Hydrolytically Deficient MutS E694A Is Defective in the MutL-dependent Activation of MutH and in the Mismatch-dependent Assembly of the MutS-MutL-Heteroduplex Complex*

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The roles of ATP binding and hydrolysis by MutS in mismatch repair are poorly understood. MutS E694A, in which Glu-694 of the Walker B motif is substituted with alanine, is defective in hydrolysis of bound ATP and has been reported to support MutL-dependent activation of the MutH d(GATC) endonuclease in a trans DNA activation assay (Junop, M. S., Obmolova, G., Rausch, K., Hsieh, P., and Yang, W. (2001) Mol. Cell 7, 1–12). Because the MutH trans activation assay used in these previous studies was characterized by high background and low efficiency, we have re-evaluated the activities of MutS E694A. In contrast to native MutS, which can be isolated in a nucleotide-free form, purified MutS E694A contains 1.0 mol of bound ATP per dimer equivalent, and substoichiometric levels of bound ADP (0.08–0.58 mol/dimer), consistent with the suggestion that the ADP-MutS-ATP complex comprises a significant fraction of the protein in solution (Bjornson, K. P. and Modrich, P. (2003) J. Biol. Chem. 278, 18557–18562). In the presence of Mg2+, endogenous ATP is hydrolyzed with a rate constant of 0.12 min−1 at 30 °C, and hydrolysis yields a protein that displays increased specificity for heteroduplex DNA. As observed with wild type MutS, ATP can promote release of MutS E694A from a mismatch. However, the mutant protein is defective in the methyl-directed, mismatch-and MutL-dependent cis activation of MutH endonuclease on a 6.4-kilobase pair heteroduplex, displaying only 1 to 2% of the activity of wild type MutS. The mutant protein also fails to support normal assembly of the MutS/MutL-DNA ternary complex. Although a putative ternary complex can be observed in the presence of MutS E694A, assembly of this structure displays little if any dependence on a mismatched base pair.

Mismatch recognition by MutS initiates mismatch repair in bacterial cells (1–3). MutS recruits MutL to the heteroduplex in an ATP-dependent fashion (4–6), and assembly of the MutL-MutS-heteroduplex ternary complex is sufficient to activate the MutH endonuclease, which incises the unmethylated strand at a hemimethylated d(GATC) strand signal (7). The ensuing strand break, which may reside either 3′ or 5′ to the mismatch, serves as the site for initiation of excision by a system comprised of DNA helicase II and an appropriate 3′ to 5′ or 5′ to 3′ single-strand specific exonuclease (8–13). This bidirectional excision capability implies that the mismatch repair system must establish the relative orientation of the mismatch and strand break that directs the reaction. This is necessary to ensure loading of a 3′ to 5′ excision system when the nick resides 3′ to the mispair and a 5′ to 3′ system when it is located 5′ to the mismatch.

In addition to its mismatch binding site, MutS has a carboxyl-terminal ATPase that is required for function of the protein in mismatch repair (7, 14, 15), and this element is conserved in MutS homologs in higher cells (16–20). Structural studies have shown that MutS is a member of the ABC (adenine nucleotide binding cassette) family (21–23), which is largely comprised of proteins that couple the energy of ATP hydrolysis to transport of molecules across biological membranes (24, 25).

Several models for ATPase function in MutS homolog action have been suggested. One class of mechanism is based on a variety of observations indicating that in the presence of ATP, MutS homologs can leave a mismatch by movement along the helix (26–29). This movement is postulated to link mismatch recognition to activation of downstream events at the strand break that directs excision, and can in principle account for the orientation-dependent loading of the excision system at the strand break that is necessitated by the bidirectional nature of the repair system. Two types of mechanisms have been proposed to explain ATP-dependent movement of MutS homologs along the DNA contour. One model posits that movement depends on ATP hydrolysis by the DNA-bound protein. The alternate molecular switch model postulates a G-protein like mechanism whereby binding of a MutS-ADP complex to a mismatch promotes ATP exchange for ADP, with the resulting MutS-ATP complex diffusing freely along the helix. Evidence consistent with both the hydrolytic model (26, 27, 29–31) and the molecular switch model (28, 32) is available.

