Studies on Adenine Nucleotide States

and their Role in Human MutSα Function

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of
Philosophy in the Department of
Biochemistry in the Graduate School
of Duke University

2012
ABSTRACT

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Abstract

Mismatch repair is a conserved pathway that corrects errors resulting from the misincorporation of bases during DNA synthesis or from the production of damaged bases. The mismatch repair pathway is initiated by MutS homologs, proteins that recognize and bind the mispaired bases and set in motion a series of steps that results in the eventual removal of the offending base. The information must be transmitted from the mismatch to a strand signal site to initiate repair and several models have been proposed to describe the mode of interaction between the mismatch and the strand signal site. These models posit either MutS homolog movement from the mismatch to initiate repair, or activation of downstream events while MutS homologs remain bound to the mismatch. In the human system, the mismatched bases are recognized by MutSα, a heterodimer of two MutS homologs, MSH2 and MSH6. Both subunits each have an adenine nucleotide binding site that is also involved in ATP hydrolysis. The role of nucleotide binding and ATP hydrolysis has been a subject of controversy with some studies suggesting that ATP hydrolysis is required for MutSα movement from the mismatch and other studies suggesting that ATP binding but not hydrolysis is necessary for movement. We, therefore, employed different methods to further elucidate the nucleotide requirements for MutSα function. Because MutSα is a heterodimer with each subunit involved in
nucleotide binding, there are 9 possible nucleotide states of the protein. We used UV cross-linking and filter-binding studies to determine the functional states of MutSα and found that MutSα can bind ADP and ATP at the same time with ADP occupying MSH2 and ATP occupying MSH6. Surface plasmon resonance studies suggest that occupancy of MSH2 by ADP is important for protein dissociation from mismatch and movement along the DNA helix, as well as for the formation of the MutSα-MutLα-mismatch ternary complex, a key step in repair initiation. Studies with poorly hydrolyzable or non-hydrolyzable ATP analogs and ATP hydrolytic constructs of MutSα also suggest that ATP hydrolysis may play a role in mismatch dissociation and ternary complex formation. Our results suggest that both the ADP occupancy of the MSH2 subunit of MutSα and ATP hydrolysis are necessary for efficient dissociation from a mismatch and for efficient ternary complex formation. Surface plasmon resonance studies with doubly-blocked DNA suggest that after initial binding of the mismatch by a MutSα dimer, additional dimers are recruited. These dimers move along the DNA helix and can displace the mismatch-bound dimer.
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1. Introduction to DNA mismatch repair

1.1 Mismatch repair and replication fidelity

Mutations are changes in DNA caused by errors that occur during cell processes, such as DNA replication and recombination, as well as by damaging agents, such as radiation and chemicals (Iyer et al., 2006). Bases misincorporated during DNA synthesis or production of damaged bases, if not corrected, can be fixed as mutations in the genome during ensuing rounds of DNA replication (Iyer et al., 2006). Mutations can be neutral, beneficial, or harmful. Neutral mutations occur in noncoding regions of DNA and result in unobserved effects. Other neutral mutations occur when a base is changed in a codon, but this change does not effect codon translation due to redundancy of the genetic code. On some occasions, even if the codon translation is changed, the function of the gene product is unaltered. In all these cases, no phenotypic effects are observed. Beneficial mutations increase the fitness of organisms by enabling them to withstand stresses from the surrounding environment. These beneficial mutations become more common through natural selection and are the driving force of evolution. Harmful mutations, on the other hand, decrease the fitness of organisms. For example, mutations that cause errors in protein sequences, resulting in partially or completely defective proteins can lead to many diseases, such as genetic disorders and cancers.
However, mutations are extremely rare, occurring at a frequency of 1 per $10^9$ – $10^{10}$ base pairs (Drake, 1991; Drake, 1999). The reason for this high fidelity is threefold. First, due to base pair geometry, DNA polymerase inserts an incorrect nucleotide at a rate of 1 per $10^4$ – $10^5$ bases (Kunkel, 2004). Second, some polymerases have a proofreading exonuclease activity, which edits the mistake in the event of an incorrect base insertion, allowing the polymerase to make a second attempt at correct synthesis. The proofreading activity increases the replication fidelity by an additional 100-fold. Finally, if there are mistakes that escape these activities, they are corrected by the mismatch repair system, which further increases fidelity by 50- to 1000-fold. This pathway recognizes mismatched bases and initiates replacement of the incorrect nucleotide in the newly synthesized strand.

The mismatch repair pathway is of great biological significance. Its inactivation raises spontaneous mutability 50 to 1000-fold (Buermeyer et al., 1999; Jiricny, 1998; Kolodner, 1996; Kolodner and Marsischky, 1999; Modrich and Lahue, 1996; Schofield and Hsieh, 2003), while defects in the pathway result in highly elevated rates of base substitution and frameshift mutations (Harfe and Jinks-Robertson, 2000; Surtees et al., 2004). In human cells, inactivation of the mismatch repair system is the cause of hereditary nonpolyposis colon cancer (HNPCC) (de la Chapelle, 2004; Kolodner, 1995; Lynch and de la Chapelle, 1999; Rowley, 2005). Defects in this system have also been implicated in the development of some
sporadic tumors that occur in a variety of tissues (de la Chapelle, 2004; Eshleman and Markowitz, 1995; Peltomaki, 2001, 2003; Rowley, 2005).

1.2 *Escherichia coli* methyl-directed mismatch repair

As mentioned above, the mismatch repair pathway recognizes and removes incorrectly paired bases. However, a key question is how this system discriminates between the parent strand and the newly synthesized strand. The best-characterized mismatch repair pathway is found in *E. coli* and is reviewed in Iyer et al. (Iyer et al., 2006). *E. coli* DNA undergoes adenine methylation at GATC sites by the Dam methylase protein. However, this modification is a post replication event with a delay in methylation of the adenine residue in the newly synthesized strand (Geier and Modrich, 1979; Lyons and Schendel, 1984; Marinus, 1976). The mismatch repair system takes advantage of this characteristic of *E. coli* DNA to distinguish between the template and the newly synthesized strands (Fig. 1).

The mismatch repair pathway is initiated by MutS, a protein that recognizes and binds the mispaired bases (Jiricny et al., 1988; Parker and Marinus, 1992; Su et al., 1988; Su and Modrich, 1986). MutL is then recruited in an ATP-dependent manner to form the MutS-MutL-mismatch ternary complex, a key intermediate in the initiation of repair (Acharya et al., 2003; Galio et al., 1999; Grilley et al., 1989b; Schofield et al., 2001; Selmane et al., 2003; Spampinato and Modrich, 2000). This complex initiates downstream activities that lead to the removal of the offending
base. The first of these events is the activation of MutH, an endonuclease that incises the unmethylated strand of the hemi-methylated GATC site (Au et al., 1992; Welsh et al., 1987). The resulting strand break is the signal that directs excision repair to the unmethylated strand. A pre-existing single strand break is also sufficient to direct excision and repair to the nicked strand bypassing the requirement for MutH and a hemi-methylated GATC site (Lahue et al., 1989; Langle-Rouault et al., 1986).

**Figure 1.** Mechanism of *E. coli* methyl-directed mismatch repair. The mechanism is described in detail in the text and is taken from Iyer et al. (Iyer et al., 2006). S and L represent MutS and MutL respectively. The green arrows represent signaling from the mismatch to the hemi-methylated site.
The incision can occur either 5’ or 3’ to the mismatch and unwinding of the DNA by DNA helicase II begins at the strand break, with a strong bias toward the mismatch (Dao and Modrich, 1998; Yamaguchi et al., 1998). The resulting single strand displaced by the helicase is then excised by exonucleases. As the incision can be either 5’ or 3’ to the mismatch, the direction of excision can be either 5’ – 3’ or 3’ – 5’, requiring the appropriate exonucleases to perform this step. When the MutH incision occurs 5’ to the mismatch, excision is dependent on ExoVII or RecJ, two exonucleases that hydrolyze with 5’ – 3’ polarity (Chase and Richardson, 1974; Cooper et al., 1993; Grilley et al., 1993; Lovett and Kolodner, 1989). When MutH cleavage occurs 3’ to the mismatch, ExoI, ExoVII, or ExoX, which support 3’ – 5’ excision, are required (Burdett et al., 2001; Chase and Richardson, 1974; Cooper et al., 1993; Grilley et al., 1993; Lahue et al., 1989; Lehman and Nussbaum, 1964; Viswanathan et al., 2001; Viswanathan and Lovett, 1999).

The single stranded gap produced by the exonucleases is stabilized by SSB, single strand binding protein. DNA polymerase III synthesizes the new strand and fills in the gap. DNA ligase seals the nick, restoring helix integrity. Finally, the newly synthesized strand is methylated, making it indistinguishable from the parent strand (Lahue et al., 1989).
1.3 Mismatch repair in human cells

Mismatch repair is highly conserved in living organisms from simple to more complex species. Many human mismatch repair proteins are homologous to the *E. coli* proteins. MutSα, a heterodimer of two MutS homologs, MSH2 and MSH6, recognizes and binds the mispair (Drummond et al., 1995; Palombo et al., 1995) and recruits MutLα, a heterodimer of two MutL homologs, MLH1 and PMS2 in humans, and MLH1 and PMS1 in yeast (Blackwell et al., 2001b; Habraken et al., 1998; Li and Modrich, 1995; Mendillo et al., 2005). Together these two proteins initiate the pathway that results in excision and repair of the incorrect base.

As in the *E. coli* repair pathway, a strand break, either 3’ or 5’ to the mismatch, is sufficient to direct repair in the human system (Fang and Modrich, 1993; Wang and Hays, 2002). *In vitro* studies with purified proteins have established several reconstituted systems that support excision and repair. The simplest of these systems is an excision pathway consisting of four proteins, MutSα, MutLα, ExoI, and RPA (Genschel and Modrich, 2003). This system is mismatch- and strand break-dependent and exclusively supports 5’ to 3’ excision. Excision in this system is independent of MutLα. However, the presence of MutLα enhances the mismatch dependence of the reaction. MutSα-activated ExoI hydrolysis of DNA is highly processive in the absence of RPA (~2000 nucleotides), but is significantly reduced in its presence (~250 nucleotides) leading to termination of excision after mismatch removal. RPA fills the single stranded gap forming a poor substrate for
ExoI, but MutSα promotes ExoI initiation at these sites provided that a mismatch is present. Therefore, upon removal of the mismatch, MutSα cannot help in ExoI initiation resulting in attenuation of excision. MutSα and MutLα act together to suppress ExoI hydrolysis in DNA that lacks a mismatch, resulting in effective termination of excision.

Studies of nick-directed mismatch repair in cell extracts have shown that polymerase δ and PCNA participate in mammalian DNA repair synthesis (Gu et al., 1998; Longley et al., 1997). Work with purified proteins has identified minimal reconstituted systems that support bidirectional excision (Dzantiev et al., 2004) and repair (Constantin et al., 2005). The excision pathway requires MutSα, MutLα, PCNA, RFC, ExoI, RPA, and a strand break, while the repair pathway also requires polymerase δ in addition to these six proteins.

These two pathways, unlike the simpler four protein system mentioned earlier that only supports 5’ to 3’ excision, support both 5’ to 3’ and 3’ to 5’ excision and repair, meaning that a strand break either 3’ or 5’ to the mismatch directs excision and repair to the nicked strand. This is puzzling because unlike the E. coli system, which has multiple exonucleases that work with either 5’ to 3’ or 3’ to 5’ polarity, ExoI, which only hydrolyzes with 5’ to 3’ polarity, is the only exonuclease thus far established to be involved in the human mismatch repair pathway. The requirement of ExoI for 3’ to 5’ excision in both nuclear extracts and the purified system led to the suggestion that ExoI may harbor a cryptic 3’ to 5’ hydrolytic
activity that is activated by other components of the repair system (Dzantiev et al., 2004; Genschel et al., 2002). However, the discovery of a latent MutLα endonuclease activity that is activated in the presence of MutSα, a mismatch, PCNA, RFC, and a pre-existing break has shed more light on the ExoI requirement for 3’ to 5’ hydrolysis (Kadyrov et al., 2006). When the strand discontinuity is 3’ to the mismatch, activated MutLα makes additional nicks in the strand containing the break. These breaks are on the distal side of the mismatch, and, for a 3’ heteroduplex, result in a product bracketed by strand breaks. These multiply nicked molecules are substrates for MutSα-activated ExoI, which then removes the DNA segment containing the mismatch by 5’ to 3’ hydrolysis (Kadyrov et al., 2006) (Fig. 2).

The role of RFC in the reconstituted bidirectional excision reaction is two-fold. It functions in PCNA loading on DNA (Pluciennik et al., 2010) and, along with PCNA, suppresses 5’ to 3’ hydrolysis from a 3’ strand break (Dzantiev et al., 2004). Studies on the role of PCNA in MutLα endonuclease activity have shown that its function is also two-fold. First, through interaction of the two proteins, PCNA activates the MutLα endonuclease, and second, due to its loading orientation, PCNA determines the strand direction of MutLα incision (Pluciennik et al., 2010). PCNA is a sliding clamp that is loaded at 3’ double strand – single strand DNA junctions with a specific orientation relative to the 3’ junction (Bowman et al., 2004; Gulbis et al., 1996; Yao et al., 2000). The two faces of PCNA are not equivalent, and MutLα
interacts preferentially with one side (Pluciennik et al., 2010). As PCNA can diffuse along the DNA (Yao et al., 1996), MutLα could be carried along the helix to dictate strand – specific incision elsewhere on the DNA (Pluciennik et al., 2010).

![Mismatch Repair Diagram](image)

**Figure 2.** Bidirectional mismatch repair in human cells. The green arrows represent signaling from the mismatch to the strand discontinuity. The red arrows represent sites of additional nicks made by MutLα in the discontinuous strand. In the case of 3' repair, the new nicks 5' to the mismatch allow for 5' to 3' hydrolysis by ExoI. Taken from Kadyrov et al. (Kadyrov et al., 2006).

Although there are similarities between the human and *E. coli* systems, there are also significant differences. A key difference is that human DNA is not adenine methylated at GATC sites, and an activity homologous to MutH has yet to be identified. Therefore, the question of strand discrimination remains. As is the case with *E. coli*, strand breaks are sufficient to direct human repair *in vitro* (Fang and
Therefore, the strand signal could be directed by discontinuities that occur naturally during DNA replication (Claverys and Lacks, 1986; Claverys and Mejean, 1988; Holmes et al., 1990). Another possible strand discrimination mechanism could be the relationship between the repair system and replication machinery (Wagner and Meselson, 1976). Interactions between the repair and replication proteins could provide the strand discrimination signal. It has also been suggested that PCNA might provide a link between repair and replication, as it interacts with several mismatch repair and replication activities, allowing DNA termini at the fork to function as strand signals (Umar et al., 1996). As described above, 3’ ends at double strand – single strand junctions dictate the orientation of PCNA loading, which in turn directs MutLα endonuclease, providing a compelling argument for DNA termini at the fork as the strand signals (Pluciennik et al., 2010).

1.4 The role of mismatch repair in DNA damage response and genetic recombination

In addition to the processing of replication errors, the mismatch repair pathway has also been implicated in the cellular response to different types of DNA damage as well as modulating the outcome of mitotic and meiotic recombination events.

Proteins of the mismatch repair pathway have been implicated in cellular response to DNA damage by activating signaling pathways that result in apoptosis.
An example of DNA damage response is the processing of DNAs containing $O^6$-methylguanine ($O^6$-MeG) by the mismatch repair system in nuclear extracts (Ceccotti et al., 1996; Duckett et al., 1999; Karran et al., 1993). The $O^6$-MeG lesion is a product of the effect of DNA methylators and can pair with either cytosine or thymine resulting in $O^6$-MeG-C and $O^6$-MeG-T base pairs (Bignami et al., 2000; Drablos et al., 2004; Patel et al., 1986a, b). Human MutSα specifically recognizes and binds both these base pairs (Duckett et al., 1996). Two models have been proposed to account for the function of MutSα and MutLα in the DNA damage response, the futile cycling model and the direct activation model. The futile cycling model posits that the lesion is on the template strand, and the mismatch repair pathway is activated once the replication fork encounters the damage. However, as the mismatch repair system is restricted to the newly synthesized strand, the lesion cannot be removed leading to multiple rounds of excision and synthesis (Goldmacher et al., 1986). The intermediates of this futile cycling could serve as a scaffold for the recruitment of damage signaling proteins that could lead to apoptosis. The direct activation model postulates assembly of MutSα, MutLα, and perhaps other activities at the damage site. These activities are sufficient to initiate damage signaling through direct interaction with signaling proteins (Duckett et al., 1996; Kat et al., 1993; Liu et al., 2010; Mello et al., 1996).

MutS and MutL homologs have also been shown to modulate the outcome of DNA recombination events, although the mechanisms of such events are not well
understood. Studies with \textit{E. coli} proteins have demonstrated that inactivation of MutS or MutL dramatically increases the frequency of homeologous recombination (recombination between quasi-homologous sequences) (Matic et al., 1994; Petit et al., 1991; Rayssiguier et al., 1989; Shen and Huang, 1989). In studies with \textit{E. coli} and \textit{Salmonella typhimurium}, two species that do not usually exchange genetic information, the presence of MutS or MutL mutants within one species allows it to incorporate the other’s DNA into its genome, indicating that these mismatch repair proteins are important in determining the species barrier in bacteria (Matic et al., 1994; Rayssiguier et al., 1989). Defective MutS\textsubscript{α} or MutL\textsubscript{α} in human cells increases the rate of gene duplication 50 – 100 fold, an effect attributed to illegitimate recombination (Chen et al., 2001). As shown in studies with \textit{E. coli} MutS and MutL and their yeast homologs, these proteins block the branch migration step of recombination by binding to the mismatches formed during the early stages of strand transfer (Chen and Jinks-Robertson, 1998, 1999; Worth et al., 1994).

