

Physical and Functional Interaction between the Mini-chromosome Maintenance-like DNA Helicase and the Single-stranded DNA Binding Protein from the Crenarchaeon *Sulfolobus solfataricus**

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Mini-chromosome Maintenance (MCM) proteins play an essential role in both initiation and elongation phases of DNA replication in Eukarya. Genes encoding MCM homologs are present also in the genomic sequence of Archaea and the MCM-like protein from the euryarchaeon *Methanobacterium thermoautotrophicum* (*Mth* MCM) was shown to possess a robust ATP-dependent 3'-5' DNA helicase activity *in vitro*. Herein, we report the first biochemical characterization of a MCM homolog from a crenarchaeon, the thermoacidophile *Sulfolobus solfataricus* (*Sso* MCM). Gel filtration and glycerol gradient centrifugation experiments indicate that the *Sso* MCM forms single hexamers (470 kDa) in solution, whereas the *Mth* MCM assembles into double hexamers. The *Sso* MCM has NTPase and DNA helicase activity, which preferentially acts on DNA duplexes containing a 5'-tail and is stimulated by the single-stranded DNA binding protein from *S. solfataricus* (*Sso* SSB). In support of this functional interaction, we demonstrated by immunological methods that the *Sso* MCM and SSB form protein-protein complexes. These findings provide the first *in vitro* biochemical evidence of a physical/functional interaction between a MCM complex and another replication factor and suggest that the two proteins may function together *in vivo* in important DNA metabolic pathways.

776 to 1017 amino acid residues), which are evolutionarily conserved especially in the central third of their polypeptide chain (3, 4). In fact, this region contains the four sequence motifs typically found in DNA helicases, including the Walker A and B boxes that are critical for nucleotide binding and hydrolysis (5). The MCM proteins are relatively abundant in proliferating cells and were purified from cell extracts of various organisms either as hetero-hexameric complexes containing all six polypeptides or as sub-assemblies of various subunit composition (such as MCM 2/4/6/7 and MCM 4/6/7 (6–10)). However, among all these multimeric complexes only the MCM 4/6/7 hexamer was demonstrated to have a weak and non-processive DNA helicase activity (11–13). The MCM 4/6/7 complex is dis-assembled *in vitro* upon addition of MCM 2 or MCM 3/5, and this causes inhibition of its DNA unwinding activity (13, 14). Based on these findings, it was proposed that the MCM 4/6/7 assembly could act as DNA unwinding factor at the replication origins, whereas the other MCM subunits could play regulatory functions. However, due to the limited processivity of their DNA unwinding activity the MCM proteins were considered poor candidates for the helicase associated with the DNA replication fork. In addition, several genetic studies have evidenced that the MCM proteins *in vivo* could interact with the Origin Recognition Complex, Cdc6, Cdc45, and Cdc7/Dbf4 kinase (15–18), but no direct evidence for their physical and/or functional interaction with any replication factor was reported so far.

More recently, it was clearly demonstrated that in *Saccharomyces cerevisiae* all six MCM genes are essential not only for the initiation but also for the elongation phase of chromosome replication (19). Furthermore, an important clue to the *in vivo* function of the MCM proteins derived from the recent biochemical characterization of the single MCM homolog from the euryarchaeon *Methanobacterium thermoautotrophicum* (*Mth* MCM). In three reports, *Mth* MCM was demonstrated to form a ring-shaped double hexamer and to possess a robust and processive 3'-5' DNA helicase activity *in vitro* (20–22). These findings reinforced the hypothesis that the MCM proteins may act as the helicase associated to the replication fork, although there is no direct evidence that *Mth* MCM is required *in vivo* for chromosome duplication (23). In addition, these studies once again pointed out that Archaea possess a replication machinery that is in several instances a simplified version of the eukaryotic counterpart and the biochemical characterization of the replication proteins from these peculiar organisms could provide an useful model to elucidate the molecular mechanisms of replication initiation, as well as replisome assembly and progression, in a context devoid of the eukaryotic regulatory complexities (24).

Mini-chromosome Maintenance (MCM)¹ genes (MCMs 2–7) were originally identified in budding and fission yeast by a genetic analysis of mutants that were unable to efficiently replicate mini-chromosomes (1, 2). Homologs of the six yeast MCM genes were subsequently identified in various other eukaryotic organisms, from *Drosophila melanogaster* to *Homo sapiens* and found to code for proteins (ranging in length from

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¹ The abbreviations used are: MCM, Mini-chromosome Maintenance; SSB, single-stranded DNA binding protein; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline, TBE, Tris-Borate-EDTA; ELISA, enzyme-linked immunosorbent assay; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATP_γS, adenosine 5'-O-(thiotriphosphate); WRN, Werner's syndrome gene; BLM, Bloom's syndrome gene; nt, nucleotide(s).

The Archaea domain is composed of two subdomains: Euryarchaeotes (including *M. thermoautotrophicum* and *Pyrococcus* species) and Crenarchaeotes (including *Sulfolobus solfataricus* and *Aeropyrum pernix* (25)). The two groups show important differences at the molecular level: only the euryarchaeal species were found to possess the hetero-dimeric family D DNA polymerase, whereas the Crenarchaeotes are believed to utilize DNA polymerases of the family B as the chromosomal replicases; the Euryarchaeotes have histone-like proteins, whereas the crenarchaeal chromatin contains a different kind of DNA binding protein (26).

We recently produced in recombinant form and biochemically characterized some putative components of the replisome from the thermoacidophilic crenarchaeon *S. solfataricus* (27), including a single-subunit family B DNA polymerase (28), two proliferating cell nuclear antigen-like sliding clamps (29), and a replication factor C-like clamp-loader (30). In addition, a SSB protein from this species (*Sso* SSB) has been recently identified and characterized (31). The *Sso* SSB (16,184 Da) is an abundant protein that exists as a monomer in solution and multimerizes upon DNA binding, forming probably tetramers. Each monomer contains a single "OB-fold" (oligonucleotide/oligosaccharide binding fold) and is able to bind 4–5 nucleotides of ssDNA.

