Molecular BioSystems

Cite this: *Mol. BioSyst.*, 2012, **8**, 1861–1864

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COMMUNICATION

Covalently trapping MutS on DNA to study DNA mismatch recognition and signaling†

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Received 12th March 2012, Accepted 16th April 2012
DOI: 10.1039/c2mb25086a

The DNA repair protein MutS forms clamp-like structures on DNA that search for and recognize base mismatches leading to ATP-transformed signaling clamps. In this study, the mobile MutS clamps were trapped on DNA in a functional state using single-cysteine variants of MutS and thiol-modified homoduplex or heteroduplex DNA. This approach allows stabilization of various transient MutS–DNA complexes and will enable their structural and functional analysis.

The DNA mismatch repair system (MMR) detects and repairs errors that escaped the proofreading function of DNA polymerases.[1] The principal protein components of the bacterial MMR system are the homodimeric ATPases, MutS and MutL. In eukaryotes the MutS and MutL-homologues (MSH and MLH) are heterodimers, *e.g.* in humans MutSα (MSH2/MSH6) and MutLα (MLH1/PMS2).[2] MMR is initiated when MutS recognizes a mismatch followed by ATP-induced complex formation with MutL.[3] This ternary complex (DNA–MutS–MutL) is a key active intermediate that couples mismatch recognition and discrimination of the template and nascent DNA strand. In *E. coli* the lack of adenine methylation in the nascent DNA strand at 5′-GATC-3′-sequences serves as a strand discrimination signal,[4] enabling the erroneous strand to be nicked by a third MMR protein, the monomeric endonuclease MutH. The nick is used by UvrD helicase and exonuclease, in the presence of single-strand DNA binding protein, to unwind and excise the erroneous strand until the mismatch is removed. DNA polymerase III and DNA ligase complete the repair process. In most bacteria and all eukaryotes that lack a MutH homologue, the strand discrimination signal is still unclear. However, it can be provided by pre-existing strand breaks or components of the replication machinery.[3]

During MMR, MutS forms several distinct complexes with DNA. First, MutS binds to DNA and searches for mismatches in a process involving linear diffusion.[5,6] Second, upon mismatch recognition MutS forms an asymmetric clamp-like complex in which the DNA is kinked by 45–60° at the mismatch region.[7–10] DNA bending/kinking has been observed by atomic force microscopy[11] or Förster resonance energy transfer (FRET).[12,13] Third, after mismatch recognition MutS undergoes ATP-induced conformational changes, finally leading to a long-lived complex with an ATP molecule bound to each subunit.[6,14,15]

This 'sliding clamp' is believed to be the active form of bacterial MutS (or eukaryotic MutSα) that binds MutL and signals mismatch recognition to downstream events.

Despite their functional importance, high-resolution structural data are not available for either the searching state or the signaling clamp state of MutS, in part due to little specific interaction between MutS and DNA in these complexes and their highly dynamic nature. To overcome these limitations we developed a covalent trapping strategy to capture MutS on DNA while searching (MutS bound to canonical DNA) or in the recognition state (MutS bound to mismatched DNA).

Various methods have been established in the past to enable covalent trapping of protein–nucleic acid complexes. The crosslinking strategy based on a thiol–disulfide exchange between a cysteine residue of a protein and DNA with a thiol-modified base is particularly attractive for its sequence specificity. This approach has been successfully used to crystallize and solve the structure of protein–nucleic acid complexes.[16–21] The reactive disulfide can also be attached to the 2′ position of a sugar moiety[22] or to a terminal phosphate (3′ or 5′).[23] However, a cross-linking strategy with DNA substrates containing a terminal S–S group for trapping proteins has not been reported thus far.

