ATP hydrolysis-dependent formation of a dynamic ternary nucleoprotein complex with MutS and MutL

Laurent Galiot, Céline Bouquet and Peter Brooks*

Genoscope, Centre National de Séquençage, BP 191, 2 rue Gaston Crémieux, 91006 Evry, France

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ABSTRACT
Functional interactions of Escherichia coli MutS and MutL in mismatch repair are dependent on ATP. In this study, we show that MutS and MutL associate with immobilised DNA in a manner dependent on ATP hydrolysis and with an ATP concentration near the solution Km of the ATPase of MutS. After removal of MutS, MutL and ATP, much of the protein in this ternary complex is not stably associated, with MutL leaving the complex more rapidly than MutS. The rapid dissociation reveals a dynamic interaction with concurrent rapid association and dissociation of proteins from the DNA. Analysis by surface plasmon resonance showed that the DNA interacting with dynamically bound protein was more resistant to nuclease digestion than the DNA in MutS–DNA complexes. Non-hydrolysable analogs of ATP inhibit the formation of this dynamic complex, but permit formation of a second type of ternary complex with MutS and MutL stably bound to the immobilised DNA.

INTRODUCTION
Single base mispairs and small insertions/deletions in Escherichia coli are removed by the long patch methyl-dependent mismatch repair system, which engages about 10 proteins to accomplish a multistep excision and resynthesis process (1–3). Detailed analysis of the bacterial system will aid understanding of the mammalian system which is mechanistically similar and includes highly homologous components; deficiencies in human mismatch repair are implicated in predisposition to multi-organ cancer (4–8). The entire repair process has been reconstituted from the DNA. The repair process has been reconstituted with purified E.coli proteins (9) and various steps in the process have been characterised. Initiation is upon mismatch recognition by MutS (10). ATP hydrolysis is required for a proposed translocation of MutS along the DNA (11), for the activation of incision by MutH of the strand bearing the incorrect base (12) and for the activation of helicase II to displace the nicked strand (13). Although the first of these ATP-dependent activities is stimulated by MutL, and the latter two require MutS and MutL, the precise role of MutL remains unknown. Activation of MutH endonuclease and translocation require 0.3 mM ATP for half-maximal activity (11,12). Here we show that a MutSL–DNA complex can be formed with ATP concentrations that correspond to the Km of the ATPase of MutS, 5–25 µM ATP (14–16), suggesting the identification of a novel partial reaction in the repair process. MutS inhibits DNase digestion of ~25 nt flanking a mismatch (10). Addition of MutL and ATP substantially expands this footprint (17) but this observation has yet to be related to other activities of MutL. In the ATP-dependent complex described here, the proteins, primarily MutL, rapidly associate and dissociate from the complex. As shown with surface plasmon resonance (SPR), it is this dynamic complex, possibly implicated in translocation, that can slow DNase digestion.

MATERIALS AND METHODS
Proteins, DNA and nucleotides
Protein concentrations of MutS and MutL preparations (GenGuard-S and GeneGuard-L; Genoscope) were assayed by dye binding (Coomassie Plus; Pierce) with BSA as standard. The proteins were >95% pure by SDS–PAGE and had no detectable nuclease activity. Complementary oligonucleotides (Operon, Alameda, CA), purified by ion exchange HPLC, including one with a 5′-biotin label, in 10 mM Na HEPES, pH 7.5, 0.1 M NaCl, 1 mM EDTA, were annealed by heating at 95°C for 5 min and slow cooling to room temperature. Fragments of 185 bp including 39 bp of the human calpain gene (GenBank accession no. X85030, positions 386–424), either wild-type or bearing a T→C mutation (position 409) were amplified from pBlueScript (Stratagene) clones. PCR in 30 µl was for 32 cycles and included Expand (Boehringer) buffer components with 1.5 mM MgCl2, 7.5% glycerol, 0.2 mM cresol red, 20 µg/ml BSA, 0.5 µM vector-specific primers (one with a 5′-biotin label), 100 µM each dNTP, 50 pg plasmid DNA and 0.8 U HiFi Expand DNA polymerase mix. PCR products were purified with HiPure (Boehringer) with final elution in 10 mM Tris–HCl pH 8.0, 1 mM EDTA and were quantified by fluorimetry with PicoGreen (Molecular Probes). Equal amounts of wild-type and mutant amplicons were mixed and annealed by adjustment to 0.2 M NaCl, incubation at room temperature for 5 min, dilution to 160 mM NaOH with adjustment to 160 mM HOAc, 40 mM Tris–HCl pH 8.0, 0.2 M NaCl and incubation at 68°C for 20 min and 37°C for 10 min. ATP analogues (Boehringer) were dissolved to 100 mM, neutralised.

