Differential Specificities and Simultaneous Occupancy of Human MutSα Nucleotide Binding Sites*

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We have examined the permissible nucleotide occupancy states of human MutSα. The MSH2-MSH6 heterodimer binds 1 mol of ADP and 1 mol of adenosine 5’-O-(thiotriphosphate) (ATPγS), with a Kᵣ for each nucleotide of about 1 μM. Anisotropy measurements using BODIPY TR and BODIPY FL fluorescent derivatives of ADP and 5’-adenosyl-β,γ-imidodiphosphate (AMPPNP) also indicate an interaction stoichiometry of 1 mol of ADP and 1 mol of triphosphate analogue per MutSα heterodimer. Di- and triphosphate sites can simultaneously be occupied as judged by sequential filling of the two binding site classes with differentially radiolabeled ADP and ATPγS and by fluorescence resonance energy transfer between BODIPY TR- and BODIPY FL-labeled ADP and AMPPNP. ATP hydrolysis by MutSα is accompanied by a pre-steady-state burst of ADP formation, and analysis of MutSα-bound nucleotide during the first turnover has demonstrated the presence of both ADP and ATP. Simultaneous presence of ADP and a nonhydrolyzable ATP analogue modulates MutS heteroduplex interaction in a manner that is distinct from that observed in the presence of ADP or nonhydrolyzable triphosphate alone, and it is unlikely that this effect is due to the presence of a mixed population of binary complexes between MutSα and ADP or a triphosphate analogue. These findings imply that MutSα has two nucleotide binding sites with differential specificities for ADP and ATP and suggest that the ADP-MutSα-ATP ternary complex has an important role in mismatch repair.

Mismatch repair corrects DNA biosynthetic errors, ensures the fidelity of genetic recombination, and participates in the cellular response to certain types of DNA damage (1–6). Assembly of MutS and MutL homologues at a mismatch or at a DNA lesion is presumed to be an early event in each of these genetic stabilization functions. In human cells the MutS homologue MSH2 forms heterodimers with MSH6 (7–10) or MSH3 (10, 11), and these activities are responsible for mismatch recognition. The MutSβ heterodimer (MSH2-MSH3) recognizes small insertion/deletion mispairs (9, 10, 12), but MutSα (MSH2-MSH6) recognizes and supports the repair of both base-base mismatches and insertion/deletion heterologies (7, 12, 13).

In the human cell lines that have been examined, the majority of the MSH2 is associated with MSH6 (11, 12, 14), and the MutS complex is present in a 6- to 10-fold excess over MutSβ.

In addition to their mismatch recognition function, MutS homologues contain a highly conserved ATP hydrolytic center near the carboxyl terminus (15). The integrity of this site is required for function in mismatch repair (15–17), and there is extensive evidence indicating that adenine nucleotides modulate the interaction between MutS homologues and DNA. ATP and nonhydrolyzable ATP analogues reduce MutS homologue affinity for a mispair (7, 8, 17–23), and nucleotide challenge of preformed protein-heteroduplex complexes provokes MutS homologue release from a mismatch (23–28).

Several models for ATPase function in MutS homologue action have been suggested. Two of these invoke ATP-dependent movement of the protein along the helix, which is postulated to link mismatch recognition to activation of downstream events at the strand signal that directs repair (24–26). Electron microscopic visualization of bacterial MutS-heteroduplex complexes has demonstrated the mismatch- and ATP-dependent extrusion of a DNA loop by the bacterial protein (24). Because nonhydrolyzable ATP analogues do not support loop extrusion and because their addition terminates ongoing loop growth, this reaction has been attributed to directional translocation along the helix in a reaction dependent on ATP hydrolysis by the DNA-bound protein. The use of streptavidin end-blocked linear DNAs has also suggested that MutS and MutSα leave a mismatch in an ATP-dependent manner, observations that have led to the suggestion that the protein may form a mobile clamp about the helix (22, 25, 26, 29). Although ATP-Mg2⁺ supports the formation of stable complexes on end-blocked heteroduplexes, AMPPNP-Mg2⁺, ATPγS-Mg2⁺, or ATP (no Mg2⁺) do not, suggesting that formation of such intermediates may depend on ATP hydrolysis by heteroduplex-bound MutSα (25). The interaction with end-blocked DNA of a mutant form of MutSα, which supports ATP binding but not hydrolysis, has led to a similar conclusion (27).

A mechanism for MutS/MutSα movement along the helix that is independent of ATP hydrolysis by DNA-bound protein has also been proposed (26, 29). This molecular switch model posits heteroduplex binding by the MutS/MutSαADP complex, mismatch-provoked exchange of ADP for ATP, and free diffusion of the MutS/MutSα-ATP complex along the helix. In this mechanism, ATP hydrolysis occurs after dissociation from the DNA to regenerate the ADP form of the protein. As in the translocation model, this movement is postulated to play an important role in activation of downstream activities that act

