Mutation in the Magnesium Binding Site of hMSH6 Disables the hMutSα Sliding Clamp from Translocating along DNA*

Ingram Iaccarinoć, Giancarlo Marra, Patrick Dufner, and Josef Jiricny§

From the Institute of Medical Radiobiology of the University of Zurich and the Paul Scherrer Institute, Auguste Forel Strasse 7, CH-8008 Zurich, Switzerland

In human cells, binding of base/base mismatches and small insertion/deletion loops is mediated by hMutSα, a heterodimer of hMSH2 and hMSH6. In the presence of ATP and magnesium, hMutSα dissociates from the mismatch by following the DNA contour in the form of a sliding clamp. This process is enabled by a conformational change of the heterodimer, which is driven by the binding of ATP and magnesium in the Walker type A and B motifs of the polypeptides, respectively. We show that a purified recombinant hMutSα variant, hMutS6 6DV, which contains an aspartate to valine substitution in the Walker type B motif of the hMSH6 subunit, fails to undergo the conformational change compatible with translocation. Instead, its direct dissociation from the mismatch-containing DNA substrate in the presence of ATP and magnesium precedes the assembly of a functional mismatch repair complex. The “translocation-prone” conformation of wild type hMutSα could be observed solely under conditions that favor hydrolysis of the nucleotide and mismatch repair in vitro. Thus, whereas magnesium could be substituted with manganese, ATP could not be replaced with its slowly or nonhydrolyzable homologues ATP-γS or AMPPNP, respectively. The finding that ATP induces different conformational changes in hMutSα in the presence and in the absence of magnesium helps explain the functional differences between hMutSα variants incapable of binding ATP as compared with those unable to bind the metal ion.

Many essential biological processes such as DNA replication, recombination, repair, and transcription, as well as protein synthesis and folding, membrane transport, and signal transduction, are driven by molecular motors that utilize the energy of purine nucleotide triphosphate hydrolysis. ATP binding motifs have been characterized in a number of proteins of different function. Of the four conserved sequences identified (1), the so-called Walker type A and B motifs, with consensus amino acid sequences (hydrophobic stretch (GXXXXGKS/T) and hydrophobic stretch (D(E/D)), respectively) are the most highly conserved. High resolution crystal structures of two members of this class of proteins, Ha-Ras (2) and HisP (3), revealed that the amino acid residues of the type A motif are involved in forming specific hydrogen bonds with the β- and γ-phosphates of the bound nucleotide; that is why this motif is often referred to as the phosphate-binding or P-loop. The absolutely conserved aspartate of motif B is thought to be part of the catalytic site of these enzymes. In the Ha-Ras structure (2), this amino acid was shown to be coordinated to a magnesium ion bound by the β- and the γ-phosphate groups of the triphosphate, activating thus the latter group toward attack by a water molecule. The proposal that this conserved amino acid residue plays a key role in nucleotide triphosphate catalysis is supported also by biochemical studies of the translocation ATPase SecA (4, 5) and of the UvrD helicase II (6) of Escherichia coli.

Mismatch binding polypeptides, homologues of the E. coli MutS protein, are important members of this superfamily of ATPases (7), but the mechanistic role of their ATP-driven motor in mismatch correction is unclear. First evidence showing that nucleotide binding could influence the function of the MutS homologues came from experiments using synthetic oligonucleotides, where the mismatch binding factors from several species could be shown to dissociate from the mismatch-containing substrates in the presence of ATP (8, 9). This biochemical feature is shared by all the functional MutS homologues examined to date, but its proposed physiological role was, until recently, based largely on our knowledge of the mismatch repair process in E. coli, which is initiated by the binding of the homodimeric MutS protein to the mismatch (10–12) and where ATP hydrolysis is supposed to drive the formation of the so-called α-loop, which is generated by threading the DNA bidirectionally through MutS. This process was visualized in electron microscopic studies (13), where it was also noted that the MutS homodimer released the mismatch during this step such that it was located predominantly in the loop. Intriguingly, this implied that ATP binding and/or hydrolysis made the MutS homodimer lose its affinity for the bound mispair, whereas its affinity for homoduplex DNA increased.

Recent studies from several laboratories showed that similar transactions take place also in eukaryotic systems (14–18), where the MutS homologues function in the form of heterodimers.

The most abundant mismatch binding factor in human cells is hMutSα, a heterodimer of the MutS homologues hMSH2 and hMSH6 (19, 20). In an earlier study, we substituted the highly conserved lysine residues in the Walker type A motifs of these two polypeptides with arginine, such that the mutant proteins were no longer able to bind the triphosphate. Examination of the ATPase and mismatch binding characteristics of the recombinant wild type and mutant hMutSα factors (15) revealed that the dissociation of hMutSα from oligonucleotide heteroduplexes required solely ATP binding, not hydrolysis. This was independently confirmed by Gradia et al. (14). We could further show that a hMutSα variant, in which the ATP binding sites of both hMSH2 and hMSH6 were mutated, could bind normally to mismatched substrates, but was not displaced from the G/T

* This work was supported in part by a Swiss National Science Foundation grant (to P. D. and J. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Imperial Cancer Research Fund Laboratories, P.O. Box 123, 44 Lincoln’s Inn Fields, London WC2A 3PX, United Kingdom.

