Nucleotides and heteroduplex DNA preserve the active conformation of *Pseudomonas aeruginosa* MutS by preventing protein oligomerization

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MutS, a component of the mismatch repair system begins the DNA reparation process by recognizing base/base mismatches or small insertion/deletion loops. We have cloned the *mutS* gene from the human opportunistic pathogen *Pseudomonas aeruginosa* and analysed the biochemical properties of the encoded protein. Complementation of the hypermutator phenotype of a *P. aeruginosa mutS* mutant strain indicated that the isolated gene was functional. When purified MutS was incubated at 37 °C in the absence of ligands, a rapid inactivation of the oligonucleotide binding capability and ATPase activity occurred. However, the presence of ATP, ADP or heteroduplex oligonucleotides, but not homoduplex oligonucleotides, prevented the protein from being inactivated. The analysis of the protein by native PAGE indicated that the active conformation state correlates with the presence of MutS dimer. Analysis by gel-filtration chromatography showed that the inactive protein formed by incubation at 37 °C in the absence of ligands corresponds to the formation of a high molecular mass oligomer. The kinetic analysis of the oligomer formation showed that the extent of the reaction was markedly dependent on the temperature and the presence of MutS ligands. However, the protein inactivation apparently occurred before the maximum extent of MutS oligomerization. Further analysis of the MutS oligomers by electron microscopy showed the presence of regular structures consisting of four subunits, with each subunit probably representing a MutS homodimer. It is concluded that MutS possesses an intrinsic propensity to form oligomeric structures and that the presence of physiological ligands, such as nucleotides or heteroduplex DNA, but not homoduplex DNA, plays an important role in keeping the protein in an active conformation by preventing protein oligomerization.

Key words: DNA binding, electron microscopy, MutS ATPase, native gel electrophoresis.

INTRODUCTION

The MutS protein is part of the MutSLH mismatch repair system (MMRS) which prevents genome alterations in prokaryotes and eukaryotes. This system avoids point mutations or small insertions/deletions by correcting replication errors, as well as chromosomal rearrangements by preventing recombination between homologous sequences [1,2].

In *Escherichia coli*, the assembly of the MMRS to repair DNA biosynthetic errors is initiated by MutS, which recognizes and binds to mispaired nucleotides. Bound MutS interacts with MutL, which is supposed to promote the translocation of the complex to a hemi-methylated d(GATC) site where MutH is bound. This interaction stimulates an endonuclease activity of MutH, which produces a nick in the newly synthesized unmethylated DNA strand. The excision of the nascent strand is catalysed by a single-strand DNA-specific exonuclease. Finally, DNA polymerase III holoenzyme and DNA ligase carry out the resynthesis and ligation steps to restore the correct complementary sequence [1–3].

A weak ATPase activity is an evolutionary conserved characteristic of MutS [4–8]. This activity appears to be relevant for its physiological function, since mutations of the conserved nucleotide-binding domain result in a dominant mutator phenotype in both bacteria and yeast [4,9,10]. Besides the ATPase activity, the binding of adenine nucleotides to MutS plays an important role in the functioning of the MMRS. It has been shown that the presence of ATP reduces the affinity of MutS for the heteroduplex and it was suggested that the ATP hydrolysis energy is used for translocation of the repair complex towards the hemi-methylated nucleotide where MutH is bound [11,12]. However, using the human MSH2–MSH6 complex a 'molecular-switch' model has been proposed (where MSH corresponds to eukaryotic MutS homologues). The hydrolysis of ATP into ADP turns the protein into an 'on' state with high affinity for the heteroduplex, and subsequent exchange of ADP for ATP generates a 'sliding clamp' conformation able to diffuse along the double helix out of the mismatch [3,8,13]. In addition to the role of nucleotide binding and the ATPase activity of MutS to modulate the affinity for different DNA structures, protein conformation may also play an important role. The dimer conformation of the protein has been shown to be the active conformation that binds heteroduplex DNA [11,14–16]. However, the isolated protein also exists as tetramers and hexamers [7,14,17,18], and the role of these structures in the MMRS, if any, is still unknown.

Although the MMRS has been extensively studied in *E. coli*, at present little is known about how this system works in the Gram-negative bacteria *Pseudomonas aeruginosa*. This species can be found ubiquitously in nature and has long been known as an opportunistic pathogen for humans. In this regard, *P. aeruginosa* can produce severe infections in cystic fibrosis patients and in immunocompromised hosts, including patients suffering from cancer, HIV infections, severe burns and wounds [19]. Recently it has been described that 11 out of 30 cystic fibrosis patients were colonized by hypermutable *P. aeruginosa* strains [20]. In four of these strains, the mutator phenotype was due to mutations in the *mutS* gene suggesting that in *P. aeruginosa*, as in other bacterial species [21], a close relation exists between pathogenesis and mutator phenotypes produced by mismatch repair deficiency.

Abbreviations used: DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani; MMRS, mismatch repair system.

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In the present study, we show that recombinant purified MutS from *P. aeruginosa* loses its ATPase and DNA binding activities by brief incubation at 37 °C. Analysis of MutS by native PAGE, light scattering and gel-filtration chromatography indicated that this effect was related to protein oligomerization. The analysis of MutS oligomers by electron microscopy indicated the presence of tetrad conformations, with each subunit being a MutS dimer. We also found that the presence of adenine nucleotides and heteroduplex DNA, but not homoduplex DNA, is important to maintain MutS in an active conformation and to inhibit protein aggregation.