Herein we report the biochemical characterization of the single MCM homolog from the crenarchaeon *S. solfataricus* (*Sso* MCM). This protein forms hexamers in solution and has ATPase and DNA helicase activity. This latter preferentially melts 5'-tailed oligonucleotides and is stimulated by the *Sso* SSB. In addition, we demonstrated that *Sso* MCM and *Sso* SSB physically interact each other. These findings provide the first direct biochemical evidence that a MCM complex functionally and physically interacts with another replication factor and have implications for other MCM proteins from higher organisms including humans.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of reagent grade. Restriction and modification enzymes were from Roche Molecular Biochemicals. Radioactive nucleotides were purchased from Amersham Biosciences, Inc. Oligonucleotides were synthesized by Primm (Milan, Italy). The anti-*Sso*MCM rabbit antiserum was kindly provided by Dr. Stephen D. Bell (Cambridge, United Kingdom).

Plasmids—The *Escherichia coli* expression vector pET19b-*Sso*MCM was constructed by Dr. M. F. White (Saint Andrews, United Kingdom). This plasmid harbors the *Sso* MCM gene cloned between the *Nco*I and *Bam*HI restriction sites of the polylinker.

The *Sso* MCM was mutated at lysine 346 to alanine by PCR-based mutagenesis (32) using the following synthetic oligonucleotides: MCM-Kpn-for (5'-ACCAGAAGAGGTACCCCTCAGGTCAGTTACC-3'), MCM-Spe-rev (AGATCTTAGCCCTACTAGTAATTTTGGT), MCM-K346A-for (AATAGGTGATCCCGGTACTGCCGCATCACAAATGCTACAGTT-TAT), MCM-K346A-rev (ATAAAGTGTAGCATTTGTGATGCCGCAGT-ACCGGGATCACCTATT). The final PCR product was subcloned back into the *Sso*MCM-pET19b vector at the unique *Kpn*I and *Spe*I sites present in the *Sso* MCM sequence. The presence of the desired mutation was checked by sequencing the *Kpn*I-*Spe*I amplified DNA fragment. This allowed us to rule out the presence of additional mutations in this construction.

The *E. coli* plasmid pET19b-*Sso*SSB expressing the recombinant *S. solfataricus* SSB was a generous gift of Dr. M. F. White (Saint Andrews, United Kingdom (31)).

Expression and Purification of Recombinant Proteins—*E. coli*BL21-CodonPlus(DE3)-RIL cells (Novagen) transformed with the plasmid pET19b-*Sso*MCM were grown at 37 °C in 2 liters of LB medium containing 100 µg/ml ampicillin and 100 µg/ml chloramphenicol. When the culture reached an $A_{600\text{ nm}}$ of 1 OD, protein expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to 0.2 mM. The bacterial culture was incubated at 37 °C for an additional 1.5 h. Then cells were harvested by centrifugation, and the pellet was stored at -20 °C until use. The pellet was thawed and resuspended in 30 ml of buffer A (25 mM Tris-HCl, pH 7.0, 2.5 mM MgCl₂) supplemented with some

protease inhibitors (50 µg/ml phenylmethylsulfonyl fluoride, 0.2 µg/ml benzamide, 1 µg/ml aprotinin). Cells were broken by two consecutive passages through a French pressure cell apparatus (Aminco Co., Silver Spring, MD) at 2000 p.s.i. The resulting lysate was centrifuged for 30 min at 30,000 rpm (Sorvall rotor 50.2 Ti) at 10 °C. The supernatant was subjected to heat treatment at 80 °C for 10 min, then incubated in ice for 10 min. The thermoprecipitated proteins were removed by centrifugation for 30 min at 30,000 rpm (Sorvall rotor 50.2 Ti) at 10 °C. The supernatant was passed through a 0.45-µm filter and loaded onto a Heparin Sepharose™ column (vol: 10 ml) pre-equilibrated in buffer A. After a washing step in buffer A, elution was carried out with 200 ml of a 0–1 M NaCl linear gradient in buffer A. 2.5-ml fractions were collected and analyzed by SDS-PAGE to detect the *Sso* MCM. Fractions containing the recombinant protein were pooled, and the pool was dialyzed overnight against buffer B (25 mM Tris-HCl, pH 8.5, 2.5 mM MgCl₂, 50 mM NaCl). The dialyzed sample was then loaded onto a MonoQ HR 10/10 column pre-equilibrated in buffer B. A linear gradient from 0.05 to 1 M NaCl in buffer B (volume, 80 ml) was applied to elute the protein. 1-ml fractions were collected and analyzed by SDS-PAGE to detect the *Sso* MCM. The peak fractions were pooled, concentrated using a Centricon 10 system (Millipore), and dialyzed overnight against buffer C (25 mM Tris-HCl, pH 8.09, 2.5 mM MgCl₂, 100 mM NaCl). The dialyzed sample was aliquoted and stored at -20 °C. The final yield of the recombinant protein after this purification procedure was of about 30 mg.

The K346A mutant *Sso* MCM was purified using the above protocol. The recombinant *Sso* SSB was purified as described (31).

Gel Filtration Chromatography—Samples of the purified *Sso* MCM (263 µg in 50 µl) were subjected to analytical gel filtration chromatography on a Superdex 200 HR 26/30 fast protein liquid chromatography column (Amersham Biosciences, Inc.) equilibrated with buffer 50 mM Tris-HCl, pH 8.0, 20 mM MgCl₂, 200 mM NaCl. The column was run at 0.25 ml/min at room temperature. 1-ml fractions were collected and analyzed by SDS-PAGE. Two sets of globular protein standards were run in the same conditions as the experiment. The first set included tyroglobulin (670 kDa), catalase (232 kDa), BSA (67 kDa). The second set included ferritin (440 kDa), aldolase (158 kDa), and ovalbumin (43 kDa).

Samples of the purified *Sso* MCM (263 µg in 50 µl) were injected into a Superose 6 HR 10/30 fast protein liquid chromatography column (Amersham Biosciences, Inc.), equilibrated in 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 100 mM NaCl. The chromatographic run was carried out at a flow rate of 0.25 ml/min at room temperature. The column was calibrated by running a set of gel filtration markers that included tyroglobulin (670 kDa), ferritin (440 kDa), and BSA (67 kDa).