A distinct role for ATP binding and hydrolysis has been proposed based on use of a trans assay for MutH activation (23). In this work, a mismatch on one oligonucleotide duplex was shown to lead to MutH activation and d(GATC) incision on a second synthetic duplex in a reaction dependent on MutL and MutS. These observations have led to the suggestion that once mismatch recognition occurs, MutS remains bound to the mispair during the course of repair, with mismatch-strand signal interaction mediated by DNA bending (23). In this proposal, ATP binding and hydrolysis by DNA-bound MutS functions in a kinetic proofreading mechanism that serves to verify mismatch recognition. Once bound to a putative mismatch, MutS...
is postulated to bind ATP. If the complex involves a misrecognition event, MutS is released and ATP is hydrolyzed to ADP.

However, if MutS resides at a bona fide mismatch, ATP binding serves to verify mismatch recognition and is sufficient for activation of downstream repair activities in the absence of hydrolysis (23). The latter feature of the model is based on the experimental observation that MutS E694A, which is defective in ATP hydrolysis, supports MutH activation in the trans assay. However, the trans activation reactions on which this conclusion is based are several hundredfold less efficient than cis activation that occurs on 6,400-base heteroduplexes that have been used to study methyl-directed repair in vivo and in vitro (23). When normalized to equivalent MutH, the ratio of the cis reaction is about 0.1 min⁻¹ per mol of MutH (7), whereas the corresponding value for the trans reaction calculated from the data of Junop et al. (23) is about 0.0002 min⁻¹ per mol. Furthermore, in contrast to the cis reaction (7), the trans activation reaction employed in these earlier studies (23, 34) is subject to high background d(GATC) cleavage in the absence of MutS or a mismatched base pair (~45% of that observed when these required repair components are present). Corresponding values for the cis assay are <3 and <10%, respectively (7). For these reasons we have re-evaluated the ability of MutS E694A to support MutH activation using the cis assay. In contrast to results obtained with the trans assay, we find MutS E694A to be defective in MutH activation, a defect that is probably due to its inability to support mismatch-dependent assembly of a MutL-MutS heteroduplex complex.

**EXPERIMENTAL PROCEDURES**

**Proteins and DNAs**—The expression plasmid MutSE694A/pET15b for a His-tagged version of *Escherichia coli* MutS E694A (23) was generously provided by Peggy Hsieh (NIDDK, National Institutes of Health). An expression plasmid for a non-tagged version of the mutant protein was constructed by insertion of a Ncol-BamHI fragment containing COOH-terminal MutS E694A sequences into Ncol- and BamHI-cleaved pVE661. The latter plasmid is a derivative of pET3a containing a 934-bp NdeI fragment that corresponds to NH2-terminal mutS sequences beginning with the initiating ATG located within the NdeI recognition site. Sequence analysis of the entire mutS gene of the resulting plasmid, SpET3a-E694A, confirmed the presence of the E694A mutation within an otherwise native sequence that lacks the His6 tag. *E. coli* strain BL21(DE3) mutS::Tn10 pLYS3 containing SpET3a-E694A was grown at 37°C in a BioFlo 4500 fermentor (New Brunswick Scientific) to an A590 of 5.6 in 22 liters of supplemented L broth (0.1% NaCl, pH 7.4, 1% (v/v) glycerol, 22 g/liter yeast extract, 11 g/liter tryptone, 10 g/liter NaCl, 4 mg/liter thymine, and 10 mg/liter thiamine) with 0.4 aeration. After addition of isopropyl-1-thio-galactopyranoside to 0.5 mM, growth was continued for 2 h at 37°C (final A590 = 13.8) and cells were harvested by centrifugation. Use of Method A the protein was incubated in potassium phosphate, pH 7.4, 100 mM KCl, 1 mM dithiothreitol, 1 mM diethiothreitol, and 1 units of bovine serum albumin.

**Nucleotide Removal from MutS E694A**—Two methods were used to deplete MutS E694A of endogenous nucleotide. In Method A the protein was incubated at room temperature for 5 min in 1.5-mL reactions containing 20 mM Tris-HCl, pH 7, 100 mM KCl, 1 mM dithiothreitol, 1 mM diethiothreitol, and 1 units of surfactant P20 (Buffer B) containing 5 mM MgCl2 and nucleotides as indicated (7). After incubation the column was dialyzed at 4°C for 2 h against 2 liters of 20 mM KPO4, pH 7.4, 150 mM KCl, 1 mM dithiothreitol, and 1 units of bovine serum albumin. Assay response was linear over ADP and ATP concentration ranges of 1.5 to 200 nM and 3 to 400 nM, respectively.