§ To whom correspondence should be addressed. Tel.: 41-1-643-8910; Fax: 41-1-643-8904; E-mail: jiricny@imr.unizh.ch.

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
mismatch-containing 34-mer oligonucleotide substrate even in the presence of 10 mM ATP. Unlike the wild type heterodimer, this hMutSa variant was unable to complement mismatch repair deficient extracts of cells lacking hMutSa. On the contrary, it inhibited the repair efficiency of a fully repair proficient HeLa cell extract, presumably by remaining bound at the mismatch site and denying access to the other components of the mismatch repair machinery.

Although the above results implied that the ATP-dependent translocation of hMutSa from the mismatch is required in the mismatch repair process, they failed to provide us with a mechanistic insight into the ATP-driven molecular transactions that take place upon mismatch binding. This came later, in the form of experiments that demonstrated that following mismatch recognition, binding of ATP and magnesium induces a conformational change in hMutSa, which allows it to travel along the contour of the DNA molecule in the form of a sliding clamp (16, 17). One model suggests that the released heterodimer then induce about the conversion of the heterodimer into a sliding clamp, which then diffuses along the DNA without ATP hydrolysis. Instead, hydrolysis of the nucleotide was suggested to be required in the recycling of the mismatch binding proficient, ADP-bound form of hMutSa (17). Whereas the role of ATP hydrolysis in the translocation process is still open to question, both models predict that if hMutSa is to leave the mispair upon ATP binding and/or hydrolysis, it would be expected to change its conformation. Using partial proteolysis, conformational changes induced by nucleotide binding to the recombinant human (17) and yeast (18) MutS were indeed observed. We now extend these findings to show that ATP binding to the human heterodimer does indeed cause a dramatic intramolecular change of conformation, but that this change is different in the presence and in the absence of magnesium. These data explain why hMutSa variants unable to bind ATP and those incapable of coordinating magnesium are phenotypically similar, even though the underlying causes of their mismatch repair defects are mechanistically very different.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Production of Recombinant Baculoviruses—A PCR1-based site-directed mutagenesis approach was used to change the conserved aspartic acid in position 1213 of hMSH6 to valine. The substitution was obtained by PCR overlap extension mutagenesis, using the following primers: X1, 5'-GGTGGCTGTGGTTGTTAGTAGGAAG-3'; X2, 5'-CTTCTCAATCAACTCCACGCC-3'; 160, 5'-GGGGCTCGAGTACAACTCCGC-3'; and 160, 5'-CTTG- TGGCCGGAATTAC-3'. The PCR fragments obtained with X1/160.27 and X2/160.28 were used as templates in a second PCR step, using 160.27/160.28 primers. The final PCR product was cloned between the AccI/AvaII sites of hMSH6/BluescriptSK+ (15). After sequencing, the hMSH6 cDNA carrying the D1213V mutation was cloned between the BamH1/XhoI sites of pFastBac (Life Technologies, Inc.) to generate clone pFastBac/hMSH6-DV6. The recombinant baculovirus was obtained using the Bac-To-Bac system according to the manufacturer's (Life Technologies, Inc.) instructions.

Production and Purification of the Recombinant Proteins—The mutant hMutSa heterodimer carrying a D1213V mutation in hMSH6 (hMutSa 6DV) was purified as described previously (15). Briefly, Spodoptera frugiperda 9 (Sf9) cells (typically 1.2 x 10^9) were infected with a mixture of the recombinant baculoviruses hMSH6-D1213V and hMSH2, at a multiplicity of infection of 10. After 72 h, the cells were collected and total extracts were prepared as described (21). The extracts were sedimented at 36,000 x g, and the supernatant was then diluted to a conductivity corresponding to that of 0.25 M NaCl with buffer A (25 mM HEPES/NaOH, pH 7.6, 1 mM EDTA, 2 mM β-mercaptoethanol), and loaded onto a 5-ml HiTrap heparin-Sepharose fast protein liquid chromatography column (Amersham Pharmacia Biotech). The protein complex was eluted with a 45 ml linear gradient from 25 to 100% buffer B (25 mM HEPES/NaOH, pH 7.6, 1 mM EDTA, 2 mM β-mercaptoethanol). The fractions containing hMutSa heterodimer (eluting at around 40% buffer B) were pooled, diluted with buffer B (final conductivity corresponding to 15% salt, and loaded onto a 1-ml Resource Q fast protein liquid chromatography column (Amersham Pharmacia Biotech). The fractions containing the pure hMutSa complex were pooled, dialyzed extensively against buffer A containing 0.11 M NaCl, 10% sucrose, 0.5 mM phenylmethylsulfonyl fluoride (storage buffer), and stored in aliquots at −80 °C. The purity of the recombinant heterodimer preparations was judged by Coomassie-stained SDS-PAGE to be in excess of 95%.