MATERIALS AND METHODS

Bacterial strains, plasmids and chemicals

E. coli Bl21 (λDE3) was obtained from Novagen, *E. coli* GM4271 (*mutS458::m*Tn*10*Kan) and GM5864 (λDE3, *mutS458::m*Tn*10*Kan) [22] were generously provided by Dr M. G. Marinus (University of Massachusetts, Worcester, MA, U.S.A.), *E. coli* RsH6 (*mutS*::Tn10) was kindly provided by Dr S. Rosemberg (Baylor College of Medicine, Houston, TX, U.S.A.), and *P. aeruginosa Hex*1*T* was isolated and characterized in our laboratory. The expression plasmid pET15b was obtained from Novagen and pET3d containing the *mutS* gene sequence from *E. coli* was kindly provided by Dr M. G. Marinus. The cloning vector pBBRIMCS-5 carrying gentamicin resistance [23] was provided by Dr G. Lucchesi (Departamento de Biología Molecular, Universidad Nacional de Río IV, Argentina). Purified human α2-macroglobulin was kindly provided by Dr G. A. Chiabrando (Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Córdoba, Argentina). Taq polymerase, DNA ligase, T4 polynucleotide kinase, calf intestinal alkaline phosphatase and restriction endonuclease were obtained from Promega. [γ-^{32}P]ATP was purchased from NEN Life Science Products. His-bind resin was obtained from Novagen. Rifampicin, ampicillin, nalidixic acid, nucleotides and nitrocellulose filters were purchased from Sigma, and oligonucleotides were from Bio-Synthesis (Lewisville, TX, U.S.A.).

Cloning of the *P. aeruginosa mutS* gene

A partial genomic *Bam*HI–*Hin*dIII library of *P. aeruginosa* constructed in pBKS (Stratagene) was used to isolate a 3.9 kbp fragment containing the *P. aeruginosa mutS* gene. The screening of the library was carried out with a ^{32}P-labelled 350 bp probe. The probe was obtained by labelling a 350 bp PCR fragment of *P. aeruginosa Hex*1*T mutS*. Primers were designed according to the nucleotide sequence of the *P. aeruginosa PAO*1 *mutS* fragment (nucleotides 6422–6770). Several *E. coli*-positive colonies containing the 3.9 kbp fragment inserted in pBKS were detected with this probe. Sequencing (Medigenomic, Martinsried, Germany) of this fragment (GenBank® accession number AF126491) revealed the presence of an open reading frame with 98 % sequence similarity to the *P. aeruginosa* PA01 *mutS* gene (GenBank® accession number AF-220055).

Subcloning of *mutS* in *E. coli* and *P. aeruginosa* vectors

In order to express the *P. aeruginosa mutS* gene in *E. coli*, the coding region of the gene (2650 bp) was subcloned in the T7 expression vector pET15b to produce the N-terminal histidine tag–MutS protein. The construction using the pET plasmid was carried out in two steps. The 3.9 kbp *Bam*HI–*Hin*dIII genomic fragment cloned in pBKS was subcloned in *Bam*HI–*Hin*dIII-digested pET15b (pETBH-*mutS*). To eliminate the 5′ untranslated region of the gene and to introduce the coding region in frame with the histidine tag of the plasmid, the following steps were carried out. The 880 bp fragment of *mutS* corresponding to the 5′ region of the coding sequence (from the start codon to the *Sal*I restriction site) was obtained by PCR using the *mutS* gene subcloned in pBKS as template, the forward primer, 5′-GGGGT-ACCATATGACCGACCTCTCCCA-3′, which contained an extra *Nde*I site and the ATG initiation codon present in the gene, and the reverse primer, which was designed using a sequence of the gene that contained a unique *Sal*I restriction site at position 880 of the coding region (5′- ATGGATCCAGGAAGTTGGG-TCCTT-3′). The amplified fragment of *mutS* was cloned into *Nde*I–*Sal*I-digested pETBH-*mutS* to obtain pET-*mutS*.

For *mutS* expression in *P. aeruginosa*, two constructions in the plasmid PBBRIMCS-5 (pMCS) [23] were carried out containing the *mutS* gene with and without the histidine tag, named pMCS-*his-mutS* and pMCS-*mutS* respectively. For the construction of pMCS-*his-mutS*, the *Xba*I–*Eco*RI fragment containing the histidine-*mutS* fusion present in pET-*mutS* was subcloned into the *Xba*I–*Eco*RI restriction sites of PBBRIMCS-5. For the construction of pMCS-*mutS* the genomic fragment *Bam*HI–*Hin*dIII containing the *mutS* gene was subcloned into the *Bam*HI–*Hin*dIII restriction sites of PBBRIMCS-5.

Expression of *P. aeruginosa mutS* in *E. coli* and purification of the recombinant protein

E. coli strain BL21 (λDE3) transformed with pET-*mutS* was grown at 37 °C in Luria–Bertani (LB) medium containing 50 μg/ml ampicillin to an absorbance at 600 nm (D_{600}) of 0.6. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.4 mM, and the cells were incubated at 37 °C for 30 min. Ampicillin (25 μg/ml) was then added and the culture was incubated for an additional 15 min. Cells were harvested by centrifugation and were washed once with 20 mM Tris/HCl (pH 7.4), 5 mM imidazole, 0.5 M NaCl and 13.3 % (v/v) glycerol. The cells were suspended in the washing buffer (0.01 vol. of the initial culture) and stored at −70 °C. Frozen cells were thawed on ice, incubated with 100 μg/ml lysozyme at 25 °C for 20 min and treated with five cycles of freezing, thawing and sonication. The cell extract was centrifuged at 100000 ***g*** for 60 min and the soluble fraction containing approx. 30 % of the total MutS was used for affinity purification. His-Bind chromatography was performed as described by the manufacturer (Novagen) except that MutS was eluted from the column with 0.4 M imidazole. Centricon S-30 was used to concentrate and equilibrate the protein in 50 mM Hepes/HCl (pH 7.4), 100 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 13.3 % (v/v) glycerol, and the protein was stored at −70 °C. Protein concentration was determined by a modified Bradford assay (Bio-Rad Laboratories) using BSA as a standard. Approx. 1.5 mg of protein was recovered from 1 litre of cell culture. Figure 1 shows a Coomassie Brilliant Blue-stained SDS/polyacrylamide gel of a representative MutS preparation. The production of MutS from the soluble fraction of extracts of uninduced and IPTG-induced *E. coli* cells, and the highly purified histidine-tagged MutS (> 90 %) obtained by nickel affinity-chromatography, are shown (Figure 1, lanes 1, 2 and 3 respectively).

