Functional characterization of the DNA mismatch binding protein MutS from *Haemophilus influenzae*

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Abstract

This investigation demonstrates DNA mismatch repair activity in *Haemophilus influenzae* cell free extracts. The *mutS* gene as well as purified protein of *H. influenzae* restored repair activity in complementation assays performed with *mutS* deficient *Escherichia coli* strain. The difference in affinity for GT and AC mismatched bases by *H. influenzae* MutS was reflected in the efficiency with which these DNA heteroduplexes were repaired in vitro, with GT being repaired well and AC the least. Unlike *E. coli* MutS, the *H. influenzae* homolog failed to give protein–DNA complex with homoduplex DNA. Interestingly, MutS was found to bind single-stranded DNA but with lesser affinity as compared to heteroduplex DNA. Apart from the nucleotide- and DNA-mediated conformational transitions, as monitored by circular dichroism and limited proteolysis, our data suggest a functional role when *H. influenzae* MutS encounters single-stranded DNA during exonucleolytic step of DNA repair process. We propose that, conformational changes in *H. influenzae* MutS not only modulate mismatch recognition but also trigger some of the down stream processes involved in the DNA mismatch repair process.

Keywords: *Haemophilus influenzae*; DNA mismatch repair; MutS; ATPase; DNA binding; Limited proteolysis; Electrophoretic mobility shift; In vivo complementation; In vitro complementation; Circular dichroism; Heteroduplex DNA

One of the most extensively studied prokaryotic mismatch repair systems is the methyl-directed mismatch repair pathway of *Escherichia coli*. The methyl-directed mismatch repair pathway of *E. coli* has been reconstituted in vitro using purified components and has been shown to require nearly 11 proteins, MutS, MutL, MutH, DNA helicase II, SSB, Rec J, exonuclease I, exonuclease VII, exonuclease X, DNA polymerase III holoenzyme, and DNA ligase [13,30]. The methyl-directed mismatch repair system processes base pairing errors within the helix in a strand-specific manner by exploiting patterns of DNA methylation [2]. Repair is initiated by the binding of MutS to the mismatched base [34,40] followed by its recruitment of MutL, the entire process being regulated by ATP [19]. Assembly of this ternary complex is adequate to activate the nicking endonuclease activity of MutH, which incises the unmethylated strand at a hemimethylated d(GATC) sequence [43]. The unwinding activity of DNA helicase II (*uvrD*/*mutU* gene product) is also initiated at the incised d(GATC) sequence and unwinds DNA towards the mismatched base [15]. The resultant single-stranded DNA is subjected to degradation by one of the several single-strand DNA exonucleases [14,20]. DNA removed in this manner is resynthesized by DNA polymerase III holoenzyme in the presence of single-strand DNA-binding protein. Eventually, DNA ligase restores covalent continuity to the repaired strand [30]. Mutant strains defective in *mutH*, *mutL*, *mutS* or *uvrD* are spontaneous mutators and are deficient in mismatch correction [34].

Haemophilus influenzae is a naturally transformable gram-negative bacterium. It is widespread in its distribution among the human population and is commonly

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seen as a secondary invader after the infection by influenza virus. The pathogenic potential of *H. influenzae* critically depends on the phenomenon of phase variation of a number of surface-expressed molecules [24]. Mono- and dinucleotide repeat tracts produce large numbers of slippage events that are corrected by the mismatch repair pathway. Inactivation of *mutS* in *H. influenzae* was found to destabilize dinucleotide repeat tracts of chromosomally located reporter constructs implicating mismatch repair pathway in generating phase variation [4]. Watson et al. [42] recently reported hypermutable *mutS* mutants of *H. influenzae* in the sputum of cystic fibrosis patients.

Sequencing of the complete genome of *H. influenzae* Rd revealed the existence of the homologs of *E. coli* DNA mismatch repair genes [16], i.e., *mutH*, *mutL*, and *mutS*. Though the *mutH* and *mutL* gene products of *H. influenzae* are functionally characterized, [17,25] the *mutS* homolog has not been studied in detail so far. It was, therefore envisaged that a comprehensive analysis of the newly obtained MutS from *H. influenzae* could potentially consolidate and even advance our knowledge about MutS and its role in DNA mismatch repair.

Materials and methods

Growth of E. coli and H. influenzae strains. Escherichia coli strains used for the preparation of cell free extracts for the mismatch repair assays were grown at 37 °C in LB broth supplemented with 0.1% glucose until late-exponential phase (OD_{600} approximately 1.0–1.2). BL21(DE3) pLysS strain transformed with the *mutS* gene construct in pET15b was grown in LB broth supplemented with 100 μg/ml ampicillin. *H. influenzae* Rd (KW20) strain was grown at 37 °C in brain heart infusion (BHI) broth supplemented with hemin (10 μg/ml) and β-NAD^+ (10 μg/ml) until late-exponential phase (OD_{600} approximately 1.0–1.2), which was used for the preparation of cell free extract.

Substrate for in vitro mismatch repair assay. The substrate for in vitro mismatch correction is a 6440-bp, covalently closed circular DNA or with a single nick on the unmethylated strand, derived from the bacteriophage f1 and contains a GT base–base mismatch located within the overlapping recognition sites for two restriction endonucleases, *Hin*dIII and *Xho*I, at position 5632. Although the presence of mispair makes this site resistant to cleavage by either of the endonuclease, repair occurring on the unmethylated or nicked strand yields an AT base pair and generates a *Hin*dIII sensitive site. The heteroduplex substrate was prepared as described [32].