Glycerol Gradient Centrifugation—A sample of the purified *Sso* MCM (186 µg in 100 µl) was applied to a 5-ml 15–35% glycerol gradient in 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 100 mM NaCl. After centrifugation at 48,000 rpm for 15 h and 20 min in a Beckman SW 65 rotor at 10 °C, fractions (250 µl) were collected from the bottom of the tube. The distribution of the *Sso* MCM protein was detected after SDS/10% PAGE and staining with Coomassie Brilliant Blue (R-250). A mixture of protein markers (tyroglobulin, 670 kDa; ferritin, 440 kDa; BSA, 67 kDa) was applied to a parallel gradient.

ATPase Assay—Standard ATPase assay reaction mixture (10 µl) contained 25 mM Hepes-NaOH, pH 7.5, 5 mM MgCl₂, 50 mM sodium acetate, 2.5 mM 2-mercaptoethanol, 100 µM [γ -³²P]ATP (0.5–1 µCi). Incubations were performed for 1 h at 60 °C in a heated-top PCR machine to prevent evaporation and stopped in ice. A 1-µl aliquot of each mixture was spotted onto a polyethyleneimine-cellulose thin layer plate (Merck), pre-run with 1 M formic acid and developed in 0.5 M LiCl, 1 M formic acid. The amounts of [γ -³²P]ATP hydrolyzed to [³²P]orthophosphate were quantitated using a PhosphorImager (Molecular Dynamics, Inc.). The rate of ATP hydrolysis was determined in the linear range of reaction time and protein concentration dependence. The amount of spontaneously hydrolyzed ATP was determined using blank reactions without enzyme and subtracted from the reaction rate values calculated as above.

Preparation of Substrate for the DNA Helicase Assay—Three DNA oligomers were synthesized and used for the preparation of helicase substrates. These included the following: a 55-mer (5'-CTTGATGCC-TGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTC-G-3'), which was fully complementary to the M13mp18(+) strand; a 64-mer (5'-TCACTTCTACTTGCATGCCTGCAGGTCGACTCTAGAGG-ATCCCCGGGTACCGAGCTCGAATTCG-3'), which had a 9-nt 5'-tail (the tail is underlined) not complementary to the M13mp18(+) strand; an 85-mer (5'-TTGAACCAACCCCTTGTAAATCACTTCTACTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGA-

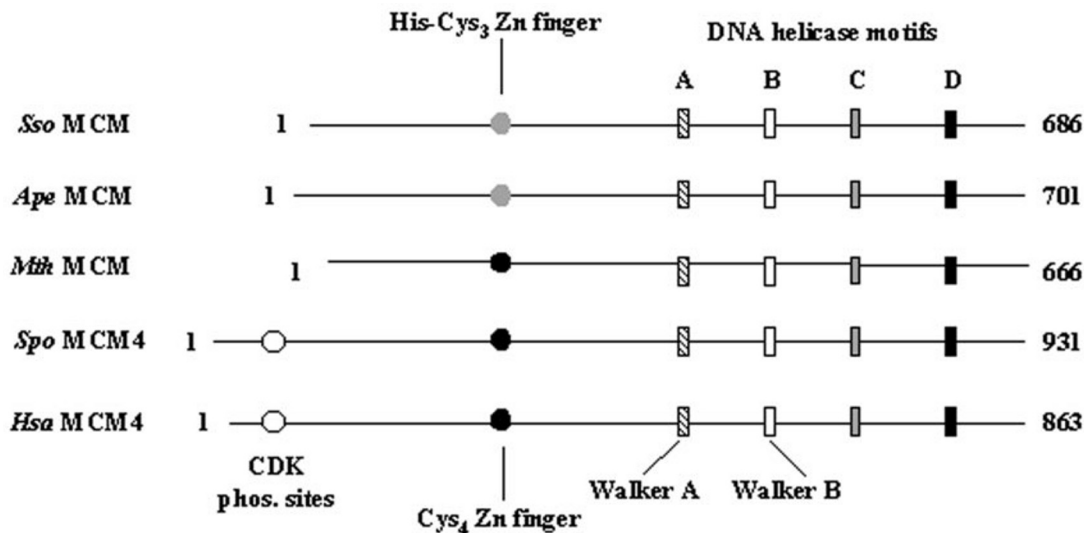


FIG. 1. Schematic representation of conserved sequence motifs in some archaeal and eukaryotic MCM proteins. The polypeptides of *S. solfataricus* MCM (*Sso* MCM), *A. pernix* MCM (*Ape* MCM), *M. thermoautotrophicum* MCM (*Mth* MCM), *S. pombe* MCM 4 (*Spo* MCM4) and *H. sapiens* MCM 4 (*Hsa* MCM4) are represented. Amino acidic boxes A, B, C, and D are typically found in DNA helicases (35). Walker A and B boxes are indicated (5). The N-terminal region of *Hsa* and *Spo* MCM 4 contains putative consensus sites for phosphorylation by cyclin-dependent kinase (3, 4).

ATTTCG), which had a 30-nt 5'-tail (the tail is underlined) not complementary to the M13mp18(+) strand. The oligonucleotides were labeled with [γ - 32 P]ATP and T4 polynucleotide kinase, and, after the labeling reaction, they were purified using Quantum Prep PCR Kleen Spin columns (Bio-Rad Laboratories), according to the manufacturer's instructions. To prepare partial duplexes, DNA molecules and mixtures containing equal molar amounts of each oligonucleotide and the M13mp18(+) strand were incubated for 5 min at 95 °C and then slowly cooled at room temperature.

DNA Helicase Activity Assay—Helicase assay reaction mixtures (20 μ l) contained 25 mM Hepes-NaOH, pH 7.5, 5 mM MgCl₂, 50 mM sodium acetate, 2.5 mM 2-mercaptoethanol, 5 mM ATP, 50 fmol of 32 P-labeled substrate (about 1×10^3 cpm/fmol). The reactions were incubated for 30 min at 70 °C in a heated-top PCR machine to prevent evaporation and stopped by addition of 5 μ l of 5 \times stop solution (0.5% SDS, 40 mM EDTA, 0.5 mg/ml proteinase K, 20% glycerol, 0.1% bromophenol blue), then run on a 8% polyacrylamide gel in TBE containing 0.1% SDS at constant voltage of 150 V. After the electrophoresis the gel was soaked in 20% trichloroacetic acid and analyzed by means of a PhosphorImager (Molecular Dynamics, Inc.). The reaction products were quantitated, and any free oligonucleotide in the absence of enzyme was subtracted.