In the present work MutS variants containing a single-cysteine residue in the clamp domain were engineered and crosslinked to G:T heteroduplex I or A:T homoduplex II in a thiol–disulfide exchange reaction between the Cys and a disulfide group of thiol modifier $RSS(CH_2)_3$– (R = $(CH_2)_3OH$)) introduced at the 3′-end

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† Electronic supplementary information (ESI) available: SDS-PAGE analysis of MutS–DNA crosslinking specificity and kinetics; tables of plasmids, proteins, oligodeoxyribonucleotides and DNA duplexes, experimental details of trapping MutS on DNA and ternary complex formation with MutL. See DOI: 10.1039/c2mb25086a

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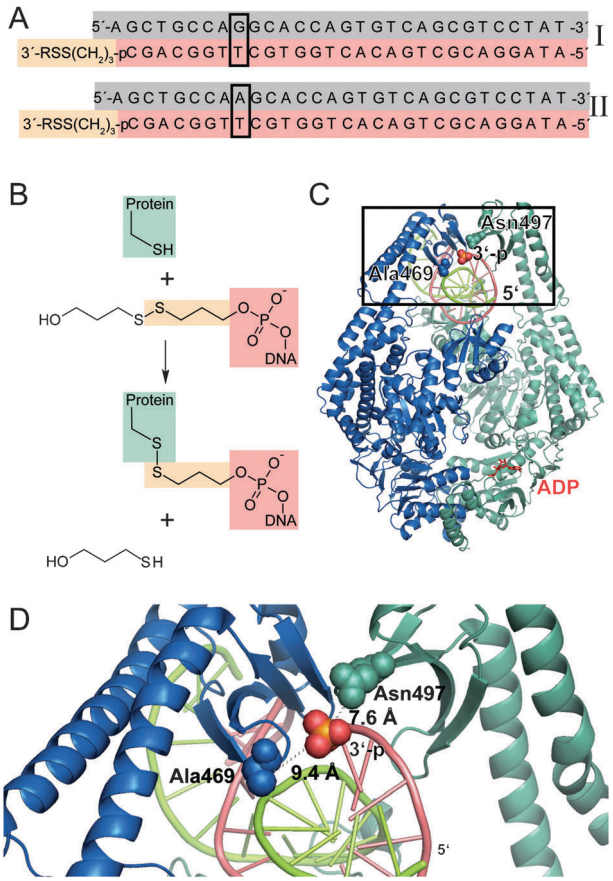

Fig. 1 Strategy for trapping MutS on DNA. (A) Sequence of the G:T heteroduplex (I) and A:T homoduplex (II) DNAs used for MutS–DNA crosslinking. The thiol-modifier RSS$(CH_2)_3$– (R = $(CH_2)_3OH$) is attached to the 3′-phosphate of the 'bottom' DNA strand. (B) Thiol–disulfide exchange reaction of a MutS cysteine residue with the asymmetric disulfide group of the thiol-modifier on DNA. (C) Crystal structure of MutS in complex with a G:T heteroduplex (PDB code 1e3m).[7] The two DNA strands of the G:T heteroduplex are colored as in (A). Subunits A and B of MutS are colored in light green and blue, respectively. Positions of residues 469 (in MutS subunit B) and 497 (in MutS subunit A) modified to Cys and the 3′-phosphate (3′p) of the 'bottom' DNA strand are shown as spheres. ADP bound to the A subunit is shown in red. Images were generated using PyMOL (8). (D) Detailed view of the structure shown in (C).

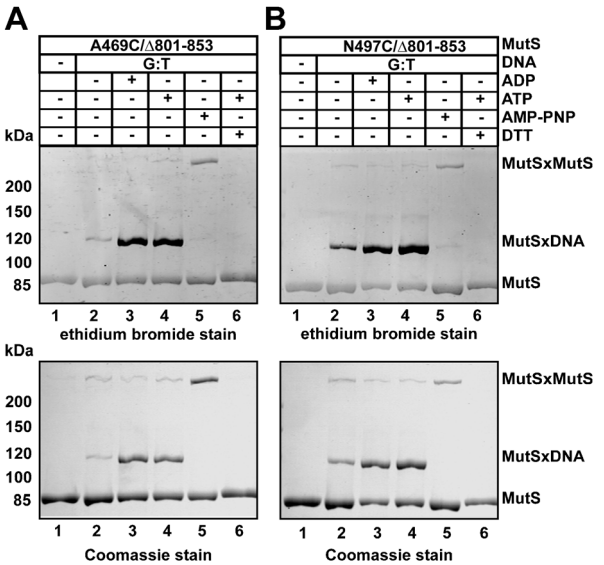

Fig. 2 Chemical crosslinking of MutS to DNA. Single cysteine variants MutS(A469C/Δ801–853) (A) and MutS(N497C/Δ801–853) (B) were crosslinked to DNA containing a thiol modifier at the 3′-end (see Fig. 1) in the absence or presence of the indicated nucleotides: ADP, ATP or adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP). All samples including a 1 μM MutS dimer were incubated in the crosslinking buffer (25 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 125 mM KCl and 0.05% (v/v) Tween-20) with 5 μM G:T heteroduplex I at 37 °C for 10 min. The reaction mixtures were analyzed by 6% SDS-PAGE followed by consecutive staining with ethidium bromide and Coomassie brilliant blue.