\textbf{1.5 Models of signaling between the mismatch and strand signal}

Once a mismatch has been recognized, information must be transmitted to initiate the repair pathway. Several models have been proposed to describe the mode of interaction between the mismatch and the strand signal sites (Fig. 3). Two of these models suggest movement of MutS homologs as well as the corresponding MutS-MutL complex along the DNA helix in an ATP-dependent manner and
postulate that this movement plays an important role in activating downstream activities that act at the strand signal (Fig. 3A) (Allen et al., 1997; Blackwell et al., 1998; Gradia et al., 1997; Gradia et al., 1999). The translocation model proposes that ATP hydrolysis is required for movement of the MutS homologs or the MutS-MutL complex along the DNA helix (Allen et al., 1997; Blackwell et al., 1998). This model is based on electron microscopy visualizations of bacterial MutS-MutL-heteroduplex complexes, which demonstrate a mismatch- and ATP-dependent formation of DNA loops. As non-hydrolyzable ATP analogs did not support loop formation, as well as terminated ongoing loop growth, it was suggested that this reaction is attributed to a directional translocation along the DNA helix, which is dependent on ATP hydrolysis (Allen et al., 1997). Studies with end-blocked linear DNAs showed that stable complexes were only formed under hydrolytic conditions and not when non-hydrolyzable ATP analogs were present, supporting the idea that ATP hydrolysis is necessary for movement away from the mismatch (Blackwell et al., 1998). MutS homologs, however, have a modest ATPase activity, and it is difficult to reconcile that ATP hydrolysis is the sole driving force of translocation.

A model has also been proposed for MutS homolog movement along the DNA that is independent of ATP hydrolysis (Gradia et al., 1997; Gradia et al., 1999). In this model, termed the molecular switch model, MutS homologs bind the mismatch only in an ADP-bound form. The mismatch provokes exchange of ADP for ATP, which results in a conformational change that allows the MutS proteins to diffuse
along the helix without hydrolyzing ATP. Hydrolysis of ATP occurs once the protein has dissociated from the DNA to regenerate the protein-ADP complex. However, homoduplex DNA also promotes ADP for ATP exchange at approximately 30% of the rate of heteroduplex, and this modest dependence of the exchange rate on a mismatch is insufficient to account for the observed mismatch-dependence of the activation of downstream events. Moreover, MutS homologs can exist in a nucleotide-free form, which binds mismatches with high affinity identical to that of the ADP-bound form (Martik et al., 2004).

![Figure 3](https://example.com/figure3.png) **Figure 3.** Models of signaling between the mismatch and strand break. The models are described in detail in the text. Taken from Iyer et al. (Iyer et al., 2006).

A third model, the static transactivation model, invokes ATPase function in a kinetic proofreading mechanism without MutS homolog movement from the mismatch (Junop et al., 2001; Wang and Hays, 2003, 2004). In this model, MutS proteins must be bound to ATP and the mismatch simultaneously to recruit MutL homologs. Downstream events are activated through DNA bending and looping (Fig. 3B). ATP binding in this model functions to verify mismatch recognition.
However, studies with the *E. coli* methyl directed mismatch repair pathway have shown an orientation dependent loading of the excision system either 3’ or 5’ to the mismatch (Dao and Modrich, 1998; Yamaguchi et al., 1998), and it is unclear how a bending mechanism would account for the heteroduplex orientation. These ideas are also incompatible with observations that ATP reduces the affinity of MutS homologs for the mismatch (Blackwell et al., 1998; Gradia et al., 1999; Hargreaves et al., 2010; Heinen et al., 2011; Mazur et al., 2006). Lastly, gel shift studies with double end blocked DNA (Blackwell et al., 2001a; Blackwell et al., 1998; Gradia et al., 1999) and studies with DNA roadblocks have suggested that MutS homologs do indeed move along the helix contour (Pluciennik and Modrich, 2007).

Finally, a model that has received less attention in the literature postulates that recognition of the mismatch by MutS homologs serves as a site for polymerization of a second protein along the DNA helix (Fig. 3C) (Modrich, 1987). These proteins could be additional MutS homologs, MutL homologs, or other proteins. Polymerization could occur in either direction and therefore establish heteroduplex orientation. Yeast MutLα does polymerize on DNA in a chain length-dependent manner, but this polymerization is abolished at physiological salt concentrations in the presence of Mg^{2+} (Hall et al., 2001). Furthermore, electron microscopy visualization of bacterial MutS and MutL on heteroduplex DNA has failed to reveal evidence of polymerization (Allen et al., 1997).
1.6 ATPase centers and their role in MutS homolog function

Human MutSα is a heterodimer of two MutS homologs, MSH2 and MSH6 (Fig. 4A). In addition to mismatch recognition and binding, MutS homologs have an intrinsic ATPase activity due to a highly conserved ATP hydrolytic center near the carboxyl terminus (Haber et al., 1988; Lamers et al., 2000; Obmolova et al., 2000; Warren et al., 2007) (Fig. 4B). While the integrity of these domains is required for function in mismatch repair, the role that ATP binding and hydrolysis play in the function of these proteins is not well understood.

The interaction between DNA binding and the ATPase centers in MutS homologs is of significant importance. Pre-steady-state studies with E. coli MutS, T. aquaticus MutS, and yeast MutSα have shown an initial burst of ADP formation in the absence of DNA or presence of homoduplex, implying fast hydrolysis relative to turnover. This burst is abolished by heteroduplex DNA inferring an increase in the lifetime of MutS-bound ATP upon mismatch recognition (Antony and Hingorani, 2003, 2004; Bjornson et al., 2000). These findings were further supported in a study with yeast MutSα using UV crosslinking (Mazur et al., 2006). Nucleotide occupancy also regulates MutS homolog interactions with DNA, with the MutS proteins undergoing conformational changes upon nucleotide binding (Lamers et al., 2004; Lamers et al., 2003).
Figure 4. Structure of human MutSα. A) The human MutSα heterodimer interacting with a DNA containing a GT mismatch. The MSH6 subunit interacts extensively with DNA, while MSH2 only makes one contact (Warren et al., 2007). B) The ATPase sites of MutSα shown here containing ADP in both subunits.

However, the modulatory effects of adenine nucleotides on the interactions between the MutS proteins and DNA are still not well understood. This may reflect the many different permissible occupancy states available for the protein. As MutS and its homologs are dimers with each subunit containing a nucleotide binding site, there are nine possible occupancy states: 1) both subunits are empty; 2) subunit 1 is empty, subunit 2 has ADP; 3) subunit 1 has ADP, subunit 2 is empty; 4) both subunits have ADP; 5) subunit 1 is empty, subunit 2 has ATP; 6) subunit 1 has ATP, subunit 2 is empty; 7) both subunits have ATP; 8) subunit 1 has ADP, subunit 2 has ATP; and 9) subunit 1 has ATP, subunit 2 has ADP. Moreover, studies with the E. coli...
MutS have shown that the protein is a tetramer in solution, further increasing the number of possible nucleotide states (Bjornson et al., 2003).

Adenine nucleotides have differing effects on the interaction of MutS homologs with DNA. The presence of ATP decreases the affinity of MutSα for mismatch, and, after mismatch binding, ATP triggers rapid dissociation from DNA (Blackwell et al., 1998; Gradia et al., 1999; Hargreaves et al., 2010; Heinen et al., 2011). On the other hand, ADP does not affect the affinity of MutSα for the mismatch (Martik et al., 2004), but it does affect the dynamics of protein-heteroduplex interactions by increasing, to a similar extent, the rates of formation and dissociation of MutSα-mismatch complexes (Blackwell et al., 1998).

Studies with *E. coli* MutS (Bjornson and Modrich, 2003), *T. aquaticus* MutS (Antony and Hingorani, 2004), yeast MutSα (Antony and Hingorani, 2003; Mazur et al., 2006), and human MutSα (Martik et al., 2004) have shown that MutS/MutSα has one high affinity site for ADP and one for ATP per dimer equivalent. In the case of the yeast protein, these high affinity sites are in the MSH2 and MSH6 subunits, respectively (Mazur et al., 2006). Filter studies with yeast MutSα have shown binding of one ATP per MutSα dimer, one ADP per dimer, and two ATPγS per dimer (Antony and Hingorani, 2003). The two ATPγS molecules bind with differing affinities, indicating that one subunit of the yeast MutSα has a higher affinity for the triphosphate than the other subunit. This result was confirmed by a UV cross-linking study with yeast MutSα in which the MSH6 subunit has the higher affinity for
the triphosphate (Mazur et al., 2006). While the ATP and ADP results have also been observed with human MutSα, only one ATPγS has been shown to bind the human MutSα dimer (Martik et al., 2004). This study with the human protein, along with studies with *E. coli* MutS (Bjornson and Modrich, 2003) and *T. aquaticus* MutS (Antony and Hingorani, 2004), also shows that the MutS proteins can exist in a state with ADP in one subunit and ATP in the other. A recent single molecule study with *Taq* MutS has demonstrated that this state of the protein, with ADP in one subunit and ATP in the other, is required for the formation of a sliding clamp that can move away from the mismatch (Qiu et al., 2012). This study also suggests that ATP hydrolysis can occur while MutS is bound to DNA, and this hydrolysis results in the ATP-MutS-ADP state.

1.7 *The MutSα-MutLα-mismatch ternary complex*

As mentioned earlier, a key step in the initiation of mismatch repair is the recruitment of MutLα after binding of the mismatch by MutSα, forming a MutSα-MutLα-DNA ternary complex (Fig. 5). This complex is mismatch-dependent and is only observed in experiments with DNAs 100 bp or longer (Blackwell et al., 2001b; Grilley et al., 1989b; Schofield et al., 2001). It has been suggested that this complex may be capable of movement along the DNA helix, carrying a signal from the mismatch to the strand discrimination signal (Acharya et al., 2003; Blackwell et al., 2001b). However, movement of the complex along the DNA is not well understood.
As shown in Figure 3, besides movement along the helix, these proteins could also polymerize along the DNA, thus transmitting the signal from the mismatch to the strand signal.

![Figure 5. Model of the ATP-dependent formation of the MutSα-MutLα-mismatch ternary complex. Integrity of the ATPase sites of MutSα is necessary for ternary complex formation, while integrity of the ATPase sites of MutLα is not.]

While the general consensus is that the ternary complex is formed in an ATP-dependent manner, the role of ATP hydrolysis has been a subject of controversy. While the poorly hydrolyzable ATP analog ATPγS supports ternary complex formation with *E. coli* MutS and MutL (Acharya et al., 2003; Grilley et al., 1989b), other studies with the human and yeast systems have shown that AMPPNP and ATPγS are much less efficient in ternary complex formation (Blackwell et al., 2001b;
Mendillo et al., 2005). Moreover, the nucleotide states of MutSα required for the ternary complex formation have not been closely studied. As is the case with MutS homolog dissociation from DNA, the nucleotide states of MutSα could play a significant role in the MutSα-MutLα-mismatch ternary complex formation.

MutL homologs also have an ATPase activity, and its role in ternary complex formation has been studied. While the ATP hydrolysis requirements for MutS homolog function are not well understood, work with the E. coli and human MutL proteins has shown that ATPase integrity of the MutL homologs is not required for assembly of the ternary complex (Acharya et al., 2003; Raschle et al., 2002; Selmane et al., 2003).
2. Nucleotide states of human MutSα

Human MutSα is a heterodimer of MutS homologs MSH2 and MSH6. It is one of the key proteins in the mismatch repair pathway due to its ability to recognize and bind mispaired bases. In addition to mismatch recognition and binding, MutS homologs have an intrinsic ATPase activity due to a highly conserved ATP hydrolytic center near the carboxyl terminus (Haber et al., 1988; Lamers et al., 2000; Obmolova et al., 2000; Warren et al., 2007). While the integrity of these domains is required for function in mismatch repair (Haber et al., 1988; Iaccarino et al., 1998; Worth et al., 1998), the role that ATP binding and hydrolysis play in the function of these proteins is not well understood, and several different models have been proposed to describe the role of ATP in mismatch repair (Allen et al., 1997; Blackwell et al., 1998; Gradia et al., 1997; Gradia et al., 1999; Junop et al., 2001; Wang and Hays, 2004).

Nucleotide occupancy also regulates MutS homolog interactions with DNA, with the MutS proteins undergoing conformational changes upon nucleotide binding (Lamers et al., 2004; Lamers et al., 2003). The modulatory effects of adenine nucleotides on the interactions between the MutS proteins and DNA, however, are still not well understood. This may reflect the nine different possible occupancy states available for the two ATPase sites in the MutS homolog dimer. Studies with *E. coli* MutS (Bjornson and Modrich, 2003), *T. aquaticus* MutS (Antony and Hingorani,
2004), yeast MutSα (Antony and Hingorani, 2003; Mazur et al., 2006) and human MutSα (Martik et al., 2004) have shown that the MutS/MutSα dimer has one high affinity site for ADP and one for ATP. Filter-binding studies with yeast MutSα have shown binding of one ATP per MutSα dimer, one ADP per dimer, and two ATPγS molecules per dimer, although one binds with high affinity and the other with reduced affinity (Antony and Hingorani, 2003). The ATP and ADP results have also been observed with human MutSα (Martik et al., 2004). However, in this study, only one ATPγS has been shown to bind the human MutSα dimer, corresponding to the high affinity binding of ATPγS to yeast MutSα. The second ATPγS with reduced binding affinity was not observed with human MutSα. The study with the human protein, along with studies with E. coli (Bjornson and Modrich, 2003) and T. aquaticus MutS (Antony and Hingorani, 2004), also shows that the MutS proteins can exist in a state with ADP in one subunit and ATP in the other. In the case of the yeast protein, these high affinity sites were in the MSH2 and MSH6 subunits, respectively (Mazur et al., 2006).

This chapter will focus on UV cross-linking and filter-binding studies used to determine the nucleotide states of human MutSα and the effect of DNA on nucleotide occupancy.


### 2.1 Materials and methods

#### 2.1.1 Proteins and protein preparations

The proteins were prepared from SF9 cells using baculovirus constructs expressing the appropriate human cDNAs as previously described (Blackwell et al., 2001b). A key step of this purification procedure is elution of the protein from a single-stranded DNA cellulose column with ATP and MgCl$_2$. The resulting protein contains 1 mole of ADP for every mole of heterodimer, a product of ATP hydrolysis. To study the nucleotide states of MutSα, it is important to start with nucleotide-free protein. Therefore, this purification procedure was modified as follows.

Ten grams of frozen SF9 cell paste were thawed at room temperature (RT) for 40 minutes. Twenty mL of room temperature Buffer A (25 mM HEPES-NaOH, 0.5 mM EDTA) containing 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL E-64, 1 μg/mL pepstatin, 1:1000 fold dilution of saturated phenylmethylsulfonyl fluoride (PMSF) in isopropanol, and 1 mM dithiothreitol (DTT)$^1$ was then added, and the cells were thawed for another 15 minutes at RT. Thirty more mL of Buffer A containing protease inhibitors was added, followed by transferring on ice and pipetting up and down 10 times to ensure that the cells are broken up and homogenized. The ionic strength was adjusted to 100 mM NaCl equivalent by the

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$^1$ The protease inhibitors (leupeptin, aprotinin, E-64, pepstatin, PMSF) and DTT are used at these concentrations throughout the purification procedure and are present in all buffers.
addition of 3 M NaCl, and the suspension was spun at 30,000g for 15 minutes. The following step were all performed at 4 °C. Using a peristaltic pump, the supernatant was loaded at 2 mL/min on a 20-mL SP-Sepharose fast flow column that was previously equilibrated with Buffer A containing 100 mM NaCl. The column was then washed with 5 column volumes of 100 mM NaCl Buffer A at 3 mL/min. The bound protein was eluted with a 10-column volume gradient of 100 to 500 mM NaCl Buffer A at a flow rate of 2 mL/min. The fractions were checked by 4-20% SDS-PAGE, and the fractions containing MutSα were pooled. The protein eluted at 200-240 mM NaCl and the ionic strength of the pooled fractions was adjusted to 200 mM NaCl equivalent with cold Buffer A. The protein-containing solution was loaded on a 10-mL single-stranded DNA cellulose column previously equilibrated with 200 mM NaCl Buffer A. The column was washed with 5 column volumes of 200 mM KCl containing Buffer B (25 mM HEPES-KOH, 0.5 mM EDTA) and bump eluted with 600 mM KCl Buffer B. The loading, washing, and elution steps on this column were carried out at a flow rate of 2 mL/min using a peristaltic pump. The protein-containing fractions, as detected by Bradford, were pooled, and the ionic strength was adjusted to 200 mM KCl equivalent with cold Buffer B. The solution was loaded on a 4-mL Q-Sepharose fast flow column followed by washing with 5 column volumes of 200 mM KCl Buffer B at 2 mL/min using a peristaltic pump. The protein was eluted drop wise with 600 mM KCl Buffer B, and 1.0-mL fractions were collected. The protein-containing fractions, as detected by Bradford, were pooled,
the ionic strength was adjusted to 200 mM KCl equivalent with Buffer B, and the solution was loaded on a 1-mL MonoQ column on the FPLC (AKTA, GE Healthcare). The column was washed with 10 column volumes of 190 mM KCl Buffer B and eluted with a 20-column volume gradient from 190 to 640 mM KCl Buffer B. The loading, washing, and elution steps were carried out at a flow rate of 0.5 mL/min. The peak fractions, as detected by UV absorbance, were checked by 4-20% SDS-PAGE, and the fractions containing MutSα were pooled and concentrated. The yield from 10 g of cell paste was approximately 1.5 mg of protein, and the resulting protein does not contain any detectable prebound ADP.

The nucleotide content was measured using an ATP bioluminescent kit from Sigma as previously described (Baitinger et al., 2003; Blackwell et al., 1998).

### 2.1.2 DNA substrates

The oligodeoxyribonucleotides were obtained from IDT Technologies. The sequence for the 21-bp duplexes was identical to the 31-bp duplexes described previously (Allen et al., 1997), with the 5 bases on either end eliminated. All duplexes were purified by native HPLC (Waters) with a gradient from 0.5 to 1.0 M NaCl in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA buffer on a 1-mL GenPak Fax column (Waters).
2.1.3 Nucleotides

All labeled nucleotides [α-32P]ATP, [γ-32P]ATP, and [35S]ATPγS were purchased from Perkin/Elmer. Labeled ADP was prepared as previously described (Mazur et al., 2006). All labeled nucleotides were diluted in unlabeled nucleotide solutions. For titration experiments, the labeled nucleotide was diluted with the highest concentration of the appropriate unlabeled nucleotide, and then serial dilutions were carried out to ensure that the ratio of labeled to unlabeled nucleotide was the same in all of the concentrations. The unlabeled nucleotides were purchased from USB Corp (ATP), Sigma (ADP), and Calbiochem (ATPγS).