Band-shift Assays—The band-shift assays were performed essentially as described (21); the protein (50 ng, 192 fmol) was incubated with the labeled oligonucleotide substrates (40 fmol) in a 20-μl total volume containing 25 mM HEPES, pH 8.0, 0.5 mM EDTA, 10% (v/v) glycerol, 0.5 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin. After 20-min incubation at room temperature, 5 μl of each sample were loaded on a 6% nondenaturing polyacrylamide gel run in TAE buffer, pH 7.5. The biotinylated substrate Gbio/Tbio was constructed by annealing the synthetic 34-mer oligonucleotide 5'-AGCTTGGCTGGTTGTTAGTAGGAAG-3' with the complementary strand 5'-ATTCTCGGGGATCGTGGCGTGGTTGTTAGGAAG-3', both 3'-biotinylated (Microsynth) and labeled at their 5'-ends with [32P]ATP and using 1 pmol of purified protein. At selected time points, 2-μl aliquots were removed, the reaction was stopped by the addition of an equal volume of formamide dye and loaded onto a 20% sequencing gel (21). The gel was run as described previously (8). For the calculation of catalytic efficiencies, Menten constants, the activities were measured in the presence of 0.5, 1, 2, 3, 4, 6, 10, and 20 μM ATP.

In Vitro Mismatch Repair Assays—The ability of the recombinant proteins to complement cytoplasmic extracts of HeLa and HCT15 cells in a mismatch repair assay was tested as described previously (15, 22).

Partial Proteolysis Experiments—Typically, 1 μg (0.85 pmol) of the recombinant protein was digested with 300 ng of V8/Endoproteinase Gluc-C (Roche Molecular Biochemicals) in 25 mM HEPES/NaOH, pH 8.0, for 30 min at 25 °C. Where indicated (Fig. 4), the reactions were supplemented with either metal chlorides (2 mM), nucleotides (0.5 mM), or with a 34-mer oligonucleotide heteroduplex (10 pmol), containing a single G/T mismatch (21). The reactions were stopped by the addition of an equal volume of SDS sample dye followed by heating at 100 °C for 5 min, and aliquots were loaded on two 10% SDS-polyacrylamide gels. After blotting on nitrocellulose or polyvinylidene difluoride membranes, the filters were stained with polyclonal antibodies against hMSH2 or hMSH6 (19) and developed using either the colorimetric or ECL methods.

Results

Expression and Biochemical Characterization of Recombinant hMutSa Containing an Asp→Val Mutation in the Catalytic Site of hMSH6—In the previous study, we mutated the conserved lysine 675 of hMSH2 and lysine 140 of hMSH6 of the Walker A motif to arginine (15). Although neither mutant protein, hMSH2 K675R or hMSH6 K140R, could bind ATP, the phenotypes of the respective recombinant variants hMutSa 2KR and hMutSa 6KR containing these mutant subunits were very different. Thus, whereas hMutSa 2KR behaved similarly to wild type, the hMSH6 K140R mutation brought about a decrease of the total ATPase activity of the hMutSo
6KR heterodimer comparable with that observed in the double mutant, hMutSa 2,6KR, where both hMSH2 and hMSH6 ATP binding sites were mutated. This implied that the ATPase activity of hMSH6 is dominant within hMutSa. Correspondingly, the hMutSa 6KR variant was severely defective in mismatch correction (15). This contrasted with the effect of ATP (in the absence of magnesium) on the stability of the hMutSa 2KR and hMutSa 6KR protein-DNA complexes, as both the heterodimers dissociated from mismatch containing oligonucleotide duplexes at similar ATP concentrations in band-shift assays. These differences suggested that whereas ATP binding in at least one subunit of the heterodimer is sufficient to bring about its dissociation from the oligonucleotide substrate, ATP hydrolysis is required in the repair process.