ELISA Method for Detection of SsoMCM-SsoSSB Protein-Protein Interaction—The *Sso* SSB was diluted to a concentration of 1.4 ng/ μ l in carbonate buffer (16 mM Na₂CO₃, 34 mM NaHCO₃, pH 9.6). This solution containing the *Sso* SSB was then added to the appropriate wells of a 96-well ELISA plate (100 μ l/well) and allowed to incubate for 2 h at room temperature. For control experiments, BSA was substituted for the *Sso* SSB in the coating step. Wells were then aspirated and washed three times with wash Buffer (PBS, 0.5% Tween 20). Blocking buffer (PBS, 0.5% Tween 20, 3% BSA) was added to appropriate wells and allowed to incubate at room temperature. Wells were aspirated and washed one time with blocking buffer. The *Sso* MCM was diluted to 1.8 ng/ μ l in binding buffer (25 mM Hepes-NaOH, pH 7.5, 50 mM NaCl, 2.5 mM 2-mercaptoethanol, 5 mM MgCl₂). The diluted *Sso* MCM was then added to appropriate wells of the ELISA plate (100 μ l/well) and allowed to incubate for 30 min at room temperature. Wells were aspirated and washed three times with binding buffer. Primary antibody (rabbit polyclonal antiserum against the *Sso* MCM protein) was diluted 1:2000 in blocking buffer, added to appropriate wells, and allowed to incubate overnight at 4 °C. Wells were then aspirated and washed four times with blocking buffer. Secondary antibody (goat anti-rabbit IgG-horse-radish peroxidase) was diluted 1:3000 in conjugate buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 1% BSA), added to appropriate wells, and allowed to incubate 30 min at room temperature. Wells were aspirated and washed five times with conjugate buffer. Complexes were detected using a colorimetric reaction with *o*-phenylenediamine dihydrochloride (Sigma Chemical Co.). The reaction was terminated after 1 min with 2.5 M sulfuric acid. Absorbance readings were taken at 490 nm. The A₄₉₀ values, corrected for background signal

in the presence of BSA, are expressed as the mean of three independent determinations.

Far Western Blotting—Far Western blotting analysis was conducted as described by Wu *et al.* (33). Each polypeptide was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher and Schuell). All subsequent steps were performed at 4 °C. Filters were immersed twice in denaturation buffer (6 M guanidine-HCl in TBS) for 10 min followed by six times for 10 min in serial dilutions (1:1) of denaturation buffer supplemented with 1 mM dithiothreitol. Filters were blocked in TBS containing 3% BSA, 0.3% Tween 20 for 30 min before being incubated in the *Sso* MCM (6 μ g/ml) in TBS supplemented with 0.1% BSA, 0.3% Tween 20, 1 mM dithiothreitol overnight at 4 °C. Filters were washed four times for 10 min in TBS containing 0.3% Tween 20, 0.1% BSA. The second wash contained 0.0001% glutaraldehyde. Conventional Western analysis was then performed to detect the presence of the *Sso* MCM using rabbit polyclonal antiserum against the *Sso* MCM as primary antibody. Anti-rabbit IgG-horseradish peroxidase conjugate was used as secondary antibody and detected by a colorimetric reaction.

RESULTS

Identification, Purification, and Hydrodynamic Properties of the Sso MCM—The analysis of the *S. solfataricus* genomic sequence revealed the presence of a single open reading frame coding for a putative homolog of the eukaryotic MCM proteins (*Sso* MCM (27)). Using the computer program ClustalW (34) we aligned the sequence of the single MCM protein from the Archaea *S. solfataricus*, *A. pernix* (*Ape* MCM), and *M. thermoautotrophicum* (*Mth* MCM), and the sequence of the MCM 4 protein from the eukaryotes *H. sapiens* (*Hsa* MCM) and *Schizosaccharomyces pombe* (*Spo* MCM) and found that the *Sso* MCM is 48, 43, 33, and 34% identical to *Ape*, *Mth*, *Hsa*, and *Spo* MCM, respectively. As schematically depicted in Fig. 1, the *Sso* MCM lacks the N-terminal extension of about 160 amino acidic residues found in the eukaryotic counterparts, and it is devoid of the cyclin-dependent kinase phosphorylation sites that are clustered in the N-terminal region of the eukaryotic MCM 4, as also found in the *Ape* and *Mth* MCM (3, 4, 22). On the other hand, the core region of the archaeal and eukaryal sequences shows a higher level of similarity, because it contains the four amino acidic motifs typically found in the DNA helicases (35). As evidenced in Fig. 1, the sequence boxes A and B correspond to the Walker A and B motifs that are responsible for nucleotide binding and hydrolysis, respectively, in the large family of ATPases associated with a