of one DNA duplex strand (Fig. 1 and Table S1, ESI†). Guided by the crystal structure of *E. coli* MutS (PDB code 1e3m) in complex with a 30 bp DNA containing a G:T mismatch, two amino acids at positions 469 and 497 that are close to the 3′-terminal modified phosphate group in the 'bottom' strand of DNA duplexes I and II (Fig. 1A) were chosen for Cys replacement. A Cys-free variant of the C-terminal truncated MutS (MutS-Δ801–853), which has been previously crystallized and biochemically characterized, was used as the starting material[24] (Table S2, ESI†).[7,9]

To demonstrate the feasibility of the single-cysteine MutS variants (A469C/Δ801–853 and N497C/Δ801–853) for crosslinking to DNA in a thiol–disulfide exchange reaction, the proteins were incubated with modified G:T heteroduplex I or modified A:T homoduplex II and samples were analyzed by SDS-PAGE (Fig. 2). Ethidium bromide was used to stain DNA (which to some extent also stains proteins in the presence of SDS[25]) followed by Coomassie brilliant blue to stain proteins.

For both variants, a species with an apparent molecular mass of 120 kDa was observed in the gel in the presence of DNA without any nucleotide (Fig. 2, lanes 2) or with ADP (Fig. 2, lanes 3) or ATP (Fig. 2, lanes 4). This species displayed intense staining by ethidium bromide and disappeared after addition of DTT prior to gel electrophoresis (Fig. 2, lanes 6), and was assigned as the crosslinked MutS–DNA complex. Other species in the gel were the MutS monomer with a molecular mass of 91 kDa (observed in all lanes), and a minor species with very low mobility in the gel, which corresponds to the MutS dimer linked *via* a disulfide bond (this >200 kDa species disappeared after treatment with DTT; Fig. S1A, ESI†).

MutS is a molecular switch. In the ATP-bound state ('off-state') it does not bind DNA. Upon ATP-hydrolysis the ADP-bound state ('on-state') is generated, which is proficient for binding DNA.[26] If ATP-hydrolysis is prevented (*e.g.*, in the absence of Mg^{2+}) or ATP is replaced with non-hydrolysable analogs such as adenosine 5′-*O*-(3-thio)triphosphate (ATPγS) or adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP), no DNA binding is observed.[26–28] Consistent with this property, when the MutS variants were pre-incubated with AMP-PNP followed by addition of DNA, no MutS–DNA crosslinked complex with an apparent molecular mass of 120 kDa was detectable. The intensity of the >200 kDa MutS dimer species was higher, consistent with ATP-induced stabilization of the MutS dimer (Fig. 2). Thus, crosslinking between MutS and DNA occurred only when MutS was able to bind to DNA. Finally, crosslinking

was highly site-specific as wild-type MutS, which contains six cysteine residues but none close to the disulfide group on DNA, was not able to form a crosslinked complex with the DNA (data not shown).

Similar crosslinking results were obtained for both MutS variants (A469C/Δ801–853, N497C/Δ801–853) when replacing the G:T heteroduplex I by A:T homopduplex II (Fig. S1, ESI†). Kinetic analysis of the crosslinking reaction revealed an about 4-fold faster complex formation between MutS(A469C/Δ801–853) and the G:T heteroduplex I compared to A:T homoduplex II (Fig. S2, ESI†). Crosslinking yields saturated at about 50% of MutS with the G:T heteroduplex I indicating that only one subunit of the MutS dimer was crosslinked to DNA (Fig. S2C, ESI†).