*To whom correspondence should be addressed. Tel: +33 1 6087 2594; Email: brooks@genoscope.cns.fr
Present addresses: +MRC Reproductive Biology Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EN3 9HT, UK and §Laboratoire de Vectorologie, Institut Gustave Roussy, 94805 Villejuif Cedex, France
and solutions stored at –80°C. Separation of ATP, ADP and AMP by PEI TLC (Merck; 1 M HCOOH, 0.5 M LiCl) showed that the nucleotide in the ATP solution was >98% ATP. To evaluate the integrity of the ATP analogues, pBluescript was transcribed with T7 RNA polymerase (Boehringer) using limiting concentrations of ATP or ATP analogues and RNA production was estimated with ethidium bromide stained agarose gels. As compared with ATP, AMP-PNP enabled 80% transcription and ATPβS was indistinguishable.

### SPR of protein binding to immobilised DNA

Biotinylated DNA substrates in 10 mM Na HEPES pH 7.5, 0.63 M NaCl, 3 mM EDTA, 2% glycerol and 0.005% Tween 20 were immobilised in one to three flow cells of streptavidin (SA)-coated sensor chips in a BIAcore 2000 (BIAcore AB). Immobilised DNA surfaces were equilibrated at 30°C with 20 µl/min running buffer, HAMG (30 mM Na HEPES pH 7.5, 220 mM NaOAc, 4 mM MgCl₂, 0.1 mM EDTA, 2% glycerol and 0.005% Tween 20). Proteins were diluted to 1.2 µM in HABG (HAMG without MgCl₂ and with 5 mM β-mercaptoethanol) and held in BIAcore thermo-racks cooled to 8°C, where their activity was stable for at least 30 h. Immediately prior to injection, proteins were diluted with HABMG (HAMG with 5 mM β-mercaptoethanol) and nucleotides as indicated. At the end of each cycle, remaining proteins were removed with two 30 s injections of HAMG containing 0.04% SDS. Each injection flowed over the four flow cells and raw data were corrected by subtraction of a kinetogram for a cell without DNA. The ratios of 1 pg protein/1 RU (18) and 1 pg DNA/0.8 RU (19) were used for estimating molar protein/DNA ratios.

### DNase protection with SPR

DNA immobilisation, protein injections and reaction conditions were as described above. DNase I (Worthington) was either mixed with proteins and nucleotides in HABMGC (HABMG with 0.5 mM CaCl₂) to 0.6 ng/ml and injected immediately at the end of the first protein injection, using the BIAcore co-injection function, or mixed with HABMGC and injected during the dissociation phase. DNA resistant to nuclease was measured when the signal was stable after the two regenerating SDS injections. Each cycle with DNase was preceded by an identical cycle without DNase. After each cycle, remaining DNA was digested by injection of excess DNase I (3 µg/ml). Neither MutS nor MutL were captured by the residual oligonucleotides (average size 6 nt). After the SDS injections, DNA was again immobilised on the same surfaces. As each cycle of DNA immobilisation exploited only a small fraction of the SA capacity, the surfaces could be regenerated by DNase and rederivatised with DNA for at least 40 cycles.

### Recovery of protein bound to DNA on magnetic beads

Biotinylated 149 bp DNA substrates (Fig. 1) were immobilised on SA-coated magnetic beads (Dynabeads M-280; Dynal) in 20 mM Tris–HCl pH 7.5, 4 M NaCl, 2 mM EDTA. The beads were then washed twice with HABMG. An aliquot of the beads was incubated with Styl to release a 141 bp fragment which was quantified by comparative ethidium bromide staining in an acrylamide gel. Reactions (40 µl in 0.5 ml polypropylene tubes) for 30 min at 30°C included HABMG, 200 µg of beads with or without 150 ng of immobilised DNA, and MutS, MutL and nucleotides as indicated. The beads were then rapidly washed at least twice with 200 µl HAMG (30°C) and held at 30°C for the times indicated. Proteins were eluted during 10 min incubations at 30°C in 30 µl of 17 mM MOPS, 17 mM Tris base, 0.033% SDS, 0.33 mM EDTA, pH 7.7, containing 150 ng ovalbumin. During association, dissociation and elution, beads were mixed every 5 min by several displacements of the tubes between two rows of magnets (BLS Magnes, Longwy, France), 1 cm in height. Magnetic gathering of the beads on the tube walls permitted removal of clarified solutions from the tube bottoms. Eluted proteins (10 µl) were separated in denaturing gels (NuPAGE 3-12%; Novex) and stained (SilverXpress; Novex). Gels dried between cellophane sheets were scanned and band intensities integrated with ImageQuant (Molecular Dynamics).