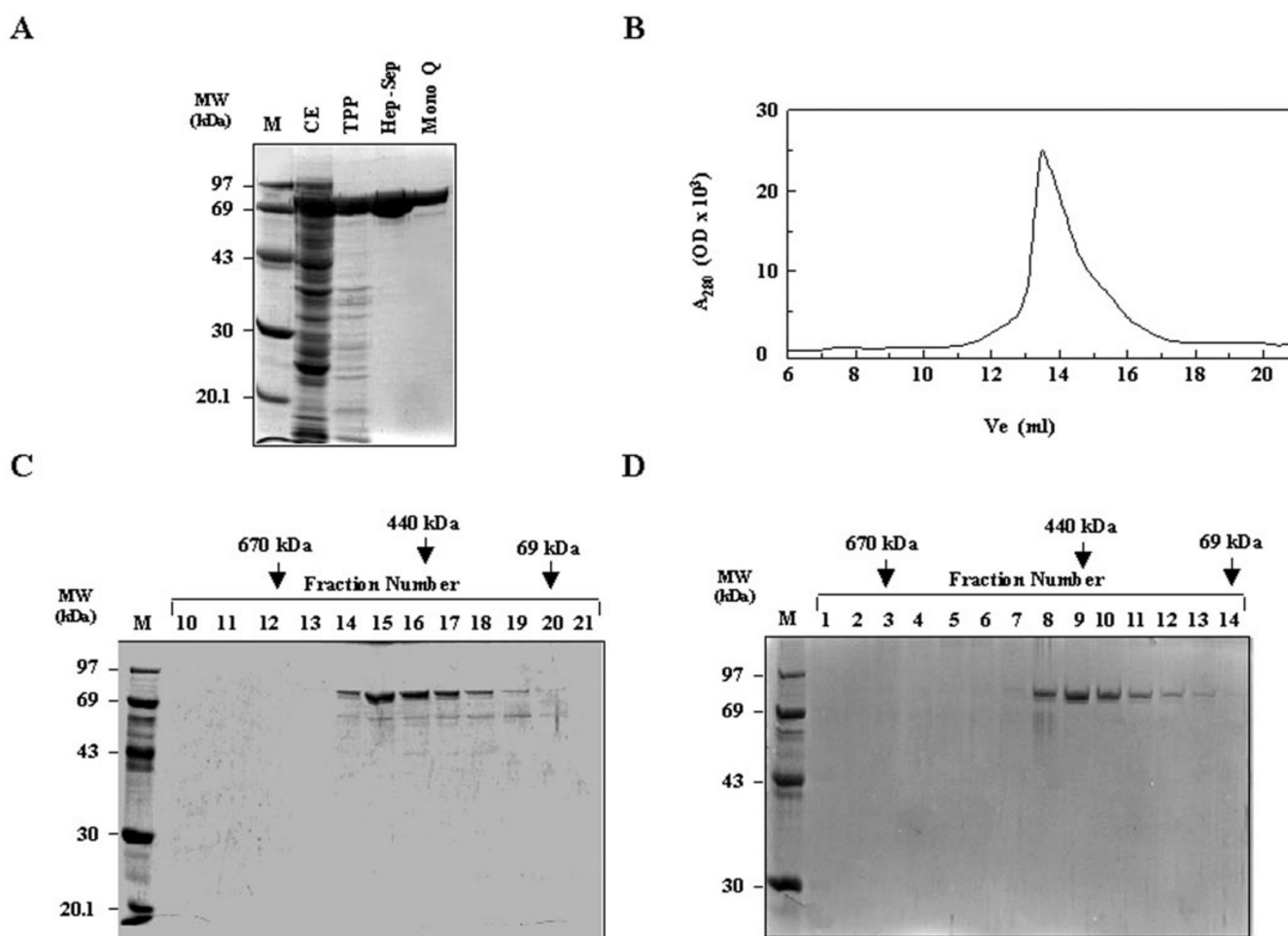


FIG. 2. Purification and hydrodynamic properties of the *Sso* MCM. A, Coomassie Blue-stained SDS-polyacrylamide gel of the *Sso* MCM protein samples after each step of the purification procedure, as described under “Experimental Procedures.” The native molecular mass of the *Sso* MCM was estimated by gel filtration on a Superdex 200 column; B, elution profile of the *Sso* MCM from the chromatographic column; C, SDS-PAGE analysis of the fractions eluted during the gel filtration experiment (arrows indicate the peak positions of some protein markers utilized to calibrate the column). D, glycerol gradient sedimentation analysis of the purified *Sso* MCM. SDS-PAGE analysis of the gradient fractions containing the *Sso* MCM protein. Arrows indicate the position of protein markers run through a parallel glycerol gradient (thyroglobulin, 670 kDa; ferritin, 440 kDa; BSA, 69 kDa).

variety of cellular activities (AAA⁺ super-family (5)). In addition, the *Sulfolobus* and *Aeropyrum* MCM sequences seem to contain a zinc finger of the His-Cys₃ type, whereas the MCM proteins from eukaryotic organisms (3, 4), *Methanobacterium*, and other euryarchaeal species (36) possess a zinc finger of the Cys₄ type.

The gene encoding the *Sso* MCM was produced in *E. coli* using the pET19b plasmid vector. The recombinant protein was found to be expressed at high level in soluble form and was purified by a procedure that included a thermal treatment of the cell extracts and chromatographic steps on heparin-Sepharose and MonoQ columns, as described under “Experimental Procedures.” The purified *Sso* MCM migrated as a Coomassie Blue-stained protein band of the predicted size (77 kDa) in a 10% SDS-polyacrylamide gel (see Fig. 2A). An additional tiny band of about 60 kDa is observed in the sample collected from the MonoQ column. N-terminal sequence analyses after electrotransfer onto a polyvinylidene difluoride membrane revealed that the 77-kDa polypeptide corresponds to the *Sso* MCM, whereas the 60-kDa band is a C-terminally truncated proteolytic fragment of the intact protein.²

To assess the oligomeric state of the recombinant *Sso* MCM, we carried out gel filtration experiments onto two columns: a Superose 6² and a Superdex 200 (Fig. 2, B and C). By either

experiment we estimated a molecular mass of about 470 kDa for the *Sso* MCM and hypothesized that it could form hexamers in solution. However, as shown in Fig. 2B, the peak eluted from the Superdex 200 column was quite broad and a portion of the protein was detected in fractions that corresponded to a molecular mass of about 75 kDa, as expected for the monomeric form of the *Sso* MCM. This result suggested that the *Sso* MCM could exist in equilibrium between an hexameric and a monomeric state. Consistent results were obtained also by glycerol gradient centrifugation experiments: as shown in Fig. 2D, the *Sso* MCM was detected by SDS-polyacrylamide gel electrophoresis in the fractions from 8 to 13 of the gradient indicating that the protein assembles into hexamers that dissociate into monomers. The oligomeric state of the *Sso* MCM was not found to be affected by addition of ATP (at 100 μM) in the buffer used to prepare the gradient or by preincubating the protein sample with forked double- or single-stranded DNA molecules before starting the ultracentrifugation.²

Characterization of ATP Hydrolysis Reaction Catalyzed by the Sso MCM—As shown in Fig. 1, Walker A and B motifs are present in the primary structure of archaeal and eukaryal MCM proteins, as well as in all known DNA helicases (5, 35). In fact, these enzymes utilize the energy deriving from the hydrolysis of ATP (or other nucleosides triphosphate) for the DNA unwinding reaction (37). Thus, we tested the ATPase activity of

² F. Carpentieri *et al.*, unpublished observations.

