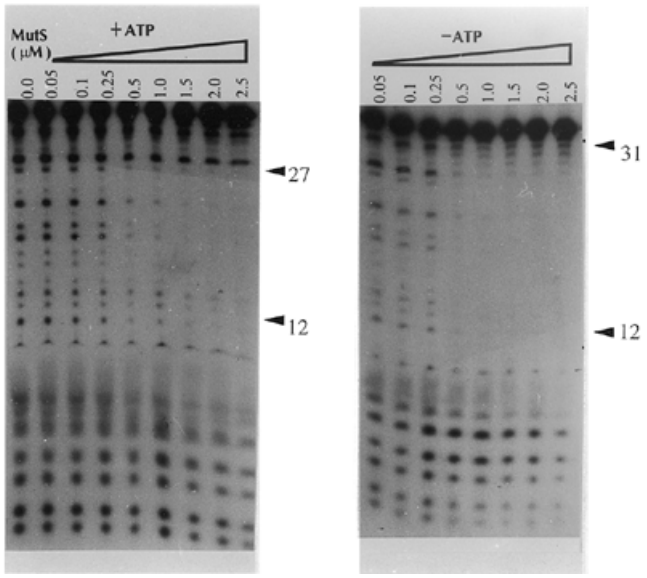

Figure 6. DNase I footprinting assay to monitor the affinity of MutS for the mismatch in the presence and absence of ATP. Standard conditions were used for binding MutS to a heteroduplex (substrate A) at 200 μM ATP, followed by DNase I footprinting. MutS concentration was varied and is expressed as μM. Location of the footprint is indicated between arrowheads. Numbers represent nucleotide positions.

of the footprints as inset). We found that the relative loss of the footprint was ~4–5-fold higher in the presence of ATP.

We confirmed the reduced stability of the complex in ATP by carrying out a binding assay. Two reactions, with and without ATP, were analyzed in parallel at various concentrations of MutS by a footprinting assay. In the reactions without ATP, a footprint manifested at 0.5 μM MutS and stabilized by ~1.0–1.5 μM, with no further change at higher concentrations (Fig. 6). A clear footprint encompassing 20 nt was revealed. In contrast, under the same conditions, reactions with ATP showed a different result. In the presence of ATP, the footprint was barely visible at 0.5–1.0 μM MutS and was stabilized only at ~2.0–2.5 μM concentration of protein. Moreover, the size of the stable footprint was ~16 nt, which was shorter than the footprint obtained without ATP by ~4 nt. These results taken together reveal that, in the presence of ATP, MutS exhibits a marginal reduction in affinity to a mismatch (by ~2-fold, at best) as well as in its footprint size.

Mismatch-dependent activation of MutS ATPase

MutS, being an ATPase, can probably use the energy of hydrolysis in a translocation process that involves duplex tracking (16). At steady-state levels, MutS that is free of ATP seems to recognize a mismatch better than one bound by ATP (Figs 5 and 6). This in turn facilitates a switch in the protein to a form that has lower affinity for the mismatch following ATP binding. Is this due to a differential affinity of MutS for ATP in its free versus bound states? We analyzed this by measuring the initial rates of ATP hydrolysis in three parallel reactions: free MutS, with homoduplex and with heteroduplex. The initial rates of hydrolysis were different, wherein mismatch bound MutS hydrolyzed ATP at a rate (9 ATP/min/protein) ~2-fold higher than that of either free MutS (4 ATP/min/protein) or homoduplex bound MutS (5 ATP/min/protein). This implies that the MutS–mismatch-DNA complex is perhaps a better target for ATP binding and hydrolysis than the free MutS itself. These observations correlate well with those seen for human MutSα, where the protein showed enhanced ATP hydrolysis in the presence of a mismatch (17,24).