Genetic complementation assay

Assays were carried out by transforming *E. coli* strains GM4271, GM5864, and RsH6 with the plasmid pET15b containing the *P. aeruginosa mutS* gene (pET-*mutS*). Overnight cultures were grown from single colonies in LB containing 100 μg/ml ampicil-

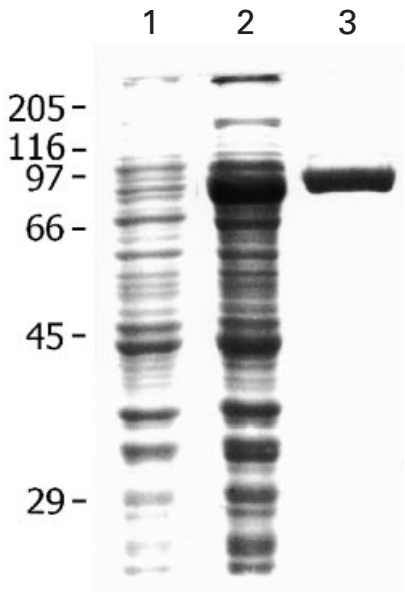

Figure 1 Purification of *P. aeruginosa* MutS expressed in *E. coli*

Analysis of an SDS/polyacrylamide gel stained with Coomassie Brilliant Blue R-250 that was loaded with the 100000 ***g*** supernatant of extracts from pET-*mutS*-transformed BL21 *E. coli* cells before (lane 1) and after (lane 2) IPTG induction. Purified protein after nickel affinity-chromatography (lane 3).

lin. To calculate the mutation frequency, appropriate dilution of cells transformed with pET15b or pET-*mutS*, were plated in LB containing ampicillin (100 μg/ml) to determine the number of viable cells, and in LB/ampicillin containing either 100 μg/ml rifampicin or 40 μg/ml nalidixic acid to score for rifampicin- or nalidixic acid-resistant cells. The mutation frequency was compared with controls carried out by transforming an *E. coli mutS*-deficient strain with the *mutS* gene from *E. coli*.

Complementation was also performed in a null *mutS* mutant of *P. aeruginosa Hex1T* (A. M. Smania, R. J. Pezza, V. de Lorenzo and C. E. Argaraña, unpublished work). This mutant was obtained by gene replacement using a cassette constructed *in vitro* containing the gene encoding kanamycin resistance flanked by the 5′ and 3′ regions of the *P. aeruginosa mutS* gene. For complementation analysis, this strain was transformed with pBBRIMCS-5 (pMCS) containing the *P. aeruginosa* genomic *mutS* (pMCS-*mutS*) sequence or the histidine-tagged *mutS* (pMCS-*his-mutS*). Transformants were selected with 30 μg/ml gentamicin. Appropriate dilutions of overnight cultures of transformed cells containing the plasmid with and without the *mutS* constructions were plated in LB/gentamicin to determine the number of viable cells, and in LB/gentamicin containing 100 μg/ml rifampicin to score for rifampicin-resistant cells. *E. coli* and *P. aeruginosa* cultures were grown at 37 and 30 °C respectively.

ATPase activity assay

This assay was performed in 20 mM Tris/HCl (pH 7.5), 3 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 100 μg/ml BSA, 200 μM ATP and 10 nCi/μl [γ-^{32}P]ATP (300 Ci/mmol) in a total volume of 100 μl. The reaction was started by the addition of 700 ng of purified MutS protein and incubated at 37 °C for 15 min. The ATPase activity of MutS was assessed by measuring the free radioactive phosphate, as described previously [24,25].

Oligonucleotide labelling and annealing

The labelling reaction was essentially performed as described by Jiricny et al. [26]; in a total volume of 10 μl, 40 pmol of single stranded 16-mer oligonucleotide, 60 units of T4-polynucleotide kinase, 5.2 pmol (16 μCi) of [γ-^{32}P]ATP, 70 mM Tris/HCl (pH 7.6), 10 mM $MgCl_2$ and 5 mM DTT were mixed and incubated at 37 °C for 30 min. The annealing of the oligonucleotides was performed in a final volume of 50 μl mixing 40 pmol of the labelled oligonucleotide and 50 pmol of its complementary strand in 20 mM Tris/HCl (pH 7.6), 5 mM $MgCl_2$, 0.1 mM DTT and 0.01 mM EDTA. The mixture was heated at 80 °C for 10 min and then left to stand at 25 °C for 2 h. The annealed duplexes were used immediately or stored at −20 °C. Duplexes were formed using the labelled oligonucleotides, 5′-GATCCG-TC**X**ACCTGCA-3′ (where **X** = G, T or C), with unlabelled 5′-TGCAGGT**N**GACGGATC-3′ (where **N** = G, A, T or C) to generate the X/N mispairs G/T, G/G, G/A, T/T, T/C, T/G, C/T, C/C and C/A, and the matched pairs G/C, T/A and C/G. Unlabelled 5′-TGCAGGTGACGGATC-3′ (15-mer) and 5′-TG-CAGGTAGGACGGATC-3′ (17-mer) were used to generate Δ1 and +1 duplexes respectively.

Binding of MutS to double-stranded oligonucleotides and DNA mobility shift assays

The binding reaction was carried out in a total volume of 25 μl containing 4 pmol of labelled oligonucleotide duplex, 1.5 μg of MutS (equivalent to 16 pmol of dimer), 20 mM Tris/HCl (pH 7.4), 1 mM DTT and 5 mM $MgCl_2$. The mixture was incubated on ice or at 37 °C for 15 min unless otherwise stated. After the incubation of MutS with the labelled oligonucleotide, 3 μl of 50 % (w/v) sucrose solution was added and the mixture was electrophoresed in a non-denaturing 5 or 6 % (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 30:1) in 40 mM Tris/acetic acid (pH 8.4), 1 mM EDTA (TAE). Electrophoresis was performed at 4 °C using TAE as a running buffer. Gels were run at 10 V/cm until the Bromophenol Blue dye migrated approximately 6 cm from the top. The radioactive complex was visualized by autoradiography.