2.1.4 UV cross-linking assays

All incubations were done on ice. For time course experiments, 0.5 μM MutSα was incubated in a 20-μL reaction containing 25 mM HEPES-KOH, pH 7.5, 150 mM KCl, 5 mM MgCl₂, and 1 mM DTT. The reactions were started by the addition of labeled ATP, either α- or γ-32P as indicated, to a final concentration of 10 μM. In the case of poorly hydrolyzable ATP analogs, [γ-35S]ATPγS was used. Either correctly paired DNA (homoduplex) or DNA with a central mismatch (heteroduplex) was present in the reactions as indicated at a concentration of 1 μM. At the indicated time points, the reactions were stopped by freezing in liquid nitrogen, and the frozen products were subjected to 10 minutes of UV irradiation in a UV Stratalinker 1800 cross-linker (Stratagene). The reactions were kept on ice
during irradiation to ensure that they remained frozen throughout the process. UV cross-linking was followed by resolving the reactions by 8% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue, destained, and dried. Radiolabeling was visualized using a Phosphorimager (Molecular Dynamics) and quantified using ImageQuant (Molecular Dynamics). The bands from the Coomassie stained gels were quantitated to normalize the amount of cross-linked nucleotide. For titration experiments, MutSα was incubated on ice for 5 minutes with the different nucleotides concentrations as indicated. The reactions were stopped by freezing in liquid nitrogen, UV irradiated, and resolved by SDS-PAGE as described above. The amount of cross-linked nucleotide was plotted and fit to a square hyperbola by nonlinear regression analysis, and apparent $K_d$ values were extracted.

### 2.1.5 Filter binding assays

Filter binding assays with ADP and ATPγS were carried out in 10-μL reactions containing 25 mM HEPES-KOH pH 7.5, 150 mM KCl, 5 mM MgCl$_2$, 1 mM DTT, and 0.5 μM MutSα. [$\alpha$-$^{32}$P]ADP and [$\gamma$-$^{35}$S]ATPγS were titrated as indicated. The reactions were incubated on ice for 5 minutes and filtered through 13-mm, 0.45-μm nitrocellulose membranes, which had been previously equilibrated and washed with 0.1 mL of room temperature reaction buffer. After application of the reactions, the filters were immediately washed with 0.1 mL of room temperature reaction buffer, dried, and counted by liquid scintillation. While the incubations
were on ice, the filtrations were carried out at room temperature. The amount of nucleotide bound was plotted and fit to a square hyperbola by non-linear regression, and the $K_d$ values were extracted.

### 2.2 Nucleotide Occupancy of MutSα

#### 2.2.1 Preparation of nucleotide free MutSα

As stated in the Materials and Methods of this chapter, the MutSα purification method developed in our lab (Blackwell et al., 2001b; Drummond et al., 1995), which will be referred to as purification procedure 1, yields highly pure protein. However, these preparations contain 1 ADP molecule for every MutSα heterodimer. To study the nucleotide states of MutSα, however, nucleotide free protein was required. Therefore, purification procedure 1 was modified as described in detail in sections 2.1.1 and is referred to as purification procedure 2. The main difference between these two procedures is the elution from the single stranded DNA cellulose column. In procedure 1, ATP and MgCl$_2$ are used to elute the protein from the column. The ADP content of the protein observed is most likely from this step. In procedure 2, ATP was eliminated from this step, and a salt gradient was instead used to elute from the single stranded DNA column. Purification procedure 2 also yields highly pure protein, with negligible amount of nucleotide (Fig. 6A), confirming that the ADP amount observed after purification procedure 1 results
from ATP hydrolysis during elution from single stranded DNA. In the studies that follow, the MutSα used was purified with procedure 2.

**Figure 6.** Purification of MutSα. A) Coomassie stained SDS gel of MutSα purified with procedure 1 or 2. The upper band is MSH6, while the lower band is MSH2. B) Measurement of the nucleotide content of MutSα purified with procedure 1 (red) or 2 (blue).

2.2.2 UV cross-linking experiments show that the MSH2 subunit of MutSa can be occupied by either ADP or ATP

UV cross-linking is a method previously used to visualize nucleotide occupancy of the two subunits of MutSα (Heinen et al., 2011; Iaccarino et al., 1998; Mazur et al., 2006). However, it does have several caveats. The efficiency of cross-linking is dependent on photoactivation of the nucleotides, geometry of the binding site, and residency time of the nucleotide in the binding pocket. The efficiency of cross-linking may also be different for each subunit and under our conditions is
quite low, with less than 1% of the nucleotide cross-linked to the subunits, bringing to question whether the cross-linking results are an accurate representation of protein populations in solution. Finally, because UV cross-linking results in covalent and irreversible attachment, it can overestimate affinity if there is non-specific binding and underestimate affinity if there is inefficient cross-linking.

With these limitations in mind, we also used this method to study the occupancy of each subunit of human MutSα by either ATP or ADP, with a key difference. The previous studies (Heinen et al., 2011; Mazur et al., 2006) were performed after incubations either on ice or at room temperature and then exposed to UV while still in solution. The results show only the endpoint of the reaction. In our studies the reactions are stopped at different time points by freezing in liquid nitrogen and exposed to UV while still frozen. This change traps the nucleotide states of MutSα and allows for observation of both endpoints and kinetic effects.
Figure 7. UV cross-linking of ATP. UV cross-linking of [γ-32P]ATP (A and B) or [α-32P]ATP (C and D) to MutSα, measured as a function of time. The reactions were started by the addition of 10 μM labeled ATP to 0.5 μM protein and stopped by freezing in liquid nitrogen at the indicated time points. The frozen reactions were exposed to UV irradiation, and the products were resolved by 8% SDS-PAGE. Panels A and C show representative gels of the cross-linking, while panels B and D show the quantitation of those experiments, respectively. The plots show the average of 3 experiments ± standard deviation.

Labeling of MutSα with either [γ-32P]ATP or [α-32P]ATP was examined as a function of time. In the case of [γ-32P]ATP, a decrease in labeling signal of both

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2 The differences in the amount of UV cross-linking observed here and in the following figures are due to the UV lamps. The bulbs lose power with age, resulting in less efficient cross-linking of the nucleotides. The results shown were obtained from experiments performed at different points in time with newer or older lamps, resulting in different UV cross-linking efficiencies. Therefore, due to this variability, the pattern of labeling is more important than the absolute values.
MSH2 and MSH6 as a function of time was observed (Fig. 7A and 7B). However, when \([\alpha^{-32}\text{P}]\text{ATP}\) replaced \([\gamma^{-32}\text{P}]\text{ATP}\) in the experiments, the labeling signal of MSH2 does not decrease with time (Fig. 7C and 7D). The simplest explanation for these observations is that the gamma phosphate is removed by hydrolysis, resulting in loss of signal when \([\gamma^{-32}\text{P}]\text{ATP}\) is used. However, as the label in \([\alpha^{-32}\text{P}]\text{ATP}\) is in the alpha phosphate, the removal of the gamma phosphate does not affect the labeling signal. Taken together, these results show that the MSH2 subunit is first occupied by ATP, and after hydrolysis has occurred, it is occupied by ADP.

Pre-incubating MutS\(\alpha\) with cold ADP and then performing the cross-linking experiments with \([\gamma^{-32}\text{P}]\text{ATP}\) showed no labeling of the MSH2 subunit (Fig. 8A and 8D), suggesting that the nucleotide site is occupied by the cold ADP. However, if cold ADP and \([\gamma^{-32}\text{P}]\text{ATP}\) are added at the same time, labeling of MSH2 is observed (Fig. 8B and 8E). The amount of nucleotide cross-linked, however, is half of that observed when no cold ADP is present (compare Fig. 8C, 8D, and 8E). These results suggest that the MSH2 nucleotide site can be occupied by either ATP or ADP with similar affinities.
Figure 8. $[^{32}\text{P}]{\text{ATP}}$ cross-linking to nucleotide-free MutSα in the absence or presence of cold ADP. A) Cross-linking of 10 μM $[^{32}\text{P}]{\text{ATP}}$ after a 5 minute incubation of MutSα with 10 μM cold ADP. B) Cross-linking of 10 μM $[^{32}\text{P}]{\text{ATP}}$ and 10 μM cold ADP added together. C) Quantitation of cross-linking of 10 μM $[^{32}\text{P}]{\text{ATP}}$ in the absence of cold ADP. D) and E) quantitation of A and B respectively. The plots show the average of 3 experiments ± standard deviation.

2.2.3 The MSH6 subunit of MutSα is labeled by ATP but not ADP

In both instances, whether using $[^{\gamma-32}\text{P}]{\text{ATP}}$ or $[^{\alpha-32}\text{P}]{\text{ATP}}$, the MSH6 subunit was labeled at significantly lower levels than MSH2 (Fig. 7A and 7C). Two possible explanations for this result are that MSH6 1) has low affinity for ATP or 2) MSH6 hydrolyzes ATP and releases the product due to lower affinity for ADP. These would result in shorter residency time of nucleotides in the site, decreasing cross-linking efficiency and causing lower labeling signal. To test this hypothesis, the cross-
linking experiments were carried out using $[\gamma^{35}S]\text{ATP}\gamma\text{S}$, a poorly hydrolyzable ATP analog (Fig. 9A and 9B).

**Figure 9.** Cross-linking of ATPγS and ADP to MutSα. A) Cross-linking of 10 μM $[\gamma^{35}S]\text{ATP}\gamma\text{S}$ to 0.5 μM MutSα. B) Quantitation of A. The 0 minute time point actually corresponds to the 0.2 minute time point from the gel. C) Cross-linking of 10 μM $[\alpha^{32}P]\text{ADP}$ to 0.5 μM MutSα. D) Quantitation of B. The plots show the average of 3 experiments ± standard deviation.

Increased labeling of MSH6 by $[\gamma^{35}S]\text{ATP}\gamma\text{S}$ is observed, indicating that MSH6 does indeed bind the triphosphate nucleotide. Moreover both subunits are labeled at similar levels (Fig. 9A and 9B) indicating that they are occupied equally by ATPγS. These results also support the notion that after hydrolysis of ATP by MSH6, the ADP produced is released by the subunit due to lower affinity.
Cross-linking experiments with [α-32P]ADP show higher labeling of MSH2, suggesting that MSH2 has higher affinity for ADP than does MSH6 (Fig. 9C and 9D). The lack of labeling in MSH6 also supports the idea that after ATP hydrolysis by MSH6, the resulting ADP dissociates due to the lower affinity of MSH6 for ADP, thus explaining the lack of labeling in MSH6 by ATP under hydrolytic conditions. Taken together, these results suggest that while ATP can occupy both MSH2 and MSH6, ADP is only observed in the MSH2 subunit. Moreover, MSH6 rapidly hydrolyzes ATP and quickly releases the resulting ADP due to its lower affinity for the diphosphate nucleotide.

2.2.4 MutSα binding affinities for ADP and ATPγS

UV cross-linking can also be used to measure apparent $K_d$ values for each subunit by carrying out nucleotide titration experiments (Fig. 10A and 10C). Titration with ADP shows saturation of the MSH2 subunit with an apparent $K_d$ of 0.8 μM. However, the MSH6 subunit only shows some labeling at the highest concentrations of ADP, indicating a lower affinity for ADP than MSH2 (Fig. 10A). Titration with ATPγS shows saturation of both subunits at similar levels with MSH2 and MSH6 having apparent $K_d$ values for ATPγS binding of 0.7 μM and 0.9 μM, respectively (Fig. 10C). Moreover, both subunits are saturated at similar levels, indicating that both MSH6 and MSH2 can be occupied equally by the triphosphate nucleotide. While these results differ from the yeast MutSα cross-linking studies in
which the MSH6 subunit was observed to have higher affinity for the triphosphate (Mazur et al., 2006), they are consistent with a study of the human protein, which shows that both subunits are labeled by ATPγS (Heinen et al., 2011).

**Figure 10.** Titrations with ADP and ATPγS. A) Titration of [α-32P]ADP as measured by UV cross-linking. MSH2 has a $K_d$ value of 0.8 μM for ADP, while the $K_d$ for MSH6 could not be determined. B) Titration of [α-32P]ADP as measured by filter binding yielding a $K_d$ value of 0.7 μM. C) Titration of [γ32S]ATPγS as measured by UV cross-linking. MSH2 and MSH6 have $K_d$ values of 0.7 μM and 0.9 μM, respectively, for ATPγS. D) Titration of [γ32S]ATPγS as measured by filter binding yielding a $K_d$ value of 1.0 μM. The $K_d$ values were determined by fitting the plots to a square hyperbola by non-linear regression analysis.

As mentioned earlier, while UV cross-linking is a good way to visualize nucleotide binding to each subunit, it does have several caveats. Because UV cross-linking results in covalent and irreversible attachment, it can overestimate affinity if
there is non-specific binding. On the other hand, inefficient cross-linking can underestimate affinity. The apparent $K_d$ values determined above, therefore, may not be an accurate representation of the affinity of each hMutSα subunit for ADP or ATPγS.

Thus, the binding of diphosphate and triphosphate nucleotides to hMutSα was also studied using filter-binding experiments. While this method does not distinguish the occupancy of each subunit, it does give information about the stoichiometry of binding. Previous filter binding studies with *E. coli* MutS (Bjornson and Modrich, 2003) and human MutSα (Martik et al., 2004) have shown that the MutS proteins bind one ADP or ATPγS molecule per dimer. Other studies with *Taq* MutS and yeast MutSα have shown two ATPγS molecules per dimer, albeit one ATPγS binds with high affinity (~3 μM) and the other with reduced affinity (~20 μM) (Antony and Hingorani, 2003, 2004). To compare our UV cross-linking results and the previous filter-binding results, we also performed filter-binding experiments to look at the nucleotide affinities of MutSα.

MutSα at a concentration of 0.5 μM was titrated with different ADP concentrations and incubated on ice for 5 minutes. The reactions were then filtered through nitrocellulose filters, and the filters were dried and counted. The results show saturation at 0.45 μM ADP bound, indicating a stoichiometry of one to one: one molecule of ADP for every molecule of MutSα dimer (Fig. 10B). These results are consistent with the yeast cross-linking studies (Mazur et al., 2006), and our
cross-linking studies (Fig. 10A), in which only the MSH2 subunit is labeled by ADP, as well as previous filter binding studies (Antony and Hingorani, 2003, 2004; Bjornson and Modrich, 2003; Martik et al., 2004).

However, when MutSα was titrated with ATPγS, a difference between the UV cross-linking and filter-binding studies was observed. UV cross-linking showed labeling of both MSH6 and MSH2 by ATPγS at similar levels, suggesting equal occupancy (Fig 9A, 9B and 10C). As mentioned earlier, the efficiency of cross-linking is very low, but if we assume that what is observed in cross-linking is a true representation of the whole MutSα population, then this result suggests 2 molecules of ATPγS per MutSα dimer (Fig. 10C). However, filter-binding shows saturation at 0.6 μM ATPγS bound, demonstrating a stoichiometry of one molecule of ATPγS per one MutSα dimer (Fig. 10D), which is consistent with previous filter-binding studies (Martik et al., 2004) but not with the UV cross-linking. A possible explanation for the inconsistencies, other than the cross-linking caveats mentioned previously, is that binding of ATPγS to one of the subunits could be weak enough that it is perturbed by filtration through the nitrocellulose membrane, and is therefore not detected. UV cross-linking covalently attaches the nucleotide to the subunit. Therefore, even a weak interaction would be observed by cross-linking. A more likely explanation is that binding of the triphosphate to one subunit of MutSα inhibits binding to the other. For example, binding of ATPγS to MSH6 inhibits binding of ATPγS to MSH2 and vice versa. As both subunits have similar Kd values
for ATPγS, 0.7 μM and 0.9 μM for MSH2 and MSH6, respectively, there would be a population of MutSα where half of the heterodimers have the triphosphate nucleotide in MSH2 with an empty MSH6, and the other half in MSH6 with an empty MSH2. This explanation would account for the one to one stoichiometry observed by filter-binding and for the labeling at similar levels of both subunits by UV cross-linking.

### 2.3 Effects of DNA on nucleotide occupancy of MutSα

#### 2.3.1 Mismatched DNA increases residency of ATP in the MSH6 subunit

The role of DNA in the UV cross-linking profiles of nucleotide to hMutSα was also studied. Time course cross-linking experiments were carried out with [γ-32P]ATP and nucleotide-free hMutSα in the presence of either homoduplex DNA or heteroduplex DNA that contains a central mismatch. In the presence of homoduplex, the labeling profile of hMutSα is similar to the labeling profile observed in the absence of DNA (compare Fig. 7A and 11A). The MSH2 subunit is labeled at higher levels at the early time points with the signal decreasing at the later time points due to hydrolysis of the gamma phosphate. As was the case in the absence of DNA, the MSH6 subunit was labeled at lower levels than MSH2 due to ATP hydrolysis and ADP release.
Figure 11. Cross-linking of $[\gamma^{-32}P]$ATP to MutSα in the presence of DNA. MutSα at a concentration of 0.5 μM was incubated with 1 μM DNA (either homoduplex or heteroduplex) for 5 minutes. The reactions were started by the addition of 10 μM $[\gamma^{-32}P]$ATP, stopped by freezing at the indicated time points, exposed to UV, and resolved by 8% SDS PAGE. A) Cross-linking in the presence of 21 bp homoduplex. B) Quantitation of A. C) Cross-linking in the presence of 21 bp heteroduplex. D) Quantitation of C. The plots show the average of three experiments ± standard deviation.

The presence of a mismatch, however, did affect the labeling profiles of the two subunits. Consistent with the yeast studies (Mazur et al., 2006), increased labeling was observed in MSH6. In fact, the labeling levels are similar to those of MSH2 (Fig. 11C and 11D). These results suggest that the presence of mismatched DNA increases the residency of the triphosphate in the MSH6 subunit. The presence of a mismatch does not completely inhibit ATP hydrolysis as indicated by the
decrease in labeling of both subunits observed in the later time points. It is possible that, as was the case with ATPγS, binding of ATP to one subunit inhibits binding to the other, and what we are observing is a mixture of MutSα heterodimers bound to the mismatch half of which have ATP in MSH6 and the other half have ATP in MSH2.