Nucleotide-dependent conformational transitions in MutS

So far, we have observed three different states of MutS–mismatch-DNA complexes in footprinting experiments: (i) a stable footprint with either ADP or no nucleotide (Figs 3C,D and 4); (ii) a footprint with ATP that is partially recessed at one end (Figs 3A and 4); and (iii) a complete loss of footprint with ATPγS (Figs 3B,D and 4). These observations clearly indicate that nucleotides influence MutS interaction with mismatched DNA. We wanted to investigate whether this modulation is a consequence of conformational transitions in MutS induced by nucleotide binding. We therefore studied limited proteolysis patterns of MutS by trypsin digestion. To make the assay more sensitive, proteolyzed MutS was detected by western blotting using a polyclonal anti-MutS antibody. Free MutS itself

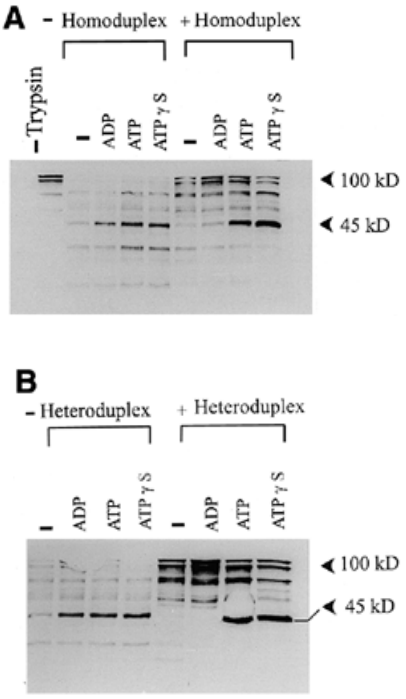

Figure 7. Conformational switch in MutS as monitored by limited proteolysis by trypsin. Binding reactions were performed as described using (**A**) homo- or (**B**) heteroduplexes (substrate A, 3 μM) and MutS (1.5 μM), followed by the addition of a nucleotide (1 mM) and further incubation at 37°C for 10 min. Regeneration was not used for the ATP reactions here. Trypsin (3 μg/ml) was added which was followed by incubation at 37°C for 2 min. The reactions were quenched by the addition of PMSF (8 mM) followed by analyses on 12% SDS–polyacrylamide gels. Following electrophoresis, the gel was blotted onto a nitrocellulose membrane and was probed using polyclonal anti-MutS antibodies (1:50 000 dilution) that was raised against the MutS protein recovered from a single band in preparative SDS–polyacrylamide gel. Standard protocol of western blotting was followed to visualize MutS fragments using diaminobenzidene as the substrate. Positions of standard markers are indicated.

showed discernible changes in the proteolysis pattern following the addition of a nucleotide: ADP, ATP or ATPγS (Fig. 7A and B). In Figure 7A and B, the left panels pertaining to minus-DNA reactions are duplicates to show the consistency of protease digestion at all sites of cleavage. The pattern of MutS cleavage observed in the absence of a nucleotide was significantly altered upon the addition of ADP, ATP or ATPγS as evidenced by an enhanced relative yield of the 45 kDa fragment. This suggests a nucleotide-dependent switch in MutS that renders the cleavage sites adjoining the 45 kDa fragment more sensitive to trypsin digestion. In the absence of a nucleotide, MutS showed an overall protection in the cleavage pattern upon the addition of DNA (homo- or heteroduplex). This manifested as relatively higher molecular weight fragments in the lanes that contained DNA (Fig. 7A and B). Moreover, heteroduplex DNA showed somewhat better protection than homoduplex DNA. The overall general protection seen with DNA persisted when ADP was added, irrespective of whether the MutS was associated with heteroduplex or homoduplex. However, this contrasted with the changes brought about by ADP in the cleavage pattern of free protein. On the other hand, when either ATP or ATPγS was added, the outcome was different. The enhanced cleavage that yielded the 45 kDa fragment recurred upon the addition of either ATP or ATPγS but not ADP (Fig. 7A and B). These results demonstrated that MutS bound to a duplex target underwent a conformational switch specifically in the presence of ATP or its analog. Surprisingly ADP, the product of hydrolysis, had no effect on the proteolytic fragmentation pattern. The implications of such a conformational switch are discussed further.