To analyze the functional properties of the trapped MutS–DNA complex we used size-exclusion chromatography to remove uncrosslinked MutS and DNA (Fig. 3). The covalently trapped complex (A_{260}/A_{280} ratio of 1.45) eluted only slightly earlier (250 kDa) than the MutS dimer (A_{260}/A_{280} = 0.66; 170 kDa) and the DNA duplex (A_{260}/A_{280} = 1.88) indicating that crosslinking neither changed the oligomeric state

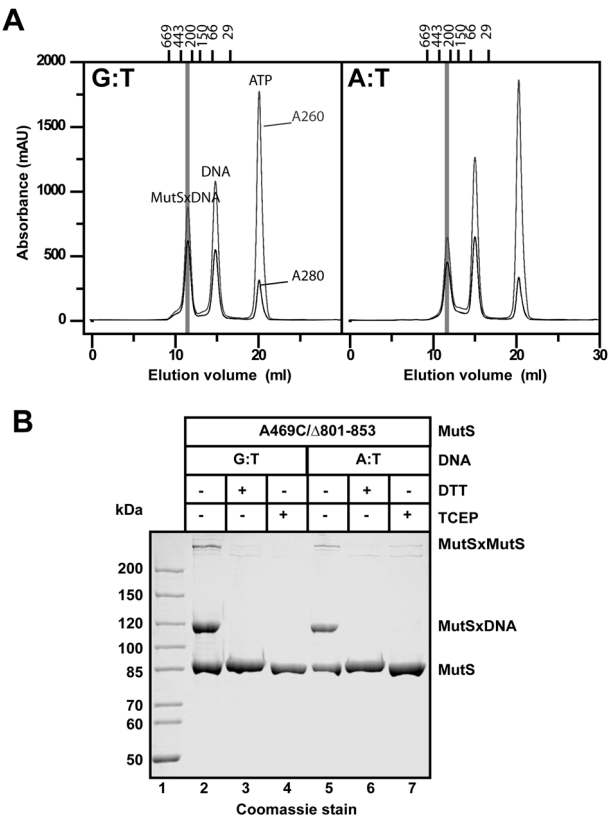

Fig. 3 Crosslinking and purification of MutS–DNA complexes. MutS (A469C/Δ801–853) (5 μM) was crosslinked to G:T heteroduplex I (10 μM, 30 min) or A:T homoduplex II (10 μM, 90 min) at 37 °C in the presence of 200 μM ADP in 500 μl of the crosslinking buffer (see Fig. 2, legend). (A) Size-exclusion chromatography elution profiles on a Superdex^{200}TM column are shown for the G:T and A:T DNA duplexes. Markers (in kDa) are indicated at the top axis. (B) Fractions eluting at Ve = 11.5 min (indicated by the grey line in panel A) were subjected to SDS-PAGE analysis before or after reduction with DTT or tris(2-carboxyethyl)phosphine hydrochloride (TCEP).

of the protein nor led to a significant formation of aggregates (Fig. 3A). Both purified complexes (with G:T heteroduplex I or A:T homoduplex II) reveal a 1 : 1 stoichiometry of crosslinked and uncrosslinked MutS (Fig. 3B).

A major characteristic of MutS function is allosteric communication between the DNA-binding and the ATPase domains. Thus, the presence of DNA stimulates the nucleotide exchange rate of MutS by more than 10-fold.[28] The purified crosslinked MutS–DNA complexes enabled us to test the effect of irreversible occupation of the DNA-binding site on the nucleotide binding/exhange activity of MutS. The fluorescent ADP analog, 2′-(or-3′)-*O*-(*N*-methylanthraniloyl)adenosine 5′-diphosphate (mant-ADP), was used to quantify the nucleotide exchange reaction, since mant-ADP fluorescence emission intensity increases on binding to MutS.[29] Release of mant-ADP from MutS was monitored by adding an excess of competitor unlabeled ADP, which blocks rebinding of mant-ADP to MutS. The nucleotide exchange kinetics were compared for MutS in the absence of DNA, in the presence of DNA and for the crosslinked MutS–DNA complexes. In the absence of DNA, the nucleotide exchange rates for all the variants were slow, k_{off}^{ADP} ranging from 0.003 to 0.007 s^{-1} (Fig. 4 and Table S3, ESI†), which is in agreement with k_{off}^{ADP} of 0.0072 s^{-1} for wild-type MutS.[30] Addition of either G:T heteroduplex I or A:T homoduplex II increased the nucleotide exchange rate to 0.046 and 0.034 s^{-1}, respectively, which again correlates with previous data.[30] Both crosslinked complexes of MutS either with G:T heteroduplex I or A:T homoduplex II displayed an increased nucleotide exchange rate similar or even higher than observed in the presence of the corresponding unmodified DNA duplexes III and IV (Fig. 4 and Tables S1 and S3, ESI†). This result indicates that the crosslink between Cys469 or Cys497 on MutS and the 3′-end of the DNA does not impair the allosteric communication between the DNA binding and the ATPase domain, and suggests that the crosslinked complex is functional and hence suitable for detailed mechanistic analysis of ATP binding, hydrolysis and ADP exchange by the MutS–DNA complex and its interaction with MutL. Indeed, we could show that the crosslinked complex is