**ATPase, Mismatch Repair Assays, and Surface Plasmon Resonance Spectroscopy**—Steady state MutS ATPase hydrolytic parameters and activation of the MutH d(GATC) endonuclease were determined as described previously (36, 37), except that ATPas reactions were performed in 25 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM MgCl2, 1 mM diethiothreitol, 0.1 mg/ml bovine serum albumin. Surface plasmon resonance spectroscopy (SPRS) was performed on a BIAcore 2000. Streptavidin SA sensor chips were chipped with ATPase activity of about 150 resonance units of bovine derivatized 41- or 201-bp heteroduplex and homoduplex as indicated. MutS or MutS E694A in 25 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 100 mM KCl, 0.05% surfactant P20 (Buffer B) containing 5 mM MgCl2 and nucleotides as indicated (7). After washing with 0.1 M glycine, pH 2.0, the chip was flowed across the sensor chip at 20 μl/min, the reaction was performed at 25°C, and samples were maintained at 4°C prior to injection. Chips were regenerated by a 20-μl injection of 0.5% sodium dodecyl sulfate. DNA binding isotherms for MutS and MutS E694A were determined by titration of chip-bound DNA with increasing concentrations of MutS or MutS E694A. Maximum binding values obtained

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1 The abbreviations used are: bis-Tris, 2-[bis(2-hydroxyethyl)aminol-2-(hydroxymethyl)propionate-1,3-diol; AMP-PNP, 5'-adenyl-β,γ-imidodiphosphate; ATPγS, adenosine 5'-O-(thiotriphosphate); SPRS, surface plasmon resonance spectroscopy.
in this manner were analyzed as a function of protein concentration as described previously (30).

RESULTS

MutS E694A Is Defective in ATP Hydrolysis and Purifies with One Bound ATP per Dimer Equivalent — It has been previously shown that substitution of Ala for Glu-694 renders His-tagged MutS defective in ATP hydrolysis (ATP) 

\[ K_m = 2.2 \mu M; k_{\text{cat}} = 0.02 \text{ min}^{-1} \text{ per monomer at } 23 ^\circ \text{C as compared with } 116 \mu M \text{ and } 2.15 \text{ min}^{-1} \text{ for the wild type protein (23)}. \]

We have confirmed the E694A hydrolytic defect for the His tag-free form of the mutant protein (\( K_m = 2 \mu M; k_{\text{cat}} = 0.040 \text{ min}^{-1} \text{ per monomer at } 30 ^\circ \text{C and } 0.060 \text{ min}^{-1} \) at 37°C for the mutant protein in the presence of 0.1 mM KCl and 5 mM MgCl\(_2\) as compared with wild type values of 24 ± 4 \mu M and 2.1 min\(^{-1}\) per monomer at 30°C and 29 ± 4 \mu M and 2.9 min\(^{-1}\) at 37°C; data not shown).

We have previously found that isolated MutS is free of ATP but can contain variable, substoichiometric quantities of bound ADP (<0.01–0.15 mol/mol of monomer) (36). Recently modified purification methods (Ref. 30 and “Experimental Procedures”) typically yield MutS isolates that are free of detectable nucleotide. Preparations isolated as described under “Experimental Procedures” contained <0.01 mol of adenine nucleotide per mol of MutS monomer. By contrast, near homogeneous preparations of MutS E694A contained 0.50 ± 0.03 mol of ATP and 0.04–0.29 mol of ADP/mol of monomer (four independent preparations; as noted under “Experimental Procedures,” chromatography on hydroxyapatite leads to partial resolution of E694A species according to their ADP content). The presence of the ATP in near homogenous isolates of the mutant protein presumably reflects the hydrolytic defect conferred by the E694A mutation. Because MutS exists as dimers and tetramers in solution and these oligomers harbor two classes of nucleotide binding sites that display specificity for ADP or ATP (22, 31, 38, 39), the simplest interpretation of the composition of bound nucleotide in MutS E694A preparations is that the triphosphate site is fully occupied in these isolates, but the diphosphate site is only partially populated. ATP and ADP in these preparations are tightly bound as 70–80% of both nucleotides were retained upon 7 h dialysis against buffers containing 1 mM EDTA and 150 mM KCl or 1 mM NaCl (not shown). However, incubation of the protein in the presence of 5 mM MgCl\(_2\) followed by dialysis (“Experimental Procedures”) resulted in the loss of 90–95% of bound nucleotide.