To study this phenomenon further, we mutated hMutSa by changing the conserved aspartate 1213 of the hMSH6 subunit to valine (Fig. 1). The rationale for selecting this particular site was the following. (i) The D1213V mutation was identified in one allele of the hMSH6 gene in a mismatch repair-deficient human lymphoblastoid cell line MT1 (23). (ii) The aspartate in question has been shown to be involved in coordinating the divalent metal ion in the catalytic site of GTPases with Walker A and B motifs that are highly homologous to those of hMSH2 and hMSH6 (2, 3). (iii) The ATPase of hMSH6 contributes more to the total activity of the heterodimer (Ref. 15; see also above); and (iv) hMSH6 was shown to be the subunit contacting the mismatched substrate in UV-cross-linking studies (8, 15, 21). Our expectation was that, unlike hMSH6 K1140R, the D1213V mutation should have no effect on the binding of the triphosphate. Indeed, the hMutS6KR variant, which changed the conserved aspartate 1213 of the hMSH6 subunit, and therefore the conformational change necessary to release the protein complex from the mismatch has to be driven entirely by hMSH2 ($K_{\text{cat(ATP)}}$ of hMutS6a 6KR is $4.7 \pm 0.2 \ \mu M$), compared with $0.4 \pm 0.2$ for the wild type hMutS6a (15)). In contrast and as anticipated, the affinity of the hMutS6a 6DV heterodimer for ATP was comparable with that of the wild type protein (hMutS6a 6DV $K_{\text{cat(ATP)}} = 0.6 \pm 0.1 \ \mu M$, wild type $K_{\text{cat(ATP)}} = 0.4 \pm 0.1 \ \mu M$), whereas its $K_{\text{cat(ATP)}}$ of 0.12 min$^{-1}$ was similar to hMutS6a 6KR ($K_{\text{cat(ATP)}} = 0.18 \ \mu M$) rather than to the wild type heterodimer ($K_{\text{cat(ATP)}} = 0.5 \ \mu M$). Taken together, these data indicated that the ATPase activity of the hMSH6 subunit could be effectively eliminated by mutations in both the Walker type A and B motifs. However, whereas the Lys $\rightarrow$ Arg mutation in the A motif affected ATP binding (15), the Asp $\rightarrow$ Val mutation in the B motif inactivated the catalytic site of hMSH6, most likely because of the inability to coordinate magnesium and thus hydrolyze the $\gamma$-phosphate of the bound ATP.

**Fig. 2. Biochemical characterization of the hMutS6a variant hMSH2/hMSH6 D1213V (hMutS6a 6DV).** A, purity of the recombinant wild type (wt) and hMutS6a 6DV variants, as analyzed by SDS-PAGE stained with Coomassie Blue. M, molecular mass standards (from top to bottom: 200, 116, 97 and 67 kDa). B, displacement of wild type and hMutS6a 6DV variants from an oligonucleotide substrate with ATP. The latter factor was displaced by ATP concentrations approximately 5-fold lower than the wild type. The figure represents a quantification of band-shift experiments carried out in triplicate. C, complementation of mismatch repair-deficient extracts of hMutS6a-deficient HCT15 cells with the recombinant factors. Whereas the wild type hMutS6a was able to fully complement these extracts, the activity of hMutS6a 6DV in this assay was severely attenuated and was comparable with the hMutS6a 6KR mutant (15).

---

**Fig. 1. Amino acid sequence alignment of Walker type B sites of several MutS homologues.** The conserved aspartate (D) responsible for magnesium coordination and substituted with valine (V) in the hMSH6 D1213V mutant is shown in bold and indicated by the arrow. MSH2 and MSH6, MutS homologues from Homo sapiens (h) and S. cerevisiae (y), respectively. MutS, mismatch binding protein from E. coli.
which inhibited repair in these extracts (15). Thus, whereas both the mutant heterodimers are clearly proficient in mismatch binding and can therefore compete with the wild type hMutSa for the mismatch, the hMutSa 2,6KR mutant inhibits the repair process presumably by remaining bound at the mismatch site (15), whereas the hMutSa 6DV heterodimer apparently dissociates from the DNA upon ATP binding and thus cannot interfere with the subsequent steps of the repair process.