Spectrophotometric light scattering measurements

Oligomerization of MutS was monitored by absorbance at 350 nm in a Shimadzu-UV-2401 PC (model 7CC-240A). Oligomerization was initiated by transferring the MutS preparation to temperature pre-equilibrated cuvettes.

Gel-filtration chromatography

Chromatography was performed at 4 °C in a Sephacryl S-400-HR (Sigma) column (33 cm × 0.5 cm) equilibrated in 20 mM Tris/HCl (pH 7.6) containing 50 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT. The flow rate was 0.15 ml/min and the elution profile was monitored by measuring the absorbance at 280 nm.

Electron microscopy

Aliquots of MutS (10 μl; 3 μg of protein) were applied on to 300 mesh carbon-coated copper grids and were negatively stained with a 2 % (w/v) aqueous solution of uranyl acetate. The samples were photographed in a JEOL 1200 EXII electron microscope at a magnification of × 60000. Figures were prepared for publication by scanning the negatives with an Agfa Duoscan 1200 scanner, and Corel PHOTO-PAINT software was used to adjust the brightness and contrast.

RESULTS

Characteristics of the *P. aeruginosa mutS* gene and general properties of the purified recombinant histidine-tagged protein

The analysis of the nucleotide sequence of the *P. aeruginosa Hex1T mutS* gene revealed an open reading frame of 2565 bp, encoding a polypeptide of 855 amino acids. The molecular mass calculated from the translated DNA sequence is approx. 95 kDa, similar to the apparent molecular mass observed by SDS/PAGE analysis of the purified recombinant protein (Figure 1). Sequence comparison of the deduced amino acid sequence of *P. aeruginosa Hex1T* MutS showed extensive similarity with MutS from *P. aeruginosa PAO1* (98% identity) and *Azotobacter vinelandii* (84% identity) and moderate similarity with MutS from *E. coli*, *Vibrio cholerae* and *Salmonella typhimurium* (58, 57 and 56% identity respectively).

In order to determine whether the recombinant MutS from *P. aeruginosa* was effective in the MMRS *in vivo*, we tested its capacity to complement a knockout *P. aeruginosa* mutant of *mutS* (see the Materials and methods section). By scoring the number of rifampicin-resistant cells, we found that the recombinant gene was able to complement the *mutS*-deficient strain, reverting the mutation frequency to the levels of the wild-type strain (Table 1). The presence of the histidine tag did not have any influence on the activity of the protein since the complementation was similar using the gene modified with the histidine tag or containing the genomic sequence in the 5′ end (Table 1).

In order to characterize the properties of the recombinant protein, we analysed the oligonucleotide binding specificity and the ATPase activity. Using band shift assays it was found that the purified MutS preferentially binds to oligonucleotide containing the G/T mismatch. The analysis of the presence of nucleotides on this reaction showed that the addition of 0.01–0.20 mM ADP produced a 3-fold activation of oligonucleotide binding. ATP also activated the binding at 0.01–0.20 mM (by 2-fold), but an inhibitory effect was observed at concentrations higher than 0.6 mM. Determination of the release of $[^{32}P]P_i$ from [γ-^{32}P]ATP by *P. aeruginosa* MutS indicated that the purified protein has an ATPase activity with a V_{max} of 2.1 μM ATP/min and a K_m of 35 μM. This activity was stimulated 2-fold by the addition of 0.1–20 μM 16 bp heteroduplex G/T or homoduplex G/C, but was inhibited at concentrations higher than 30 μM. These results show that, except for the moderate activation of oligonucleotide binding observed on the addition of ATP or ADP, the properties of MutS are similar to those described for MutS of other bacterial species [4,27,28].

Table 1 Effect of *P. aeruginosa mutS* gene expression on the frequency of spontaneous mutation in *P. aeruginosa*

Mutator assays were carried out as described in the Materials and methods section. Results are the means from three independent cultures (±S.D.).

P. aeruginosa strain	Number of rifampicin-resistant mutant cells/10^8 cells
Wild-type	3.9 ± 2.5
Wild-type (PMCS)	5.8 ± 4.5
Wild-type (PMCS-*mutS*)	7.0 ± 3.5
Wild-type (PMCS-*his-mutS*)	7.5 ± 3.5
Δ*mutS*	(1.1 ± 0.5) × 10^3
Δ*mutS* (PMCS)	(1.5 ± 0.3) × 10^3
Δ*mutS* (PMCS-*mutS*)	3.2 ± 1.5
Δ*mutS* (PMCS-*his-mutS*)	5.1 ± 2.0

In the absence of ligands, the incubation of MutS produces inactivation of ATPase activity and oligonucleotide binding capability

We found that the oligonucleotide binding capability and the ATPase activity of MutS are rapidly inactivated by brief incubation at 37 °C in the absence of ligands (Figure 2, left-hand panel). Further analysis (see below) showed that this result was not due to protein degradation. Preincubation of the protein for 15 min at different temperatures (Figure 2, right-hand panel) showed that the binding capability of MutS is lost at a temperature higher than 30 °C, and the ATPase activity started to decrease above 20 °C. Although both activities of MutS are lost, the ATPase activity is apparently more rapidly inactivated and temperature-sensitive than the DNA binding capability. We found that the inactivation process is not an exclusive property of MutS from *P. aeruginosa* but it also is shared by MutS from *E. coli* (results not shown).