The latter observation suggested that release of endogenous nucleotides may depend on hydrolysis of bound ATP. Indeed, incubation of MutS E694A in the presence of Mg\(^{2+}\) resulted in the hydrolysis of the bound ATP with the concomitant appearance of ADP. As shown in Fig. 1, this event is well described by a single exponential with a rate constant of 0.12 min\(^{-1}\) at 30°C. The simplest interpretation of this value is that it corresponds to the intrinsic ATP hydrolytic rate constant for the mutant protein. This parameter is almost identical to the \( k_{\text{cat}} \) for steady state ATP hydrolysis by MutS E694A at 30°C (see above), which is 0.08 min\(^{-1}\) when expressed per dimer, the minimum MutS functional unit (22, 26, 37, 38). The similarity of these values indicates that hydrolytic chemistry is largely rate-limiting for turnover by MutS E694A. This differs from wild type MutS, for which ATPase turnover in the absence of DNA is governed by product release (36).

\[ k_{\text{cat}} = 0.06 \text{ min}^{-1} \text{ at } 37 ^\circ \text{C for MutS E694A as compared with } 2.9 \text{ min}^{-1} \text{ for wild type MutS expressed per monomer).} \]

Mismatches Recognition by MutS E694A and Nucleotide Effects on MutS E694A DNA Interaction — MutS E694A has been shown to recognize mismatched base pairs with modest specificity (28), but affinities of the protein for heteroduplex and homoduplex DNAs have not been reported (23, 34). As shown in Table I, wild type MutS displays high affinity (\( K_m \approx 15 \) nM) and a 10–20-fold preference for a 41 bp G-T heteroduplex over an otherwise identical A-T homoduplex when binding is performed in the presence or absence of Mg\(^{2+}\). Heteroduplex affinities of MutS E694A isolates determined in the absence of Mg\(^{2+}\) are

FIG. 1. Hydrolysis of endogenous ATP in a MutS E694A preparation. MutS E694A (2 \mu M as monomer, containing 0.50 mol of ATP/mol and 0.04 mol of ADP/mol) was incubated at 30°C in 0.5 ml of 20 mM Tris-HCl, pH 7.6, 100 mM KCI, 1 mM diithiothreitol, 5 mM MgCl\(_2\). Samples (70 \mu l) were withdrawn as indicated, and the reaction quenched by addition of EDTA to 8 mM and chilling to 0°C. ATP (a) and ADP (c) content was determined by luciferase assay (“Experimental Procedures”). Lines shown correspond to nonlinear regression fits to the equations: [ATP] = [ATP]_0 e^{-kt} and [ADP] = [ADP]_0 + [ATP]_0(1-e^{-kt}), where [ATP]_0, [ADP]_0, and \( k \) were allowed to float in the fitting routine. The fit to this model was excellent in each case (\( r = 0.994 \) and 0.997, respectively). Parameters returned from these fits were: [ATP]_0 = 0.43 ± 0.015 mol/mol monomer (ATP fit) and 0.48 ± 0.018 mol/mol (ADP fit); [ADP]_0 = 0.023 ± 0.012 mol/mol (ADP fit); and \( k \) = 0.12 min\(^{-1}\) (ATP fit) and 0.11 min\(^{-1}\) (ADP fit). The latter values correspond to hydrolytic \( k_{\text{cat}} \) of about 6 min.

a C. Baitinger and P. Modrich, unpublished observations.
about 10-fold less than that of the wild type protein, and this is associated with limited heteroduplex specificity, particularly in isolates with low ADP content. However, analysis of binding in the presence of Mg$^{2+}$ resulted in a significant increase in heteroduplex affinity, and in the case of low ADP preparations, enhanced specificity as well.