Mismatch repair assay using H. influenzae or E. coli cell-free extracts. The bacterial cells were collected by centrifugation at 4 °C and resuspended in 2 ml of ice cold buffer containing 50 mM Tris–HCl (pH 7.6), 10% sucrose, 1.2 mM DTT, and 150 mM KCl. The cells were treated with 0.23 mg/ml of lysozyme for 1 h followed by a heat shock at 37 °C for a time sufficient to yield a final suspension temperature of 20 °C. After centrifugation at 37,000 rpm at 4 °C for 1 h, the supernatant was subjected to ammonium sulfate precipitation at 70% saturation and the precipitate was collected by centrifugation.

Haemophilus influenzae. Rd cell extract, before subjecting to ammonium sulfate precipitation, was loaded on to phospho-cellulose column (15 ml bed volume) equilibrated with 25 mM potassium phosphate buffer (pH 7.6) containing 50 mM KCl, 0.1 mM EDTA, and 2 mM DTT. The column was washed with twice the bed volume of the same buffer. The wash was collected and subjected to ammonium sulfate precipitation at 70% saturation and the precipitated proteins were collected by centrifugation. The pellet was scraped into dialysis bag and dialyzed against buffer containing 25 mM Tris–HCl (pH 7.6), 0.1 mM EDTA, 100 mM KCl, and 2 mM DTT for 5 h at 4 °C with an intermittent buffer change. The dialysate was split into 20 μl aliquots and stored at −20 °C. The stored cell-free extracts were active up to 1 month.

A typical mismatch repair reaction (final volume, 15 μl) contained 50 mM Hepes–potassium salt (pH 8.0), 5 mM $MgCl_2$, KCl (adjusted to a final concentration of 90 mM taking into consideration the contribution of salt from added cell free extract), 1.5 mM ATP, 0.5 mM β-NAD^+, 1 mM glutathione, 100 mM (each) of the four deoxyribonucleoside-triphosphates, 200 ng of covalently closed or open circular DNA heteroduplex substrate and cell free extract (150 μg total protein) [32]. After incubation for 1 h at 37 °C, 45 μl of 25 mM EDTA was added and the samples were extracted twice with phenol and twice with water saturated diethyl ether. DNA collected by ethanol precipitation was subjected to *Hin*dIII/*Cla*I double digestion in a reaction volume of 20 μl and the digestion products were separated by electrophoresis on a 1% agarose gel in 89 mM Tris–borate/2 mM EDTA at 5.3 V/cm for 4 h. The gel was stained with ethidium bromide and viewed on a UV-transilluminator. The DNA bands were quantitated using UVI Band V. 97 software.

Over expression and purification of His_6-MutS protein. BL21(DE3) pLysS cells transformed with pET15b containing the *mutS* gene (pNJ6) were grown overnight, picked from a single colony, at 37 °C in 3 ml LB broth containing 100 μg/ml ampicillin (Amp). Six hundred milliliters of LB-Amp medium was inoculated with 3 ml of the overnight culture and allowed to grow to an OD_{600} of 0.8 at 37 °C. The expression of the MutS protein was achieved by the addition of IPTG to a final concentration of 0.2 mM and the cells were allowed to grow for another 3 h at 37 °C. The *H. influenzae* MutS was purified as described [25]. The protein was dialyzed extensively against the buffer containing 40 mM Tris–HCl (pH 7.6), 1 mM DTT, 200 mM KCl, 0.1 mM EDTA, 13.3% glycerol, and stored in the same buffer with 50% glycerol at −20 °C. The purity of the protein was analyzed on 0.1% SDS–10% polyacrylamide gel. The protein concentration was determined by using the theoretical extinction coefficient ($\varepsilon = 63{,}010\ M^{-1}\ cm^{-1}$) [36]. Typically, approximately 1 mg *H. influenzae* MutS protein of over 95% purity was obtained from 100 ml culture.

Complementation assay. Cell extracts were prepared, as described before, from isogenic strains of *E. coli*, which exhibited wild type (CC106) or *mutS* mutant (TX2929) phenotype. In vitro complementation assays were carried out using the cell free extract, prepared from the *E. coli mutS* deficient strain (RK1517), which was complemented by the addition of the purified *H. influenzae* MutS protein exogenously (1 μg MutS in 15 μl reaction). Since glycerol is a potent inhibitor of the mismatch repair activity [41], the purified MutS protein was dialyzed against the buffer (40 mM Tris–HCl (pH 7.6), 1 mM DTT, 200 mM KCl, and 0.1 mM EDTA) without glycerol before use in the mismatch repair assay.

DNA labeling and annealing. The oligonucleotide sequences used for the preparation of different heteroduplexes are the following. The master oligonucleotide (5′-CGAGTACCGCAATGAACTTAGCACC GAGTGAATGC-3′) when annealed with the complementary oligonucleotides 5′-GCATTCACTCGGTGCT **( )** ATTGCGGTACTCG-3′ (the sequence in the square brackets differs) incorporates a mismatched base which, in all cases, is present in the same sequence context as far as possible. The base mismatch (underlined) and the corresponding sequence in the square bracket are as follows: **GC**, 5′-AA GTTC-3′; **GT**, 5′-AAGTTT-3′; **GA**, 5′-AAGTTA-3′; **GG**, 5′-AAG TTG-3′; **AA**, 5′-AAGTAC-3′; **AC**, 5′-AAGTCC-3′; **CT**, 5′-AATTTC-3′; **CC**, 5′-AACTTC-3′; **TT**, 5′-ATGTTC-3′; and **ΔT**, 5′-TAAGTTC-3′. To study the effect of sequence context on the interaction of MutS with GT mismatch, the sequence in the square bracket for the master and complementary oligonucleotides used are: 5′-ATATGTATA-3′ and

5′-TATATATAT-3′ for AT-rich sequence context; 5′-GCGCGCGCG-3′ and 5′-CGCGTGCGC-3′ for GC-rich sequence context, respectively. One strand of each duplex (3 pmol) was labeled at the 5′-end with [γ-^{32}P]ATP (3 pmol) by using polynucleotide kinase as described by Sambrook et al. [37].