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‡ The abbreviations used are: AMPPNP, 5’-adenosyl-β,γ-imidodiphosphate; ATPγS, adenosine 5’-O-(thiotriphosphate); SPRS, surface plasmon resonance spectroscopy; RU, resonance unit; PEI, polyethyleneimine; P-γp, P-glycoprotein; MRP1, multidrug resistance protein 1.
A). After washing with 40 ml of starting buffer, the column was eluted in pH 7.5, 0.1 mM EDTA, and protease inhibitors as above. MutS from *Escherichia coli* accounted for the observed mismatch dependence of activation of MutL. This proposal stipulates that, once mismatch recognition occurs, MutS/MutS remains bound to the mismatch during the course of repair (34). In this model, interaction of the mismatch and the strand signal is mediated by DNA bending, and ATP binding by DNA-bound MutS/MutS functions in a kinetic proofreading mechanism that serves to verify mismatch recognition prior to initiation of repair. Although this model has several attractive features, it is inconsistent with the finding that bacterial MutS/MutL and human MutS/MutLα complexes leave the mismatch by movement along the helix contour (29, 35, 36) and studies of *in vivo* assembly of bacterial MutS and MutL foci at mispairs, which suggest that the two proteins migrate from a mismatch in the cell (37).

To further clarify the role of the human MutSα ATPase, we have determined the possible states of nucleotide occupancy of the protein. The stoichiometry of MutSα interaction with ADP and nonhydrolyzable ATP analogues indicates that the two nucleotide binding sites within the MSH2/MSH6 heterodimer display different specificities for ADP and ATP. These studies also show that di- and triphosphate binding sites can be simultaneously occupied at nucleotide concentrations in the low micromolar range.

**EXPERIMENTAL PROCEDURES**

**Proteins and Oligonucleotides—** Human MutSα was purified from SF9 cells infected with a baculovirus expressing construct by a modification of the previously described procedure (7, 36). Eluates from the Mono Q column (30–40 mg, about 1 mg/ml) were diluted to a conductive activity equivalent to 100 µM KCl and loaded at 2 ml/min onto an 8-ml Mono S column (Amersham Biosciences) equilibrated with 25 mM HEPES-KOH (pH 7.5), 100 mM KCl, 0.1 mM EDTA, and protease inhibitors (1 µg/ml each Pefabloc, E64, aprotinin, leupeptin, and pepstatin A). After washing with 40 ml of starting buffer, the column was eluted with a 60-ml gradient of KCl (100 to 370 mM) in 25 mM HEPES-KOH, pH 7.5, 0.1 mM EDTA, and protease inhibitors as above. MutSα fractions, which eluted at about 200 mM KCl, were supplemented with 1 mM dithiothreitol, pooled, and dialyzed against 25 mM HEPES-KOH, pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol containing protease inhibitors and 1 mM KCl, for 5 h, and then against the same buffer containing 0.2 mM KCl for 1 h. After spin concentration at 4 °C using a Centrifrplex YM-30 (Millipore), samples were frozen in liquid N$_2$ and stored at −70 °C. Concentrations of the protein are expressed as homodimer equivalents and were determined from the absorbance at 280 nm using an extinction coefficient calculated from the primary amino acid sequences of the MSH2 and MSH6 subunits (38). Nucleotide content of MutSα preparations was determined by bioluminescent assay as described previously (22). Parallel gel filtration procedures described above results in a significant reduction in the ADP level in MutSα isolates. The preparations used in this study contained about 0.07 µM of ADP per mole of homodimer and were free of detectable ATP.

5′-Biotinylated and nonbiotinylated oligonucleotides were obtained from Oligos Etc. (Wilsonville, OR). 5′-bi G-T helicoduplex and AT homoduplex-substi-tuted DNA were expressed as heterodimer equivalents and were determined from the absorbance at 280 nm using an extinction coefficient calculated from the primary amino acid sequences of the MSH2 and MSH6 subunits (38). Nucleotide content of MutSα preparations was determined by bioluminescent assay as described previously (22). Parallel gel filtration procedures described above results in a significant reduction in the ADP level in MutSα isolates. The preparations used in this study contained about 0.07 µM of ADP per mole of homodimer and were free of detectable ATP.

**Surface Plasmon Resonance Measurements—** MutSα-DNA interaction was monitored by surface plasmon resonance spectroscopy (SPRS) using a Biacore 2000 optical biosensor system. Streptavidin sensor chips (SA chip, Amersham Biosciences) were prepared according to recommendations of the manufacturer and derivatized with ~100 resonance units (RU) of biotinylated heteroduplex or homoduplex DNA. MutSα was injected at a flow rate of 20 µl/min in buffer comprised of 10 mM HEPES-ROH (pH 7.5), 1 mM EDTA, 5 mM MgCl$_2$, 150 mM NaCl, 0.005% surfactant P20. Experiments were performed at 25 °C, and samples were maintained at 4 °C prior to injection. Adenine nucleotide effects on association of MutSα with DNA were monitored by preincubation of the protein with indicated nucleotides at 4 °C for 5 min prior to injection.

Filter binding constants for interaction of MutSα and MutSα-nucleotide complexes with heteroduplex DNA were determined by SPRS by titration of chip-bound DNA with increasing concentrations of MutSα or MutSα-nucleotide complexes in the buffer system described above. Maximum binding values obtained after completion of the association phase of the reaction were plotted as a function of MutSα concentration, data were fit to a square hyperbola by nonlinear regression analysis, and the K$_D$ value was extracted (22).