**Figure 12.** Cross-linking of [$\alpha$-32P]ADP to MutSα in the absence or presence of DNA. For experiments performed in the presence of DNA, MutSα was first incubated with DNA and the reactions were started by the addition of [$\alpha$-32P]ADP and stopped at the indicated time points. A) Cross-linking 10 μM [$\alpha$-32P]ADP to 0.5 μM MutSα in the absence of DNA. B) Cross-linking 10 μM [$\alpha$-32P]ADP to 0.5 μM MutSα in the presence of 1 μM 21-bp homoduplex. C) Cross-linking of 10 μM [$\alpha$-32P]ADP to 0.5 μM MutSα in the presence of 1 μM 21-bp heteroduplex. Top panels show representative gels of the cross-linking, while the bottom panels show the quantitation of these experiments. The plots show the average of 3 experiments ± standard deviation.

While heteroduplex DNA affected ATP occupancy of MutSα, it did not have any effect in ADP binding (Fig. 12). Time course UV cross-linking experiments with labeled ADP show cross-linking at high levels only in the MSH2 subunit. This result
held true whether the experiments were carried out in the absence of DNA (Fig. 12A) or in the presence of homoduplex or heteroduplex DNA (Fig. 12B and 12C), suggesting that DNA does not affect time-dependent ADP occupancy of the MSH2 subunit of MutSα.

2.3.2 MutSα can be occupied by ADP and ATP at the same time

UV cross-linking experiments with $[^{32}\text{P}]\text{ATP}$ after MutSα has been incubated with excess cold ADP show little to no labeling of the MSH2 subunit in the absence of DNA (Fig. 13A), indicating that the MSH2 site is occupied by ADP. While the presence of homoduplex DNA does not affect the labeling of the two subunits (Fig. 13B), an increase in labeling of MSH6 is observed in the presence of DNA containing a mismatch (Fig. 13C). Moreover, an increase in labeling of MSH2 is also observed at the later time points.
Figure 13. Cross-linking of [γ-32P]ATP to MutSα after cold ADP incubation in the absence or presence of DNA. For experiments performed in the presence of DNA, MutSα was first mixed with DNA and then incubated with cold ADP. The reactions were started by the addition of [γ-32P]ATP and stopped at the indicated time points. A) UV cross-linking of 10 μM [γ-32P]ATP to 0.5 μM MutSα after a 5 minute incubation with 10 μM cold ADP in the absence of DNA. B) UV cross-linking of 10 μM [γ-32P]ATP to 0.5 μM MutSα after a 5 minute incubation with 10 μM cold ADP in the presence of 1 μM 21-bp homoduplex. C) UV cross-linking of 10 μM [γ-32P]ATP to 0.5 μM MutSα after a 5 minute incubation with 10 μM cold ADP in the presence of 1 μM 21-bp heteroduplex. Top panels show representative gels of the cross-linking, while the bottom panels show the quantitation of these experiments. The plots show the average of 3 experiments ± standard deviation.

As the UV cross-linking experiments only track the labeled nucleotide, the above experiments were also performed with labeled ADP and cold ATP. MutSα was incubated with [α-32P]ADP for 5 minutes prior to the addition of cold ATP. In the absence of DNA or presence of homoduplex (Fig. 14A and 14B, respectively), MSH2 is labeled by ADP, and the labeling does not significantly change as a function of
time. In the presence of heteroduplex DNA, however, a decrease in labeling of MSH2 is observed during the time course (Fig. 14C). This decrease in ADP labeling along with the increased ATP labeling observed in Figure 13C indicates that in the presence of a mismatch, the bound ADP in MSH2 is slowly replaced by ATP.

**Figure 14.** Cross-linking of [α-32P]ADP to MutSα after addition of cold ATP. 0.5 μM MutSα was incubated with 10 μM [α-32P]ADP in the absence or presence of 1 μM 21-bp DNA (either homoduplex or heteroduplex) for 5 minutes. For the DNA containing experiments, MutSα was first mixed with DNA and then incubated with hot ADP. The reactions were started by the addition of 10 μM cold ATP and stopped by freezing at the indicated time points. A) In the absence of DNA. B) In the presence of homoduplex. C) In the presence of heteroduplex. Top panels show representative gels of the cross-linking, while the bottom panels show the quantitation of these experiments. The plots show the average of 3 experiments ± standard deviation.

Assuming that the UV cross-linking results represent the true population of MutSα in solution, these results, along with the finding that ATP occupies the MSH6
subunit, indicate that in the absence of DNA or presence of homoduplex or heteroduplex DNA, MutSα can bind both ATP and ADP at the same time, with ATP in MSH6 and ADP in MSH2. However, the decrease in ADP labeling and increase in ATP labeling of MSH2 at the later time points of the experiments carried out in the presence of heteroduplex indicate that the mismatch causes the MSH2 to become more dynamic and undergo slow nucleotide exchange. This idea is explored further below.

2.3.3 Mismatched DNA makes the MSH2 nucleotide site more dynamic

To further examine the effects of DNA on ADP dissociation from MutSα, 1 μM protein was incubated with 1 μM [α-32P]ADP in the absence or presence of DNA and then chased with 10 μM cold ADP. The reactions were sampled as a function of time and analyzed via either cross-linking or filter binding methods (Fig. 15). In the absence of DNA or presence of homoduplex DNA, chase with cold ADP does not affect the amount of [α-32P]ADP cross-linked to the MSH2 subunit as a function of time. The amount of cross-linked ADP remains constant (Fig. 15A and 15B). In the presence of heteroduplex, however, the amount of [α-32P]ADP cross-linked to MSH2 decreases as a function of time upon challenge with cold ADP (Fig. 15C). These results indicate that in the absence of DNA or presence of homoduplex DNA, the prebound ADP remains stably bound to the MSH2 site. The presence of a mismatch causes the site to become more dynamic. The prebound ADP is either released into
solution or replaced by the surrounding cold ADP. However, as experiments carried out in the presence of DNA with labeled ADP alone (Fig. 12) do not show a decrease in ADP cross-linking as a function of time, the decrease observed here is attributed to replacement of the labeled ADP with the cold ADP and not to release alone.

**Figure 15.** ADP exchange in MutSα. 1.0 μM MutSα was incubated with 1 μM [α-32P]ADP for 5 minutes prior to chase with 10 μM cold ADP. The remaining bound [α-32P]ADP was measured by UV cross-linking (A, B, C) or filter binding (D). A) In the absence of DNA. B) In the presence of 1 μM homoduplex. C) In the presence of 1 μM heteroduplex. In A, B, and C, cross-linking to MSH2 is shown in blue and cross-linking to MSH6 in red. For the experiments performed in the presence of DNA, MutSα was first mixed with homoduplex or heteroduplex and then incubated with hot ADP. The reactions were started by the addition of cold ADP and at the indicated time points were either stopped by freezing (A, B, C) or filtered through nitrocellulose membranes (D).

Filter-binding studies yielded similar results with the total amount of bound ADP decreasing as a function of time in the presence of heteroduplex DNA and cold
ADP but remaining unchanged in the absence of DNA or presence of homoduplex DNA (Fig. 15D). Based on the UV cross-linking studies showing that most of the ADP is expected to be bound to the MSH2 subunit, the filter-binding results are consistent with the cross-linking results and support the finding that the presence of a mismatch causes the MSH2 nucleotide site to become more dynamic.

2.4 Summary and discussion

UV cross-linking is a method previously used to visualize nucleotide binding to the two subunits of MutSα (Heinen et al., 2011; Iaccarino et al., 1998; Mazur et al., 2006). This technique is a good way to visualize nucleotide occupancy of MutSα, but, as mentioned in section 2.2.2, it also has several caveats. While keeping these in mind, we used UV cross-linking along with other techniques to determine the functional nucleotide states of human MutSα. Unlike the studies with yeast MutSα (Mazur et al., 2006), our cross-linking experiments show that both MSH2 and MSH6 are labeled at similar levels by ATPγS. In the yeast studies, binding of ATPγS to MSH2 as judged by UV cross-linking was reported to be over 500-fold weaker than ATPγS binding to MSH6 (K_d of ~84 μM for MSH2 as compared to ~0.13 μM for MSH6)(Mazur et al., 2006). Our results, however, show that ATPγS binding, as judged by UV cross-linking, was similar for both subunits (K_d of ~0.7 μM for MSH2 and ~0.9 μM for MSH6). However, when looking at nucleotide occupancy by filter-binding studies, the triphosphate results are inconsistent with the cross-linking
results. A stoichiometry of one ATPγS per dimer was observed through filter binding, while cross-linking showed labeling of both subunits, suggesting a stoichiometry of two ATPγS molecules per dimer. Other than the UV cross-linking caveats, a possible explanation for these differences is that binding of ATPγS to one subunit inhibits binding of ATPγS to the other subunit, which would result in a mixture of MutSα in solution where half of the dimers have ATPγS in MSH2 and half in MSH6. In this case, a stoichiometry of one ATPγS per dimer would be observed via filter binding, whereas both subunits would show up as labeled when looked at by cross-linking.

UV cross-linking experiments with ADP only show labeling of the MSH2 subunit, consistent with the yeast MutSα studies. Moreover, filter-binding studies showed a stoichiometry of one ADP per dimer, indicating that of the two subunits MSH2 has a higher affinity for ADP than MSH6. Using nucleotide-free hMutSα and both α- and γ-labeled ATP, we determined that the MSH2 subunit exists first in an ATP-bound state, and after hydrolysis it is in an ADP-bound state. Labeling of MSH6 was observed when the poorly hydrolyzable ATPγS was used but not under hydrolytic conditions, suggesting hydrolysis of ATP by MSH6 and release of the resulting ADP due to the low affinity of MSH6 for the diphosphate. This hydrolysis is reduced by the presence of DNA containing a central mismatch, resulting in labeling of MSH6 by ATP under hydrolytic conditions.
By first incubating MutSα with cold ADP and then carrying out the UV cross-linking with [γ-32P]ATP, the labeling of MSH2 by ATP was lost. These results suggest that the MSH2 site is occupied by ADP, thus allowing no room for ATP binding. These studies also show that in the absence of DNA or the presence of homoduplex or heteroduplex DNA, MutSα can be occupied by both ATP and ADP in MSH6 and MSH2, respectively. Heteroduplex, however, makes the MSH2 site more dynamic, allowing for nucleotide exchange to occur. However, as the experiments with DNA are carried out with short DNAs, only 21 base pairs long, it is possible that the release of ADP occurs after the protein has dissociated from DNA. As was the case with Taq MutS (Qiu et al., 2012), MutSα with ATP in MSH6 and ADP in MSH2 could form a sliding clamp that leaves the mismatch and slides along the helix to dissociate from DNA. The release of ADP from MSH2 could occur after the protein is off the DNA. The significance of the nucleotide states of MutSα and ADP occupancy in MSH2 will be further explored and discussed in chapters 4 and 5.
3. Characterization of MutSα ATPase mutants

MutSα is a member of the ABC transporter family, which is characterized by the conserved Walker A and B motifs with the hydrophobic stretches (G/AXXXGKS/T) and (DE/D), respectively (Haber et al., 1988). Several studies have looked at adenine nucleotide processing by MutS proteins mutated in the Walker A motif. An early study with S. typhimurium MutS containing a lysine to alanine mutation in the Walker A motif showed a drastic reduction in ATPase activity (Haber and Walker, 1991). Other studies with the same mutations have shown similar results with the yeast MutSα (Antony et al., 2006) and human MutSα (Heinen et al., 2011). However, as the mutation to alanine also affects nucleotide binding, these studies cannot differentiate between ATP hydrolysis and ATP binding.

An earlier study with yeast RAD3, a member of the ABC transporter family, showed that mutating the conserved lysine to arginine abolished its ATPase activity but not its ability to bind ATP (Sung et al., 1988). This mutation was used in a study with human MutSα, in which it was inferred that this mutation also reduces ATP binding to the protein (Iaccarino et al., 1998). However, nucleotide binding was not examined closely as this study only shows UV cross-linking with [α-32P]ATP and does not show any filter-binding data.
Other studies with mutant forms of MutSα have been carried out. A UV cross-linking study with yeast MutSα examined mutation of the lysine to methionine (Mazur et al., 2006). However, this mutation also seems to significantly reduce nucleotide binding. Another study with human MutSα looked at a mutation in MSH2 where the glycine adjacent to the conserved lysine was replaced by alanine, a change that reduces nucleotide binding (Geng et al., 2012). This same study also looked at a mutation in MSH6 near the ATPase site, which fails to couple nucleotide binding and mismatch recognition.

Therefore, as we are interested in the different effects that ATP binding and hydrolysis have in the function of MutSα, we constructed mutant forms of MutSα containing the lysine to arginine mutation in MSH2, MSH6, and both subunits and characterized their nucleotide binding, ATP hydrolysis, and DNA binding activities.

### 3.1 Material and Methods

#### 3.1.1 Proteins and protein preps

The proteins were prepared from SF9 cells using baculovirus constructs expressing the appropriate human cDNAs as previously described (Blackwell et al., 2001b). The Bac-to-Bac Baculovirus Expression System (Invitrogen, Life Technologies) was employed to generate the appropriate baculovirus constructs. One of the key processes of this system is to generate a pFastBac vector that
contains the appropriate cDNAs. In the case of MutSα, the two subunits were cloned into the pFastBac Dual vector (Blackwell et al., 2001b). The pFastBac Dual plasmid containing the wild type MutSα was used to construct vectors containing the mutant MutSα sequences.

The mutant MutSα constructs have a lysine to arginine substitution in either subunit or in both. In MSH2 the substitution is at residue 675, while in MSH6 it is at residue 1140. Different methods were used to generate each mutant. The MSH6 mutant was generated using QuikChange site-directed mutagenesis (Agilent Technologies) and the pFastBac Dual plasmid containing wild type MutSα. The forward primer used was 5’-GGA CCA AAT ATG GGG GCC CGG TCT ACG CTT ATG AGA CAG GCT-3’, and the reverse primer was its complement 5’-AGC CTG TCT CAT AAG CGT AGA CCG GCC CCC CAT ATT TGG TCC-3’. The thermal cycling was carried out as described in the QuikChange site-directed mutagenesis protocol with the modification of increasing the extension time from 1 minute per kilobase of DNA to 3 minutes per kilobase of DNA. The PCR product was transformed into XL-10 Gold super competent cells, and the mutation was confirmed via sequencing.

The QuikChange method was not successful for the construction of the MSH2 mutant, possibly because the primers were not optimal for this procedure. Therefore, a two-step PCR method was employed. Two unique restriction sites, one on either side of the mutation site, were located. The two sites were NheI (5’ to the mutation site) and AgeI (3’ to the mutation site). Primers were designed as follows
to perform PCR from the Nhel site to the mutation site, and from the mutation site to the AgeI site: 1) 5’-GCA TGC TAT GCA TCA GCT GCT AGC ACC ATG GGC ACT GAC AGT-3’; 2) 5’-ACT GGC CCC AAT ATG GGA GGT CGG TCA ACA TAT ATT CGA CAA-3’; 3) 5’-TTG TCG AAT ATA TGT TGA CCG ACC TCC CAT ATT GGG GCC AGT-3’; 4) 5’-ACA TTT ATC AGG ACC TCA ACC GGT TGT TGA AAG GCA AAA AGG-3’. Primers 1 and 2 were used for PCR from the Nhel site to the mutation site, resulting in a 929 base pair sequence. Primers 3 and 4 were used for PCR from the mutation site to the AgeI site, resulting in a 1,337 base pair sequence. The two PCR products and primers 1 and 4 were then used to generate a 2,266 base pair sequence with the mutation near the middle and the Nhel and AgeI restriction sites on either end. The pFastBac Dual plasmid containing the wild type MutSα sequence was digested with the Nhel and AgeI restriction enzymes, as was the PCR product. The plasmid digestion results in a 2,228 base pair fragment and a 10,114 base pair fragment. The PCR digestion results in a 2,228 base pair fragment and two 19 base pair fragments. The digestion products were run on a low-melt 0.8% agarose gel, and the 10,114 base pair fragment from the plasmid digestion and the 2,228 base pair fragment from the PCR digestion were cut out and ligated using in-gel ligation (Methods in Molecular Biology). The resulting construct was transformed into XL-10 Gold super competent cells, and the mutation was confirmed via sequencing.

To make the double mutant construct, the two pFastBac Dual plasmids generated above and containing the MSH2 and MSH6 mutations were used. The two
plasmids were double digested with NheI and AgeI and run on a low-melt 0.8% agarose gel. The bands were cut out and the 10,114 base pair fragment from the MSH6 digestion was ligated to the 2,228 base pair fragment from the MSH2 digestion using in-gel ligation (Methods in Molecular Biology). The product was transformed into XL-10 Gold super competent cells, and the mutations were confirmed via sequencing.

The vectors containing the appropriate mutations were used to generate the baculovirus constructs according to the Bac-to-Bac Baculovirus Expression System (Invitrogen, Life Technologies). The proteins were expressed in SF9 cells and purified using purification procedure 2 described in section 2.1.1.

3.1.2 DNA substrates

The oligodeoxyribonucleotides were obtained from IDT Technologies. The sequence for the 21-bp duplexes was identical to the 31-bp duplexes described previously (Allen et al., 1997), with 5 bases on either end removed.

The 202-bp DNAs were generated as previously described by PCR amplification of base pairs 5531-5732 of bacteriophages f1MR1 and f1MR3 (Blackwell et al., 1998). Single strands were isolated from the PCR products by HPLC using a GenPak Fax column (Waters) under denaturing conditions (0.25 M Tris-HCl adjusted to pH 12.4 with NaOH). Each PCR product was diluted with denaturing buffer containing 0.5 M NaCl and injected onto the column at a flow rate
of 0.55 mL/min. DNA was eluted with a gradient of NaCl (0.5 – 1.0 M), and each strand eluted as a distinct peak with the viral strand eluting first and the complementary strand second. Each eluted strand was supplemented with 1/10 volume of 3 M sodium acetate, pH 5.2 and 2 volumes of ethanol for precipitation. The viral strand from f1MR1 was combined with the complementary strand from f1MR1 to form homoduplex DNA and with the complementary strand from f1MR3 to form the heteroduplex. The annealed products were purified by native HPLC (Waters) with a gradient of 0.5 to 1.0 M NaCl in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA buffer on a 1-mL GenPak Fax column (Waters).