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays were carried out as described [25]. Unlabeled poly(dI)·poly(dC) (50 ng/reaction) was included in the reaction to reduce non-specific binding along with 1.5 nM [γ-^{32}P]-labeled DNA. Densitometric quantitation of the results was carried out using UVI Band V.97 software.

ATPase assay. ATPase activity of *H. influenzae* MutS was assayed in 15 μl of the reaction buffer containing 20 mM Tris–HCl (pH 8.0), 90 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, and ATP (concentrations as indicated) [3]. [α-^{32}P]ATP was included in the assay as a tracer. Unless otherwise specified, *H. influenzae* MutS was included at a concentration of 1 μM (monomer). When present, ADP or ATPγS was at a concentration of 5 mM. Reactions were carried out at 37 °C for 5 or 30 min as described [25]. Kinetic data of ATP hydrolysis were obtained with ATP concentrations varying from 5 μM to 3 mM. Kinetic constants were derived from an Eadie–Scatchard plot of $v/[S]$ vs. v. The results are average of at least three independent experiments.

Partial proteolysis. The limited proteolysis of *H. influenzae* MutS (2.5 μM) with trypsin (0.3 μg/ml) was carried out at room temperature for 5 min in a 20 μl reaction. When present, ADP or ATP was at 1 mM and DNA, 0.8 μM. The reactions were quenched by the addition of PMSF (1 mM). The proteolytic products were separated on 12% SDS–polyacrylamide gel and silver stained.

Circular dichroism measurements. Far-UV CD spectra (200–240 nm) were measured at 24 °C with a Jasco J-810 spectropolarimeter using a 0.2-cm path-length cell. The samples were prepared in 20 mM Tris–HCl (pH 7.6), 90 mM KCl, 1 mM $MgCl_2$, 5 mM β-ME, and 5% glycerol. *H. influenzae* MutS was used at a concentration of 1 μM (monomer) and ADP or ATP at 0.1 mM. DNA, when present, was 0.07 μM. The spectra plotted are the average of three independent scans.

Results and discussion

Haemophilus influenzae cell free extract is mismatch repair proficient

We have utilized the in vitro assay for mismatch correction to identify and characterize the mismatch repair pathway in *H. influenzae*. When the repaired GT heteroduplex is subjected to *Hin*dIII/*Cla*I double digestion, it is cleaved in to 3.3 and 3.1 kb fragments designated as the mismatch repair (MMR) products. The unrepaired GT heteroduplex would fail to give the MMR products; instead get linearized to a 6.4 kb fragment. *H. influenzae* cell free extract prepared in the same manner as for *E. coli* [32] did not give the expected mismatch repair products in the in vitro assay carried out using GT heteroduplex. Instead, two bands with different mobility were obtained (Fig. 1A, lane 2). It is likely that the cell free extract contains an unknown endonuclease. Incubating the substrate with the crude cell extract yielded a single band corresponding to the linearized substrate (Fig. 1A, lane 3), confirming the above possibility. To get rid of the contaminant, the *H. influenzae* cell extract was passed through phospho-cellulose column (see Materials and methods). This dialyzed cell extract was highly proficient in repairing the mismatches giving expected mismatch repair products of 3.3 and 3.1 kb (Fig. 1A, lane 4).