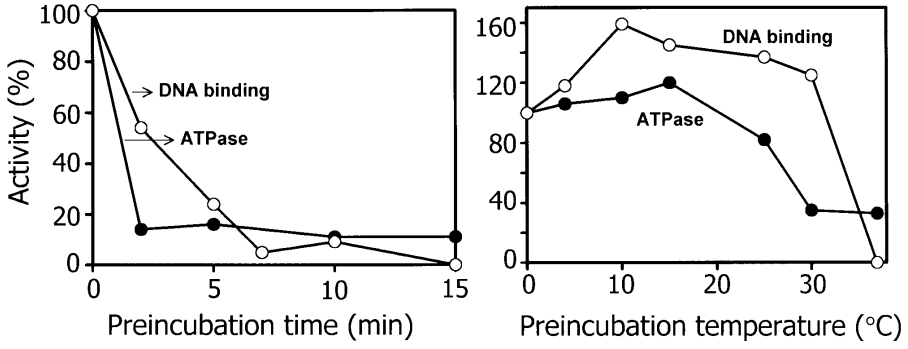

Figure 2 Effect of preincubation time and temperature on oligonucleotide binding and ATPase activity of MutS

Left-hand panel: MutS was preincubated at 37 °C in the oligonucleotide binding or ATPase buffer for different periods. After preincubation, the binding of ^{32}P-labelled 16 bp oligonucleotide G/T to MutS (○) and the ATPase activity (●) were assayed as described in the Materials and methods section. The oligonucleotide binding values were obtained by densitometric scanning of the gel autoradiogram. The ATPase and the oligonucleotide binding activities are expressed as the percentage of the activity determined with MutS without preincubation. Right-hand panel: after preincubation of the protein for 15 min at different temperatures, complex formation between the ^{32}P-labelled 16 bp oligonucleotide G/T and MutS (○) and the ATPase activity of MutS (●) were determined as described above.

Table 2 Nucleotides and heteroduplex oligonucleotides protect the ATPase activity of MutS from inactivation

MutS (700 ng) was preincubated for 15 min at 37 °C in the ATPase buffer (see the Materials and methods section) in the absence or presence of nucleotides, oligonucleotide homoduplex (G/C) or oligonucleotide heteroduplex (G/T). After preincubation, ATPase activity was measured as described in Materials and methods section, and activities are presented as means ± S.D. The ATPase activity of MutS without preincubation was measured in the absence or presence of the nucleotide or oligonucleotide as indicated.

Assay	Preincubation	Preincubation period addition	Incubation period addition	ATPase activity (pmol of ATP hydrolysed)
1	+	–	–	70 ± 28
2	–	–	–	380 ± 17
3	+	50 μM ATP	–	510 ± 27
4	–	–	50 μM ADP	210 ± 26
5	+	50 μM ADP	–	300 ± 27
6	–	–	1.6 μM G/C	680 ± 16
7	+	0.16 μM G/C	–	100 ± 8
8	+	1.60 μM G/C	–	120 ± 14
9	–	–	1.6 μM G/T	700 ± 18
10	+	0.16 μM G/T	–	530 ± 14
11	+	1.60 μM G/T	–	1000 ± 13

Nucleotides and heteroduplex DNA preserve the ATPase activity of MutS

We examined whether the presence of nucleotides during preincubation might affect the inactivation of the ATPase activity. The preincubation of MutS for 15 min at 37 °C caused a loss of approx. 80 % of the ATPase activity (Table 2, assay 1 compared with assay 2; also see Figure 2). However, the addition of ATP during the preincubation period completely preserved the enzyme activity (Table 2, assay 3). We also assayed the ATPase activity of MutS after preincubation with different compounds (Table 2, assays 5, 7, 8, 10 and 11) and compared it with the enzyme activity without preincubation but in the presence of the same compounds (Table 2, assays 4, 6 and 9). ADP preserved the enzyme from inactivation (Table 2, assay 5) although a partial inhibition of the activity was produced (Table 2, assay 4). The effect of DNA on the inactivation of MutS ATPase was also investigated. The presence of homoduplex G/C oligonucleotide increased enzyme activity moderately (Table 2, assay 2 compared with assay 6), but it did not protect MutS during preincubation (Table 2, assays 7 and 8). The presence of mismatch G/T oligonucleotide also produced an activating effect (Table 2, assay 2 compared with assay 9), but, unlike the homoduplex, it preserved the ATPase activity from inactivation at 37 °C (Table 2, assays 10 and 11). It should be noted that when the MutS ATPase was preserved from inactivation, except for ADP, the enzyme activity was slightly higher than controls, probably due to an effect of the ligands on the protein conformation during preincubation which makes the enzyme more efficient at catalysing ATP hydrolysis.

Nucleotides prevent the inactivation of the oligonucleotide binding capability of MutS

We found that the inactivation of the oligonucleotide binding capability of the protein was also completely prevented by the presence of ATP or ADP (0.1 mM) during the preincubation (Figure 3A, lanes 3 and 4 respectively). However, under these conditions no activation of oligonucleotide binding was detected, as in the case with non-preincubated MutS mentioned above. We also found that in a similar way to MutS from *P. aeruginosa*, the

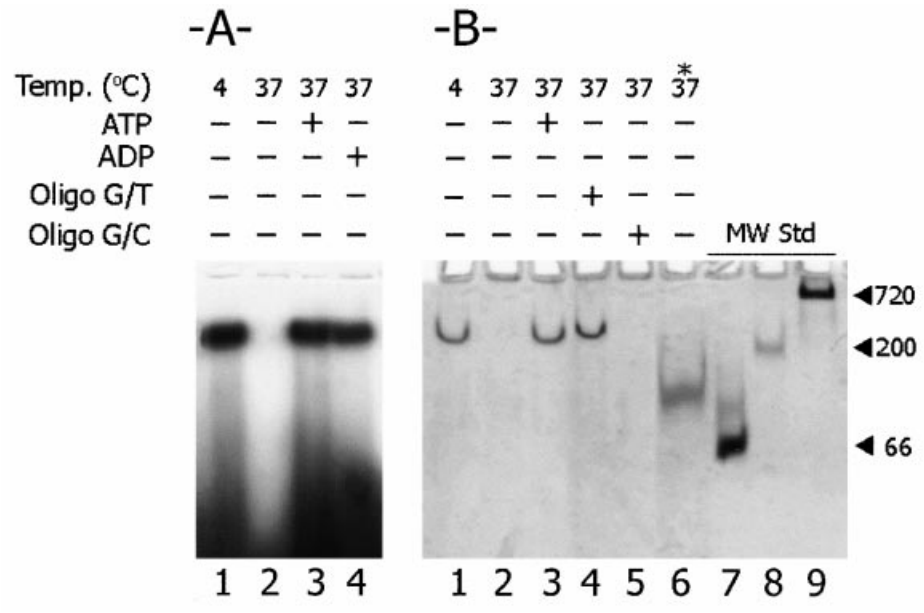

Figure 3 Effect of different preincubation conditions on MutS oligonucleotide binding capability and protein aggregation analysed by non-denaturing PAGE