For the 41-bp duplexes, the mismatch was in the center, and the 20 base pair flanking sequences on either side were identical to the sequences flanking the mismatch in the 202-bp duplexes. The 21- and 41-bp duplexes were purified by native HPLC (Waters) with a gradient of 0.5 to 1.0 M NaCl in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA buffer on a 1-mL GenPak Fax column (Waters). For the 41- and 202-bp DNAs used in Surface Plasmon Resonance measurements, one of the strands in the duplex was 5'-biotinylated as described previously.

3.1.3 Nucleotides

Labeled [α-32P]ATP, [γ-32P]ATP, and [γ-35S]ATPγS were purchased from Perkin/Elmer. Labeled [α-32P]ADP was prepared as previously described (Mazur et al., 2006). All labeled nucleotides were diluted in unlabeled nucleotide solutions.
For titration experiments, the labeled nucleotide was diluted with the highest concentration of the appropriate unlabeled nucleotide, and then serial dilutions were carried out to ensure that the ratio of labeled to unlabeled nucleotide was the same in all of the concentrations. The unlabeled nucleotides were purchased from USB Corp (ATP), Sigma (ADP), and Calbiochem (ATPγS).

3.1.4 UV cross-linking assays

UV cross-linking studies were done as described in section 2.1.4 with the exception that only one time point (5 minutes) was scored. After the 5-minute incubation, the reactions were stopped by freezing in liquid nitrogen and the frozen products were treated and visualized as described in section 2.1.4.

3.1.5 Filter binding assays

Filter binding assays with ATPγS and the ATP hydrolytic mutants were carried out in 10-μL reactions as described in section 2.1.5.

3.1.6 Surface plasmon resonance measurements

MutSα-DNA interactions were monitored via surface plasmon resonance spectroscopy (SPRS) using a Biacore 2000 optical biosensor system as previously described (Blackwell et al., 2001a; Blackwell et al., 1998; Martik et al., 2004). Streptavidin sensor chips (SA chip, GE Healthcare) were derivatized with ~150
resonance units (RUs) of biotinylated DNA, either correctly paired or with a central mismatch. The proteins were injected at a flow of 20 µL/min in buffer comprised of 25 mM HEPES-KOH, 5 mM MgCl₂, 150 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.005% surfactant P20. Experiments were performed at 20 °C, and the samples were kept at 4 °C prior to injection. The chip was regenerated with a 20-µL injection of 0.1% sodium dodecyl sulfate (SDS).

3.1.7 ATPase assays

Each protein at 200 nM was incubated in 25 mM Hepes-KOH, pH 7.5, 150 mM KCl, 5 mM MgCl₂, and 1 mM DTT at room temperature. The reactions were started with the addition of [α-³²P]ATP at concentrations of 5, 10, 20, 50, 100, and 200 µM and were quenched every 5 minutes in 100 mM NaOH and 20 mM EDTA. The resulting ADP was separated from ATP by thin layer chromatography as follows. One µL of the quenched reaction was spotted on a PEI-cellulose plate and resolved in 0.3 M KPi buffer, pH 7.0. As ADP migrates faster than ATP, the amount of ADP formed was calculated and plotted as a function of time for each starting ATP concentration. These plots yield steady state rates for each concentration. Plotting these rates of hydrolysis as a function of concentration results in a Michaelis-Menten curve, which was fit to a square hyperbola by non-linear regression to calculate the kinetic parameters for the proteins.
3.2 Biochemical studies of MutSα mutants

3.2.1 Purification of mutant forms of MutSα

As mentioned in section 2.2.1, we have two purification procedures for MutSα preparations and both yield highly pure protein. The key step in purification procedure 1 is the ATP elution from the single stranded DNA cellulose column. However, as the mutant constructs are expected to be defective in ATPase activity, this purification procedure was not deemed suitable. Purification procedure 2, however, does not include an ATP elution step and was therefore used as the procedure to purify the MutSα mutant constructs. This procedure, described in detail in section 2.1.1, yielded highly pure protein of all the mutated constructs (Fig. 16A). Moreover, because they are all purified identically, it allows for direct comparison of the wild type MutSα with the mutant constructs. Finally, as was the case for wild type MutSα, this procedure resulted in proteins with negligible amount of nucleotide bound, 0.05 moles of nucleotide per mole of MutSα dimer or less (Fig. 16B).
Figure 16. Purification of the wild type and mutant forms of MutSα with procedure 1. A) Coomassie stained gel of the different proteins. Upper band is MSH6, while lower band is MSH2. B) Measurement of nucleotide content of wild type MutSα (red), 2KR MutSα (blue), 6KR MutSα (green), and 2,6KR MutSα (brown).

3.2.2 ATPase activity of the mutant forms of MutSα

The ATPase activity of the MutSα proteins was measured by incubating 200 nM of each protein at room temperature with increasing concentrations of [α-\(^{32}\)P]ATP and stopping at different time points. The ADP produced was plotted as a function of time, and the steady state rates for each concentration were calculated. Plotting the hydrolysis rates against ATP concentration yielded a Michaelis-Menten curve. Fitting the data to a square hyperbola by nonlinear regression analysis allows for the derivation of the kinetic parameters (Fig. 17 and Table 1).
Figure 17. ATPase activity of MutSα proteins at room temperature. The kinetic parameters for wild type MutSα (black), 2KR MutSα (red), 6KR MutSα (green), and 2,6KR MutSα (blue) are summarized in Table 1.

Table 1. Kinetic parameters of wild type and mutant MutSα at room temperature

<table>
<thead>
<tr>
<th>Proteins</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μM ADP/min)</th>
<th>$k_{cat}$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTSα</td>
<td>5.5±2.2</td>
<td>0.13±0.01</td>
<td>0.65±0.02</td>
</tr>
<tr>
<td>2KRSα</td>
<td>7.7±1.8</td>
<td>0.012±0.001</td>
<td>0.06±0.005</td>
</tr>
<tr>
<td>6KRSα</td>
<td>11.0±2.5</td>
<td>0.013±0.001</td>
<td>0.065±0.005</td>
</tr>
<tr>
<td>2,6KRSα</td>
<td>3.3±0.9</td>
<td>0.0057±0.001</td>
<td>0.029±0.005</td>
</tr>
</tbody>
</table>

As summarized in Table 1, the lysine to arginine mutations in the Walker A motif of MutSα significantly alter the ATPase activity of the protein. The $k_{cat}$ values decrease 10-fold for the single site mutants and 20-fold for the double mutant as
compared to wild type MutSα. The $K_m$ values, though, are very similar, only differing by 2-fold in the most extreme case (WTSα compared to 6KRSα).

### 3.2.3 The ATPase mutations do not affect MutSα-mismatch binding

DNA binding was studied using surface plasmon resonance spectroscopy (SPRS) in a Biacore 2000 instrument. This method measures mass bound to ligand, in our case MutSα bound to DNA. Changes in mass bound are represented as resonance units and an increase or decrease in these units corresponds to an increase or decrease in the amount of protein bound to DNA. Resonance units and mass are used interchangeably when describing the SPRS results.

DNAs of either 41 base pair or 202 base pair lengths with a central mismatch were attached to Biacore chips via a biotin-streptavidin interaction. The four proteins (WTSα, 2KRSα, 6KRSα, and 2,6KRSα) were titrated over the DNAs, and the amount bound, represented by resonance units, was measured. The maximum binding values obtained after the association phase of the reaction at each concentration were plotted as a function of protein concentration, and the data were fit to a square hyperbola by nonlinear regression analysis that provided the binding constants for each protein (Table 2). Figure 18 shows an example with wild type MutSα and a 41-basepair DNA.
Figure 18. Example of how the binding constants of MutSα for DNA at 20 °C are determined using SPRS. The protein is first loaded on DNA, washed with buffer, and challenged with 1 mM ATP. A) Titration of MutSα on homoduplex DNA. B) Titration of MutSα on heteroduplex. C) Plot and fit of the maximum binding values after the loading phase to determine the binding constants in Table 2. Heteroduplex binding is shown in red while homoduplex in blue.

Table 2. Binding constants (nM) of the wild type and mutant MutSα proteins for 41- and 202-bp DNAs with central mismatches measured at 20 °C.

<table>
<thead>
<tr>
<th></th>
<th>41-bp</th>
<th>202-bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTSα</td>
<td>28.8±4.3</td>
<td>71.4±6.3</td>
</tr>
<tr>
<td>2KRSα</td>
<td>31.8±4.4</td>
<td>83.7±2.5</td>
</tr>
<tr>
<td>6KRSα</td>
<td>27.5±3.7</td>
<td>87.6±3.4</td>
</tr>
<tr>
<td>2,6KRSα</td>
<td>35.3±4.1</td>
<td>91.3±4.5</td>
</tr>
</tbody>
</table>

The measured $K_d$ values are similar for all four proteins for both DNA lengths, indicating that the mismatch binding activity of MutSα is not affected by the lysine to arginine mutations in the Walker A site of the protein. For the 41-bp DNA,
the $K_d$ values range from 28 to 35 nM, while for the 202-bp DNA, they range from 83 to 92 nM.

3.2.4 Nucleotide binding is not affected by the Walker A mutations

To determine whether the lysine to arginine mutations have an effect in the ability of MutSα to bind diphosphate or triphosphate nucleotide, we used UV cross-linking and filter-binding methods. For the cross-linking experiments, 0.5 μM MutSα was incubated for 5 minutes with 10 μM [$\alpha^{32}$P]ADP, [$\gamma^{32}$S]ATPγS, or [$\gamma^{32}$P]ATP, either in the absence of DNA or in the presence of 1 μM 21-bp homoduplex or heteroduplex. After the incubation, the reactions were stopped by freezing in liquid $N_2$, UV irradiated and resolved by 8% SDS-PAGE (Fig. 19).

UV cross-linking with [$\alpha^{32}$P]ADP results in similar labeling of all four proteins, with only the MSH2 subunit being labeled (Fig. 19, upper panels). While expected for wild type MutSα and 6KR MutSα, in which the MSH2 subunit is not mutated, the results with 2KR MutSα and 2,6KR MutSα indicate that the lysine to arginine mutation in the Walker A motif does not affect ADP cross-linking to MSH2. Moreover, the cross-linking in MSH2 is not affected by the presence DNA, whether homoduplex or heteroduplex.
Figure 19. Cross-linking of [α-32P]ADP (top panels), [γ-35S]ATPγS (middle panels), and [γ-32P]ATP (bottom panels) to wild type or mutant constructs of MutSα. The first lane in each set shows cross-linking in the absence of DNA, the second lane in the presence of 21-bp homoduplex, and the third lane in the presence of 21-bp heteroduplex. MutSα at 0.5 μM was mixed with 1 μM DNA where indicated, and then incubated for 5 minutes with 10 μM nucleotide as indicated. The reactions were started by the addition of nucleotide and stopped by freezing after the 5-minute incubation.

The four proteins are also labeled similarly by [γ-35S]ATPγS (Fig. 19, middle panels), with both MSH2 and MSH6 subunits of each protein being labeled similarly, suggesting equal occupancy of both subunits by ATPγS. These results also indicate that the lysine to arginine mutation in the Walker A motif does not effect the UV cross-linking of ATPγS to MutSα, whether DNA is present or absent. If we assume that UV cross-linking is a true representation of the MutSα nucleotide states and
that UV cross-linking correlates to nucleotide binding, then these results suggest that these mutations do not affect the ability of MutSα to bind ADP or ATPγS.

Labeling with \([\gamma^{-32}P]ATP\), however, differs for each protein. As expected for the wild type protein, the MSH2 subunit is labeled at a higher level than MSH6 in the absence of DNA or presence of homoduplex. In the presence of heteroduplex, an increase in MSH6 labeling is observed, indicating increased residency of ATP in MSH6 similar to the results discussed in 2.3.1.

The labeling pattern of the 2KR mutant with \([\gamma^{-32}P]ATP\) is similar to that of the wild type protein. The MSH2 subunit is labeled at a higher level than MSH6 and an increase in labeling of MSH6 is observed in the presence of heteroduplex DNA. The labeling of MSH2 suggests that the mutation does not affect ATP cross-linking to the subunit. The lower labeling of MSH6 as compared to the MSH2 subunit indicates loss of the gamma phosphate and that ATP hydrolysis occurs in this subunit. Because ADP cross-linking was not observed in the MSH6 subunit of 2KR MutSα (Fig. 19, upper panels), this result also suggests that as with wild type MutSα, the resulting ADP is released. The presence of heteroduplex increases residency of ATP in MSH6 similar to the observations with wild type MutSα.

In 6KR MutSα, the MSH6 subunit is labeled higher than MSH2 by \([\gamma^{-32}P]ATP\), suggesting that the mutation in MSH6 increases the residency of ATP in MSH6. This increase in residency is probably due to inhibition of ATP hydrolysis because of the lysine to arginine mutation. The lower labeling in MSH2 as compared to the labeling
of the MSH6 subunit of 6KR MutSα is possibly due ATP hydrolysis in this subunit. The cross-linking results with the poorly hydrolyzable ATPγS (Fig. 19, middle panels), in which both subunits of 6KR MutSα are labeled at similar levels, support this idea. Labeling of MSH2 by ATPγS suggests that the lack of labeling in MSH2 by ATP is due to the loss of the gamma phosphate, indicating ATP hydrolysis. The presence of DNA, homoduplex or heteroduplex, does not seem to affect the residency of ATP in MSH2.

The labeling of the 2,6KR mutant by \([\gamma^{32P}]ATP\) is similar to the labeling of the wild type and mutant proteins by \([\gamma^{35S}]ATP\gammaS\), suggesting that the mutation increases ATP residency in both subunits regardless of the presence or absence of DNA. These results also suggest that both subunits are occupied equally by ATP. Assuming that UV cross-linking is a true representation of the MutSα nucleotide states and that UV cross-linking correlates to nucleotide binding, then the results with the ATP hydrolytic mutants suggest that the lysine to arginine mutations while inhibiting ATP hydrolysis, do not affect ATP binding. However, these results represent only a 5-minute time point and a more detailed kinetic study is necessary.

The binding of ATPγS was also studied using filter-binding experiments. The four proteins were titrated with increasing concentrations of ATPγS in the absence of DNA (Fig. 20).
Figure 20. Binding of ATPγS to MutSα as measured by filter-binding. Each protein at 0.5 μM was incubated with increasing concentrations of [γ-35S]ATPγS for 5 minutes and collected on nitrocellulose filters. The amount of ATPγS bound was determined for each protein: wild type MutSα (blue), 2KR MutSα (red), 6KR MutSα (black), and 2,6KR MutSα (green). Binding results are summarized in Table 3.

As seen in Figure 20 and Table 3, the mutations in the Walker A site do not significantly affect ATPγS binding to MutSα. All four proteins bind ATPγS with similar affinity with K_d values ranging from 1.4 to 3.2 μM. Moreover, the saturation values for all proteins are approximately 0.5 μM ATPγS bound. As the proteins concentration is also 0.5 μM, this indicates a stoichiometry of 1 ATPγS for every MutSα heterodimer. Because UV cross-linking showed labeling of both subunits by ATPγS (Fig. 19, middle panels), the filter-binding results suggest that, as was the
case for wild type MutSα, binding of triphosphate to one subunit inhibits binding to the other.

Table 3. Binding of ATPγS to 0.5 μM wild type and mutant MutSα

<table>
<thead>
<tr>
<th>Protein</th>
<th>K_d (μM)</th>
<th>Total ATPγS bound (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTSα</td>
<td>1.5±0.3</td>
<td>0.61±0.03</td>
</tr>
<tr>
<td>2KRSα</td>
<td>2.8±0.2</td>
<td>0.51±0.01</td>
</tr>
<tr>
<td>6KRSα</td>
<td>3.3±0.8</td>
<td>0.52±0.03</td>
</tr>
<tr>
<td>2,6KRSα</td>
<td>2.3±0.5</td>
<td>0.56±0.03</td>
</tr>
</tbody>
</table>

3.3 Summary and discussion

A key question in mismatch repair is whether the ATPase activity of MutSα is necessary for its function or whether nucleotide binding alone is sufficient. Studies with MutS proteins mutated in the conserved Walker A motif have tried to answer this question (Antony et al., 2006; Geng et al., 2012; Heinen et al., 2011; Mazur et al., 2006). However, these mutations were found to not only be defective in ATPase activity but also in nucleotide binding, making it difficult to differentiate the two. Therefore, we focused on a mutation (lysine to arginine) that has been shown to affect hydrolysis but not nucleotide binding in other proteins with a Walker A motif (Sung et al., 1988). We made constructs with the mutation in MSH2 alone, MSH6 alone, and both MSH2 and MSH6.
We found that the ATPase activity was considerably reduced by the lysine to arginine mutations in the Walker A motifs. The $k_{\text{cat}}$ values decreased 10-fold for the single mutants and 20-fold for the double mutant suggesting that for efficient ATP hydrolysis the ATPase centers of both subunits must be intact.

While the ATPase activity was significantly reduced by the mutations, the ability to bind DNA was not affected. All four proteins, wild type and the three mutants, bind 41-bp and 202-bp DNAs with a central mismatch similarly, indicating that the mutations did not significantly alter the ability of MutSα to bind to mismatches.