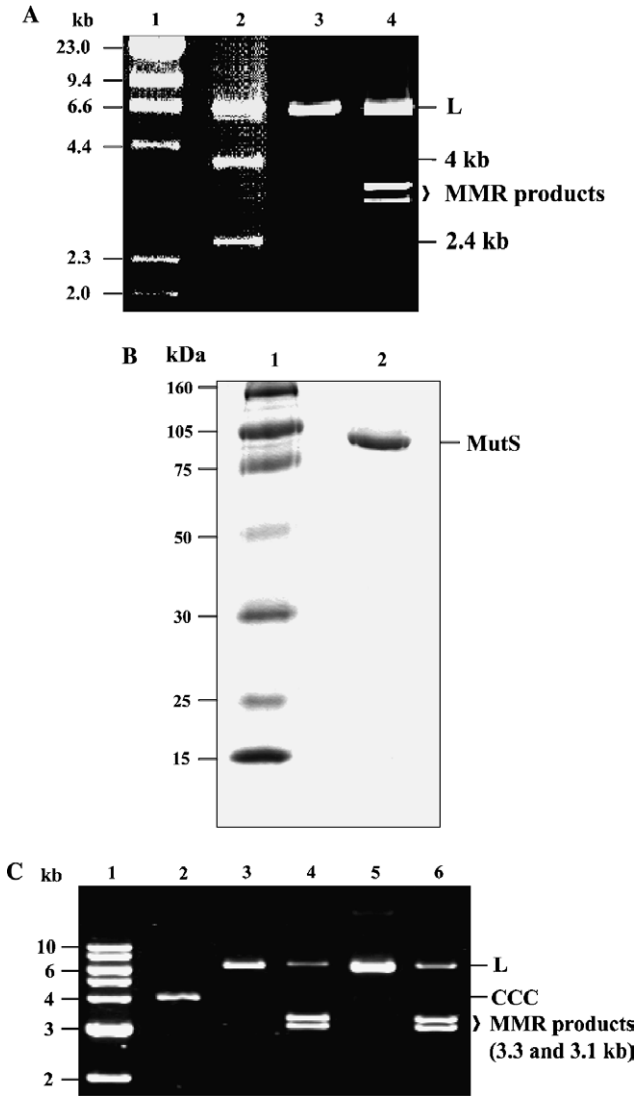

Fig. 1. In vitro mismatch repair assays. (A) Mismatch repair assays using cell free extracts from *H. influenzae*. Cell extract before and after passing through the phospho-cellulose column was used for the mismatch repair assay. Lane 1, molecular weight markers; lane 2, open circular heteroduplex DNA incubated with cell extract before passing through phospho-cellulose column and subsequent digestion with *Hin*dIII and *Cla*I; lane 3, same as lane 2 but not subjected to *Hin*dIII/*Cla*I digestion and lane 4, same as lane 2 but cell extract passed through phospho-cellulose column. (B) SDS–PAGE analysis of purified recombinant His_6-tagged MutS protein of *H. influenzae* visualized by staining with Coomassie blue. Lanes 1 and 2 are molecular weight markers and purified MutS, respectively. (C) In vitro complementation assay using purified *H. influenzae* MutS protein. Lane 1, 1 kb ladder; lane 2, untreated CCC heteroduplex; lane 3, same as lane 2, but digested with *Hin*dIII and *Cla*I. Lanes 4 and 5 are reactions with the cell extracts prepared from AB1157 and RK1517 strains, respectively. Lane 6, repair assay with the cell extract of RK1517 complemented by the exogenous addition of purified *H. influenzae* MutS protein (1 μg in 15 μl reaction) (CCC, covalently closed circular; OC, open circular; and L, linear).

Optimum repair activity was observed when the salt concentration was maintained between 50 and 90 mM KCl (data not shown). The final concentration of KCl in the reaction was calculated taking into consideration the contribution of salt from the reaction buffer and the added cell free extract.

Complementation assay of H. influenzae mutS gene product

Earlier studies on complementation of *mut* deficient *E. coli* strains with *mut* genes from other bacteria reported successful complementation in the case of the closely related *mutL* and *mutS* genes from *Salmonella typhimurium*, reduced complementation with the *mutS* gene of *Pseudomonas putida* and no complementation with *mutS* or *mutL* genes from more distantly related organisms like *Thermus aquaticus* and *Streptococcus pneumoniae* [6,21,29]. Therefore, it was of interest to find whether the addition of purified MutS protein could confirm its functional role in mismatch repair. For in vitro complementation assays, the purified recombinant *H. influenzae* MutS protein (Fig. 1B) was exogenously added to the cell extract prepared from *E. coli* RK1517 strain (Fig. 1C). Assays with the cell extract from wild-type *E. coli* strain was repair proficient (Fig. 1C, lane 4) whereas extract from the mutant was not (Fig. 1C, lane 5). Addition of the purified *H. influenzae* MutS exogenously (1 μg MutS) resulted in the mismatch repair deficient cell extracts regaining their ability to correct the base mispairs (Fig. 1C, lane 6). The repair bands were quantitated densitometrically using UVI Band V.97 software. The degree of complementation was observed to be high (up to approximately 80%), considering the amount of mismatch repair products obtained in the repair assays.

Affinity of MutS for heteroduplexes containing different base mismatches

Band shift analysis was used to study the interaction between *H. influenzae* MutS and synthetic DNA fragments containing all possible DNA mismatches as well as one-nucleotide loop (represented as Δ*T*) (Fig. 2A,

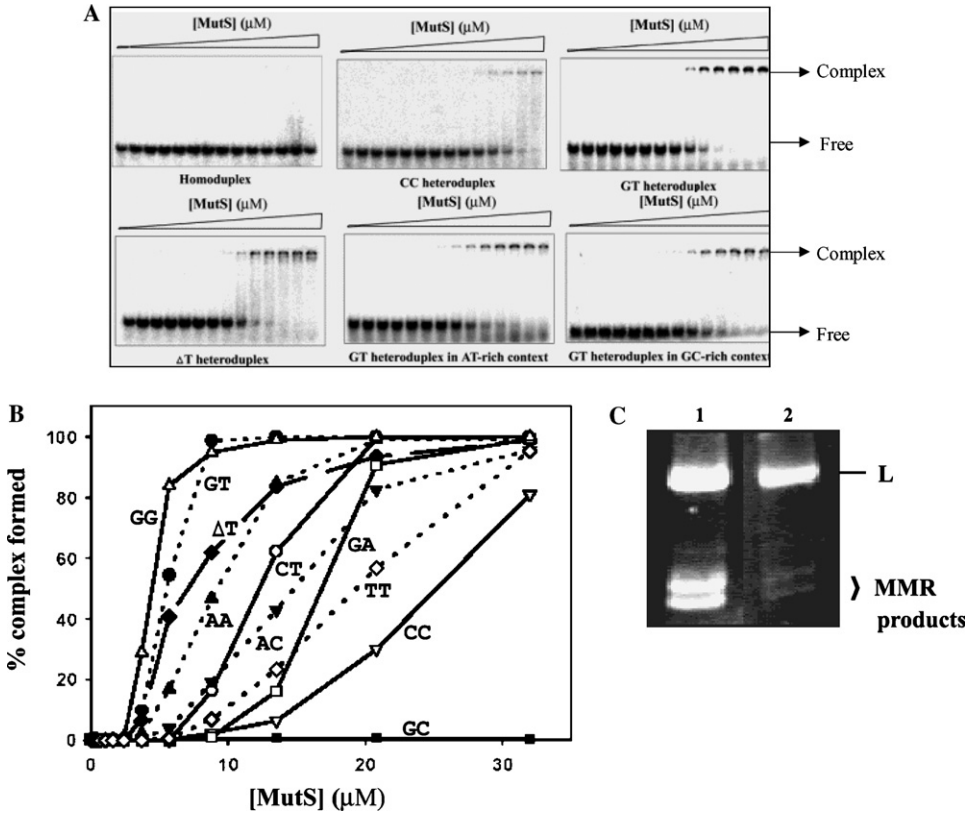

Fig. 2. (A) Gel mobility shift analysis of interaction between *H. influenzae* MutS and DNA duplexes containing mismatched bases carried out as described in Materials and methods. Endlabeled DNA (1.5 nM) was incubated with increasing concentrations of MutS (0–32 nM as monomer). The individual mismatches are shown below each panel. (B) MutS binding plot derived from the gel mobility shift assays are shown in (A). The data were obtained by a quantitative analysis of the phosphor images using UVI Band V.97 software. The plotted data are the average of at least three independent experiments. The symbols used are as follows: GC (black square), GG (open triangle), GT (black circle), AC (inverted black triangle), AA (black triangle), TT (open diamond), Δ*T* (black diamond), GA (open square), CT (open circle), and CC (inverted open triangle). (C) *H. influenzae* cell free extract repairs different mismatches with varying efficiencies. A representative repair assay using GT (lane 1) and AC (lane 2) DNA heteroduplexes (L, linear).