**Filter Binding Assays—** Binding of ADP, ATP, and AMPPNP to MutSα was determined in 20 µl reactions containing 20 mM HEPES-KOH (pH 7.6), 150 mM KCl, 1 mM MgCl$_2$, MutSα, and [3H]ADP (31.9 Ci/mmol, PerkinElmer Life Sciences), [32P]ATP (65 Ci/mmol, PerkinElmer Life Sciences) or [35S]ATP ([35S]ATP, 60 Ci/mmol, ICN Biomed Inc.) as indicated. Labeled nucleotides were diluted prior to use with the corresponding unlabeled compound (ADP, U. S. Biochemical Corp.; ATP, Calbiochem-Novabiochem; or AMPPNP, Calbiochem-Novabiochem) to achieve the desired specific activity. Reactions were incubated for 4 min at 30 °C, diluted to 1 ml with room temperature reaction buffer, and immediately filtered through 15-mm, 0.45-µm nitrocellulose membranes (Schleicher & Schuell, BA-45), which had been pre-washed with reaction buffer. Filters were dried and radioactivity determined by liquid scintillation counting. For analysis of sequential binding of di- and triphosphates, MutSα was first incubated with a near saturating concentration of one nucleotide for 4 min prior to titration with the second nucleotide. Background nucleotide hydrolysis was less than 4%, and data shown were corrected for background values.

Human MutSα hydrolyzes ATP without at a low rate (17), potentially compromising interpretation of filter binding experiments using this nucleotide. To evaluate the significance of ATP hydrolysis, 8 µM MutSα was incubated under filter binding conditions (0 °C in binding buffer) with 8 µM [32P]ATP; the reaction was sampled as a function of time, samples were analyzed by chromatography on PEI-cellulose plates (EM Science, Gibbstown, NJ) as described (40), and radioactivity was quantitated using an Amersham Biosciences PhosphorImager. This analysis indicated that hydrolysis under filter-binding conditions occurred at a rate of 1.6% per min. In a second approach 8 µM MutSα was preincubated with 8 µM [32P]ATP for 4 and 8 min at 0 °C, and MutSα-[32P]ATP complexes were collected on filters. Filter-bound nucleotide was eluted by 2-h incubation in 10 mM EDTA and 0.2% sodium dodecyl sulfate at room temperature, and samples were analyzed by thin layer chromatography as described above. Recovered radioactivity was >80% ATP/S for both 4- and 8-min incubations. In similar experiments, 10 µM MutSα was incubated at 0 °C with 50 µM [32P]AMPPNP. No hydrolysis of the ATP analogue was detected after 30 min of incubation.

**ATP Hydrolysis by MutSα and Nucleotide Occupancy under Steady-State Conditions—** Reactions (20 µl) contained 10 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 10 µM MutSα, 100 µM [α-32P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences) preincubated with 1 µM each Pefabloc, E64, aprotinin, leupeptin, and pepstatin A. After washing with 40 µl of starting buffer, the column was eluted under filter binding conditions (0 °C in binding buffer) with 8 µM [32P]ATP; the reaction was sampled as a function of time, samples were analyzed by chromatography on PEI-cellulose plates (EM Science, Gibbstown, NJ) as described (40), and radioactivity was quantitated using an Amersham Biosciences PhosphorImager. This analysis indicated that hydrolysis under filter-binding conditions occurred at a rate of 1.6% per min. In a second approach 8 µM MutSα was preincubated with 8 µM [32P]ATP for 4 and 8 min at 0 °C, and MutSα-[32P]ATP complexes were collected on filters. Filter-bound nucleotide was eluted by 2-h incubation in 10 mM EDTA and 0.2% sodium dodecyl sulfate at room temperature, and samples were analyzed by thin layer chromatography as described above. Recovered radioactivity was >80% ATP/S for both 4- and 8-min incubations. In similar experiments, 10 µM MutSα was incubated at 0 °C with 50 µM [32P]AMPPNP. No hydrolysis of the ATP analogue was detected after 30 min of incubation.
RESULTS

MutS Binding of MutS in Nonhydrolyzable ATP Analogues

Fluorescent Measurements—Affinities of MutS for the fluorescent nucleotide derivatives BODIPY TR (2′ or 3′)-ADP and BODIPY FL (2′ or 3′)-AMPPNP (Molecular Probes, Inc.) were determined by anisotropy measurement. Reactions contained MutS and nucleotide as indicated in 20 mM HEPES-KOH (pH 7.5), 150 mM KCl, 5 mM MgCl2, and 10% (v/v) glycerol. Measurements were performed at 4°C using an SLM-AMINCO 8100 Series 2 spectrofluorometer fitted with calcite polarizers using the L-format. The G factor was determined once for each experiment. BODIPY FL (2′ or 3′)-AMPPNP was excited at 480 nm, and maximal emission was observed at 514 nm. Excitation of BODIPY TR (2′ or 3′)-ADP was at 590 nm with peak emission occurring at 620 nm. Fluorescence anisotropy was calculated using software provided with the instrument.