**Magnesium Binding Determines the Mode of hMutSa Dissociation from a Mismatch**—We (8, 15) and others (14) have reported earlier that hMutSa can dissociate from short, mismatch-containing oligonucleotide duplexes in the presence of ATP, under conditions where no hydrolysis takes place, i.e. in the absence of Mg$^{2+}$. However, these experiments could not distinguish between the two alternative modes of dissociation of the heterodimer from DNA: direct dissociation or translocation along the DNA contour until the complex falls off the end of the short substrate. More recent studies by Blackwell et al. (17) presented evidence showing that both modes of dissociation are possible in vitro, depending on whether magnesium is present or not. We decided to use a similar experimental strategy, namely, to employ mismatch-containing oligonucleotide duplexes with streptavidin-blocked ends, which prevent the heterodimer from sliding off the ends of the substrate if it dissociates via the translocation mode, but which do not affect the direct dissociation of hMutSa from DNA. Of particular interest in this experiment was the behavior of the wild type hMutSa and the hMutSa 6DV mutant heterodimer in the presence of ATP and in the absence of Mg$^{2+}$. As shown in Fig. 3, both wild type hMutSa and hMutSa 6DV dissociated freely from the unblocked G/T mismatch containing oligonucleotide substrate, as well as from the probe bound by only a single streptavidin, and the presence of the divalent cation appeared to make little or no difference. In contrast, when both ends of the oligonucleotide were blocked with streptavidin, the wild type heterodimer could dissociate completely only in the absence of magnesium. In its presence, more than half of the protein remained trapped on the heteroduplex. This failed to happen in the case of hMutSa 6DV, which dissociated with a similar efficiency from the blocked substrate in the presence and in the absence of Mg$^{2+}$. As can be seen from Fig. 3, both wild type hMutSa and hMutSa 6DV dissociated freely from the unblocked G/T mismatch containing oligonucleotide substrate, as well as from the probe bound by only a single streptavidin, and the presence of the divalent cation appeared to make little or no difference. In contrast, when both ends of the oligonucleotide were blocked with streptavidin, the wild type heterodimer could dissociate completely only in the absence of magnesium. In its presence, more than half of the protein remained trapped on the heteroduplex. This failed to happen in the case of hMutSa 6DV, which dissociated with a similar efficiency from the blocked substrate in the presence and in the absence of Mg$^{2+}$, as would be anticipated from the finding that this mutant is unable to bind the metal ion. These data thus show that for dissociation from a mismatch by the translocation mode, hMutSa requires the occupancy of both Walker type A and B sites, by ATP and magnesium, respectively, whereas the direct mode requires solely the nucleotide binding.

**ATP Binding in the Presence and Absence of Magnesium Brings about Distinct Conformational Changes in hMutSa**—In the light of the above data, it must be anticipated that hMutSa can adopt two distinct conformational states upon ATP binding, depending on whether a divalent cation such as magnesium or manganese is present. In the absence of the metal ion, the conformational change leads to a direct dissociation of the heterodimer from the mismatch and from DNA, whereas in its presence, hMutSa adopts a sliding clamp conformation and leaves the mismatch site by sliding along the DNA contour. Evidence demonstrating that the human (17) or *Saccharomyces cerevisiae* (24) MutSa does indeed undergo conformational changes upon nucleotide binding came from partial proteolysis studies, where the degradation patterns of the ADP-, ATP$\gamma$S- (17), or the ATP-bound forms (24) could be shown to be different from that of the free form. In the experiments described below, we further extend these findings. The purified, recombinant hMutSa variants were subjected to partial proteolysis with chymotrypsin or V8 protease, either in the absence or in the presence of the selected cofactors, the reaction mixtures were loaded on SDS-denaturing polyacrylamide gels, and the separated fragments were transferred onto nylon membranes, which were hybridized either with anti-hMSH2 or anti-hMSH6 antibodies (see “Experimental Procedures”). As can be seen from Fig. 4A, the proteolysis of hMSH2 varied little under the different experimental conditions. In some instances, a different band pattern could be observed in the presence of ATP, but only small or no further changes were observed upon the addition of magnesium. The hMSH2 digests are therefore omitted in the remaining figures. In contrast, the proteolytic degradation pattern of hMSH6 varied dramatically, depending on the cofactors present in the reaction. In the presence of ATP and magnesium, V8 protease digest of hMutSa gave rise to three prominent polypeptides originating from hMSH6, of approximately 108, 84, and 54 kDa, referred to as bands A, B, and C, respectively (Fig. 4A). In the absence of the metal ion, only band B was prominent. (Similar differences were observed also in the pattern produced by chymotrypsin, but these tended to be less informative. For this reason, the following section will focus solely on the V8 digest.) That this band pattern was dependent on the binding of both the cofactors to the heterodimer, rather than being due to some nonspecific effect of magnesium on the activity of the protease or on the folding of the polypeptides was demonstrated in the experiments shown in Fig. 4B. Here the proteolytic degradation pattern of the hMutSa 2,6KR mutant that does not bind ATP in either of its subunits (15) can be seen to remain unaltered in the absence or presence of the triphosphate or the metal ion.

![Fig. 3](image_url)
Fig. 4. hMutSα undergoes distinct conformational changes following the binding of various nucleotide and metal ion cofactors. A, conformational changes in wild type hMutSα as detected by the partial proteolytic digest with the proteases V8 and chymotrypsin. The figure is a Western blot of SDS-PAGE gels (see "Experimental Procedures"). The upper panel shows changes in hMSH6 as detected by a polyclonal anti-hMSH6 antibody, whereas the hMSH2 changes are shown in the lower panel. The positions of the molecular mass markers are indicated on the left of the figure in kilodaltons. B, comparison of the partial proteolytic patterns of wild type hMutSα with the hMutSα 2,6KR variant (15), which is unable to bind ATP. C, effect of various divalent metal ions on the ATP-dependent conformational change of the hMutSα. D, effect of various nucleotide cofactors on the ATP-dependent conformational change of the hMutSα. E, hMutSα 6DV is unable to undergo the magnesium-dependent conformational change in the presence of ATP. This panel also shows that neither hMutSα variant appears to change its conformation when bound to the G/T oligonucleotide substrate in the absence of added cofactors. The characteristic diagnostic bands A, B, and C in the hMSH6 digests are indicated by asterisks.