data shown for only GT, ΔT, CC mismatches). In all cases, the addition of MutS caused decrease in the mobility of DNA to different extents. These changes were dependent on the concentration of MutS, such that in several instances almost no free DNA was evident at the highest protein concentration used. We measured the disappearance of free DNA with increasing concentrations of His_6-MutS from which the percentage of retarded complex was calculated. The quantitative analysis for the heteroduplexes yielded the binding plots shown in Fig. 2B. Though *E. coli* MutS is shown to use a common binding mode to recognize a wide range of mismatches [35], reports have shown that it has variable affinity for different mismatches, the order being $\Delta T > GT > GG > AA \approx TT \approx TC > CA > GA > CC > GC$ [12]. From the analysis of our binding data, it can be concluded that the *H. influenzae* MutS exhibited differences in its affinities for the various mismatched bases and the order of affinity observed being $GG > GT > \Delta T > AA > AC \approx CT > TT > GA > CC > GC$. In contrast to *E. coli* MutS [12], we did not find any retarded complex when the radiolabeled Watson–Crick homoduplex was used in the absence (data not shown) or presence of unlabeled poly(dI).poly(dC) (Fig. 2A, panel Homoduplex). This could be due to the poor stability of the MutS-homoduplex complex compared to its binding to the heteroduplex DNA. The mismatch binding hierarchy is different to that of MutS from other organisms but this could be a reflection of MutS function in *H. influenzae* such as dinucleotide tract recognition. These differences might be associated with the role of mismatch repair in hyper mutability of contingency loci of *H.influenzae*.

To evaluate the repair efficiency of heteroduplex containing different base mismatches, assay was carried out using substrates containing either GT or AC base mismatch. Both these mismatches were located at the same position within the heteroduplex molecules and were embedded within the same sequence environment. In AC heteroduplex, the mismatch is present in the recognition sequence of *Xho*I instead of *Hin*dIII [32]. Both these substrates were corrected with different efficiencies in vitro (Fig. 2C). Analysis of the results have shown that GT was corrected more efficiently (Fig. 2C, lane 1), with AC being repaired poorly compared to GT mispair (Fig. 2C, lane 2).

Escherichia coli mismatch repair system has been shown to correct transition mismatches better than transversions, with GT and AC mismatches being repaired at a rate of 43–61% of that observed for GT mismatch, depending on the strand on which the repair occurred [39]. It was, therefore, surprising to note that the *H. influenzae* MutS binds AC mismatch to a lesser extent than GT. The hierarchy of apparent affinities of MutS for at least two mispairs (GT and AC) does correlate well with the efficiencies with which these mispairs are replaced in the in vitro assay. We have analyzed the effect of sequence context on binding of *H. influenzae* MutS to the DNA mismatch. Heteroduplexes containing a GT mismatch in two environments, surrounded by alternating A and T or G and C, were used for the binding assays. It was observed that under sub-saturating concentrations of *H. influenzae* MutS, the binding was marginally better when the GT mismatch was embedded in an AT rich sequence rather than GC (Fig. 2A). It is interesting to recall that the *H. influenzae* genome is AT rich (61%) in contrast to the *E. coli* genome, which has a GC content of 53% [11]. *E. coli* MutS protein, on the other hand, binds to GT mismatch flanked by GC rich sequences better than to GT mismatch flanked by AT rich regions [12]. The pathogenicity islands in *H. influenzae* are found to have higher GC content [23] when compared to the bulk of the genome. It could be speculated that the less affinity of *H. influenzae* MutS to mismatched bases in GC-rich sequences could lead to decreased efficiency of repair in such regions and hence contributing to high level of variability. The in vivo significance of this observation, in *H. influenzae*, is yet to be studied in detail.

Haemophilus influenzae MutS was found to be capable of binding to single-stranded DNA (Fig. 3A, lanes 2–7) and the DNA–protein complex could be competed out when excess of unlabeled single-stranded DNA was included in the reaction (Fig. 3A, lanes 9–14). This observation of *H. influenzae* MutS binding to single-stranded DNA is surprising, though its affinity is much less compared to that for GT or GG heteroduplex DNA. Blackwell et al. [9] reported the binding of hMutSα to high molecular weight ssDNA. It is quite possible that MutS encounters single-stranded DNA regions during the exonucleolytic cleavage step elicited upon recognition of the mismatched base. The in vivo significance of this observation at present is unclear.

Effect of nucleotide on the mismatch binding by H. influenzae MutS

In addition to the mismatch binding activity, *E. coli* MutS is capable of hydrolyzing ATP. The nucleotide hydrolytic center of MutS is necessary for the biological activity of this protein in vivo [22]. In the presence of ATP, MutS was found to dissociate quickly from linear heteroduplexes or heteroduplexes blocked at only one end [38]. MutS dimers were found to assemble into higher order oligomers in a concentration-dependent manner and ATP binding shifted this equilibrium in favor of the assembly [7,8].