Because there is significant spectral overlap between the emission spectrum of BODIPY FL (2′ or 3′)-AMPPNP and the excitation spectrum of BODIPY TR (2′ or 3′)-ADP (41), simultaneous binding of the two nucleotides to MutS was evaluated by energy transfer. Energy transfer between BODIPY FL (2′ or 3′)-AMPPNP and the BODIPY TR (2′ or 3′)-ADP acceptor was monitored by exciting at 490 nm and determining the emission at 620 nm. Reaction conditions were as described above for anisotropy measurements using protein and nucleotide concentrations as indicated. Efficiency of energy transfer was determined by the Ratio A method (42, 43) as described previously (41) except that the experimentally determined value for the term e490 R0 TR(490 nm)/R0 TR(590 nm) under the buffer conditions used in this work was 0.048. A value of for the Förster radius, R0, which is the distance where energy transfer is 50% efficient, was determined to be 38.1 Å (41). This R0 value and the measured efficiency of energy transfer were used to estimate the distance between 4- and triphosphate binding centers of MutS as described (43).

results show that MutS also binds ADP and the nonhydrolyzable ATP analogue AMPPNP with similar high affinities.

MutS Has One ADP and One ATP Binding Site per Heterodimer—Because each subunit of the MSH2-MSH6 heterodimer contains a Walker nucleotide binding consensus (45–47), the protein may potentially exist in nine different nucleotide occupancy states (48). To address this issue we have examined the stoichiometry of the interactions of the protein with ADP and nonhydrolyzable ATP analogues. In these experiments a fixed nucleotide concentration was titrated with MutS (Fig. 2) or a fixed MutS concentration was titrated with nucleotide (Fig. 3). In both types of experiment, the concentration of the fixed component was well above the corresponding Kd to ensure that binding was largely stoichiometric. Data were fit to Equation 1 or 2, which assume a single class of binding site. Given the knowledge of the Kd and the total concentration of the fixed component, the stoichiometry of binding can be determined. For example, in a titration of a fixed ADP concentration with MutS, this analysis yields a value for A0, the apparent ADP concentration at the equivalence point. Division of A0 by the total nucleotide concentration yields the stoichiometry of interaction.

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As shown in Fig. 2, Equations 1 and 2 provided excellent fits to the binding behavior observed when fixed concentrations of ADP, ATPγS, BODIPY TR (2′ or 3′)-ADP, or BODIPY FL (2′ or 3′)-AMPPNP were titrated with MutSa. Good fits to the experimental data were also obtained when MutSa was titrated with ADP and ATPγS (Fig. 3). The stoichiometries of interaction determined by this method are 0.98–1.13 mol of ADP, 0.96–1.03 mol of ATPγS, 1.16 mol of BODIPY TR (2′ or 3′)-ADP, and 0.82 mol of BODIPY FL (2′ or 3′)-AMPPNP per mol of the MSH2-MSH6 heterodimer (Figs. 2 and 3). Inasmuch as each subunit of MutSa contains one nucleotide binding site (17), these observations show that only half of the expected number of binding sites are available for high affinity occupancy by ADP or nonhydrolyzable ATP analogues. Previous studies have shown that isolates of human MutSα can contain significant amounts of bound ADP (25). However, the preparations used in this work contained only 0.07 mol of ADP per mol of MutSa and were free of detectable levels of ATP (see “Experimental Procedures”). Consequently, the nucleotide binding stoichiometries observed here can not be due to the fact that one binding site is already occupied in MutSa preparations used.

To test the possibility that these binding stoichiometries might be due to differential specificity of the two MutSa nucleotide binding sites, the protein was tested for its ability to simultaneously bind ADP and ATPγS. As shown in Fig. 3, a binding site for ATPγS was available on the MutSa heterodimer after the ADP binding site had been filled, and an ADP binding site was available when the triphosphate site was filled. Thus, MutSa heterodimer contains two nucleotide binding sites that display differential specificities for ADP and ATP. Because we have been unable to determine the affinities of MutSa for ADP and for ATPγS under conditions where the other nucleotide binding site is occupied, it is possible that occupancy of the triphosphate site modulates the affinity of the diphosphate site, and vice versa, as has been observed with bacterial MutS (41). However, the data of Fig. 3 imply that binding affinities for the second nucleotide must also be in the low micromolar range.

The two MutSa nucleotide binding sites display differential specificities for adenine nucleoside di- and triphosphate, but it is possible that high concentrations can drive ADP into the triphosphate site and vice versa. This question was addressed by titrating 10 μM MutSa with ADP or ATPγS. As judged by filter binding assay, occupancy in each case was restricted to a single site at nucleotide concentrations as high as 100 μM (Fig. 4). Although these findings indicate that one binding site is highly specific for ADP and the other highly specific for triphosphate, it is important to note that ADP-MutSa-ADP and