### RESULTS

#### SPR analysis of nucleoprotein complexes produced with MutS and MutL

Biotinylated duplex oligonucleotides of 25 or 39 bp, either perfectly paired or containing a T-G mismatch, were immobilised on a SA surface for kinetic SPR analysis. Binding of MutS to these substrates enabled evaluation of the protein’s affinity (apparent Kd = 50 nM), protein/DNA ratio (about two monomers of MutS per molecule of DNA) and discrimination (heteroduplex to homoduplex ratio of 5:10) (data not shown). These values agree with those previously reported for MutS–DNA interactions as determined with SPR (11). With immobilised 149 bp substrates (Fig. 1), about twice as much MutS associated with the heteroduplex bearing a T-G mismatch as with the homoduplex control DNA (Fig. 2A). However, the molar ratio of bound protein to mismatch (after correcting for the binding to homoduplex) was similar to that observed with oligonucleotide substrates (Fig. 2A and data not shown). Thus, the decline in discrimination for the heteroduplex DNA, as compared with oligonucleotide substrates, might simply reflect the greater number of non-specific binding sites in the longer DNA molecule.

MutL, at concentrations ranging from 0.1 to 1 µM, in the presence or absence of ATP, did not associate with any of the substrates (not shown). Injection of MutS and MutL without ATP over the surfaces with the 149 bp substrates produced kinograms similar to those observed with injection of MutS alone (Fig. 2B). However, when MutS, MutL and ATP (0.5 mM) were injected together, binding increased substantially (Fig. 2B).
Figure 2. MutSL-dependent dynamic protein binding to DNA requires ATP hydrolysis. DNA substrates (149 bp; Fig. 1), either homoduplex or heteroduplex, with a T-G mismatch were immobilised for SPR analysis. Protein injections for 10 min and surface regeneration were as described in Materials and Methods. Kinetograms represent data points collected each second; symbols are for curve identification. All surfaces had 690 ± 10 RU of DNA. (A) MutS (50 nM) without nucleotide. (B) MutS (50 nM) and MutL (50 nM) with or without 0.5 mM ATP. (C) MutS (50 nM) and, as indicated, MutL (50 nM) and 0.5 mM ATP or 0.5 mM AMP-PNP.

Interaction with homoduplex DNA was also enhanced, such that the discrimination factor of about two was unchanged as compared to binding by MutS alone (Fig. 2A and B). Replacement of ATP with ATPγS or AMP-PNP, inclusion of either of these non-hydrolysable analogues in addition to ATP or injection with ATP in the absence of MgCl₂ did not permit enhanced binding (Fig. 2C and data not shown).

Most of the enhanced binding in the MutSL- and ATP hydrolysis-dependent complex was not stable such that half of the protein dissociated in 2–5 min. Therefore, during the association phase protein was rapidly exchanged between the bound and free states. The rate of dissociation gradually slowed until it approached the rate of dissociation of the MutS–DNA complex (half-life ~0.5–1 h) (Fig. 2). Due to a multiphasic dissociation of the dynamic binding, kinetic dissociation constants were not readily determined.

Enhanced binding occurred only when MutS, MutL, and ATP were injected together over the DNA surfaces. Sequential injections of the individual proteins, with or without ATP, with the second injections either during the dissociation phase following the first injection or immediately following the first injection, failed to produce enhanced binding (not shown). Furthermore, the continued presence of all three components was necessary to maintain the dynamic complex. At the end of the MutSL/ATP injection, immediate sequential injections of either of the proteins or ATP or of any pair-wise combination of the components failed to stabilise the complexes, which decayed with rates similar to that observed when running buffer followed the end of the first injection (not shown). Only a sequential injection with ATP alone slightly retarded the rate of dissociation (not shown).

To determine if the single strand interruptions in the 149 bp substrate were required for dynamic binding, the experiments were repeated with hybrid PCR products. An enhanced and dynamic binding dependent on MutS, MutL, and ATP also occurred (Fig. 3), thus indicating that nicks are not implicated in the interaction.

Maximum levels of enhanced binding were achieved with 10–20 µM ATP and varied little with increasing concentrations of ATP to 2 mM (Fig. 4). The half-maximal binding level corresponds to an ATP concentration between 1 and 5 µM (Fig. 4).

The dynamic nucleoprotein complex includes MutS and MutL.