To study the effect of nucleotide on the mismatch binding activity of *H. influenzae* MutS, the assays were carried out in the presence of ATP at 4 °C as well as room temperature (24 °C). Upon prolonged electrophoresis (upto 6 h), two distinct complexes, C1 and C2, were

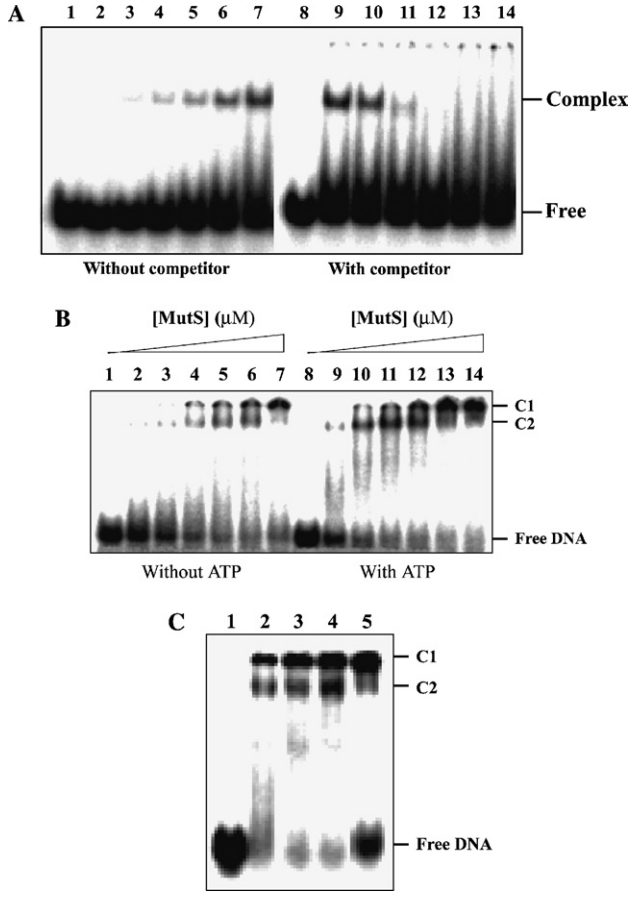

Fig. 3. (A) *Haemophilus influenzae* MutS binding to single-stranded DNA. End-labeled ssDNA (1.5 nM) was incubated with increasing concentrations of MutS (0–50 μM as monomer) (lanes 1–7). Lanes 8–14 represent assays with 1.5 nM labeled ssDNA incubated with 50 μM MutS and increasing concentrations of unlabeled ssDNA (0–500 nM). Lane 8, no protein control; lane 9, with MutS but no competitor; lanes 10–14 are reactions with 2, 15, 60, 250, and 500 nM of unlabeled competitor ssDNA, respectively. Samples were analyzed as described in Materials and methods. (B) Effect of nucleotide on the mismatch binding property of *H. influenzae* MutS. Gel mobility shift assay in which 1.5 nM of radio-labeled GT heteroduplex was incubated with increasing concentration of MutS (0–50 μM) in the absence (lanes 1–7) or presence (lanes 8–14) of 1 mM ATP and the samples were analyzed on 6% native polyacrylamide gel. (C) MutS binding assays in the absence of any nucleotide (lane 2); in the presence of 1 mM ADP (lane 3) or ATP (lane 4) or ATPγS (lane 5). Lane 1, no protein control.

obtained (Figs. 3B and C). The percentage of protein–DNA complex in the presence of ATP was higher than in the absence of ATP (Fig. 3B, compare lanes 3–7 with lanes 10–14 and Fig. 3C, compare lane 2 with lane 4) or in the presence of ADP (Fig. 3C, lane 3). From the differences in the mobility of the complexes formed, we suggest that complex C1 could be a tetramer or higher order oligomer whereas complex C2 could be a dimer of MutS. The presence of the non-hydrolyzable ATP analog, ATPγS, greatly reduced the amount of DNA–protein complex in complex C2 (Fig. 3C, lane 5). From the above observations it is suggested that, ATP favors the formation of *H. influenzae* MutS dimers which later, in a protein concentration-dependent manner, result in higher order oligomerization. Sedimentation velocity and gel filtration studies by Modrich and co-workers [7,8] indicated that *E. coli* MutS dimers assembled into higher order structures in the presence of ATP and this dimer-to-tetramer assembly promoted DNA binding by MutS.

ATPase activity of H. influenzae MutS

Thin layer chromatography analysis of *H. influenzae* MutS ATPase activity was carried out using [α-^{32}P]ATP as a tracer along with unlabeled ATP. Spontaneous hydrolysis of ATP in the absence of any protein was less than 5%.

Using Eadie–Scatchard plot, kinetic parameters were calculated (Fig. 4A). The k_{cat} of *H. influenzae* MutS ATPase was found to be $19.4 \pm 0.5\ min^{-1}$ and K_m (ATP) was 359.4 ± 35.7 μM at pH 8.0 and 37 °C. The kinetic parameters for *E. coli* MutS ATPase reported so far vary considerably; reported K_m values for ATP vary from 8.7 to 116 μM and that for k_{cat} falls in the range of 0.008–13.2 min^{-1} [5,7,27,31].

The kinetics of ATP hydrolysis by *H. influenzae* MutS in the presence of DNA was also determined (Fig. 4A). In the presence of GT heteroduplex, the MutS ATPase showed a K_m of 375.1 ± 8.2 μM ATP and the maximal hydrolysis rate ($k_{cat)}$ was $37.6 \pm 1\ min^{-1}$. When ssDNA was included in the reaction, the K_m and k_{cat} were 517.6 ± 15.2 μM ATP and $63.2 \pm 1.6\ min^{-1}$, respectively. *H. influenzae* MutS ATPase activity is stimulated 2-fold in the presence of GT heteroduplex and about 3.5-fold in the presence of single-stranded DNA. Interestingly, in the presence of DNA, substrate inhibition at higher concentrations of ATP was observed, which was highly significant when ssDNA was included in the reaction (data not shown). This could be indicative of the differential regulation of the activities of MutS upon encounter with single-stranded or duplex DNA during the repair process.