The ability of MutSα to bind nucleotides was also not affected by the mutations, as shown by the cross-linking results with ADP, ATPγS, and ATP and by the filter-binding results with ATPγS. In all cases, cross-linking of ADP was only observed in MSH2, and the extent of labeling was not significantly altered by the mutations or by the presence of DNA. ATP cross-linking showed labeling of the mutated subunits, suggesting increased residency of ATP in the subunit due to inhibition of hydrolysis. Cross-linking with ATPγS showed labeling of both subunits and was not affected by the mutations or DNA. The similar levels of labeling for both subunits suggest equal occupancy by ATPγS. However, filter-binding results showed only one ATPγS per MutSα heterodimer, indicating that, as was the case for wild type MutSα, binding of the triphosphate to one subunit inhibits binding to the other.
These mutations, because they affect the ATPase activity of MutSα but not its ability to bind nucleotides and DNA, will allow us to differentiate between the roles that nucleotide binding and ATP hydrolysis play in the function of MutSα and are explored further in chapters 4 and 5.
4. MutSα dissociation from DNA

Once MutSα recognizes the mismatch, the information must be transmitted to initiate the repair pathway. Several models have been proposed to describe the mode of transmission. The translocation model proposes ATP hydrolysis-dependent movement of MutS homologs as well as the corresponding MutS-MutL complexes along the DNA helix (Allen et al., 1997; Blackwell et al., 1998). MutS homologs, however, have a modest ATPase activity, and it is difficult to reconcile that ATP hydrolysis is the sole driving force of translocation.

The molecular switch model proposes ATP hydrolysis-independent movement of MutS homologs and their corresponding MutS-MutL complexes along the DNA helix. This model postulates that the mismatch provokes ADP for ATP exchange in the MutS proteins, resulting in a conformational change that allows diffusion along the helix without ATP hydrolysis (Gradia et al., 1997; Gradia et al., 1999). However, homoduplex DNA also promotes ADP for ATP exchange at approximately 30% of the rate of heteroduplex. This modest dependence on a mismatch is insufficient to account for the mismatch-dependence of the activation of downstream events.

While the translocation and molecular switch models propose movement from the mismatch along the DNA, a third model, the static transactivation model, invokes a mechanism without MutS homolog movement from the mismatch. MutS
proteins are bound to ATP and a mismatch simultaneously, and downstream events are activated through DNA bending and looping, with ATP binding functioning to verify mismatch recognition (Junop et al., 2001; Wang and Hays, 2003, 2004). However, studies with the *E. coli* methyl directed mismatch repair have shown an orientation dependent loading of the excision system either 3’ or 5’ to the mismatch (Dao and Modrich, 1998; Yamaguchi et al., 1998), and it is unclear how a bending mechanism would account for the heteroduplex orientation. The bending model is also incompatible with observations that ATP reduces affinity of MutS homologs for the mismatch (Allen et al., 1997; Blackwell et al., 1998; Gradia et al., 1999; Hargreaves et al., 2010; Heinen et al., 2011; Mazur et al., 2006). Studies with double blocked DNAs have demonstrated the formation of stable MutSα-DNA complexes in the presence of ATP when both ends of the DNA are blocked. These complexes are significantly reduced when one or both ends of the DNA are free, suggesting that MutSα moves along the helix and away from the mismatch in the presence of ATP (Blackwell et al., 1998; Gradia et al., 1999). Also, studies with DNA roadblocks have shown that several mismatch repair proteins may move along the helix to initiate repair (Pluciennik and Modrich, 2007).

A final model that has received less attention in the literature postulates that recognition of the mismatch by MutS homologs serves as a site for polymerization of a second protein along the DNA helix (Modrich, 1987). The second protein could be another MutS homolog, a MutL homolog, homologs of both, or an entirely different
protein. However, electron microscopy visualization of bacterial MutS and MutL on heteroduplex DNA has failed to reveal evidence of polymerization (Allen et al., 1997).

To shed more light on the different models, we have further explored the effects of nucleotide occupancy and ATP hydrolysis on MutSα dissociation from DNA using poorly hydrolyzable and non-hydrolyzable ATP analogs and our ATP hydrolytic mutants, which support nucleotide binding.

4.1 Materials and Methods

4.1.1 Proteins, DNAs and nucleotides

Wild type MutSα was purified using either purification procedure 1 or 2 as described in section 2.1.1, while the mutant forms of MutSα were purified using procedure 2. The wild type MutSα purified using procedure 1 contains 1 mole of ADP per mole of heterodimer, while the proteins purified using procedure 2 contain no significantly detectable amount of ADP (Fig. 6B and 16B).

The 202-bp DNAs were generated as previously described by PCR amplification of base pairs 5531-5732 of bacteriophages f1MR1 and f1MR3. (Blackwell et al., 1998). In SPRS studies the DNA is attached to a streptavidin chip (SA chip, GE Healthcare) via a biotin-avidin interaction. Therefore, the reverse primer in the PCR amplification has a 5’ biotin modification, resulting in DNA with a
biotinylated strand. Attachment to the chip yields a DNA with one blocked end and one free end and is referred to as singly-blocked DNA. To make a doubly-blocked DNA, the reverse primer was biotinylated, while the forward one was modified by the addition of a 5’ digoxigenin tag. After attachment of the DNA on the chip, addition of digoxigenin antibody results in a nonreversible block on the previously free end (Heinen et al., 2011). Oligonucleotides to construct the 59-bp DNAs were obtained from IDT Technologies with one of the strands modified with a 5’ biotin and the other with a 5’ digoxigenin. The sequence for the 59-bp duplexes was identical to the 59 central base pairs of the 202-bp duplexes. The sequences for the 31-bp DNAs were identical to those described previously (Allen et al., 1997), with the 5’ biotin and 5’ digoxigenin modifications on either strand.

Radio-labeled \([γ^{32}P]ATP\) and \([^{35}S]ATPγS\) were purchased from Perkin/Elmer, while unlabeled nucleotides were purchased from USB Corp (ATP), Sigma (ADP), and Calbiochem (ATPγS).

4.1.2 Surface plasmon resonance studies

DNA dissociation was studied by surface plasmon resonance spectroscopy (SPRS) in a Biacore 2000 instrument (GE Healthcare) as previously described (Blackwell et al., 2001a; Blackwell et al., 1998; Martik et al., 2004). Streptavidin sensor chips (SA chip, GE Healthcare) were derivatized with ~150 resonance units (RUs) of biotinylated DNA, either correctly paired or with a central mismatch. The
proteins were injected at a flow of 20 μL/min in buffer comprised of 25 mM HEPES-KOH, 5 mM MgCl₂, 150 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.005% surfactant P20. Experiments were performed at 20 °C, and the samples were kept at 4 °C prior to injection. The proteins were loaded on DNA in the absence or presence of nucleotide as indicated. For doubly-blocked DNA experiments, 150 μL of 50 nM digoxigenin antibody (α-DIG) were injected onto the chip prior to MutSα loading, resulting in DNA with blocks on both ends. After the MutSα binding phase, the chip was washed briefly with buffer and then challenged with 60 μL of 1 mM ATP or ATP analog as indicated. The chip was finally regenerated by a 20-μL injection of 0.1% sodium dodecyl sulfate (SDS). This step removes all proteins bound to the DNA but does not perturb the α-DIG-digoxigenin interaction.

4.1.3 ATPase assays

ATPase assays were performed at room temperature as described in section 3.1.7 with the following exceptions. [γ-³²P]ATP or [γ-³⁵S]ATPγS were used instead [α-³²P]ATP, resulting in phosphate production as opposed to ADP. The resolving buffer (0.3 M KPi, pH 7.0) contained 2 mM DTT. Also, MutSα was at 200 nM for ATP hydrolysis and at 1 μM for ATPγS hydrolysis. The resulting phosphate was separated and analyzed as described in section 3.1.7.
4.2 Dissociation of MutSα from DNA

4.2.1 Nucleotide states of MutSα affect dissociation from DNA

Dissociation of MutSα from mismatched DNA was studied via SPRS in a Biacore 2000 instrument. Binding of the protein to homoduplex is minimal, as seen in Figure 21A and most dissociates during the buffer wash step. As mentioned in section 2.2.1, preparations of MutSα by purification procedure 1 yield protein that has one ADP molecule per MutSα heterodimer, while preparations using purification procedure 2 result in nucleotide-free protein. From the cross-linking and filter-binding experiments discussed in Chapter 2, we concluded that the ADP in the former case occupied the MSH2 subunit. The two preparations of MutSα bind mismatched DNA with similar affinity, indicating that ADP occupancy of MSH2 does not affect the affinity of the protein for a mismatch. However, a difference was observed in the dissociation of these two forms of MutSα from mismatched DNA after the ATP challenge step (Fig. 21B).
Figure 21. MutSα binding to and dissociating from a 202-bp DNA at 20 °C. ADP-bound MutSα from purification procedure 1 is shown in blue. Nucleotide-free MutSα from purification procedure 2 is shown in green. The black trace shows nucleotide-free MutSα after incubation with stoichiometric amounts of ADP. The different phases of SPRS are indicated. A) Homoduplex DNA. B) Heteroduplex DNA.

Almost all of the MutSα that has one ADP bound per heterodimer dissociates upon challenge with 1 mM ATP (Fig. 21B, blue trace). However, only about half of the nucleotide-free MutSα dissociates from DNA upon ATP addition (Fig. 21B, green trace). To test whether this difference was due to a nucleotide effect or whether it was due to the differences in purification, ADP was added back to the nucleotide-free MutSα. This was achieved either by first incubating the protein with stoichiometric amounts of ADP or by incubating with ATP and allowing hydrolysis to occur prior to the SPRS experiments. Both methods yielded similar results, but only the ADP incubation is shown here (Fig. 21). Addition of ADP to the nucleotide-free protein resulted in almost complete dissociation of MutSα from the DNA (Fig.
21B, black trace), similar to the results observed with the ADP-bound protein. These results indicate that ADP in MSH2 is necessary for efficient dissociation of MutSα from DNA. These results also suggest that the addition of ATP results in a state of MutSα where MSH6 is occupied by ATP and MSH2 by ADP and this form of the protein is the preferred state for release from the mismatch and dissociation from the DNA. These results are consistent with a single molecule study with Taq MutS which suggests that MutS, with ATP in one subunit and ADP in the other is a required step for protein release from the mismatch and sliding along the DNA helix (Qiu et al., 2012). Alternatively, exchange of ADP for ATP in MSH2 resulting in a conformational change in the protein could be necessary for efficient dissociation.

An explanation for the 50% release of the nucleotide-free MutSα is that upon ATP challenge the protein could be in a state with ATP in both subunits, and this state of the protein does not efficiently dissociate from the mismatch. Another possible reason is that binding of ATP to one subunit blocks binding of ATP to the other subunit. Therefore, half of the MutSα dimers would have ATP in MSH6 and the other half in MSH2, and it is possible that only one of these states can lead to efficient dissociation.

4.2.2 Role of ATP hydrolysis in MutSα dissociation from DNA

While the nucleotide states of MutSα are important for dissociation from DNA, we were also interested in the role ATP hydrolysis may play in dissociation.
Therefore, ATP was replaced with poorly hydrolyzable and non-hydrolyzable ATP analogs in the challenge step of the SPRS studies (Fig. 22 and Table 4).

**Figure 22.** Dissociation of ADP-bound MutSα at 20 °C from a 59-bp DNA after challenge with 1 mM ATP or ATP analogs. Challenge with ATP is shown in blue, challenge with the poorly hydrolyzable ATPγS is shown in green, and challenge with the non-hydrolyzable AMPPNP is shown in black. The different phases of SPRS are indicated. A) Homoduplex DNA. B) Heteroduplex DNA.

**Table 4.** Dissociation constants of MutSα from DNA upon 1 mM ATP or ATP analog challenge at 20 °C

<table>
<thead>
<tr>
<th></th>
<th><strong>ATP</strong></th>
<th><strong>ATPγS</strong></th>
<th><strong>AMPPNP</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>t½ (sec)</td>
<td>3.6±0.3</td>
<td>17.6±0.5</td>
<td>57.9±1.5</td>
</tr>
<tr>
<td>k (sec⁻¹)</td>
<td>0.2±0.01</td>
<td>0.04±0.001</td>
<td>0.012±0.001</td>
</tr>
<tr>
<td>Plateau (RU)</td>
<td>94.65</td>
<td>150.7</td>
<td>294.5</td>
</tr>
</tbody>
</table>

Because the most efficient dissociation was observed with ADP-bound MutSα (Fig. 21), these studies were carried out with protein purified using procedure 1, which results in MutSα that contains prebound ADP. Very little MutSα binds to
homoduplex DNA, and, as was observed with the 202-bp homoduplex, most of the protein dissociates during the buffer wash phase (Fig. 22A). As MutSα contains prebound ADP, the challenge with the ATP analogs should initially result in a state of the protein with diphosphate in MSH2 and triphosphate in MSH6. If this state of MutSα were the sole requirement for dissociation from DNA, then in all three cases (ATP, ATPγS, or AMPPNP) the dissociation would be expected to be similar. However, as can be seen from Figure 22B and Table 4, this is not the case. Dissociation with ATP is fast and almost all the protein comes off, with a plateau value of approximately 100 RUs. However, when ATPγS is used instead of ATP, MutSα is longer lived on DNA with a 5-fold increase in half-life. The MutSα-DNA complexes are even more stable with AMPPNP and demonstrate an even longer half-life. The plateau value with AMPPNP is approximately 300 RUs, indicating that not all of the protein dissociates from DNA. These results indicate that while the nucleotide state of MutSα with diphosphate in MSH2 and triphosphate in MSH6 is important for DNA dissociation, hydrolysis also may play a role.

The dissociation of MutSα due to ATPγS can also be explained by hydrolysis. ATPγS is a poorly hydrolyzable analog and not a non-hydrolyzable analog like AMPPNP. It has been reported that it is hydrolyzed at about 1/10 of the rate of ATP (Iaccarino et al., 1998). We looked at hydrolysis of ATPγS as compared to that of ATP and found that ATPγS is hydrolyzed approximately 5-fold slower than ATP (Table 5), which is consistent with the 5-fold difference in MutSα dissociation from
DNA, indicating that not only nucleotide occupancy but also ATP hydrolysis may play a role in dissociation from DNA.

**Table 5. Hydrolysis of ATP and ATPγS at room temperature**

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>ATPγS</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (μM/min)</td>
<td>0.42±0.05</td>
<td>0.39±0.08</td>
</tr>
<tr>
<td>$K_M$ (μM)</td>
<td>8.1±0.8</td>
<td>11.3±1.2</td>
</tr>
<tr>
<td>$k_{cat}$ (min⁻¹)</td>
<td>2.1±0.3</td>
<td>0.39±0.11</td>
</tr>
</tbody>
</table>

AMPPNP, however, also promotes dissociation from DNA, albeit at a slower rate. The half-life of MutSα on DNA after the AMPPNP challenge is over 3-fold longer than that after ATPγS challenge and over 16-fold longer than after ATP challenge. A possible explanation is that dissociation in the presence of AMPPNP may occur via direct release from the mismatch into solution without sliding along the helix. To test this possibility, we placed a digoxigenin tag on the unbiotinylated end of the 59-bp DNA. Addition of a digoxigenin antibody would result in doubly-blocked DNA, which would allow us to distinguish between the two modes of MutSα release from DNA. Direct release would not be inhibited by the second block, while sliding along the helix would be trapped on the DNA when the second block is present. As can be seen in Figure 23, the addition of the second block inhibits the fast dissociation of MutSα upon nucleotide challenge. Any dissociation observed seems to be a continuation of the buffer wash phase. Moreover, the dissociation is similar whether ATP, ATPγS, or AMPPNP is used in the nucleotide challenge phase. These results indicate that dissociation from the mismatch occurs via movement.
along the DNA helix. These results also suggest that MutSα may dissociate from DNA under poorly hydrolytic conditions, albeit less efficiently than under optimal hydrolytic conditions.

Figure 23. Dissociation of ADP-bound MutSα at 20 °C from a doubly-blocked 59-bp DNA upon challenge with 1 mM ATP or ATP analogs. Challenge with ATP is shown in blue, challenge with the poorly hydrolyzable ATPγS is shown in green, and challenge with the non-hydrolyzable AMPPNP is shown in black. The different phases of SPRS are indicated. A) Homoduplex DNA. B) Heteroduplex DNA.

The dissociation studies with ATP, ATPγS, and AMPPNP challenge were also carried out with 202-bp DNAs and the results of dissociation from heteroduplex are summarized in Table 6. Nucleotide-free, ADP-bound MutSα, and nucleotide-free MutSα that was first incubated with ADP were used in these experiments. As can be seen in Table 6, the rates of dissociation for nucleotide-free and ADP-bound MutSα are similar for the ATP challenge studies. However, the ADP-bound form of the protein dissociates more efficiently, as indicated by the lower plateau values. The
Dissociation rates are approximately 10-fold slower when ATPγS replaces ATP in the challenge phase. The presence of pre-bound ADP does not affect the rate of dissociation but it does affect the extent of dissociation as indicated by the plateau values. The lower plateau values indicate that more protein dissociates from heteroduplex DNA when ADP is first bound to MutSα. AMPPNP decreases the rate of dissociation another 3-fold, and the presence of pre-bound ADP results in more efficient dissociation from DNA.

**Table 6.** Dissociation constants of wild type MutSα from 202-bp heteroduplex upon challenge with 1 mM ATP or ATP analog at 20 °C

<table>
<thead>
<tr>
<th></th>
<th>ADP-MutSα</th>
<th>Nucleotide-free MutSα</th>
<th>Nucleotide-free MutSα + ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$ (sec)</td>
<td>2.9±0.3</td>
<td>2.4±0.2</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>$k$ (sec$^{-1}$)</td>
<td>0.23±0.07</td>
<td>0.29±0.04</td>
<td>0.32±0.05</td>
</tr>
<tr>
<td>Plateau (RU)</td>
<td>111.9</td>
<td>303.0</td>
<td>158.0</td>
</tr>
<tr>
<td>ATPγS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$ (sec)</td>
<td>20.2±1.4</td>
<td>22.0±2.3</td>
<td>21.9±3.1</td>
</tr>
<tr>
<td>$k$ (sec$^{-1}$)</td>
<td>0.034±0.008</td>
<td>0.031±0.005</td>
<td>0.032±0.007</td>
</tr>
<tr>
<td>Plateau (RU)</td>
<td>124.3</td>
<td>255.5</td>
<td>195.0</td>
</tr>
<tr>
<td>AMPPNP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$ (sec)</td>
<td>57.3±5.3</td>
<td>68.6±5.9</td>
<td>66.3±4.9</td>
</tr>
<tr>
<td>$k$ (sec$^{-1}$)</td>
<td>0.012±0.005</td>
<td>0.010±0.008</td>
<td>0.010±0.007</td>
</tr>
<tr>
<td>Plateau (RU)</td>
<td>195.6</td>
<td>316.3</td>
<td>274.0</td>
</tr>
</tbody>
</table>

To further examine the role of ATP hydrolysis in MutSα dissociation from DNA, we carried out the SPRS studies with the ATPase mutant forms of MutSα. We found that while DNA binding was not affected by the mutations in MutSα, dissociation was affected, albeit at different levels (Fig. 24). Binding to homoduplex
was significantly lower than heteroduplex and most of the protein dissociated during the buffer wash step.