To determine if the dynamic binding observed with SPR involved inclusion of MutL into a ternary complex or was due to a MutL-dependent activity resulting in accumulation of additional
MutS, the same 149 bp biotinylated DNA substrates used for SPR were immobilised on SA-coated magnetic beads. The beads were incubated with MutS, MutL and ATP under conditions faithfully mimicking the SPR experiments, including washing with SPR running buffer. DNA-bound proteins were eluted with SDS and resolved by SDS–PAGE. In the absence of ATP, MutS bound to the heteroduplex DNA with a molar ratio ranging from 2 to 4, similar to the proportions observed with SPR (Fig. 5A and data not shown). MutL was indeed a component of the protein–DNA complex formed with MutSL and ATP. MutL was not bound when MutL alone or MutS and MutL without ATP were incubated with the beads (Fig. 5A). Substantially less protein was captured by the beads (Fig. 5A). The amount of protein bound to heteroduplex DNA (not shown), as was observed with SPR.

To simulate the dissociation phase of the SPR binding experiments, beads with nucleoprotein complexes were held in the wash buffer (same as the SPR running buffer) for varying times before elution of the proteins with SDS (Fig. 5B). The relative recoveries of the two proteins after increasing wash times revealed that MutL dissociated more rapidly than MutS (Fig. 5B, compare MutS and MutL intensities, lanes 3, 5, 7 and 9). The rapid initial rate of loss of MutL (Fig. 5C) resembles the rapid decay of the dynamic complex observed by SPR (Figs 2 and 3). We infer that the dynamic complex observed by SPR analysis includes MutL and, while ATP-dependent recruitment of additional MutS into the complex is apparent, the rapid decay is primarily due to the departure of MutL.

**Ternary complex formation enabled by ATP binding**

When ATP was replaced with ATP$_\gamma$S in incubations with DNA immobilised on the SA beads, both MutS and MutL were bound to the DNA (Fig. 5B). The complex resembled the dynamic complex formed with hydrolysable ATP in that the amounts of the proteins were similar to each other, but differed in that these amounts were substantially reduced. The complex was also more stable, with no rapid dissociation of MutL (Fig. 5B and C).

**Protection of DNA from nuclease digestion by the dynamic MutSL–DNA complex**

To determine if the additional protein in the MutSL–DNA complex was accumulating on a foundation of MutS bound to DNA without increasing the number of protein–DNA contacts or was interacting with a larger region of DNA, the capacity of the dynamic complex to protect the bound DNA from nuclease digestion was analysed by SPR. Nucleoprotein complexes were first established with a 15 min injection of MutS, MutL and ATP (Fig. 6A and B). The same mixture with or without DNase I was immediately injected (Fig. 6B). The duration of this second injection (Fig. 6B and C) and the concentration of DNase were chosen to enable a gradual digestion of most of the DNA in control experiments when neither MutS nor MutL were present. Subsequent injection of SDS removed the remaining bound proteins (Fig. 6D) and incubation with running buffer continued until the re-establishment of a stable response signal. Then the remaining DNA, representing biotinylated fragments surviving the random DNase I cleavage, was measured (Fig. 6E). The values were corrected for any loss of DNA in the absence of DNase: the results from several experiments are compiled in Table 1. In the absence of both MutS and MutL, 90% or more of the DNA was digested for immobilisation levels ranging from 30 to 250 RU. Formation of a complex with MutS and MutL but without ATP slowed DNase digestion such that up to twice as much DNA remained as compared with that remaining when DNase alone was injected. Injections of MutS, MutL and ATP substantially increased the level of protection. ATP at 0.5 mM provided no significant additional delay to nuclease digestion than ATP at 20 µM (Table 1), a concentration that enables a maximal level of dynamic binding (Fig. 4). We conclude that the MutL-dependent dynamic binding involves protein interaction with sites on the DNA in addition to those interacting with MutS in the binary MutS–DNA complex.

Neither replacing ATP with ATP$_\gamma$S nor allowing dissociation of the dynamic complex before exposure to DNase permitted enhanced protection (Table 1). In these two cases, MutL is found in the complexes (Fig. 5B) and thus the simple presence of MutL is not sufficient to delay DNase digestion. The facility of challenging the immobilised DNA–protein complex with DNase after removal of unbound proteins shows an advantage of such protection experiments as compared with classic solution footprinting where unbound proteins are generally present and active during nuclease treatment. We infer that an expanded gel footprint would not be expected if unbound MutS and MutL were removed before DNase digestion.