Genetic analysis has indicated that the nucleotide hydrolytic center of bacterial MutS is required for mismatch repair in vivo [31], and poorly hydrolyzed ATP analogues have been shown to inhibit steps of the mismatch repair reaction in vitro [33]. ADP, ATPγS (Fig. 4B) and AMP–PNP (data not shown) were able to inhibit the enzyme activity of *H. influenzae* MutS ATPase. Inhibition of ATPase activity by ADP was reported in *E. coli* MutS as well [7]. It was interesting to observe, that the *H. influenzae* MutS ATPase inhibition by ADP (product inhibition) was considerably reduced in the presence of DNA (Fig. 4B, compare bar 3 with bar 4). Blackwell et al. [10] have shown that *E. coli* MutS has the highest mismatch specificity in the absence of nucleotide or in the presence of ADP.

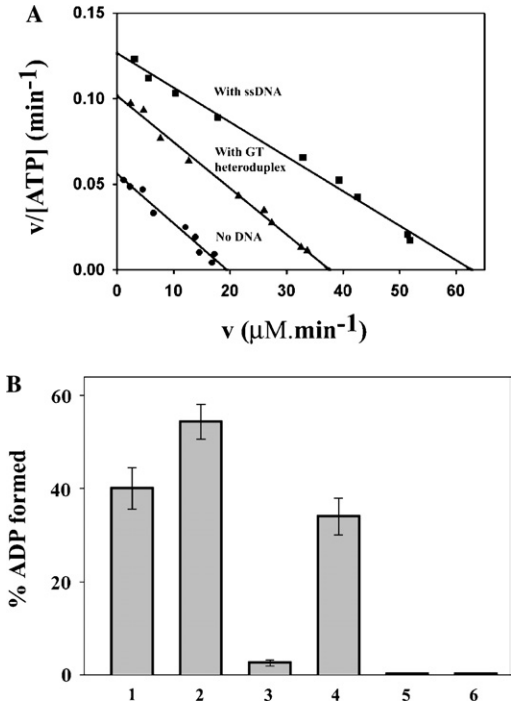

Fig. 4. (A) Eadie–Scatchard plot of *H. influenzae* MutS ATPase. The assays were done in the absence or presence of GT heteroduplex or ssDNA. The data points of v/[ATP] vs. v for the assay in the absence of DNA were fitted to a straight line with a slope of −0.003 and y-intercept of 0.0562 ($R^2 = 0.9628$). When present, DNA is used at a DNA to protein ratio of 1:3. Data points obtained for reactions in the presence of GT heteroduplex were fitted to a straight line with a slope of −0.0027 and y-intercept of 0.1018 ($R^2 = 0.9925$). For reactions with single-stranded DNA, the data points obtained were fitted to a straight line with a slope of −0.002 and y-intercept of 0.1266 ($R^2 = 0.9906$). Kinetic parameters such as K_m and k_{cat} were calculated from the Eadie–Scatchard plots. The results are the average of at least three independent experiments. (B) Effect of nucleotide analogs on MutS ATPase activity. The assays were done for 30 min at 37 °C with 1 μM MutS, 750 μM ATP and in the absence or presence of ssDNA, the ratio of DNA to protein being 1:3. When present, ADP or ATPγS was 5 mM. Bar 1, protein alone; bar 2, protein with ssDNA; bar 3, protein with ADP but no ssDNA; bar 4, protein with ADP and ssDNA; bar 5, protein with ATPγS but no ssDNA; bar 6, protein with ATPγS and ssDNA. The data were obtained by a quantitative analysis of the phosphor images using UVI Band V.97 software. Results are the average of at least three independent trials. Standard deviations are indicated by error bars.

Although our experiments in this study do not address which step is rate-limiting, we suggest that the rate-limiting step of MutS ATP hydrolysis activity could be the product (ADP) release and this is likely to be facilitated by the binding of *H. influenzae* MutS to DNA. This is reflected in the increased turnover of ATP hydrolysis by *H. influenzae* MutS in the presence of DNA (Fig. 4A). Both the Hingorani [1] and Modrich [7] laboratories have pre steady-state analyses that suggest which in the presence of ATP and mismatched DNA, a step after ATP binding but at or before chemistry becomes rate-limiting. It has been demonstrated that the level of ATP hydrolysis by *H. influenzae* MutS was higher when it encounters DNA ends [25] or in the presence of single-stranded DNA (Fig. 4A). DNA binding is shown to stimulate exchange of ADP for ATP [7,18] and ATP binding decreases the affinity for heteroduplexes [10,27]. Repeated binding of MutS to DNA with the concurrent exchange of ADP for ATP and the subsequent dissociation from DNA ends or at single-stranded DNA regions coupled with ATP hydrolysis could explain the alleviation of ADP inhibition of *H. influenzae* MutS ATPase in the presence of DNA.

DNA and nucleotide-dependent conformational changes in H. influenzae MutS

The conformation of *Thermus thermophilus* MutS in solution was found to be influenced by the presence of nucleotide, where in the ATP-bound form has the most compact conformation, the ADP-bound form was largely stretched and the nucleotide-free MutS attains a conformation intermediate between the two [28]. Joshi et al. [26] have shown that *E. coli* MutS undergoes conformational transition upon binding ATP or ADP or ATPγS, but in the presence of DNA, only ATP or ATPγS was able to bring about similar effect.