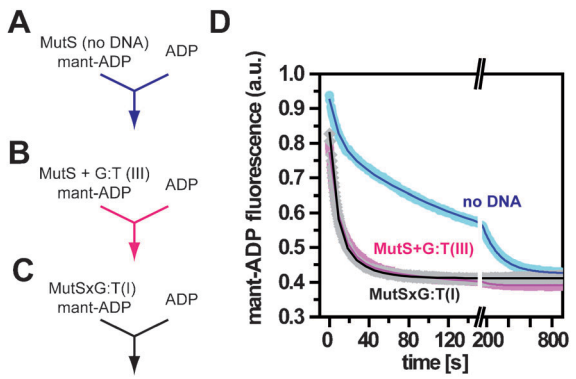

Fig. 4 Fast ADP-exchange of MutS crosslinked to DNA. ADP-exchange was measured by release of mant-ADP (500 nM) bound to MutS (0.5 μM dimer) (A) in the absence of DNA (blue), (B) in the presence of unmodified G:T duplex III (red) or (C) MutS crosslinked with G:T duplex I (black), when mixed with an excess of unlabeled ADP competitor (1 mM). (D) Change in mant-ADP fluorescence is plotted against time. Note, unbound mant-ADP has 40% fluorescence intensity compared to MutS-bound mant-ADP.[30]

still able to interact with MutL in an ATP-dependent manner using a recently developed method for chemical trapping of the MutS–MutL complex[31] (Fig. S3 in ESI†).

Conclusions

In the present work we have developed a new approach for efficient trapping of a protein–nucleic acid complex based on a variation of the thiol–disulfide exchange reaction, using a commercially available thiol modifier attached to the 3′-end of DNA. This approach will be useful for characterizing structure–function relationships of the DNA mismatch repair sensor and signaling protein MutS that forms multiple transient complexes with DNA during the reaction. Our results indicate that high yield crosslinking can be achieved allowing the purification of covalently linked MutS–DNA complex. Initial characterization of the purified complex revealed that the allosteric communication between the DNA binding and the ATPase domains is not impaired upon crosslinking and that the complex undergoes DNA-stimulated ADP exchange as well as ATP-induced ternary complex formation with MutL. In conclusion crosslinking of MutS with DNA is a promising starting point for a variety of structural and functional studies of MutS on homoduplex DNA (searching) and the ATP-activated clamp at the mismatch (signaling) or to trap the dynamic ternary complex formed between DNA, MutS and MutL. The specificity and efficiency of the crosslinking approach presented in this study is also applicable for the structural and functional characterization of other proteins that form mobile, dynamic or non-specific complexes with DNA. Even in the absence of structural information the disulfide exchange reaction can be used to map protein–DNA interaction or to trap proteins on DNA by a simple variation of the method. As an example, lysine residues, which are often part of protein–DNA interfaces, can be thiolated (*e.g.* *N*-succinimidyl *S*-acetylthioacetate).[32] Thiolated proteins that are still able to bind and crosslink to the DNA can be identified by SDS-PAGE combined with in-gel trypsin digestion/mass spectrometry analysis.

Acknowledgements

This work was supported by European Community Seventh Framework Program FP7/2007–2013 under Grant HEALTH-F4-2008-223545 (to P.F., I.W., and R.J.H.), Deutsche Forschungs-gemeinschaft Grant GRK 1384 (to P.F. and R.J.H.) and Russian Foundation for Basic Research Grant RFBR-DFG 11-04-91336 (to T.O. and S.S.).

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