DISCUSSION

In this study, we discuss how ATP modulates MutS function in the mismatch repair pathway. We examined the status of ATP-dependent MutS interaction with the heteroduplex by gel shift and DNase I footprinting assays. To maintain the ATP concentration in spite of continual hydrolysis, we carried out all the ATP experiments in the presence of an ATP regeneration system. We observed that, specific complexes formed between MutS and heteroduplex DNA in the absence of a nucleotide became dissociation prone upon the addition of ATP, in the gel shift assays (Fig. 2). We wanted to assess whether the ATP-induced dissociation of MutS from the heteroduplex was an apparent effect of the non-equilibrium nature of the assay, and hence failed to capture specific complexes, if any. To rule out this possibility, we probed MutS–DNA complexes under equilibrium conditions using DNase I footprinting assay. In contrast to the gel shift assay, DNase I footprinting showed a high steady-state level of MutS–mismatch-DNA complexes in spite of the presence of ATP (Figs 3A,D and 4). The complexes that were destabilized with as little as 50 μM ATP in the gel shift assays, showed stable DNase I footprints even at concentrations as high as 0.4–1.0 mM ATP. Hence we used equilibrium assays such as DNase I and trypsin footprinting (limited proteolysis approach) to study the conformational states of MutS–DNA interactions in the presence of various nucleotides. As discussed below, MutS complexes appear to be sensitive to ATP/ADP interactions that result in both DNA binding as well as protein conformational changes (Figs 3 and 7).

Modulation of MutS–mismatch interaction in the presence of ATP

The changes in MutS–DNA interactions associated with nucleotide binding were analyzed on both strands of the heteroduplex using DNase I footprinting. In the presence of ATP, the initial (minus nucleotide) footprint was altered specifically at the 3′ end of the bottom strand (Fig. 3A). This alteration appeared as a shortening of the footprint from 19 to ~16 nt, which reflects on the partial opening up of the complex probably due to a conformational change in the protein mediated by ATP. We wanted to assess the stability of these altered ATP–MutS–DNA complexes and hence studied them further by competition assays. Although the complex was stable enough in the absence of any nucleotide, addition of ATP converted it to a form that could be easily titrated out by excess competitor simply by mass action effect. When several-fold molar excess of cold heteroduplex competitor was added to a preformed complex containing MutS and labeled heteroduplex, a new equilibrium was established, as monitored by a loss of the initial footprint (Fig. 5, inset). Quantification of the loss revealed that the dissociation of MutS–mismatch-DNA

complexes was ~4–5-fold higher in the presence of ATP at this new equilibrium (Fig. 5). Based on the stable footprint observed, one can assume that the association rate constant of MutS with heteroduplex is largely unaffected in the presence of ATP. On the other hand, the competition experiment that showed a 4–5-fold loss of footprint suggests that ATP results in an equivalent fold enhancement in the dissociation rate constant of MutS from the mismatch. Such dissociation can be attributed to the reduced binding affinity of MutS for the mismatch in the presence of ATP (Fig. 6). An enhancement in the dissociation rate of complexes inferred above is consistent with the reduction in the binding affinity of MutS to the mismatch. One must note that this marginal reduction in affinity may also stem from the reduced footprint size of MutS–mismatch-DNA complexes in ATP. It is therefore possible that such complexes are rendered unstable under non-equilibrium assay conditions such as electrophoretic migration during a gel shift assay (Fig. 2A).

The effects of ATP described above could principally stem from either ATP hydrolysis and/or the product of hydrolysis, namely ADP. Hence, we used a poorly hydrolyzable analog of ATP, namely ATPγS, as well as ADP in separate controls to test these components. We observed that, unlike ATP, ATPγS brought about a complete dissociation of the complex (Figs 3B,D and 4). In contrast, ADP did not alter the stability of the complex in any way (Figs 3C,D and 4). An 'ADP-alone' control would perhaps represent a post-hydrolysis scenario where the added ADP binds to the site of hydrolysis without subsequent exchange by ATP. These experiments taken together suggest that the hydrolysis of ATP and/or the presence of ADP are important to maintain a minimal stability of MutS–heteroduplex complexes, in the absence of which binding of ATP alone (ATPγS) leads to dissociation of the complexes.

Conformational changes in MutS in response to nucleotide co-factors

In order to study the conformational transitions that the protein might undergo upon nucleotide binding, we carried out limited proteolysis assays. We observed that binding of either ATP, ATPγS or ADP brings about a similar conformational change in the free protein (Fig. 7A and B). However, in the presence of DNA, only ATP/ATPγS retains the ability to bring about such a conformational change. This may be because ADP does not appreciably bind to MutS that is associated with DNA. Such reasoning is consistent with observations made by Gradia *et al.* (17,20), where it was demonstrated that the exchange rate of ADP to ATP on MutS was negligible in the absence of DNA, but increases dramatically when DNA is present. Based on relative yields of the 45 kDa fragment, which is indicative of the conformational switch described above, we conclude that MutS, when bound to DNA, has a higher affinity for ATP *vis a vis* ADP than when it is free in solution. Thus, MutS may primarily exist in an ATP-bound state when complexed with DNA. This finding was also substantiated by a time-course analysis of ATP hydrolysis. The initial rates of hydrolysis suggested that the mismatch-bound MutS binds and hydrolyzes ATP two to three times better than free MutS. This observation is consistent with the notion that MutS that is mismatch bound has a higher affinity for ATP than one that is free.