In order to understand the influence of DNA and nucleotide on the conformational changes acquired by *H. influenzae* MutS, we studied the limited proteolysis pattern by trypsin digestion. The proteolytic products obtained for free MutS as well as in the presence of ADP or ATP were largely similar when analyzed on 12% SDS–polyacrylamide gel using silver staining. (Fig. 5A, lanes 7–9). It is quite clear that, in the presence of nucleotide and DNA, MutS undergoes drastic conformational change, which results in the absence of several proteolytic products in the size range of 25–65 kDa (Fig. 5A, marked by arrowheads) as visualized on the polyacrylamide gel (Fig. 5A, compare lanes 3–5 with lanes 7–9). Circular dichroism spectra of *H. influenzae* MutS in the absence or presence of nucleotide reveals that the protein is structurally disordered (Fig. 5B). The spectra for MutS in the presence of nucleotide and/or DNA have been subtracted from those for DNA and or nucleotide alone. The nucleotide concentration in the samples had to be limited to 0.1 mM, as higher concentrations of it resulted in increased HT[V] that interfered with the recording of the CD spectra. The structural perturbation and disorder of MutS were observed when homoduplex DNA was included irrespective of the presence or absence of nucleotide (Fig. 5C). This clearly indicates that the ordered and compact functional conformation of MutS can be achieved only through the concerted effects of both ATP and heteroduplex DNA. The presence of ATP and heteroduplex DNA conferred MutS with a more

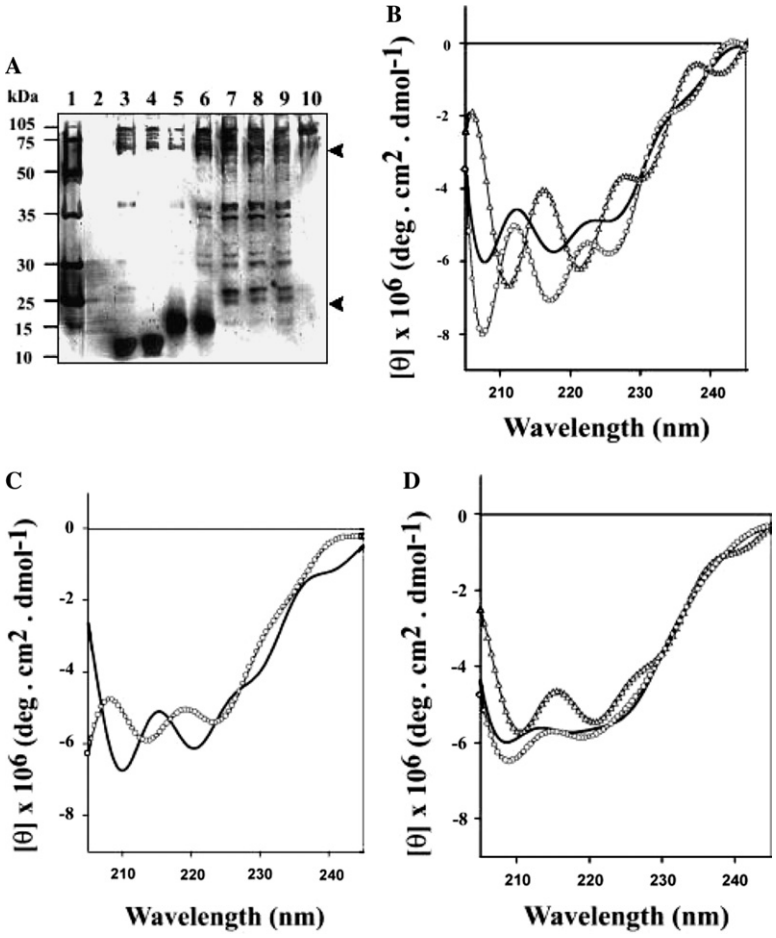

Fig. 5. Conformational changes in *H. influenzae* MutS. (A) Limited proteolysis. Partial tryptic digestion (0.3 μg/ml) of MutS (2.5 μM) was performed as described in Materials and methods. Lane 1, molecular weight markers; lane 2, trypsin alone; lane 3, MutS trypsinized in the presence of ssDNA and ATP; lane 4, MutS trypsinized with ssDNA and ADP; lane 5, MutS trypsinized with GT heteroduplex and ATP; lane 6, MutS trypsinized with GT heteroduplex and ADP; lane 7, MutS with trypsin treatment in the absence of DNA and nucleotide; lane 8, MutS trypsinized in the presence of ATP; lane 9, MutS trypsinized in the presence of ADP; lane 10, untreated MutS. (B) Far-UV CD spectra of *H. influenzae* MutS in the absence or presence of nucleotides. No nucleotide (thick line), 0.1 mM ADP (triangle) or 0.1 mM ATP (circle) was added to the samples. (C) MutS CD spectra with homoduplex DNA in the absence (thick line) or presence (circle) of 0.1 mM ATP. (D) MutS CD spectra in the presence of GT heteroduplex DNA with no nucleotide (thick line), 0.1 mM ADP (triangle) or 0.1 mM ATP (circle) added to the samples. The contributions of DNA or nucleotide to ellipticity were subtracted.

ordered structure. This is reflected in the increase in its ellipticity compared to conditions where MutS and heteroduplex were present without nucleotide or when ADP was included in the sample (Fig. 5D).

A challenging problem in the DNA mismatch repair pathway is to delineate the consequences of conformational changes of MutS upon mismatch/nucleotide binding thereby bringing about the recruitment of other protein components to the repair site. It could be anticipated that the conformational switch of MutS dimer helps in the formation of its MutL-interaction domain. Identification of the amino acid residues that mediate MutS–MutL interaction in conjunction with the binding of nucleotide can lead to the understanding of the coupling of initial mismatch recognition signal with the down stream processes of DNA mismatch repair.

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