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**Fig. 2.** MutSa has one ADP and one ATP binding site per heterodimer. Binding of MutSa to [3H]ADP (panel A) and [35S]ATPγS (panel B) was determined by filter binding assay, while binding to BODIPY TR (2′ or 3′)-ADP (panel C) or BODIPY FL (2′ or 3′)-AMPPNP (panel D) was determined by fluorescence anisotropy (“Experimental Procedures”). Nucleotide concentration was 10 μM for [3H]ADP, [35S]ATPγS, and BODIPY TR (2′ or 3′)-ADP (note that the abscissa for panel C differs from the others). Because these concentrations are 6 to 50 times the Kd of the nucleotides (Fig. 1), interaction is near stoichiometric. Error bars are ± one S.D. (three determinations). After correction for retention efficiencies (Fig. 1), data with radiolabeled nucleotides were fit to Equation 1 using Kd values from Fig. 1 with RE set to 1.0 and A0 floated. Results obtained with fluorescent nucleotides were fit to Equation 2 with A0 and with amplitude floated. This analysis yielded values for A0 of 9.8 μM (ADP, actual = 10 μM), 9.6 μM (ATPγS, actual = 5 μM), and 8.2 μM (BODIPY FL (2′ or 3′)-AMPPNP, actual = 10 μM). These values therefore correspond to stoichiometries of 0.98 mol of ADP per mol of MutSa (heterodimer equivalent), 0.96 mol of ATPγS per mol, 1.16 mol of BODIPY TR (2′ or 3′)-ADP per mol, and 0.82 mol of BODIPY FL (2′ or 3′)-AMPPNP per mol.
were fit to Equation 1 as described in Fig. 2 except that ATP analogues display differential specificities for ADP and nonhydrolyzable

\[ \text{ATP} \]

under filter binding conditions (but ATP

\[ \text{Kd} \]

Fig. 1. As described in the text, this assumption may be invalid, because

\[ \mu \text{mol of ADP per mol of the MSH2}/H18528 \]

experiments using this nucleotide. The experiments described

\[ \text{Kd} \]

drolysis does not compromise the interpretation of binding

\[ \text{mol of ATP} \]

that, in titrations with a second nucleotide, the concentration of the first

\[ \text{M} \]

alternate order of nucleotide addition is shown in the

\[ \text{error bars} \]

Experimental Procedures

\[ \text{Error bars} \]

Sequential binding with the second nucleotide also employed

\[ \text{one S.D.} \]

MutS

\[ \text{s} \]

nucleotide binding sites.

\[ \text{binding ex} \]

Simultaneous occupancy of the MutS

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MutS

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Simultaneous Occupancy of the MutS\text{ADP and ATP Binding Sites by Fluorescence Resonance Energy Transfer—The filter binding studies described demonstrate differential specificities of the two MutS\text{ nucleotide binding sites and indicate that the di- and triphosphate sites can be simultaneously occupied. The possibility of simultaneous occupancy was confirmed by application of fluorescence resonance energy transfer. MutS binds BODIPY TR (2' or 3')-ADP and BODIPY FL (2' or 3')-AMPPNP with high affinity and specificity (Figs. 1 and 2). We have exploited the fact that these two fluorescent derivatives form an energy transfer pair (41) to confirm the idea that the di- and triphosphate binding sites of MutS can be simultaneously occupied. Because energy transfer is proportional to the inverse sixth power of the distance separating the two probes, interaction between the fluorophores is detectable only if the molecules in question are in proximity (49). Emission spectra of a solution of 5 \( \mu \text{M} \) BODIPY FL (2' or 3')-AMPPNP (donor) and 5 \( \mu \text{M} \) BODIPY TR (2' or 3')-ADP (acceptor) were determined upon donor excitation at 490 nm in the presence and absence of 5 \( \mu \text{M} \) MutS. As shown in Fig. 5 (upper panel), the presence of MutS decreased the emission intensity of the donor at 514 nm and enhanced emission at 620 nm, indicative of sensitized emission of the acceptor, and these effects were abolished by inclusion of excess ATP (Fig. 5, lower panel). These observations indicate energy transfer between the two fluorophores and imply simultaneous occupancy of MutS di- and triphosphate binding sites. The efficiency of energy transfer in this system is 33%, corresponding to a distance of 46 Å between the fluorophores when bound to MutS in the absence of DNA (“Experimental Procedures”). Because calculation of this distance assumes 100% occupancy of di- and triphosphate, this value is an upper limit for the separation distance of the fluorescent tags on the AMPPNP donor and the

\[ \text{MutS} \]
ADP acceptor. These results are similar to those that have been obtained with bacterial MutS (41).

MutSα Nucleotide Occupancy under Hydrolytic Conditions—Because filter binding and energy transfer experiments described above indicate that the di- and triphosphate binding sites of MutSα can be simultaneously occupied by ADP and a nonhydrolyzable ATP analogue, we have examined the nature of nucleotide occupancy during the first ATP hydrolytic turnover. Hydrolysis of 50 μM (not shown) or 100 μM ATP (Fig. 6) by 10 μM MutSα at 0 °C was characterized by a pre-steady-state burst of 0.462 ± 0.14 mol of ADP/mol of MutSα (50 μM ATP, four determinations) or 0.464 ± 0.18 mol of ADP/mol of MutSα (100 μM ATP, three determinations). The burst was followed by a steady-state hydrolytic phase with a kcat of 0.50 ± 0.04 min⁻¹ (50 μM ATP) or 0.45 ± 0.04 min⁻¹ (100 μM ATP). The nature of these results was independent of the manner in which the reaction was initiated; identical results were obtained upon mixing of MutSα with ATP-Mg²⁺ or when preformed MutSα-ATP complexes were mixed with Mg²⁺ (not shown).