Figure 24. Dissociation of the MutSα ATPase mutants at 20 °C from a 202-bp DNA with a central mismatch upon challenge with 1 mM ATP. A) Dissociation of 2KR MutSα. B) Dissociation of 6KR MutSα. C) Dissociation of 2,6KR MutSα. The binding and dissociation from heteroduplex DNA of the nucleotide-free proteins are shown in blue, while binding and dissociation of the proteins after incubation with stoichiometric amounts of ADP to generate the ADP-bound forms are shown in green. Binding and dissociation from homoduplex DNA of the nucleotide-free and ADP-bound proteins are shown in black and pink, respectively. Dissociation from the mismatch is summarized in Table 7.

After binding to a 202-bp DNA containing a central mismatch, the proteins were challenged with 1 mM ATP. As compared to the nucleotide-free form of wild type MutSα (Fig. 21, green trace), 2KR MutSα dissociated at similar levels from DNA, with about half of the protein amount coming off (Fig. 24A, blue trace). The rate of dissociation is also slower than the wild type MutSα, with approximately 10-fold difference in half-life (Tables 6 and 7). However, while incubation of wild type MutSα with ADP prior to the experiment to generate the ADP-bound form resulted in almost complete dissociation of the protein from DNA (Fig. 21B, black trace),
incubation of 2KR MutSα with ADP did not have an effect (Fig. 24A, green trace). Only half of the protein dissociated from DNA. This result is not due to a deficiency in ADP binding, because, as shown in the UV cross-linking studies in Chapter 3, Figure 19, the ATP hydrolytic mutants bind ADP in a similar manner to wild type MutSα.

Table 7. Dissociation constants of MutSα mutants from 202-bp heteroduplex upon 1 mM ATP challenge at 20 °C

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k$ (sec$^{-1}$)</th>
<th>$t_{1/2}$ (sec)</th>
<th>Plateau</th>
</tr>
</thead>
<tbody>
<tr>
<td>2KRSα</td>
<td>0.04±0.003</td>
<td>19.3±1.3</td>
<td>387.1</td>
</tr>
<tr>
<td>2KRSα+ADP</td>
<td>0.04±0.002</td>
<td>19.1±1.6</td>
<td>351.6</td>
</tr>
<tr>
<td>6KRSα</td>
<td>0.04±0.004</td>
<td>19.3±2.1</td>
<td>185.4</td>
</tr>
<tr>
<td>6KRSα+ADP</td>
<td>0.04±0.003</td>
<td>17.4±1.3</td>
<td>127.7</td>
</tr>
<tr>
<td>2,6KRSα</td>
<td>0.013±0.005</td>
<td>49.6±2.4</td>
<td>379.1</td>
</tr>
<tr>
<td>2,6KRSα+ADP</td>
<td>0.014±0.002</td>
<td>49.2±3.1</td>
<td>342.5</td>
</tr>
</tbody>
</table>

6KR MutSα dissociated at levels comparable to the ADP-bound wild type protein in the presence of ATP, although more slowly (Fig 21). Interestingly, unlike wild type MutSα (Fig. 21B), the amount of protein dissociating from DNA is not dependent on ADP occupancy of MSH2. 6KR MutSα dissociated almost completely from DNA whether ADP is bound to MSH2 or not. However, the rate of dissociation due to ATP is slower for 6KR MutSα as compared to the wild type protein. It is very similar to the rate observed in the case of wild type MutSα and ATPγS with half-lives of 20 seconds for wild type MutSα and ATPγS and 19 seconds for the 6KR MutSα and ATP (Tables 6 and 7).
The levels of 2,6KR MutSα dissociation are similar to those observed with 2KR MutSα, with only half of the protein coming off the DNA. However, the rate of dissociation is considerably slower and the half-life is longer (Fig. 24C and Table 7). In fact, the dissociation constants for 2,6KR MutSα are similar to those observed for wild type MutSα and AMPPNP (Tables 6 and 7). The different modes of dissociation for each mutant suggest that the two subunits play different roles in MutSα release from DNA. The dissociation observed could be due to ATP hydrolysis, because as shown in chapter 3, the lysine to arginine mutations reduce hydrolysis but do not completely abolish it (Table 1). These results also suggest that while MutSα can dissociate from DNA under poorly hydrolytic conditions, this dissociation is less efficient than that observed in the presence of optimal ATP hydrolysis. Moreover, the ATPase activity of both subunits of MutSα must be intact for efficient dissociation.

4.2.3 Dissociation of MutSα from a mismatch

To further analyze the modes of MutSα dissociation from a mismatch we performed more SPRS studies with doubly-blocked DNAs. The DNAs used in SPRS experiments already have one end blocked due to the biotin-avidin attachment on the chip. To examine the mode of dissociation, we also blocked the other end of the DNA by adding a digoxigenin tag. As mentioned earlier, addition of digoxigenin
antibody (α-DIG) results in an irreversible double end blocked DNA, which would allows us to further characterize the modes of MutSα release from a mismatch.

Figure 25. ADP-bound MutSα binding to and dissociation from 202-bp DNA at 20 °C. A and C show MutSα on homoduplex DNA, while B and D on heteroduplex DNA. A and B were carried out prior to α-DIG block, while C and D after the block. ADP-bound MutSα at 200 nM was loaded on DNA in the absence of nucleotide (blue), presence of 100 μM ATP (green), or presence of 100 μM ATPγS (black).³

When carrying out the SPRS experiments with doubly-blocked DNA, a curious result was observed (Fig. 25). The ADP-bound form of MutSα was loaded on

³ The units have been normalized to RU s bound per RU of DNA on the chip. This normalization was done to simplify the y-axis to make the comparisons easier.
DNA, and the binding to and dissociation from DNA with no α-DIG block was similar to those observed for the 202-bp DNA (compare the blue traces from Fig. 25B and Fig. 21). While the units are different, the profiles of binding and dissociation are similar. The binding is mismatch-dependent, as very little non-specific binding is observed on homoduplex DNA. The addition of the α-DIG block results in a significant increase in mass bound to the DNA (compare blue traces from Fig. 25B and 25D). This increase is biphasic with a fast binding early phase and slower second phase. This increase in mass is also mismatch-dependent, as it is not observed with homoduplex DNA (compare blue traces in Fig. 25A and 25C). Moreover, the lack of mass increase in the homoduplex experiments indicates that this increase is not due to MutSα interaction with the digoxigenin antibody. These results imply that the increase in mass is due to the binding of additional MutSα heterodimers after mismatch recognition, which are then trapped on the doubly-blocked DNA.

Loading MutSα on DNA in the presence of 100 μM ATP results in an even greater increase in resonance units when the second block is present (Fig. 25, green traces), representing additional MutSα heterodimers on the DNA. These results suggest that after MutSα binds to the mismatch, the presence of ATP causes release and sliding along the DNA. The α-DIG block traps these molecules on the DNA. The free mismatch site is now available for new molecules of MutSα to bind and slide along the DNA in the presence of ATP, resulting in the increase of mass.
Loading MutSα in the presence of 100 μM ATPγS yielded results similar to those observed in the absence of nucleotide (Fig. 25, black traces). Both the shapes of the curves and the mass increase levels were similar, indicating that efficient ATP hydrolysis is necessary for dissociation from the mismatch, sliding along the DNA, and accumulation of MutSα on the double blocked DNA.

**Figure 26.** DNA length dependence of the mass increase observed with ADP-bound MutSα. A) Binding to 31-bp DNA. B) Binding to 59-bp DNA. C) Binding to 202-bp DNA. Heteroduplex binding is shown in black and pink for singly- and doubly-blocked DNA, respectively. Homoduplex binding is shown in green and blue for singly- and doubly-blocked, DNA respectively.4

The increase in mass is observed at different extents depending on the length of DNA used (Fig. 26). The increase in resonance units is minimal when the 31-bp DNA is doubly-blocked (Fig. 26A). However, there is a 2- to 3-fold increase in mass when the 59-bp and 202-bp DNAs are used (Fig. 26B and 26C). These results

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4 The difference in RU is due to the amount of DNA on the chip. As the DNAs have different sizes and therefore different mass, the same amount of RUs of DNA bound to the chip results in a different number of DNA molecules.
suggest that after the initial binding of a MutSα dimer, a second one is recruited that can move along the DNA and is trapped by the second block. This possibility is discussed further below.

The increase in resonance units could also be a $K_d$ effect because the second block on DNA may affect the on and off rates of MutSα on DNA. To test whether that is why the increase in mass was observed, we carried out titration experiments with the ADP-bound form of MutSα with DNA that is either singly- or doubly-blocked (Fig. 27).

The titration experiments show that for most of the concentrations of protein there is approximately a 2-fold increase in resonance units on heteroduplex DNA (Fig. 27B and 27D), while no significant increase is observed with homoduplex (Fig 27A and 27B). Plotting the maximum resonance units for each concentration results in the graph shown in Figure 27E. The $K_d$ values obtained for the singly-blocked and doubly-blocked DNAs are similar, with only a 2-fold difference, and as indicated by the asymptotic values from Figure 27E, twice as much protein binds the doubly-blocked DNA as compared to the singly-blocked DNA. Because the resonance units with the doubly-blocked DNA are not saturating, but still increasing, these results suggest that in the absence of ATP, two or more MutSα dimers bind the DNA, with one of the dimers binding the mismatch and the others sliding along the DNA. The secondary dimers either slide off the free end or are trapped if the end is blocked. These dimers can bind on either side of the first dimer and can displace the
mismatch-bound dimer. The first dimer would then slide along the DNA, while a second one binds the mismatch.

Figure 27. Titration of ADP-bound MutSα on 202-bp DNA at 20 °C. A) Titration on singly-blocked homoduplex. B) Titration on singly-blocked heteroduplex. C) Titration on doubly-blocked homoduplex. D) Titration on double-blocked heteroduplex. E) Plotting the maximum binding values after the association phase as a function of concentration and fitting to a square hyperbola by nonlinear regression analysis yielded $K_d$ values of 92 nM and 44 nM and asymptotic values of 4.3 and 9.3 for singly-blocked and doubly-blocked DNA, respectively.

4.3 Summary and discussion
The SPRS results indicate that the presence of ADP in MSH2 is important for efficient MutSα dissociation from DNA. Approximately half of MutSα dissociated from DNA when the nucleotide-free protein was used. However, in the experiments where MutSα already has ADP in the MSH2 subunit, all of the protein dissociated from DNA upon ATP challenge. A possible explanation for the observed dissociation with the nucleotide-free protein is that binding of ATP to one subunit inhibits ATP binding to the other subunit. Therefore, ATP binding to MSH2 inhibits binding to MSH6 and vice-versa, resulting in a mixture of MutSα in which half of the dimers have ATP in MSH6 and half in MSH2. It is possible that only one of these states can lead to efficient dissociation from the mismatch. The following could be a possible mechanism that explains why only 50% of the nucleotide-free protein dissociates from DNA. After hydrolysis in dimers with ATP in MSH6, the resulting ADP is released due to low affinity of the subunit for the nucleotide, forming a nucleotide-free MutSα state that remains bound to the mismatch. Hydrolysis in dimers with ATP in MSH2 results in the ADP bound form of MutSα, which may then bind ATP in MSH6 to create an ADP-MutSα-ATP state that releases the mismatch and moves along the helix. This model would explain why only 50% of nucleotide-free MutSα dissociates from DNA and would also suggest that ATP hydrolysis occurs while MutSα is bound to the mismatch. This hydrolysis can result in a protein state where MSH6 is occupied by ATP and MSH2 by ADP. This state of the protein could be
important for MutSα dissociation from the mismatch, and would be consistent with results for Taq MutS release from DNA (Qiu et al., 2012).

Experiments with poorly hydrolyzable and non-hydrolyzable ATP analogs and MutSα ATPase mutants also suggest that ATP hydrolysis is required for optimal dissociation from DNA. Dissociation with ATP analogs is observed, albeit at slower rates than that observed with ATP. In the case of ATPγS, the dissociation rate was 5-fold lower than that of ATP corresponding to the 5-fold slower MutSα hydrolysis of ATPγS as compared to ATP hydrolysis, indicating that the dissociation observed may be due to ATP hydrolysis. However, AMPPNP also promotes release from the mismatch suggesting that MutSα can dissociate from a mismatch under non-hydrolytic conditions, albeit less efficiently. The ATPase mutant results show different levels of dissociation from DNA depending on which subunit contains the mutation. MutSα with the MSH2 subunit intact dissociates with the highest efficiency, suggesting that MSH2 may play a greater role on protein movement on DNA. The ATPase mutant results also suggest that MutSα can dissociate from DNA under poorly hydrolyzable conditions, although less efficiently than under optimal hydrolysis. These results suggest that both occupancy of MSH2 by ADP and ATP hydrolysis by MutSα are required for efficient DNA dissociation. Moreover, the ATPase of both subunits must be intact for optimal dissociation.

The increase in mass observed with doubly-blocked DNAs indicates additional binding of MutSα heterodimers after the initial recognition of the
mismatch. In the absence of ATP or ATP hydrolysis, MutSα could bind as a dimer and recruit a second dimer, which then moves along the helix possibly scanning the DNA for other mismatches or the strand signal. The second dimer can bind on either side of the first dimer and is capable of displacing the mismatch-bound dimer. The first dimer would then slide along the helix and either dissociate through the free end or be trapped by the block. If the dimer moving along the helix runs into a block, it can bounce off and come back towards the mismatch where it can displace the mismatch-bound dimer. This would explain why the mass increase is observed with the doubly- but not singly-blocked DNA. In the presence of ATP the increase in mass is even greater, suggesting that after mismatch binding, MutSα leaves the mismatch in an ATP-dependent manner by sliding along the DNA. The mismatch site is consequently free for other MutSα molecules to bind, which also slide along the DNA in the presence of ATP. These molecules are then trapped on the DNA due to the second block, resulting in the observed increase in resonance units. Models for these observations are discussed in more detail in Chapter 6.
5. The MutSα-MutLα-mismatch ternary complex

After recognition and binding of the mismatch by MutS proteins, MutL homologs are recruited to form a MutS-MutL-DNA ternary complex. This complex is mismatch dependent and is only observed in experiments with DNAs of 100 base pairs or longer (Blackwell et al., 2001b; Grilley et al., 1989a; Schofield et al., 2001). This complex may be capable of movement along the DNA helix carrying information from the mismatch to the strand discrimination signal (Acharya et al., 2003; Blackwell et al., 2001b).

The general consensus is that the ternary complex is formed in an ATP-dependent manner, but the role of ATP hydrolysis has been a subject of controversy. Both MutS and MutL homologs have an ATPase activity, and work with E. coli and human MutL proteins defective in ATPase function has shown that ATPase integrity of the MutL homologs is not required for assembly of the ternary complex (Acharya et al., 2003; Raschle et al., 2002; Selmane et al., 2003).

Although the ATPase integrity of the MutL homologs is not required, studies to determine the ATP requirements for complex formation with ATP analogs have yielded differing results. While studies with E. coli MutS and MutL have shown that the poorly hydrolyzable ATP analog ATPγS supports ternary complex formation (Acharya et al., 2003; Grilley et al., 1989b), studies with the human and yeast
systems have shown that AMPPNP and ATPγS are much less efficient in ternary complex formation (Blackwell et al., 2001b; Mendillo et al., 2005).

The nucleotide states of MutSα required for the ternary complex formation have not been closely studied. Studies with Taq MutS (Qiu et al., 2012) and the studies with human MutSα discussed in chapter 4 suggest that the ADP-ATP state of the MutS homologs is important for protein release from the mismatch. This state or other nucleotide states could also play a significant role in the MutSα-MutLα-mismatch ternary complex formation. We therefore examined the role of nucleotide occupancy as well as the role of ATP hydrolysis in ternary complex formation.

5.1 Material and methods

5.1.1 Proteins and DNAs

Wild type MutSα was prepared with either purification procedure 1 or 2 as described in section 2.1.1. Mutant constructs of MutSα were prepared using purification procedure 2. MutLα was prepared as previously described (Blackwell et al., 2001b).

PCR-derived 202-bp heteroduplex and homoduplex DNAs were prepared after strand separation by denaturing HPLC as previously described (Blackwell et al., 1998).
Adenine nucleotides were purchased from USB Corp (ATP), Sigma (ADP), and Calbiochem (ATPγS).

5.1.2 Ternary complex studies

Streptavidin sensor chips (SA chip, GE Healthcare) were derivatized with ~150 resonance units (RUs) of biotinylated 202-bp DNA, either correctly paired or with a central mismatch. MutSα and/or MutLα at concentrations of 200 nM each were loaded on DNA in the presence of either 1 mM ATP or ATPγS at a flow of 20 μL/min in buffer comprised of 25 mM HEPES-KOH, 5 mM MgCl₂, 150 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.005% surfactant P20. Experiments were performed at 20 °C, and the samples were kept at 4 °C prior to injection. The chip was regenerated by a 20-μL injection of 0.1% sodium dodecyl sulfate (SDS).

5.2 Requirements for MutSα-MutLα-mismatch ternary complex formation

5.2.1 Nucleotide occupancy requirement for ternary complex formation

We used SPRS to examine MutSα nucleotide occupancy requirements for the formation of the MutSα-MutLα-mismatch ternary complex. The formation of ternary complex requires both proteins, ATP, and a mismatch (Fig. 28). As has been mentioned previously, purification of wild type MutSα by two different procedures
yields either protein that is nucleotide-free, or protein that contains 1 ADP molecule per MutSα heterodimer, with the ADP being bound to MSH2. We used these two forms of MutSα to study the nucleotide requirements for ternary complex formation (Fig. 28).