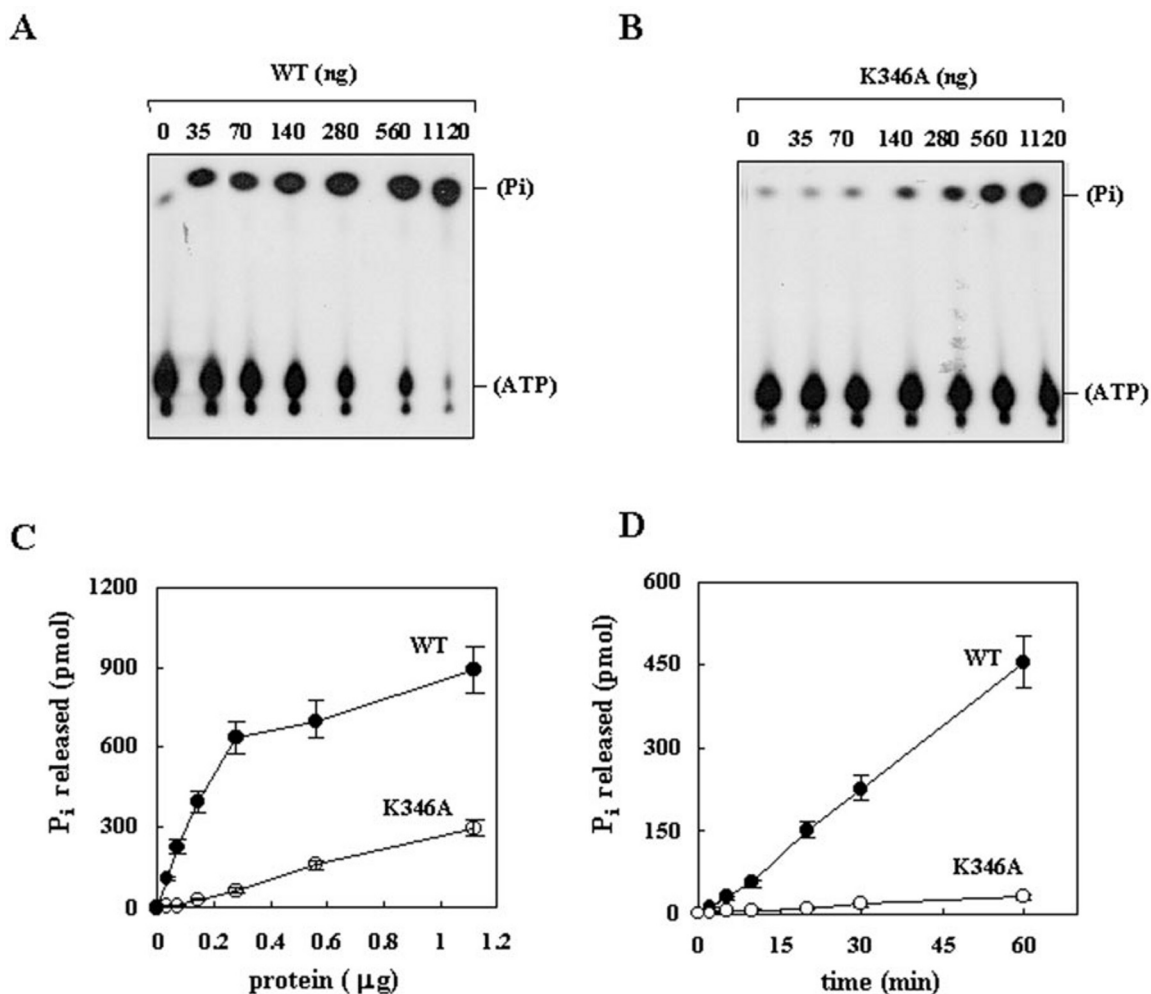


FIG. 3. ATPase activity of the wild type and the K346A mutant *Sso* MCM. *A* and *B*, autoradiography of the thin layer chromatography plates used to test the ATPase activity of increasing amounts of wild type and mutant *Sso* MCM, respectively. Reactions were carried out at 60 °C for 1 h in the presence of radiolabeled [γ - 32 P]ATP. *C*, the orthophosphate released during the ATP hydrolysis reaction described in *A* was quantitated by phosphorimaging and plotted versus the amount of wild type and mutant *Sso* MCM. *D*, the ATPase activity of wild type and mutant *Sso* MCM was plotted versus the incubation time. Reactions were carried out at 60 °C using 140 ng of each homogeneous protein. Data reported in *C* and *D* are mean values of at least three independent determinations.

the wild type and K346A mutant *Sso* MCM in which the lysine residue of the Walker A motif was replaced by alanine. The release of [32 P]orthophosphate from [γ - 32 P]ATP was measured using a thin layer chromatography method, as described under "Experimental Procedures." The reaction mixtures were incubated at 60 °C and not at the optimal growth temperature for *S. solfataricus* (87 °C) to limit the thermally induced autohydrolysis of ATP. As shown in Fig. 3, the *Sso* MCM (1.8 pmol of monomer in a 20- μ l reaction volume) is able to hydrolyze ATP with a steady-state rate of about 8 pmol/min, whereas the activity of the K346A mutant was noticeably reduced, although not completely abolished.

The ATPase activity of various DNA helicases is greatly stimulated by DNA (37). This prompted us to assess the effect of nucleic acid molecules on the ATP hydrolysis catalyzed by the *Sso* MCM. We did not observe any significant stimulation upon addition of various kinds of single- or double-stranded DNA molecules to the ATPase assay reaction mixture.² The *Sso* MCM is able to utilize (and thus to hydrolyze) other nucleosides triphosphate, in addition to ATP, as energy source for the unwinding reaction (see below).