MutSα nucleotide complexes were collected on nitrocellulose membranes over the course of the first 2 min (1.4 half-lives) of hydrolysis, and composition of MutSα-bound nucleotide was determined. As shown in Fig. 6, the molar ratio of bound ADP:ATP was essentially invariant over this period, with a mean value of 1.61 ± 0.21. Because the burst kinetics observed imply that the rate-limiting step for turnover occurs after hydrolysis, these findings strongly suggest that ADP and ATP are simultaneously bound to a substantial fraction of MutSα heterodimers under hydrolytic conditions.

Adenine Nucleotide Effects on MutSα Affinity for Heteroduplex DNA—Previous studies have shown that ADP does not significantly affect the affinity of MutSα for a mismatch, but the affinity of the protein for a mispair is reduced in the presence of ATP or nonhydrolyzable ATP analogues (7, 8, 17, 19–21, 23, 25). Because the experiments described here permit prediction of nucleotide occupancy of the free protein, we have evaluated the potential consequences of distinct nucleotide occupancy states on MutSα-heteroduplex interaction. In these studies, the protein was equilibrated with the indicated nucleotide(s) at 4 °C for 5 min prior to analysis of interaction at 25 °C with a 31-bp G-T heteroduplex or A-T homoduplex by surface plasmon resonance spectroscopy.

As shown in Fig. 7 and Table I, the affinity of MutSα for a 31-bp G-T heteroduplex remains constant at 20 ± 6 nM over an ADP concentration range of 15–1000 μM, an affinity identical to that observed in the absence of nucleotide (Fig. 7 and Table I). By contrast, heteroduplex affinity of the protein was reduced substantially in the presence of ATPγS (Kα = 130 ± 22 nM for nucleotide concentrations of 15–1000 μM) or in the presence of AMPPNP (Kα = 380 ± 64 nM for AMPPNP concentrations of 50–1000 μM). A reduction in steady-state heteroduplex affinity was also observed under hydrolytic conditions in the presence of a near saturating ATP concentration (Kα of 100 ± 18 nM for concentrations of 100–500 μM). Effects of preincubation of MutSα with equivalent concentrations of ADP and AMPPNP or with ADP and ATPγS were also assessed. Under these conditions heteroduplex affinities observed were intermediate between those obtained in the presence of only ADP or only the ATP analogue. (Table I, 88 ± 11 nM for ADP and ATPγS, and

Fig. 6. MutSα nucleotide occupancy under hydrolytic conditions. Hydrolysis of ATP (●) 100 μM) by MutSα (10 μM) was performed at 0 °C (Experimental Procedures). The magnitude of the pre-steady-state burst was 4.7 μM, and the steady-state rate of hydrolysis 0.08 μM s⁻¹ (least squares fit). Parallel reactions were sampled, and MutSα-nucleotide complexes were collected on nitrocellulose membranes. After elution from filters, nucleotide composition was analyzed as described under “Experimental Procedures,” and the ratio of bound ADP/ATP (□) was determined. Filter binding assay was used to monitor total MutSα-bound nucleotide over the course of experiments like those shown. Assuming filter retention efficiencies of 0.59 for MutSα-ADP and MutSα-ATP complexes (“Results”), total bound nucleotide was essentially invariant over the 15- to 120-s time period at 17 ± 0.6 μM.
MutSα Nucleotide Binding Sites

**Fig. 7. Effects of adenine nucleotides on MutSα interaction with hetero- and homoduplex substrate.** MutSα binding to a 31-bp G-T heteroduplex (○) or an otherwise identical A-T homoduplex (□) was examined by SPRS as a function of MutSα concentration in the presence of 250 μM adenine nucleotides as indicated (“Experimental Procedures”): A, ADP; B, ATP; C, AMPPNP; D, ATPγS; E, ADP and AMPPNP (250 μM each); F, ADP and ATPγS (250 μM each). Curves shown for heteroduplex data were obtained by nonlinear least squares regression fit to a square hyperbola. $K_D$ values obtained from fits to these data sets and from data sets of related experiments are summarized in Table I. Dashed lines in E and F correspond to expected heteroduplex binding profiles if the potential formation of the ternary ADP-MutSα AMPPNP (or ADP-MutSα ATPγS) complex is ignored, and nucleotide binding by MutSα is restricted to formation of binary MutSα ADP and MutSα AMPPNP (or MutSα ATPγS) complexes, with these species binding independently and competitively to heteroduplex. These profiles were calculated as follows: In the SPRS method used in these experiments, the total concentration of free MutSα and its nucleotide complexes after achievement of equilibrium corresponds to the input concentration of the protein $[M]_{total}$. If nucleotide binding to MutSα is restricted to formation of binary complexes with ADP or a triphosphate analogue, then $[M]_{total} = [M] + [MD] + [MT]$, where $[M]$, $[MD]$, and $[MT]$ correspond to free concentrations of MutSα, MutSα ADP, and MutSα AMPPNP (or MutSα ATPγS), respectively. This relationship permits calculation of $[M]$, $[MD]$, and $[MT]$ as:

$$[M] = \frac{K_D K_T [M]_{total}}{K_D K_T + K_D [D] + K_T [T]}; [MD] = \frac{K_D K_T [M]_{total}}{K_D K_T + K_D [D] + K_T [T]}, \text{ and } [MT] = \frac{K_T [T] [M]_{total}}{K_D K_T + K_D [D] + K_T [T]}.$$