The differential modulation of heteroduplex affinity and specificity of wild type MutS by adenine nucleotides suggests an explanation for these observations. Whereas the wild type protein exhibits high mismatch affinity and specificity in the absence of nucleotide or in the presence of ADP, affinity and specificity are reduced substantially when binding is scored in the presence of nonhydrolyzable AMP-PNP-Mg$^{2+}$ or ATP (no Mg$^{2+}$) (30). As described above, isolated MutS E694A contains 1 mol of bound ATP per dimer and variable amounts of bound ADP. We think it likely that the low heteroduplex affinity of MutS E694A that is observed in the absence of Mg$^{2+}$ reflects the presence of bound ATP and that specificity differences observed with different isolates are because of differential occupancy of the MutS ATP site in accord with the different levels of the diphosphate in different preparations. Because MutS E694A slowly hydrolyzes endogenous ATP when Mg$^{2+}$ is present (Fig. 1), the increased heteroduplex affinity and specificity observed in the presence of this divalent cation is probably because of hydrolysis of endogenous ATP. This is a reasonable possibility because the average MutS E694A sample was incubated in the presence of Mg$^{2+}$ for about 1 h at 4 °C prior to automated injection into the BIACore used for binding constant determination (“Experimental Procedures”). Indeed, nucleotide-depleted samples of MutS E694A (<0.05 mol of adenine nucleotide/mol of dimer), prepared by hydrolysis of endogenous nucleotide or by phosphatase treatment (“Experimental Procedures”) display heteroduplex affinities and specificities that differ from those of wild type protein by only a factor of two, irrespective of the presence of Mg$^{2+}$ (Table I).

Previous studies have demonstrated that the interaction of MutS with DNA is modulated by adenine nucleotides (4, 26, 30), and as expected, the converse is also true (36, 41). We have therefore employed SPRS analysis to compare adenine nucleotide effects on MutS E694A- and MutS-DNA interaction. MutS heteroduplex complexes formed in the absence of nucleotide or in the presence of ADP-Mg$^{2+}$ undergo rapid and complete dissociation upon subsequent challenge with ATP-Mg$^{2+}$ (30). This effect is reproduced in Fig. 3 (panels A and B), and it can be seen that wild type MutS and MutS E694A respond in a similar manner to triphosphate challenge. MutS heteroduplex complexes formed in the presence of ATP (±Mg$^{2+}$) behave differently. The presence of the triphosphate reduces the specificity of MutS-DNA interaction because of a reduction in mismatch affinity, and in contrast to MutS heteroduplex complexes that assemble in the presence of ADP or absence of nucleotide, those formed in the presence of ATP are resistant to dissociation upon buffer wash and subsequent challenge with ATP-Mg$^{2+}$ (30). Fig. 3 (panels C and D) demonstrates that complexes of MutS E694A with heteroduplex DNA behave like wild type MutS in this respect. These observations confirm a previous study (30) that has shown that the lifetime of MutS heteroduplex complexes upon ATP challenge depends upon the nature of the nucleotide present during the binding phase of the reaction. This effect requires the presence of a physical barrier on at least one end of the heteroduplex, as is the case in the BIACore experiments of Fig. 3 in which the heteroduplex is attached to the sensor chip via biotin-streptavidin linkage.

Thus, although slightly compromised with respect to heteroduplex affinity, MutS E694A is clearly capable of mismatch recognition, and assembly of MutS E694A-heteroduplex complexes responds to adenine nucleotides in a manner similar to that of wild type MutS. Consequently, the causative basis for the failure of the mutant protein to support MutH activation is not evident at the level of its interaction with a mismatch.

MutS E694A Fails to Support Mismatch-dependent Assembly of a MutL-MutS-DNA Ternary Complex—A ternary complex comprised of heteroduplex DNA, MutS, and MutL is believed to be a key intermediate in the initiation of mismatch repair. This ternary complex has been visualized in the bacterial and human systems by DNase footprinting, SPRS, magnetic bead assay, and gel shift analysis (4, 5, 42). In the SPRS assay, this complex is manifested as an enhanced mass of DNA-bound protein when chip-bound heteroduplex is simultaneously exposed to MutS and MutL (5) or MutS$\alpha$ and MutL$\alpha$ (42) in the presence of ATP. The behavior of wild type MutS and MutL in this assay is illustrated in panels A and B of Fig. 4. In the presence of ATP, the mass of G-T heteroduplex-bound protein was increased dramatically when MutS and MutL are both present as compared with that observed in the presence of MutS alone (panel A). In the absence of other proteins, MutL does not bind at detectable levels to heteroduplex under these conditions (5).3 These results are identical to those reported by Gallo et al. (5), who showed that this mass enhancement is because of MutS-dependent assembly of a MutS-MutL complex on heteroduplex DNA. Control experiments with an A-T homoduplex demonstrated a strong dependence of ternary complex assembly on a mismatched base pair (compare panels A and B). The substantial increase in heteroduplex-bound mass observed in the presence of both MutS and MutL, as compared with that observed with MutS alone (Fig. 4A), might suggest that each MutS molecule recruits multiple copies of MutL to the heteroduplex. This is not necessarily the case, however, because MutL may enhance the steady-state level of heteroduplex-bound MutS. The nature of the BIACore assay does not permit distinction between these possibilities.