Characterization of the *Sso* MCM DNA Helicase Activity—The purified recombinant *Sso* MCM was then tested for DNA helicase activity by a strand-displacement assay performed at

70 °C. The substrates utilized in these assays were prepared by annealing to single-stranded M13mp18 DNA 32 P-5'-end-labeled synthetic oligonucleotides of 55 or 64 or 85 nucleotides, which gave rise to partial duplexes having no tail (no-tail substrate) or a 9-nt 5'-tail (5'-mis-9) or a 30-nt 5'-tail (5'-mis-30), respectively. Unwinding of these DNA substrates was measured as a function of the amount of the *Sso* MCM protein utilized (Fig. 4). The *Sso* MCM was almost completely unable to unwind the no-tail DNA substrate: only about 2 fmol of the 55-mer oligonucleotide was displaced by 280 ng of homogeneous protein (about 600 fmol of hexamer) after an incubation of 30 min at 70 °C. On the other hand, when the substrate contained a single-stranded 5'-tail, the helicase activity was noticeably stimulated: The figure for the unwinding reaction rose to about 20 fmol of oligo displaced with 600 fmol of *Sso* MCM hexamer. The analysis shown in Fig. 4C indicated that increasing the length of the 5'-tail from 9 to 30 nucleotides did not further stimulate the *Sso* MCM DNA helicase activity.

Like all known DNA helicases (37), the *Sso* MCM requires both nucleotide binding and hydrolysis for its activity. As shown in Fig. 5, DNA duplex unwinding is effected by the *Sso* MCM only when nucleoside triphosphates are present in the reaction mixture. Indeed, the K346A mutant enzyme, whose ATPase activity is greatly impaired, is also almost completely

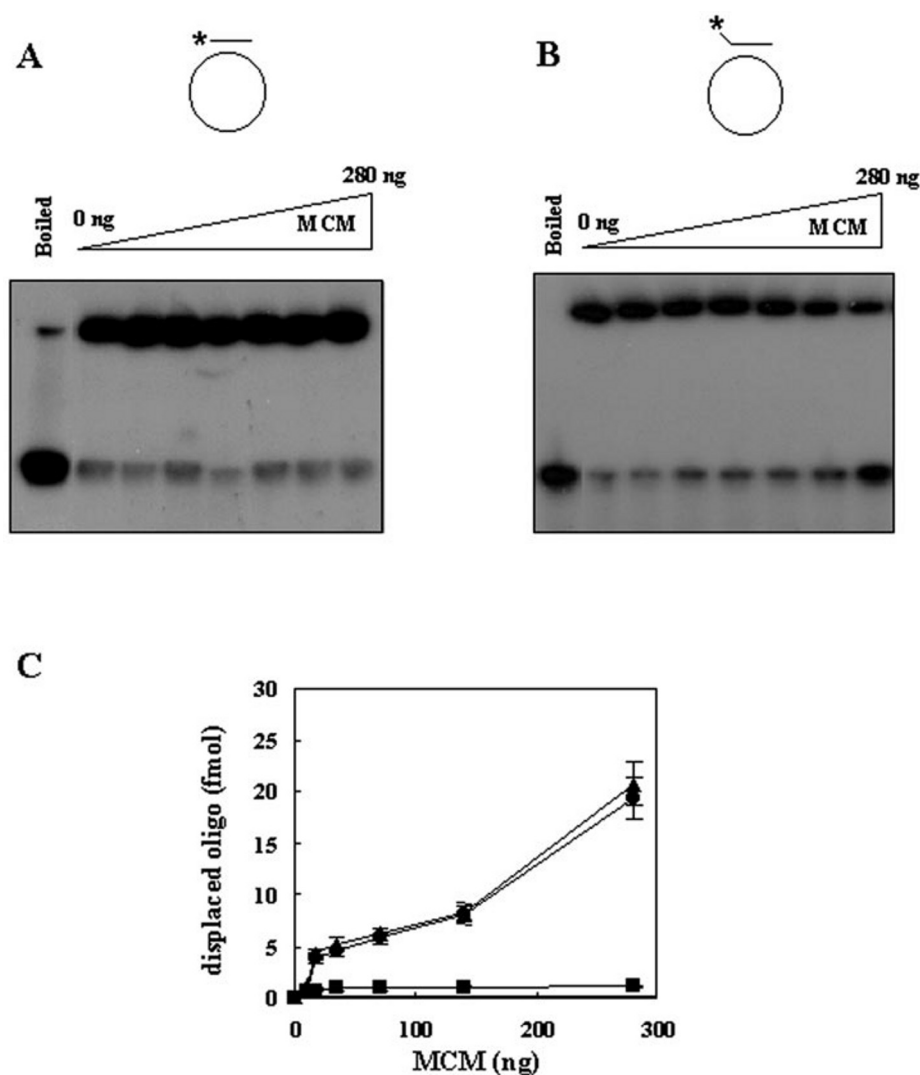


FIG. 4. DNA helicase activity of the *Sso* MCM on various DNA substrates. DNA helicase activity assays were carried out with increasing amounts of the *Sso* MCM in 20 μ l at 70 $^{\circ}$ C for 30 min, as described under "Experimental Procedures," using 42 fmol of the no-tail (A) or the 5'-mis-9 (B) or the 5'-mis-30 (not shown) substrates, which are schematically depicted in the upper part of each panel. C, the oligonucleotide melted by the *Sso* MCM was quantitated by phosphorimaging and plotted versus the amount of pure protein in each activity assay. The partial duplexes DNA utilized had no 5'-tail (squares), a 9-nt (circles), or a 30-nt (triangles) 5'-tail. Data shown are mean value of at least three independent experiments.

devoid of helicase activity. Oligonucleotide displacement by the *Sso* MCM on ss-M13 DNA was measured as a function of the ATP concentration and in our assay conditions the maximal helicase activity was observed with 5 mM ATP. Besides, a variety of nucleotides and deoxynucleotides were tested for their ability to support the DNA unwinding reaction catalyzed by the *Sso* MCM and only ATP and dATP were found to be efficient co-factors. Quite interestingly, in the presence of ATP γ S the *Sso* MCM helicase activity was diminished but not completely abolished, as also reported for the *Mth* MCM (22).