**Figure 28.** MutSα-MutLα-mismatch ternary complex formation at 20 °C. In all cases the proteins are loaded on 202-bp DNA in the presence of 1 mM ATP. A) Ternary complex with nucleotide-free MutSα. B) Ternary complex with ADP-bound MutSα. C) Ternary complex with nucleotide-free MutSα, which was incubated with a stoichiometric amount of ADP prior to the ternary complex experiments. Blue traces show MutLα on DNA, black traces MutSα, and green traces MutSα and MutLα together. The top panels show ternary complex on homoduplex DNA, while the bottom panels show ternary complex on heteroduplex DNA.

Some ternary complex is observed on homoduplex DNA (Fig. 28, top panels), suggesting that MutLα is recruited to form the complex provided that MutSα is
already bound on DNA. However, the extent of ternary complex observed on homoduplex is less than that observed with heteroduplex, most likely due to less MutSα bound on homoduplex DNA as compared to heteroduplex DNA.

Using nucleotide-free MutSα results in a 2-fold increase in resonance units when MutLα is also included (Fig. 28A, green and black traces). This increase indicates formation of a ternary complex and is not due merely to MutLα binding to DNA. MutLα alone does not bind DNA (Fig. 28, blue traces). However, when performing the experiments with ADP-bound hMutSα, a 4-fold increase in resonance units was observed when MutLα was included in the reaction (Fig. 28B). As discussed in section 4.2.1, to test whether this variance between nucleotide-free and ADP-bound MutSα was due to the purification differences or due to ADP occupancy, ADP was added back to the nucleotide-free MutSα prior to the SPRS experiments. A 3.8-fold increase in resonance units was observed when MutLα was included in the reaction (Fig. 28C), similar to the results observed with ADP-bound MutSα. These results demonstrate that, as was the case for the dissociation studies, the occupancy of ADP in MSH2 is necessary for optimal ternary complex formation.

5.2.2 Role of ATP hydrolysis in ternary complex formation

While ternary complex was observed in the presence of ATP (Fig. 29A, lower panel), substituting ATPγS for ATP yielded no such results (Fig. 29B, lower panel). There is a very modest increase in resonance units when the ADP-bound form of
MutSα and MutLα are injected in the presence of ATPγS, but that increase could be due to low levels of non-specific MutLα binding to DNA (Fig. 28, blue traces). A small increase in resonance units is observed with homoduplex DNA in the presence of ATP (Fig. 29A, upper panel), suggesting some ternary complex formed. This increase disappears in the presence of ATPγS (Fig. 29B, upper panel).

**Figure 29.** Ternary complex in the presence of ATP or ATPγS at 20 °C. ADP-bound MutSα at 200 nM is loaded on 202-bp DNA either alone (blue) or with 200 nM MutLα (green) in the presence of A) 1 mM ATP, B) 1 mM ATPγS, or C) 0.5 mM ATPγS and 0.5 mM ADP. The top panels show ternary complex on homoduplex DNA, while the bottom panels on heteroduplex DNA. MutSα alone is shown in blue, while MutSα and MutLα together are shown in green.

A possible explanation for the absence of ternary complex could be that as the experiments are carried out with 1 mM ATPγS, MutSα could be in a state with
ATPγS in both subunits, and it is possible that this state of the protein is not capable of forming a ternary complex with MutLα. The ADP in MSH2 could be displaced by the excess ATPγS, and the occupancy of ADP in MSH2 is required for the ternary complex. While the displacement of ADP was shown to be slow (Fig. 15), those experiments were carried out with only a 10-fold excess of competing nucleotide. Because MutSα has one ADP bound per dimer, the amount of ADP in the experiments is equal to the amount of MutSα (200 nM). As the ternary complex studies are done with 1 mM ATPγS, there is a 5000-fold excess of ATPγS over ADP, possibly resulting in faster displacement of ADP in MSH2. To discern this possibility, we performed ternary complex experiments in the presence of equal amounts of both ADP and ATPγS and at vast excess over the protein concentrations (Fig. 29C). At these nucleotide amounts, both subunits of MutSα have equal opportunity to bind either of the nucleotides. This also resulted in no observed ternary complex. Together with the ATP findings, these results strongly suggest that while ADP occupancy in MSH2 is necessary for optimal formation of the MutSα-MutLα-mismatch ternary complex, ATP hydrolysis is also required.

Studies with the mutant forms of MutSα also suggested that hydrolysis is required for formation of the MutSα-MutLα-DNA ternary complex. In the presence of ATP, wild type MutSα readily forms a ternary complex with MutLα and mismatched DNA (Fig. 28). However, this complex is not observed when either of the ATPase mutant forms of MutSα is used in place of the wild type protein (Fig. 30).
Interestingly, while the ATPase mutants dissociated from DNA, albeit at differing levels, (Fig. 24), neither of the mutant forms of MutSα supported the formation of a ternary complex with MutLα and DNA.

![Graphs showing ternary complex formation](image)

**Figure 30.** Ternary complex with nucleotide-free MutSα ATP hydrolytic mutants at 20 °C. In all cases the proteins are loaded on 202-bp DNA in the presence of 1 mM ATP. A) 2KR MutSα; B) 6KR MutSα; C) 2,6KR MutSα. The top panels show ternary complex on homoduplex DNA, while the bottom panels on heteroduplex DNA. MutSα alone is shown in black, MutLα alone in blue, and MutSα and MutLα together in green.

The ATP hydrolytic mutants used in these experiment are nucleotide-free and studies with the ADP-bound forms have not been performed. However, because nucleotide-free wild type MutSα does support some ternary complex formation with a two-fold increase in resonance units (Fig. 28A), and no such increase is observed
with the mutant forms of MutSα, the ADP-bound forms of the mutants are not expected to efficiently form ternary complexes. However, these studies must be performed before such conclusions are reached.

The ATPase mutant results are consistent with the previous findings in which ternary complex was not observed in the presence of ATPγS (Fig. 29), suggesting that ATP hydrolysis is required for formation of the MutSα-MutLα-mismatch complex. These results are also consistent with a recent study with other mutant forms of MutSα in which the protein either has a partial defect in nucleotide binding or fails to couple nucleotide binding and mismatch recognition (Geng et al., 2012). However, unlike that study, our work directly addresses the roles of the ATPase sites of MutSα. These mutants, while defective in ATP hydrolysis, are not defective in nucleotide binding and do not uncouple nucleotide binding and mismatch recognition.

### 5.3 Summary and discussion

As was the case with the DNA dissociation studies, the SPRS results indicate that the occupancy of MSH2 by ADP is important for efficient ternary complex formation. The mass increase due to ternary complex with ADP-bound MutSα is twice as great as that observed with nucleotide-free MutSα. The observed ternary complex with nucleotide-free MutSα could be due to ATP hydrolysis resulting in
ADP occupancy in MSH2 and ATP in MSH6. This state of the protein may then be recognized by MutLα to form the complex.

However, the state of the protein alone is not sufficient for the ternary complex to form. Results with ATPγS and the ATPase mutant forms of MutSα suggest that ATP hydrolysis is also required for the MutSα and MutLα interactions on DNA. Although MutSα does dissociate from DNA in the presence of ATPγS, albeit at a slower rate than that observed with ATP, even with ADP in MSH2, ATPγS, unlike ATP, did not support MutSα-MutLα-DNA ternary complex formation.

While the ATPase mutant forms of MutSα did dissociate from DNA to some extent, neither of these mutants supported formation of the MutSα-MutLα-mismatch ternary complex. Together with the ATPγS results, the ATPase mutant findings suggest that although ADP occupancy of MSH2 is necessary for optimal ternary complex formation, ATP hydrolysis by MutSα is also probably required. Moreover, the ATPase centers of both subunits must be intact for efficient formation of the MutSα-MutLα-mismatch complex.


6. Conclusions and future directions

6.1 Conclusions

Adenine nucleotides play an important role in the function of MutS proteins, and this role has been studied extensively (Allen et al., 1997; Antony and Hingorani, 2003, 2004; Bjornson and Modrich, 2003; Blackwell et al., 1998; Gradia et al., 1999; Heinen et al., 2011; Iaccarino et al., 1998; Lamers et al., 2004; Martik et al., 2004; Mazur et al., 2006; Qiu et al., 2012). However, questions still remain, and we have used various methods to further elucidate the role of adenine nucleotides in the function of human MutSα.

We have used UV cross-linking studies to visualize the occupancy states of each of the MutSα subunits. However, as mentioned in section 2.2.2, this method has several caveats, and interpretation requires several assumptions. Filter-binding methods were also used to confirm some of the cross-linking results. We found that the MSH2 subunit of MutSα is occupied by ATP and after hydrolysis it is in an ADP-bound state, while the MSH6 subunit is occupied by ATP. The protein can then exist in a state with ADP in MSH2 and ATP in MSH6. The filter-binding and UV cross-linking studies together also suggest that binding of triphosphate nucleotide to one subunit prevents binding of triphosphate nucleotide to the other subunit. Therefore, if ATP binds in MSH2, the MSH6 site remains empty until hydrolysis has
occurred and MSH2 is occupied by ADP. ATP can then bind in MSH6 forming a
MutSα state with ADP in MSH2 and ATP in MSH6.

A recent study with Taq MutS has shown that the ADP-MutS-ATP state is a
required step for movement from the mismatch and along the DNA (Qiu et al.,
2012). Our results suggest that this state also may be required for human MutSα.
Our UV cross-linking and filter-binding studies show that a mismatch makes the
MSH2 subunit more dynamic, allowing for nucleotide exchange to occur. The bound
ADP is replaced either by ATP or ADP in solution as shown by the increase in ATP
cross-linking to MSH2 after cold ADP incubation in the presence of a mismatch (Fig.
13C) and the loss of ADP cross-linking after addition of cold ATP in the presence of a
mismatch (Fig. 14C). However, these experiments were performed with short
DNAs, only 21 base pairs long, and it is possible that the exchange occurs after
MutSα has dissociated from DNA.

Two possible models can explain the dissociation of ADP-bound MutSα from
mismatched DNA (Fig. 31A). Binding and hydrolysis in MSH6 may promote ADP for
ATP exchange in MSH2, resulting in a conformational change that allows MutSα to
move away from the mismatch. Because of the low affinity of MSH6 for ADP, MSH6
would be nucleotide-free after ATP hydrolysis. The ATP in MSH2 is then hydrolyzed
while away from the mismatch or after dissociation from DNA, and the ADP-bound
state of MutSα is regenerated (Fig. 31A, upper pathway). It is also possible that
MutSα in an ADP-ATP state leaves the mismatch and dissociates from DNA while
hydrolyzing ATP in MSH6 (Fig. 31A, lower pathway). After dissociation, the ADP in MSH2 is replaced by ATP. This ATP is then hydrolyzed to regenerate the ADP-bound form of MutSα. The exchange of ADP was only observed in the presence of a mismatch, suggesting that MutSα undergoes a conformational change upon mismatch binding that allows for the ADP exchange upon dissociation. The lack of exchange with homoduplex DNA suggests that MutSα does not undergo the conformational change when bound to correctly paired DNA, which allows it to continue to scan the DNA for mismatches.

Figure 31. Models of MutSα dissociation from DNA. T represents ATP, while D represents ADP. A) Dissociation of ADP-bound MutSα. B) Dissociation of nucleotide-free MutSα. The models are described in the text.
SPRS studies show that for efficient dissociation of MutSα from longer DNAs, ADP must be bound to the MSH2 subunit. The protein could dissociate from the mismatch by either of the pathways shown in Figure 31A. However, when nucleotide-free MutSα is used, only half of the protein dissociates upon ATP challenge. A likely explanation is that binding of ATP to one subunit inhibits binding to the other subunit (Fig. 31B). Therefore, ATP binding to MSH2 inhibits binding to MSH6 and vice-versa, resulting in a mixture of MutSα in which half of the dimers contain ATP in MSH6 (Fig. 31B, upper pathway) and half in MSH2 (Fig. 31B, lower pathway). After ATP hydrolysis in MSH6, the resulting ADP is released due to the low affinity of MSH6 for ADP, resulting in a nucleotide-free MutSα state that does not dissociate from the mismatch (Fig. 31B, upper pathway). ATP hydrolysis in MSH2 results in the ADP bound form of MutSα (Fig. 31B, lower pathway), which would then dissociate as in Figure 31A. Because ATP can bind each subunit with equal probability, only half of the heterodimers would have ATP in MSH2, which would then hydrolyze to ADP, resulting in half of protein dissociating from DNA. It is also possible that the two forms of MutSα bound to DNA (Fig. 31B), with ATP in either MSH2 or MSH6, do not dissociate from a mismatch in the same way. For example, MutSα with ATP in MSH6 may release from the mismatch, whereas MutSα with ATP in MSH2 may remain bound to the mismatch and vice-versa, resulting in only half of the protein dissociating from DNA.
Studies with poorly hydrolyzable and non-hydrolyzable ATP analogs and MutSα ATPase mutant constructs suggest that both occupancy of MSH2 by ADP and ATP hydrolysis are required for efficient MutSα dissociation from DNA. The different modes of dissociation with the ATP hydrolytic mutants suggest that the two subunits of MutSα play different roles in protein dissociation from DNA and suggest that MutSα can dissociate from DNA even under poor hydrolytic conditions, albeit less efficiently. Moreover, for optimal dissociation from DNA, the ATPase activity of both subunits must be intact.

SPRS studies with a doubly-blocked DNA show a greater increase in resonance units than that observed with singly-blocked DNA. This increase is mismatch dependent, indicating that it is not due to MutSα interaction with the antibody. These studies suggest that in the absence of ATP or ATP hydrolysis, MutSα could bind the mismatch as a dimer and recruit a second dimer, which then moves along the helix, possibly scanning the DNA for other mismatches or the strand signal (Fig. 32A). The second dimer can bind on either side of the first dimer and is capable of displacing the mismatch-bound dimer. The first dimer would then slide along the helix and either dissociate via the free end or be trapped by the block. If the dimer moving along the helix runs into a block, it can bounce off and return towards the mismatch where it can displace the mismatch-bound dimer. This model would explain why the mass increase is only observed with the doubly-blocked DNA but not with the singly-blocked DNA (Fig. 32A).
Figure 32. Models for the observed increase in mass with blocked DNAs.  A) In the absence of ATP.  B) In the presence of ATP.

In the presence of ATP, the increase in mass is even greater, suggesting that after mismatch binding MutSα leaves the mismatch in an ATP- and ATP hydrolysis-dependent manner by sliding along the DNA. The mismatch site is consequently free for other MutSα dimers to bind, which also slide along the DNA in the presence of ATP. These molecules are then trapped on the DNA due to the second block, resulting in the observed increase in resonance units (Fig. 32B).

SPRS studies also show that ADP occupancy of MSH2 is necessary for formation of the MutSα-MutLα-mismatch ternary complex, suggesting that the ADP-ATP form of MutSα is the protein state recognized by MutLα. However, unlike with the DNA dissociation studies, ATP hydrolysis is absolutely required for the MutSα-MutLα interaction. Moreover, the ATPase activity of both subunits of MutSα is required for formation of the MutSα-MutLα-mismatch complex.
6.2 Future directions

The increase in mass observed in SPRS experiments with doubly-blocked DNA is curious and quite interesting. While we attribute this effect to the loading of multiple MutSα heterodimers, it does need further investigation. Another method to study this phenomenon would be gel shift assays. Proteins bound to doubly-blocked or singly-blocked DNAs would be run on a gel to determine the band shifts and whether the second block results in greater retardation of the DNA in the gel. To determine whether the increase in mass is due to the second block and not the type of block, the double blocked DNAs could have either biotin on both ends, or biotin on one end and digoxigenin on the other.

The MutSα-MutLα-mismatch studies showed a 4-fold increase in mass when MutSα and MutLα are added together as compared to MutSα alone, suggesting multiple heterodimers on the DNA. However, the nature of these dimers is not known. They could be either additional MutSα binding after the complex is formed, additional MutLα, or both. To determine the identity of the proteins responsible for the increase in mass, bead assays could also be used. DNAs with biotin and digoxigenin tags on either end could be used with avidin magnetic beads to trap the ternary complex on DNA. Running the samples on gels and performing Western analyses with MutSα or MutLα antibodies would reveal the nature and amount of the proteins on DNA.
Our studies show that the ATPase sites of both subunits must be intact for formation of the MutSα-MutLα-mismatch ternary complex. MutLα has been shown to possess a latent endonuclease activity that is activated in a mismatch-, MutSα-, RFC-, PCNA-, and ATP-dependent manner (Kadyrov et al., 2006). It would be interesting to examine whether this activity is dependent on MutSα ATP hydrolysis. The MutSα mutants could be used in MutLα nicking assays to determine which of the subunits, if either, is important for MutLα endonuclease activation. These experiments would also shed light on the importance of the ternary complex observed via SPRS.

Another protein in the mismatch repair pathway is ExoI, which when activated by MutSα is highly processive, hydrolyzing \(~2000\) nucleotides. This processivity is reduced in the presence of RPA (Genschel and Modrich, 2006). This study also showed that ATP and ATP hydrolysis are required for mismatch specificity when MutSα and MutLα are included in the reaction. As both proteins have ATPase activity, the MutSα ATP hydrolytic mutants would help investigate the role of MutSα ATP hydrolysis in ExoI activation.

Finally, the mutants could be used to look at MutSα ATP hydrolysis requirements in mismatch repair assays. While the abovementioned studies with MutLα and ExoI would address the separate steps in mismatch repair, including the mutants in repair assays with nuclear extracts would address the role of MutSα ATP hydrolysis in the overall repair reaction.
References


Biography

Lored Asllani was born on May 18, 1979 in the small town of Peshkopi in northeastern Albania. He moved to Holden, Massachusetts in June 1994 where he attended Wachusett Regional High School. After graduating in 1998, he matriculated at Clark University where in 2002 he earned his Bachelor of Arts degree in Biochemistry. Lored entered the Duke University Biochemistry Department’s graduate program in the fall of 2002 and in spring of 2003 he joined the lab of Paul Modrich. He began research in the adenine nucleotide states of the mismatch repair protein MutSα, and has presented his work at departmental research forums and retreats.