3 C. Baitinger, L. Blackwell, and P. Modrich, unpublished observations.
MutS E694A supports mismatch recognition in the presence of Mg$^{2+}$

Binding isotherms were determined by SPRS assay (30) as described under “Experimental Procedures.” Apparent $K_d$ values were determined by non-linear least squares fit to a hyperbola. Heteroduplex binding promotes MutS dimer to tetramer assembly, resulting in cooperative binding behavior (37). Since the deviation from hyperbolic behavior is limited under the conditions used here (37), apparent $K_d$ values obtained by hyperbolic fit provide valid estimates of the relative binding affinities of MutS and MutS E694A. Specificity was calculated at the ratio of the $K_d$ value for homoduplex to that for heteroduplex. Errors shown are ± 1 SD.

### Table I

| Protein          | G-T $K_d$ $-\text{Mg}^{2+}$ | +Mg$^{2+}$ | AT $K_d$ $-\text{Mg}^{2+}$ | +Mg$^{2+}$ | Specificity |
|------------------|-----------------------------|-----------|---------------------------|-----------|-------------|
| MutS             | 14 ± 3                      | 15 ± 4    | 170 ± 26                  | 350 ± 40  | 12 ± 3.2    |
| MutS E694A       | 130 ± 13                    | 82 ± 8    | 530 ± 96                  | 440 ± 120 | 4.1 ± 0.8   |
| High ADP$^a$     | 130 ± 20                    | 73 ± 10   | 230 ± 93                  | 530 ± 100 | 1.8 ± 0.8   |
| Nucleotide-depleted | 34 ± 6                     | 35 ± 7    | 190 ± 48                  | 320 ± 62  | 5.6 ± 1.7   |
| MutS E694A$^a$  |                            |           |                           |           |             |

$^a$ The several preparations tested contained 1.0 mol of ATP and 0.2–0.4 mol of ADP/mol of dimer.

$^b$ The preparation contained 1.0 mol of ATP and 0.08 mol of ADP/mol of dimer.

MutS samples depleted by endogenous hydrolysis and dialysis, or by phosphatase treatment and purification (“Experimental Procedures”). Average nucleotide content was 0.04 mol of ATP and 0.01 mol of ADP/mol of dimer.

Although enhanced protein binding to DNA was also observed in the presence of MutS E694A and MutL, an effect that was ATP-dependent, the extent of binding to heteroduplex and homoduplex DNAs did not differ significantly (Fig. 4, panels C and D). Thus, whereas the mutant protein supports the apparent formation of a ternary MutL-MutS E694A-DNA complex, assembly of this structure does not depend on the presence of a mismatched base pair. The ternary complexes that are assembled on heteroduplex and homoduplex DNA in the presence of MutS E694A also differ from those obtained with wild type MutS with respect to behavior upon subsequent buffer wash. As can be seen, MutS E694A ternary complexes dissociate from DNA more slowly than those assembled in the presence of the wild type protein (compare panel A of Fig. 4 with panel C, and panel B with panel D). The inability of MutS E694A to support normal assembly of the MutL-MutS-DNA ternary complex provides a simple explanation for the failure of the mutant protein to support MutL-dependent activation of the MutH endonuclease as described above.