where $[D]$ is the concentration of ADP, $[T]$ the concentration of AMPPNP (or ATPγS), and $K_D$ and $K_T$ are dissociation constants for the MutSα-ADP and the MutSα-AMPPNP/ATPγS complexes, respectively (Fig. 1). For a model invoking independent and competitive binding of MutSα, MutSα ADP, and MutSα AMPPNP/ATPγS to heteroduplex, the extent of DNA binding in resonance units is given by Equation 3,

$$RU = RU_{max} \times \frac{K_D K_T [M] + K_D K_T [MD] + K_D K_T [MT]}{K_D K_T [M] + K_D K_T [MD] + K_D K_T [MT]} \quad (Eq. 3)$$

where $RU_{max}$ is the maximum observed binding from hyperbolic fit of experimental data, $K_D$ is the equilibrium constant governing dissociation of the MutSα-ADP complex from heteroduplex DNA (panel A and Table I), $K_T$ is the equilibrium constant governing dissociation of the MutSα-AMPPNP/ATPγS complex from DNA (panels C and D, and Table I), and $K_D$ is the dissociation constant of the MutSα-heteroduplex complex (Table I). Substitution of $[M]$, $[MD]$, and $[MT]$ values from the equations above into this relationship for RU yields the predicted binding profiles shown by dashed lines in panels E and F.

122 ± 10 nM for ADP and AMPPNP where di- and triphosphates were present equal concentrations over the range of 100–1000 μM.

We think it unlikely that the intermediate heteroduplex affinities observed in the presence of ADP and a triphosphate analogue are due to production of a mixed population of MutSα ADP and MutSα AMPPNP (or MutSα ATPγS) binary complexes under these conditions. If this were the case, then binding profiles in the presence of the two nucleotides could be calculated based on the independent binding of MutSα ADP and MutSα AMPPNP (or MutSα ATPγS) complexes to heteroduplex DNA. As shown in Fig. 5 (E and F), the binding behavior predicted for a mixed population of binary MutSα nucleotide complexes deviates significantly from experimental binding
curves obtained in the presence of ADP and AMPPNP (or ATPγS). For example, if it is assumed that in the presence of equivalent, saturating concentrations of ADP and AMPPNP, nucleotide binding by MutSα is restricted to formation of MutSα:ADP and MutSα:AMPPNP complexes, then the affinities of the protein for these two nucleotides (1.0 and 5.0 μM, respectively (Fig. 1)) predict that 80% of MutSα will be present as the ADP complex, with the remainder largely MutSα:AMPPNP. Because the heteroduplex affinity of the MutSα:ADP complex is 20 times that of MutSα:AMPPNP (Table 1), this model predicts that nearly all the heteroduplex binding observed in the presence of the two nucleotides would be due to MutSα:ADP, which is characterized by a mean dissociation constant of 20 ± 6 nM. This value is considerably less than that observed in the presence of both nucleotides (mean value 122 ± 10 nM). Similar arguments apply to the deviation of experimental binding isotherms observed in the presence of ADP and ATPγS from those predicted by a model that restricts nucleotide occupancy to formation of MutSα:ADP and MutSα:ATPγS. Whereas difficult to reconcile with a model that invokes a mixed population of binary complexes of MutSα with ADP or a triphosphate analogue, the experimental binding curves obtained in the presence of the two nucleotides can be understood in terms of the results described above, which indicate that the ADP-MutSα:AMPPNP-ATPγS ternary complex is highly populated in solution when ADP and a nonhydrolyzable triphosphate analogue are both present.

### DISCUSSION

Structural analysis of the bacterial MutS-heteroduplex complex has indicated intrinsic asymmetry in the dimeric protein, with one subunit preferentially interacting with the mismatch (50, 51). In the complex obtained with the *Escherichia coli* protein, the nucleotide binding site of mismatch recognition subunit is occupied by ADP and the other site is empty (50). Analysis of nucleotide binding by *E. coli* MutS has demonstrated that this asymmetry is also evident in solution in the absence of DNA: the two nucleotide binding sites within the dimer display differential specificity for ADP or nonhydrolyzable ATP analogues, and di- and triphosphate sites can be simultaneously occupied (41). Furthermore, isolates of hydrolytically defective MutS E894A have been shown to contain 1 mol of bound ATP per dimer equivalent and sub-stoichiometric quantities of ATP (0.1–0.6 mol/mol), suggesting that the ATP-MutS-ADP complex may occur within the cell (39). The results described here indicate that differential specificity and the potential for simultaneous occupancy are conserved in human MutSα.

While this report was in preparation, Antony and Hingorani (52) demonstrated that yeast MutSα binds only one molecule of ADP but is capable of binding two molecules of ATPγS with different affinities, although simultaneous occupancy of di- and triphosphate sites was not addressed in these studies. At sub-stoichiometric concentrations, AMPPNP has been shown to potentiate ADP binding by bacterial MutS, but high concentrations of the triphosphate analogue can displace ADP from the protein (41), indicating that, as in the case of yeast MutSα, bacterial MutSα may also be able to bind two molecules of ATP with different affinities. By contrast, we have been unable to detect binding of more than one molecule of ATPγS to human MutSα using a filter assay similar to that employed by Antony and Hingorani with the yeast protein (52). However, as noted under “Results,” it is possible that weak binding of a second ATPγS molecule to the human MutSα:ATPγS complex might go undetected in our experiments due to potential perturbation of the interaction during collection of the complexes on filters.