Fig. 4C shows that magnesium can be substituted with manganese, but not calcium or zinc. When the effects of several nucleotides and nucleotide homologues that bind in the active site of both the proteins were examined, it became apparent that only ATP in the presence of magnesium could give rise to the characteristic ABC pattern of bands (Fig. 4D). Thus, when hMutSα was incubated with magnesium together with AMP or ADP, the most prominent band was A, with band C being weak and band B hardly detectable. In the presence of magnesium and ATPγS or AMP-PNP, only band B was observed, a pattern very similar to that obtained with ATP in the absence of a divalent cation. These latter findings predict that in the presence of the above ATP homologues, hMutSα would dissociate from mismatched substrates irrespective of whether magnesium was present or not. Interestingly, this hypothesis was already substantiated by Blackwell et al. (16), who showed that ATPγS and AMP-PNP brought about a direct dissociation of hMutSα from the substrate, even when blocked at both ends.

hMutSα thus appears to adopt two distinct conformations, "dissociation-prone" and "translocation-prone," whereby the
latter comes about solely in the presence of ATP and magnesium or manganese. The hMSH6 subunit appears to alter its structure to a greater degree during this transition, which would appear to be consistent with the fact that this polypeptide contacts DNA (15) and is apparently involved in mismatch recognition (25). hMSH2 also undergoes structural alterations upon nucleotide and/or metal ion binding (Fig. 4A). However, these tended to be less diagnostic in our hands and are therefore omitted from this study.

The above data demonstrate that, within the context of hMutSα, the constituent subunits undergo distinct conformational changes upon ATP binding, depending on whether Mg$^{2+}$ or Mn$^{2+}$ is present or not. Such changes are consistent with the coordination of the divalent cation to the γ- and β-phosphate groups of the nucleotide, as was observed in the crystal structure of p21 Ha-Ras (2). To confirm that the magnesium effect does indeed involve the Walker type B motif, the study was further extended to include the hMutSα 6DV mutant. We have already shown that the D1213V mutation of hMSH6 does not affect the binding of the nucleotide cofactor to the mutant protein, but that hydrolysis of ATP is severely attenuated (see text above and Fig. 2B), presumably because of the inability to coordinate the metal atom at the Walker type B motif. The partial proteolysis patterns confirm these results. As shown in Fig. 4E, wild type hMutSα and hMutSα 6DV yielded similar patterns of protein bands (with band B predominating) in the presence of ATP. Addition of the nucleotide and magnesium resulted in the appearance of the characteristic ABC bands in the digest of the wild type factor, whereas the hMutSα 6DV pattern remained unaltered. This implies that the observed structural transitions involve the binding of magnesium within the Walker type B motif. Fig. 4E further shows that the partial proteolysis of hMutSα is largely unaffected by mismatch binding and thus that the conformational changes induced by the binding of the nucleotide and the metal ion discussed above are likely to be similar in the presence and in the absence of DNA.

Taken together, our data obtained with the Walker type A and B motif mutants show that under physiologically relevant experimental conditions, i.e., in the presence of ATP and magnesium, hMutSα assumes a conformation that allows it to leave the mispair and to travel along the DNA contour. The occupancy of both the motifs is required for this change to take place.