### DISCUSSION

Previous work using a *trans* assay for MutH activation has led to the conclusion that hydrolytically defective MutS E694A supports MutL-dependent activation of MutH (23, 34). However, use of a *cis* assay for MutH activation indicates that MutS E694A is defective in this regard, with the magnitude of the defect comparable with the observed reduction in the $k_{cat}$ for ATP hydrolysis. Differences in the experimental conditions used in these two cases suggest a possible explanation for this discrepancy. The *cis* assay for MutH activation is typically performed in the presence of 2.4 nM heteroduplex, 1 nM MutH, 24 nM MutL, and 30–300 nM MutS (7, 37) (Fig. 2). By contrast, the *trans* assays that led to the conclusion that MutS E694A is active in MutH activation employed much higher DNA and protein concentrations: 50 nM DNA, 1,000 nM MutH, 500 nM MutL, and 250–500 nM MutS or MutS E694A (23). The high MutH and MutL concentrations used in the *trans* reactions...
may permit assembly of a MutH activation complex by a nonbiological pathway that bypasses key assembly step(s) that may otherwise depend on a functional MutS ATP hydrolytic center. That this may be the case is suggested by the low efficiency and high background characteristic of the trans assay employed in the previous study. When normalized to input MutH, the efficiency of trans activation is less than 1% of that observed with the cis reaction (7, 23). Whereas conditions for more efficient trans activation have been described (40), analysis of MutS E694A for its ability to support this reaction has not been reported. The finding that MutS E694A is defective in cis MutH activation thus raises questions concerning the validity of the conclusion based on the trans assay that ATP binding by MutS is sufficient for activation of downstream activities as postulated by the mismatch verification model (23).

As discussed above, the two other models for MutS homolog ATPase function invoke nucleotide-dependent movement of the protein from the mismatch along the DNA contour (26–29). These two models differ in that one invokes ATP binding and hydrolysis by DNA-bound MutS/MutS during the course of movement (26, 27), whereas the alternate molecular switch model postulates mismatch recognition by the MutS/MutS-ADP complex, exchange of ATP for ADP, and diffusion of the MutS/MutS-ATP complex along the helix (28). These models derive from the direct visualization of the temporal evolution of MutS-DNA complexes (26) and study of the dynamics of MutS interaction with linear heteroduplexes (27–30). Use of the latter approach has shown that ATP-Mg$^{2+}$ greatly reduces the steady-state level of heteroduplex-bound MutS (or MutS$\alpha$) and promotes dissociation of preformed complexes from a linear heteroduplex. However, placement of streptavidin blocks at the heteroduplex termini prevents ATP-promoted dissociation resulting in elevated steady-state levels of MutS(MutS$\alpha$)-heteroduplex complexes.

Results of the MutS E694A experiments described here can be considered in the context of the hydrolysis-dependent translocation and molecular switch models for MutS ATPase function. The finding that the mutant protein is defective in MutL-dependent activation of MutH and mismatch-dependent assembly of the MutS-MutL-DNA ternary complex suggests that ATP hydrolysis by DNA-bound MutS may be necessary for its function in mismatch repair. Analysis of the interaction of human MutSo with linear heteroduplexes derivatized with avidin end blocks has led to similar conclusions. Whereas ATP-Mg$^{2+}$ challenge of pre-formed complexes of MutSo with an end-blocked heteroduplex leads to formation of a long lived complex, challenge with AMP-PNP-Mg$^{2+}$ or ATP'yS-Mg$^{2+}$ results in dissociation (27). A similar observation has been made by Iaccarino et al. (29) using a hydrolytically defective form of human MutSo (Val substitution for Asp-1213 in MSH6) that retains the ability to bind ATP. In contrast to wild type MutSo, pre-formed complexes of MutSo(MSH6 D1213V) with an end-blocked heteroduplex dissociate upon ATP-Mg$^{2+}$ challenge. Whereas results obtained with nonhydrolyzable ATP analogues, and the abnormal behavior of MutS E694A and MutSo(MSH6 D1213V) might be attributed to conformational effects unrelated to an inability to hydrolyze triphosphate, the simplest explanation for this set of observations is that ATP hydrolysis by DNA-bound MutS/MutSo$\alpha$ is required for function of the proteins in mismatch repair.

The MutS dimer displays intrinsic asymmetry (21, 22), and the nucleotide binding sites within E. coli MutS dimers and tetramers are of two types that display differential specificities for ADP and nonhydrolyzable ATP analogues (22, 31, 37, 39). Our finding that the triphosphate binding site is fully occupied (1 mol of ATP per dimer equivalent) and the diphosphate site partially populated (0.1 to 0.6 mol/dimer) in isolated MutS...
E694A is consistent with the previous demonstration that the two classes of nucleotide binding site within wild type MutS can be simultaneously occupied by ADP and a nonhydrolyzable ATP analogue (31). In particular, the presence of bound ATP and ADP in the isolates of the mutant protein is in agreement with the suggestion that the ternary ADP-MutS-ATP complex comprises a substantial fraction of the protein in solution. The existence of this ternary complex is incompatible with two-state models for MutS action that restrict the protein to only the ADP or ATP forms (28).

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