**DISCUSSION**

The amount of protein bound to heteroduplex and homoduplex substrates after incubation with MutS, MutL and ATP was proportional to the amount of MutS bound to these substrates in the absence of nucleotides. Hence no improvement in specificity for the designed mismatch was provided by MutL and so we infer that the dynamic complex represents a partial reaction that is subsequent to mismatch recognition. Indeed, the enhanced protection from nuclease shows that the interaction is not limited to the
vicinity of the mismatch. Although we cannot exclude protein interactions with the double-stranded ends of the substrates, strand nicks are not implicated in the dynamic binding. Thus the dynamic complex appears to represent a partial reaction occurring between mismatch recognition and incision of the incorrect strand.

In a manner dependent on ATP hydrolysis and stimulated by MutL, MutS leaves the mismatch and apparently translocates along the DNA helix; both proteins were identified by crosslinking in a nucleoprotein translocation complex (11). Stimulation by MutL of a translocation activity of MutS might correspond to our observation of MutL-dependent rapid association and dissociation. Such instability would be expected for proteins translocating on the DNA, necessarily involving sequential fixation and release from sequential sites on the DNA. In addition, the dynamic complex is implicated in events following mismatch recognition, just as the translocation complex is no longer bound to the mismatch site. However, the $K_{m, ATP}$ for translocation is 0.3 mM (11), whereas the ATP concentration for half-maximal dynamic complex formation is between 1 and 5 µM, near the solution $K_{m}$ for the ATPase of MutS (14–16). (We cannot exclude participation of the ATPase activity of MutL, but its $K_{m}$ is 90 µM; 20.) The dynamic complex may represent an intermediate in the translocation process. A model for translocation of MutS or hMutSα involves cycling of several protein conformational states, with the different conformations depending on binding or hydrolysis of ATP (11,21,22). An alternative model has been proposed where mismatch recognition provokes adenosine nucleotide exchange and induces a conformational transition of MutS homologs into a hydrolysis-independent sliding clamp similar to G protein signaling systems (23–25). In this model, the MutLs may associate and alter the MutS sliding clamp in the presence of ATP. The complex reported here with stably bound MutL could participate in either of these models.

The addition of MutL and ATP (0.5 mM) to a MutS binding reaction has been shown by gel footprinting to expand the region of protection of mismatch-bearing DNA from DNase digestion (17). This expanded protection was established with ATP or with ATPγS (17), whereas the protection provided by the dynamic
Figure 6. SPR analysis of protection from nuclease by nucleoprotein complexes. Experiments were as described in Materials and Methods. The resonance signal was set at 0 RU before immobilisation of DNA. (A) Injection of MutS, MutL and ATP. (B) Injection identical to (A) but with (lower curve) or without (upper curve) DNase I. (C) End of DNase injection. (D) Protein removal by SDS. (E) Remaining immobilised DNA.

The concentration required for half-maximal activation of MutH endonuclease by MutSL and ATP hydrolysis (12) and the $K_m$ of ATP for an ATP binding-dependent stimulation of MutH endonuclease by MutL (16) are both $\sim0.3$ mM ATP. The MutSL interactions reported here might represent partial reactions toward configuration of MutS and MutL to activate MutH. Other activities dependent on ATP that might be relevant include stimulation of the helicase activity of UvrD by MutS and MutL (13) and enhancement by MutL of the ability of MutS to inhibit RecA-catalysed strand exchange (15,27).

Table 1. Expanded DNase I protection dependent on MutS, MutL and ATP hydrolysis

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<tr>
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Experiments were performed as described in Materials and Methods and in Figure 6.

Initial levels of DNA are those at point A, Figure 6, just before protein injection (90–120 nM) and final amounts at point E, Figure 6, after DNase and recovery from SDS elution. Measurements were taken after the signal stabilised to a variation of $\pm0.5$ RU.

For the determination of the protection efficiency, which is the percent DNA remaining after DNase, the loss of DNA due to DNase was corrected for any losses occurring in the immediately preceding control injections without DNase.

*DNase injection was during the dissociation phase, 10 min after the end of protein injection.
ATP-independent binding of MutL to DNA has been reported (28), whereas we did not detect any association of MutL alone with DNA. This discrepancy is not understood but may be related to our use of a physiological ionic strength. As observed with electrophoretic mobility shift assays and using a limiting concentration of MutS, MutL in the absence of added ATP reduced the apparent concentration of MutS, MutL in the absence of added ATP (29). In contrast, our SPR kinetograms for incubations with MutS or with MutS and MutL but without ATP were similar, with comparable rates of protein dissociation.

ATP hydrolysis is required to form a repair initiation complex in human cell extracts that includes MSH2, MLH1, PMS2 and PCNA (30). Perhaps the ATP-dependent dynamic complex documented here is involved in a similar interaction with the β-subunit of DNA polymerase III. As yet, no other eukaryotic DNA might be useful to reveal such interactions.

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REFERENCES