As observed previously with bacterial MutS and yeast MutSα (40, 52), we have shown that ATP hydrolysis by the human protein is accompanied by a pre-steady-state burst of ADP formation, implying that the rate-determining step for turnover occurs after the chemical step. In contrast to results obtained with the yeast protein, where an ADP burst of 1 per MSH2-MSH6 heterodimer has been reported (52), the burst described here is sub-stoichiometric, 0.5 mol of ADP per mol of heterodimer. Because the human MutSα preparations we have used bind ADP and nonhydrolyzable ATP analogues with a stoichiometry near unity, we think it unlikely that this effect is due to the presence of a significant fraction of inactive protein. However, these results may indicate that the functional form of human MutSα is a higher oligomer, as has been suggested for bacterial MutS (53).

MutS homologues are members of the ABC superfamily. Nonequivalence of the nucleotide binding domains of P-glycoprotein (P-gp) and multidrug resistance protein 1 (MRP1), which are also members of this family, has been documented. However, the apparent asymmetry of the nucleotide binding centers within these two classes of drug transporters is thought to reflect distinct modes of action. The two nucleotide binding sites of P-gp are believed to be functionally equivalent, alternately hydrolyzing ATP during the catalytic cycle, and inactivation of one binding site abolishes ATPase activity of the transporter (54, 55). By contrast, the nucleotide binding domains of MRP1 do not appear to contribute equally to activity of the protein: inactivation of nucleotide binding domain 2 abolishes drug transport, but inactivation of nucleotide binding domain 1 results in only partial loss of activity (56, 57). It is noteworthy in this regard that, like MutS/MutSα, MRP1 has been shown to bind di- and triphosphate simultaneously (56, 57), and, as observed with MRP1, inactivation of the nucleotide binding site within the MSH6 subunit of human MutSα results in a more severe ATPase defect than does inactivation of the nucleotide binding center within MSH2 (17). These observations suggest that the functional roles of ATP binding and

### Table 1

| Nucleotide | $K_d$ (μM) |
|------------|------------|
| None       | 19 ± 1     |
| ADP        | 15 ± 2     |
| 60 μM      | 18 ± 2     |
| 100 μM     | 27 ± 6     |
| 250 μM     | 27 ± 2     |
| 1 mM       | 15 ± 2     |
| ADP and ATPγS | 73 ± 4  |
| 100 μM each| 91 ± 4     |
| 250 μM each| 100 ± 15   |
| 500 μM each| 88 ± 8     |
| 1 mM each  | 109 ± 8    |
| ADP and AMPPNP | 60 ± 8 |
| 100 μM each| 126 ± 6    |
| 250 μM each| 130 ± 13   |
| 500 μM each| 130 ± 25   |
| 1 mM each  | 109 ± 10   |
| ATP        | 84 ± 15    |
| 100 μM     | 109 ± 8    |
| 250 μM     | 120 ± 15   |
| 500 μM     | 150 ± 4    |
| 1 mM       | 120 ± 22   |
| ATPγS      | 150 ± 11   |
| 50 μM      | 310 ± 24   |
| 250 μM     | 420 ± 70   |
| 1 mM       | 420 ± 49   |
hydrolysis by MutS may be more similar to those of the MRP1 class of ABC transporters than those exemplified by P-gp.

There is extensive evidence indicating interaction of the ATP hydrolytic centers and the heteroduplex binding site of MutS homologues. Thus, heteroduplex, but not homoduplex, DNA alters the rate-limiting step for ATP hydrolytic turnover with E. coli MutS and yeast MutSα (40, 52). Although ADP does not significantly alter the affinity of human MutSα for a mispair, the nucleotide enhances the kinetics of heteroduplex association and dissociation (21, 25). Moreover, the affinity of MutS homologues for a mismatch is reduced in the presence of ATP or nonhydrolyzable ATP analogues (7, 8, 17). MutS homologues for a mismatch is reduced in the presence of di- and triphosphate forms of MutS/MutSβ, whereas di- and triphosphate forms of the protein (Fig. 7), an effect we have demonstrated that, in solution, human MutSα readily forms a ternary complex with ADP and a nonhydrolyzable ATP analogue, with affinities for each nucleotide being in the low micromolar range. We have also shown that, in the presence of equimolar concentrations of ADP and a nonhydrolyzable ATP analogue, MutSα interacts with heteroduplex in a manner distinct from that expected for a mixed population of di- and triphosphate forms of the protein (Fig. 7), an effect we attribute to the high population of ADP-MutSα-AMPNNP and ADP-MutSα-ATPγS species in solution. Interestingly, the heteroduplex affinities observed for these ternary complexes are similar to those obtained with MutSα under conditions of ongoing ATP hydrolysis (Table I), conditions where the ADP-MutSα-ATP complex comprises a significant fraction of the protein in solution (Fig. 6). These findings indicate that the ADP-MutSα-ATP complex has a finite lifetime, and hence that this species should be considered as a significant intermediate during ATP hydrolytic turnover by the free protein and, perhaps, during the course of its interaction with DNA.

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