**DISCUSSION**

One of our principal interests is to elucidate the mechanistic role of the ATP-driven molecular motor of hMutSα in the process of postreplicative mismatch repair. In earlier studies it could be demonstrated that ATP binding was necessary and sufficient to displace the bound heterodimer from a mismatch, under conditions where no hydrolysis could take place, i.e., in the absence of magnesium, or when slowly or nonhydrolyzable ATP analogues were used (14, 15). Although these early results suggested that ATP hydrolysis was not required to bring about the conformational change that hMutSα must undergo to leave its preferred substrate, later experiments showed that the heterodimer can dissociate from DNA by two distinct modes, either directly or by sliding along the contour of the DNA (16, 17), and it became clear that gel-shift experiments using short oligonucleotides could not distinguish between these alternatives. Blocking of the ends of the oligonucleotides with streptavidin effectively hinders the heterodimer from leaving the substrates via the translocation mode, and this system was employed to demonstrate that whereas the direct dissociation mode did indeed require solely ATP binding, addition of magnesium forced hMutSα into the translocation mode. Our present results support these findings by demonstrating that in the presence or absence of magnesium, the ATP-bound form of hMutSα does indeed undergo two distinct conformational changes. Moreover, the asymmetry of the mismatch recognition complex was further emphasized in this study, inasmuch as the magnesium effect was controlled principally by the occupancy of the Walker type B motif of hMSH6 by the metal ion. Mutation of the homologous position in hMSH2 had very little effect on mismatch repair. This underscores the dominant role of hMSH6 in the function of the mismatch binding factor. Our earlier work showed that hMSH6 contributed more to the total ATPase activity of the heterodimer (15) and that a mutation in the ATP binding site of this polypeptide was associated with a more severe phenotype than the corresponding mutation in hMSH2 (15). Furthermore, only hMSH6 polypeptide could be cross-linked to heteroduplex DNA (8, 15, 21), which lead to the prediction that hMSH6 is the hMutSα subunit responsible for mismatch recognition. This notion was recently confirmed by showing that a single amino acid change (phenylalanine to alanine) in the amino terminus of the MSH6 homologues in both human and *S. cerevisiae* (25) could abolish mismatch binding.

The current findings also help us understand why the ATP binding-deficient mutant hMutSα 2,6KR (15) displayed a “dominant negative” phenotype in an *in vitro* mismatch repair assay, whereas addition of a large excess of the hMutSα 6DV mutant to repair-proficient extracts was without effect (data not shown). The underlying reason for these phenotypic differences lies in the fact that the former factor remains bound at the mismatch site because of its inability to undergo the necessary ATP-driven conformational change and thus interferes with the loading of the wild type factor and the assembly of the repairosome. In contrast, the hMutSα 6DV mutant can still compete with the wild type factor for mismatch binding, but fails to interfere with the repair process because of its direct dissociation (as opposed to translocation) from DNA following ATP binding.

The hMutSα molecular motor thus appears to drive the conformational change required to switch the heterodimer from a mismatch-bound form to a homoduplex-bound sliding clamp that is free to diffuse along the DNA contour. What is the role of this translocation process in mismatch correction? The principal task of the mismatch repair system is to eliminate DNA polymerase errors that escape detection by the proofreading activity of the enzyme. This is achieved through the exonucleolytic degradation of the newly synthesized strand, a process that initiates at DNA termini such as nicks or gaps which define this strand, rather than at the mismatch itself (see Refs. 26 and 27 for review). The mismatch repair system thus requires the ability to signal the presence of a replication error to the nearest strand interruption, often several hundred nucleotides distant from the mismatch site. Our model suggests that the hMutSα sliding clamp acts as this signal, the arrival of which at a DNA terminus will result in the recruitment of the other members of the repairosome, namely the MutL homologue hMLH1/hPMS2, as well as PCNA, polymerase-δ, DNA helicase(s), exonuclease(s), single-strand binding protein RPA, and other polypeptides either known or predicted to be part of the mismatch repair machinery (27, 28).

The final point that remains to be addressed is the role of the ATPase of hMutSα. Our data show that the translocation-prone conformation of the heterodimer is poised for ATP hydrolysis. However, the energy of this process can be envisaged to play a part in two possible scenarios. Blackwell *et al.* (16)...

---

2. P. Dufner and J. Jiricny, unpublished data.
3. P. Dufner and J. Jiricny, manuscript in preparation.
suggest that ATP hydrolysis is required during the translocation process, in which hMutSo translocation results in the formation of the α-loop structure as predicted from the E. coli model (13). It is attractive to think that the ATP-driven motor winds the DNA through the sliding clamp as a molecular ratchet (16), a notion compatible with the proposed functions of several ABC transporter ATPases that function in various processes such as the opening/closing of transmembrane pumps (3). Experimental evidence showing that the ATPase activity of hMutSo is the rate-limiting step of the mismatch repair process (16, 29) provide a compelling argument in support of this hypothesis. The other scenario suggests that, following mismatch binding and the concomitant ADP → ATP exchange, hMutSo will freely diffuse along the DNA, until it encounters the repairosome assembly point (17). Hydrolysis of the triphosphate would then recycle the ADP-bound, mismatch binding-proficient form of hMutSo. The precedent for this model might be considered to be PCNA. This trimeric ring-shaped factor is loaded on the DNA by means of the ATP-driven clamp loader RFC, whereupon the free PCNA diffuses along the helix contour in an energy-independent manner. This latter mechanism is attractive for one additional reason. As noted above, hMutSo has evolved to act in the repair of replication-associated mismatches. But certain types of mismatches that are excellent substrates for hMutSo can arise in a replication-independent manner. The best example of this type is the G/T mispair that arises through the spontaneous hydrolytic deamination of 5-methylcytosine in double-stranded DNA. It must be assumed that hMutSo will load at these mismatches and that it will be transformed into the sliding clamp. However, as these mismatches are not flanked by strand interruptions, the sliding clamp would travel along the DNA abortively and perhaps interfere with other biological processes. As many hMutSo molecules can be loaded successively at a single mismatch site (17), the deamination-associated G/T mispair could create serious problems for DNA metabolism. It is intriguing to think of the ATPase activity of hMutSo as a molecular clock that will bring about the dissociation of sliding clamp molecules, which have failed to locate a strand discontinuity within a given time frame and thereby evade problems associated with loading of hMutSo at inappropriate sites.