(**A**) Autoradiogram of the ^{32}P-labelled 16 bp oligonucleotide G/T–MutS complex analysed by native PAGE (see the Materials and methods section). Preincubation of MutS prior to the binding reaction was performed for 15 min at 37 °C in the binding buffer in the absence or presence of 0.1 mM ATP or ADP. (**B**) Gel electrophoresis was performed as described in (**A**). MutS (3 μg) was incubated, prior to electrophoresis, in 25 μl of oligonucleotide-binding buffer for 15 min under the conditions indicated. The concentration of ATP was 0.1 mM, and the concentration of mismatch G/T oligonucleotide (oligo G/T) or homoduplex G/C oligonucleotide (oligo G/C) was 1.6 μM. *After incubation, the sample was incubated for 5 min at 90 °C in the presence of 5% (w/v) SDS. Molecular mass standards: BSA, 66 kDa; β-amylase from sweet potato, 200 kDa; and human α2-macroglobulin, 720 kDa. The gel was stained with Coomassie Brilliant Blue R-250.

presence of ATP and ADP preserved *E. coli* MutS from inactivation (results not shown). However, it was observed that the addition of nucleotide (0.1–1 mM ATP or ADP) after preincubation did not recover the binding capability of any of the proteins (results not shown). It was also determined that the addition of ATP at concentrations as low as 1 μM during the preincubation period was sufficient to preserve the binding capability of MutS. The nature of the compounds that preserve the oligonucleotide binding of MutS seems to be restricted to adenine nucleotides since the addition of 0.1 mM GTP or GDP to the preincubation mixture did not produce any effect. Similarly, the presence of 100 μg/ml albumin, a common additive used in MutS buffers, or the increase of the ionic strength by the addition of up to 200 mM NaCl, did not affect the inactivation of MutS (results not shown).

Inactivation of MutS correlates with changes in protein aggregation

In order to investigate if MutS inactivation by incubation at 37 °C was produced by a change in the aggregation state of MutS, we analysed the protein migration (Figure 3B) in the same native gel system used for the MutS–oligonucleotide complex. The migration of the protein without incubation at 37 °C (Figure 3B, lane 1) was similar to the migration of the MutS–heteroduplex (Figure 3A, lane 1). In both cases migration corresponds to an apparent molecular mass slightly higher than 200 kDa, according to the migration of the molecular mass standards (MW Std; Figure 3B, lanes 7–9). Taking into account that it has been demonstrated that the dimer is the active conformation of MutS [11,14–16], we conclude that the protein band observed in Figure 3(B), lane 1, corresponds to the dimer conformation. As previously mentioned, MutS preincubated at 37 °C in the absence of ligands fails to form a complex with heteroduplex DNA (Figure 3A, lane 2) and, as shown in Figure 3(B), lane 2, the protein band is absent. This suggests the formation of a high molecular mass aggregate that is not able to enter into the gel. To verify this, after the incubation of MutS in the absence of ligands, SDS was added in order to disaggregate the MutS oligomers and the protein was then analysed by gel electrophoresis. This treatment produced a faster migrating band with an apparent molecular mass of approx. 110 kDa, probably corresponding to a denatured monomer species of MutS (Figure 3B, lane 6). This result clearly demonstrated that the inability of the protein to enter into the gel (lane 2) was due to the formation of a high aggregation state of MutS during incubation in the absence of ligands.

The incubation of MutS in the presence of 0.10 mM ATP resulted in a protein species that migrated with a similar mobility to that without incubation (Figure 3B, lanes 3 and 1 respectively). A similar migration was observed when MutS was incubated in the presence of 0.1 mM ADP (results not shown) or 1.6 μM heteroduplex DNA (Figure 3B, lane 4); however, homoduplex DNA was not able to avoid protein oligomerization (Figure 3B, lane 5). We found that ATP prevents MutS aggregation within the 0.001–0.100 mM range, whereas ADP at the same concentration also prevents aggregation, but less efficiently than ATP. The non-hydrolysable nucleotide adenosine 5′-[β,γ-imido]triphosphate also prevents MutS oligomerization at 0.01 mM, but not at higher concentrations (results not shown). The addition of ATP or ADP (0.01–1.00 mM) after the incubation of the MutS in the absence of ligands did not revert protein aggregation (results not shown). These results indicated that the presence of ATP, ADP or heteroduplex DNA are important to maintain the protein in a dimeric active conformation and that the oligomeric MutS formed in the absence of ligands is not disassembled by the addition of these compounds.

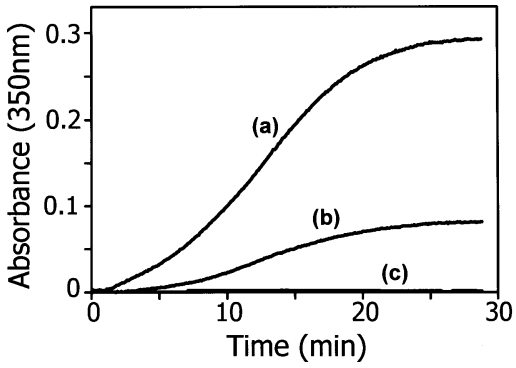

Figure 4 Time course of MutS oligomerization monitored by light scattering

The oligomerization reaction was initiated by adding a purified sample of MutS in 20 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA and 1 mM DTT to temperature pre-equilibrated cuvettes. Unless indicated, the reaction was carried out for 15 min at 37 °C. a, 2 μM MutS; b, 0.2 μM MutS; and c, 0.2 μM MutS in the presence of ATP or ADP (0.1 mM), or incubated at 30 °C.