MutS–mismatch-DNA complexes: a comparison with current models

The current status of knowledge in the field pertaining to ATP modulation of MutS–mismatch-DNA complexes encompasses two distinct models which are derived from studies on *E.coli* MutS as well as its eukaryotic counterparts (16–20,25). In the translocation model, that has been proposed for *E.coli* MutS, the protein departs from the mismatch upon ATP binding and uses the energy of hydrolysis to translocate along the DNA contour in search of hemimethylated sites (16,25). In the molecular switch model, ATP binding and not hydrolysis, serves to switch the protein to a conformational state that dislodges MutS from the mismatch (17,18,20). This model also invokes an active role for ADP in mismatch recognition by hMSH2–hMSH6. Upon ADP to ATP exchange, the protein switches to a diffusible clamp which can then slide along the duplex in a diffusion-controlled manner that is independent of energy consumption (ATP hydrolysis) and serves as a signal for the assembly of repair complex. Blackwell *et al.* (25) reported an ATP-promoted dissociation of hMutSα–mismatch-DNA complexes using gel shift assays. However, the complexes were stabilized when the ends of the duplexes were blocked by bulky physical barriers thereby preventing the translocation-mediated departure of the protein from the ends. Our results of ATP versus ATPγS show that although ATPγS brings about a complete dissociation of the protein from the heteroduplex, ATP hydrolysis still keeps it intact on the heteroduplex. This happens in spite of the free ends of the short duplexes studied. So it is important to note that MutS is stable enough on mismatched duplexes even when ATP is present and results in a 'footprintable' complex (Figs 3A,D and 4). The same complexes dissociate completely during gel shift assays due to significant perturbation of the binding equilibrium during the assay conditions (Fig. 2). When hydrolysis of ATP is prevented (ATPγS), MutS goes into an irreversible 'dissociation mode'. These findings, taken together, reinforce the view that hydrolysis is an important component of MutS action. The observations also point to an altered conformation of MutS in the presence of ATP, which might represent a dynamic 'tracking-competent' mode as opposed to the more stable 'mismatch-binding' mode. The presence of ADP seems largely insignificant because the effects seen with ADP in DNA-binding and footprinting assays are identical to those obtained without a nucleotide (Figs 3C,D and 4). This is further substantiated by an observation where the proteolysis pattern of MutS associated with DNA (homo- or heteroduplex) remained unchanged upon the addition of ADP (Fig. 7A and B). Thus, the DNA and protein footprinting analyses revealed that neither free nor bound MutS underwent any measurable alterations when ADP was added, which is in agreement with reports for the yeast MSH2–MSH6 proteins (26). This, however, contrasts with reports on hMutSα–mismatch-DNA complex (17,25). Two opposing effects were reported with ADP, one where an increase in the ADP/ATP ratio stimulated hMutSα binding to mismatches (17), and the other where ADP destabilized most of the complexes that were preformed in the absence of any nucleotide (25). The assay systems used were quite different and deviation from equilibrium conditions might have contributed to the observed effects. The results described here are from an 'in-solution' assay where presumably the binding equilibrium

is least perturbed and the effects seen reflect true modulations of MutS–mismatch-DNA complexes. Future studies are focussed on trying to unravel the relationship between the ATP-induced altered form of MutS–mismatch-DNA complex reported here and the 'tracking mode' that MutS achieves following the initial recognition of a mismatch.

ACKNOWLEDGEMENTS

We would like to thank Dr Leroy Worth, for providing the MutS clone. We are grateful to Mr Dandekar for his help in PhosphorImager quantitation of gels and Jatin for the photographic prints. We thank Karthikeyan Gopalakrishnan and Mahendra Wagle for helpful discussions.

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