Acknowledgments—We gratefully acknowledge the expert technical assistance of Darinka Bohrer and Christine Hemmerle. We also thank Gray Crouse and Zuzanna Storchova for critical reading of the manuscript.

REFERENCES

1. Gorbalenya, A. E., and Koonin, E. V. (1990) J. Mol. Biol. 213, 583–591
2. Pai, E. F., Rahs, W., Krengel, U., Holmes, K. C., John, J., and Wittinghofer, A. (1989) Nature 341, 209–214
3. Hung, L. W., Wang, I. X., Nokado, K., Liu, P. Q., Ames, G. F., and Kim, S. H. (1990) Nature 396, 703–707
4. van der Wall, J. P., Klose, M., de Wit, J. G., den Blaauwen, T., Freund, R., and Driessen, A. J. (1995) J. Biol. Chem. 270, 18975–18982
5. Sato, K., Mori, H., Yoshida, M., and Mizushima, S. (1996) J. Biol. Chem. 271, 17439–17444
6. Brush, R. M., Jr., and Matson, S. W. (1996) J. Biol. Chem. 271, 25365–25368
7. Eisen, J. A. (1998) Nucleic Acids Res. 26, 4291–4300
8. Hughes, M. J., and Jiricny, J. (1992) J. Biol. Chem. 267, 23876–23882
9. Iacarino, L., Palombo, F., Drummond, J., Totty, N. F., Hsuan, J. J., Modrich, P., and Jiricny, J. (1996) Curr. Biol. 6, 484–486
10. Su, S. S., and Modrich, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5057–5061
11. Jiricny, J., Su, S. S., Wood, S. G., and Modrich, P. (1988) Nucleic Acids Res. 16, 7845–7853
12. Haber, L. T., and Walker, G. C. (1991) EMBO J. 10, 2707–2715
13. Allen, D. J., Makhov, A., Grilley, M., Taylor, J., Thresher, R., Modrich, P., and Griffith, J. D. (1997) EMBO J. 16, 4467–4476
14. Gradia, S., Acharya, S., and Fishel, R. (1997) Cell 91, 995–1005
15. Iacarino, L., Marra, G., Palombo, F., and Jiricny, J. (1998) EMBO J. 17, 2677–2686
16. Blackwell, L. J., Martik, D., Bjornson, K. P., Bjornson, E. S., and Modrich, P. (1998) J. Biol. Chem. 273, 32055–32062
17. Gradia, S., Subramanian, D., Wilson, T., Acharya, S., Makhov, A., Griffith, J., and Fishel, R. (1999) Mol. Cell. 3, 255–261
18. Studamire, B., Quach, T., and Alani, E. (1998) Mol. Cell. Biol. 18, 7590–7601
19. Palombo, F., Gallinari, P., Iacarino, L., Lettieri, T., Hughes, M., D’Arrigo, A., Truong, O., Hsuan, J. J., and Jiricny, J. (1995) Science 268, 1912–1914
20. Drummond, J. T., Li, G. M., Longley, M. J., and Modrich, P. (1995) Science 268, 1909–1912
21. Jiricny, J., Hughes, M., Corman, N., and Rudkin, B. B. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8860–8864
22. Thomas, D. C., Roberts, J. D., and Kunkel, T. A. (1991) J. Biol. Chem. 266, 3744–3751
23. Papadopoulos, N., Nicolaides, N. C., Liu, B., Parsons, R., Lengauer, C., Palombo, F., D’Arrigo, A., Markowitz, S., Willson, J. K., Knizler, K. W., Jiricny, J., and Vogelstein, B. (1995) Science 268, 1915–1917
24. Ross MacDonald, P., and Roeder, G. S. (1994) Cell 79, 1069–1080
25. Bowers, J., Sokolsky, T., Quach, T., and Alani, E. (1999) J. Biol. Chem. 274, 16115–16125
26. Modrich, P., and Lahue, R. (1996) Annu. Rev. Biochem. 65, 101–133
27. Jiricny, J. (1998) EMBO J. 17, 6427–6436
28. Kolodner, R. (1996) Genes Dev. 10, 1433–1442
29. Blackwell, L. J., Bjornson, K. P., and Modrich, P. (1998) J. Biol. Chem. 273, 32049–32054