Analysis of MutS oligomerization

To obtain information about the kinetics of the oligomerization reaction, we analysed the time course of the protein aggregation by light scattering (Figure 4, traces a and b). It was observed that the reaction followed a typical oligomerization curve where the time lag is probably due to some co-operative structural protein change that takes place before the proper oligomer formation. The oligomerization process did not proceed when the reaction was performed below 30 °C or in the presence of 0.1 mM ATP or ADP (Figure 4, trace c). One additional observation was that the pre-formed oligomer was not disassembled by the addition of nucleotides or shifting the temperature from 37 to 20 or 4 °C for a period of 30 min (results not shown). These results are in agreement with those previously obtained by native PAGE analysis.

We also investigated whether the histidine tag present in the recombinant MutS had any effect on protein oligomerization. Treatment of MutS with thrombin (60 ng/ml thrombin/μg of MutS, incubated for 8 h at 8 °C) removed more than 80 % of the histidine tag as evaluated by immunoblotting (results not shown), using an anti-(poly histidine) antibody (His-probe H15; Santa Cruz Biotechnology). Following this treatment, the protein showed a similar extent of oligomerization as determined by absorbance at 350 nm and native PAGE analysis (results not shown). This result is in agreement with the expectation that slight modifications at the N-terminal region of the protein might not alter MutS oligomerization, since it has been shown that the domain involved in this process is localized at the C-terminal region of the protein [16,22,29].

In order to verify that the protein was forming high molecular mass oligomers we performed gel-filtration chromatography of MutS after incubation at 37 °C and compared the elution profile with that of the protein without incubation. Using this methodology it has been described that native purified MutS preparations without any treatment, contain molecular mass species of 200 and 560 kDa, probably corresponding to a dimer and hexamer oligomers respectively [18]. For this reason we used a gel matrix (Sephacryl S-400 HR; see the Materials and methods

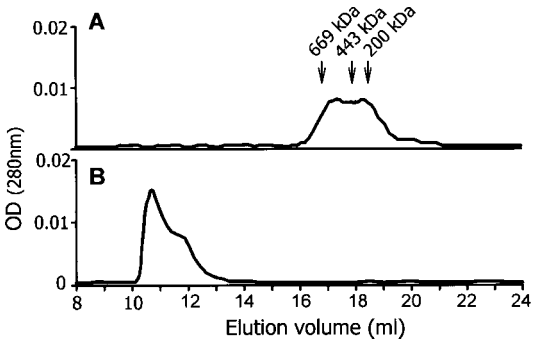

Figure 5 Gel-filtration chromatography analysis

MutS (3 μM) in 20 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA and 1 mM DTT was chromatographed as described in the Materials and methods section. Molecular mass standards used were: tyroglobulin, 669 kDa; apoferritin, 430 kDa; and β-amylase, 200 kDa. (**A**) Elution profile of MutS without incubation. (**B**) Elution profile of MutS preincubated at 37 °C for 15 min.

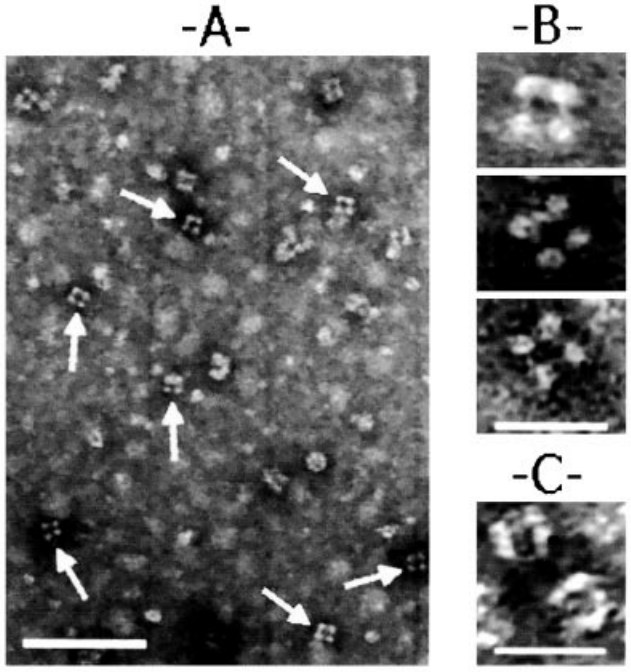

Figure 6 Transmission electron microscopy of MutS oligomers

Oligomerization of MutS was carried out by incubation of purified MutS (2 μM) in 20 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA and 1 mM DTT, for 15 min at 37 °C. Electron microscopy was carried out as described in the Materials and methods section. (**A**) Arrows indicate the MutS tetrad oligomeric structures. The white bar corresponds to 100 nm. (**B**) Three individual MutS oligomers at high magnification. (**C**) The image of human α2-macroglobulin tetramer. The white bars in (**B** and **C**) correspond to 25 nm.

section) able to separate compounds in the range of 20–8000 kDa for globular proteins and 2–2000 kDa for dextrans. Figure 5(A) shows that the protein without incubation eluted in a broad peak, probably corresponding to the described species of 200 and 560 kDa. On the other hand, after incubation at 37 °C, the protein eluted as apparently two overlapped peaks close to the void volume of the column (Figure 5B), indicating the presence of higher molecular mass oligomers than those observed in Figure 5(A).

Analysis by electron microscopy of a MutS oligomer preparation after incubation at 37 °C revealed the presence of regular structures (Figures 6A and 6B), whereas in MutS samples without incubation or incubated at 37 °C in the presence of ATP no regular protein structures were observed (results not shown). These regular structures form a tetrad arrangement with a diameter of 17.3 ± 1.3 nm, where each subunit is apparently not superposed with each other but symmetrically separated (Figure 6B). Since the more stable structure of non-oligomerized MutS appears to be a dimer, we assume that each oligomer subunit is composed of a dimer of MutS (190 kDa), and, in this case, the calculated molecular mass of the oligomer should be at least 760 kDa. To analyse this possibility, we compared by electron microscopy the MutS oligomer dimensions with a protein of a similar molecular mass, i.e. human α2-macroglobulin, which is a globular tetramer with a molecular mass of 720 kDa formed by four identical subunits [30]. Figure 6(C) shows that the diameter of this protein is of a comparable magnitude (13.5 ± 1.2 nm) to that of the MutS oligomer. It should be pointed out that the absence of a protein peak corresponding to a molecular mass of 760 kDa in the oligomerized MutS sample analysed by gel-filtration chromatography could be explained by assuming that the tetrad structure observed by electron microscopy constitutes part of a more complex structure not observed by this methodology or that each tetrad subunit represents more than one MutS dimer.

DISCUSSION

In the present study, we found that MutS from *P. aeruginosa* is inactivated by a process related to protein oligomerization. The DNA binding capability and the ATPase activity of MutS decreased after incubation for a short period at 37 °C. The time course of inactivation and the analysis of the temperature of inactivation showed that the ATPase activity is more unstable than the DNA binding capability of the protein. We also found that the DNA binding capability and the ATPase activity of the *E. coli* protein are inactivated by preincubation at 37 °C (results not shown), which suggest that this property is not inherent to *P. aeruginosa* MutS but could be a general property of bacterial MutS.

Interestingly, we found that both the ATPase activity and the DNA binding capability are preserved from inactivation by the addition of physiological ligands. The ATPase activity was preserved by the addition of ATP, ADP or oligonucleotides. However, whereas both homo- and hetero-duplex DNA were able to stimulate ATPase activity, only the heteroduplex DNA was efficient at protecting the ATPase activity, probably due to the different affinity of MutS for each oligonucleotide [31]. We also found that the addition of 0.001–0.100 mM ATP or ADP during preincubation preserved the binding activity of MutS. However, the inactivation of the ATPase activity and the DNA binding capability were not reversed if the nucleotides were added after preincubation.

The analysis of the mobility of MutS by native gel electrophoresis, after incubation at 37 °C in the absence of ligands or in the presence of homoduplex DNA, showed that it was present only at the top of the gel, indicating that MutS was forming a high molecular mass oligomer. On the other hand, the mobility of MutS without incubation or incubated in the presence of ATP or heteroduplex oligonucleotide was similar to the mobility of the DNA–MutS complex, with an apparent molecular mass of approx. 200 kDa, indicating that the protein migration observed would correspond to the active dimer. Our results are in agreement with observations from different laboratories, which

reported that the dimer form of bacterial MutS is the active conformation of the protein [11,14–16,29]. Even when preincubation of the protein with ATP or ADP (0.1 mM) maintains most of the protein in the dimer state, the addition of nucleotides (0.1–1.0 mM) after preincubation did not reverse the oligomer conformation. Further support to the striking effect of nucleotides on the conformational changes of *P. aeruginosa* MutS were obtained by experiments of limited trypsin digestion of the protein in the absence and presence of ATP or ADP. This treatment produces different peptide patterns (results not shown), in agreement with results obtained in similar experiments carried out with MutS from *E. coli* and *Thermus aquaticus* [32,33]. Although the presence of nucleotides has been mainly involved in the modulation of the MutS–DNA interaction, our results showed that they also modulate protein oligomerization.

Analysis of MutS by electron microscopy after incubation at 37 °C showed the presence of regular tetrad structures in contrast with non-assembled MutS where these structures were not observed. Although previous reports have described the presence of dimer and oligomer structures in bacterial purified MutS samples [7,14,17,18,34], to our knowledge, the present study is the first report in which the structure of a high order oligomer of MutS is visualized. The tetrad structure of *P. aeruginosa* MutS observed in the present study showed an apparent molecular mass higher than human α2-macroglobulin (720 kDa), as determined by native gel electrophoresis and in agreement with the relative dimensions of both proteins observed by electron microscopy. Therefore this MutS oligomer should contain at least 3.8 dimers, which is compatible with the assumption that each of the four subunits present in the oligomer may represent a dimer of MutS (molecular mass of dimer = 190 kDa). The presence of high molecular mass oligomers in protein samples incubated at 37 °C was confirmed by gel-filtration chromatography; however, the molecular mass of the oligomer based on this result is difficult to ascertain since the frictional coefficient of the protein structure is not known.

Although we clearly demonstrate that the MutS oligomer is inactive, the comparison of the kinetics of inactivation of the ATPase activity and DNA binding capability of MutS with the velocity of oligomerization analysed by light scattering showed that a high percentage of inactivation takes place at the beginning of the reaction, long before the oligomerization reaches its maximum. It could be assumed that during the time lag of the oligomerization process, MutS underwent a conformational change that is sufficient to transform the protein into an inactive conformational state. One important characteristic of the oligomerization process is the strict dependence of the reaction on the temperature, since under the conditions of our experiments the oligomerization of MutS only takes place at a temperature higher than 30 °C. Furthermore, analysis of the secondary structure of MutS as a function of temperature by IR spectroscopy (R. J. Pezza, G. G. Montich and C. E. Argaraña, unpublished work) showed that the native secondary structure remains unchanged up to a temperature of 42–45 °C, indicating that the MutS oligomerization at 37 °C described in the present study does not occur due to extensive unfolding of the protein.

The results obtained in the present work allow us to demonstrate that under conditions where oligomer formation of MutS is favoured, inactivation of the oligonucleotide binding capability and of the ATPase activity of the protein take place. We also found that the presence of heteroduplex DNA, as well as ATP or ADP in a specific concentration range, is important to restrain the protein in an active conformation and to regulate its oligomerization state. However, it is important to point out that if this high order conformation of MutS represents a physiological state of the protein in equilibrium with the dimer conformation, additional factors would be necessary to establish this equilibrium, since heteroduplex DNA or nucleotides do not revert the oligomer state of the protein. It is also conceivable that co-assembly of MutS with some other factor could produce an active oligomer. In this sense, we are currently investigating if MutL or some other factor present in the cell extract is able to reverse the oligomer state of MutS or to confer some physiological activity in the MMRS to this structure.

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