Stimulation of the *Sso* MCM DNA Helicase Activity by the *Sso* SSB—SSBs play a critical role in DNA replication, recombination, and repair reactions. In fact, they not only are responsible for the stabilization of DNA in the single-stranded form but often also effect a regulatory function by stimulating or inhibiting the catalytic activities of the enzymes involved in the above DNA transactions (38). All that considered, we decided to test the effect of the SSB recently purified from *S. solfataricus* (31) on the DNA helicase activity of the MCM protein from the same species. To this end, the strand displacement activity of the *Sso* MCM was assayed in the presence of increasing amounts of SSB using as substrates the partial duplexes with no tail (Fig. 6A), with a 9-nt 5'-tail (Fig. 6B), and with a 30-nt 5'-tail.² As reported in the plot of Fig. 6C, when the *Sso* SSB was added at a concentration of 4.4 ng/ μ l, a stimulation of about 20-fold was observed on the unwinding of the no-tail oligonucleotide, whereas the effect on the duplexes having a

9-nt 5'-tail (or a 30-nt 5'-tail²) was less striking (about 2-fold). The plot of Fig. 6C also shows that oligonucleotide displacement by the *Sso* SSB alone was negligible. It should be observed that the *Sso* SSB was present in these assays at concentrations that are from 40 to 10 times lower than required to saturate all single-stranded DNA binding sites, if we assume that a single tetrameric molecule of the *Sso* SSB is able to bind about 20–25 nt (31). This allowed us to rule out that the *Sso* SSB could act by merely coating the single strands produced during the helicase reaction and preventing their re-annealing. However, we did not observe any effect of the *Sulfolobus* SSB on the ATPase activity of the *Sso* MCM either in the presence or absence of DNA molecules. This suggests that the stimulation of the helicase activity is not due to an increased rate of the ATP hydrolysis reaction catalyzed by the *Sso* MCM.

Physical Interaction between the *Sso* MCM and the *Sso* SSB—The stimulation of the *Sso* MCM helicase activity by the *Sso* SSB suggested that the two proteins could physically interact with one another. ELISAs were used to test for a protein-protein interaction. In these experiments increasing amounts of the homogeneous MCM protein were incubated with a fixed aliquot of SSB that had been cross-linked to polystyrene microtiter wells. Then, the bound *Sso* MCM was detected using a specific polyclonal rabbit anti-serum. As shown in Fig. 7, the colorimetric signal is dose-dependent and gives a hyperbolic saturation curve if plotted against the *Sso* MCM protein concentration. An apparent dissociation constant (K_d)

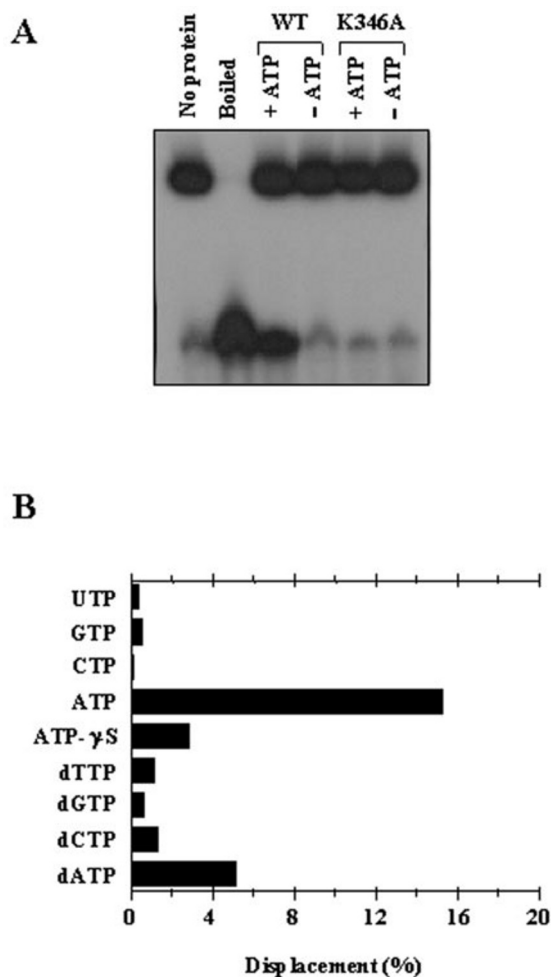


FIG. 5. Nucleotide effect on the *Sso* MCM DNA helicase activity. *A*, DNA helicase assays were carried out at 70 °C for 30 min using 140 ng of wild type and K346A mutant *Sso* MCM protein with and without ATP (5 mM) using as a substrate the partial duplex with a 9-nt 5'-tail (25 nm). The analysis of the gel by a PhosphorImager indicated that the wild type protein in the presence of ATP is able to unwind about 8 fmol of oligonucleotide from the ss-M13 DNA template, whereas its activity is completely abolished, when ATP is omitted; the K346A mutant has no helicase activity either with or without ATP. *B*, the bar graph shows the oligonucleotide displacement activity of the *Sso* MCM in the presence of the indicated nucleotides (5 mM) as co-factors. DNA helicase assays were carried out as described in *A*.

of 45 nm was calculated for the binding reaction by a Hill plot analysis of the data.²

Furthermore, the interaction between the *Sso* MCM and the *Sso* SSB was investigated by a far Western blot experiment. The SSB protein was transferred onto a nitrocellulose membrane, which was then incubated with the homogeneous DNA helicase. At the same time in a control experiment a second identical filter was incubated with the buffer alone. The filters were then extensively washed, and the bound *Sso* MCM was detected by Western blot analysis using an anti-MCM-specific rabbit antiserum. As shown in Fig. 8, the *Sso* MCM was detected at the position corresponding to the SSB protein band, whereas the signal was absent from the control blot that had been incubated with the buffer alone.

Based on the ELISAs and far Western blot analysis we inferred that the *Sso* MCM physically interacts with the *Sulfolobus* SSB.

DISCUSSION

In this report we describe the biochemical features of the single MCM homolog from the thermoacidophilic crenarchaeon

S. solfataricus. The protein was produced in *E. coli* in soluble form with high yield and purified to homogeneity by a procedure that included a thermal treatment of the bacterial cell extract and chromatographies on heparin-Sepharose and MonoQ columns. Quite interestingly, a multiple alignment of the MCM amino acidic sequence from various species revealed that the proteins from the crenarchaea *S. solfataricus* and *A. pernix* both contain a zinc finger motif of the His-Cys₃ type, whereas the homologs from *M. thermoautotrophicum* and other euryarchaeal species, and four out of the six eukaryotic MCM proteins (MCM 2, 4, 6, and 7) possess a Cys₄ type zinc finger. In a recent report by Poplawski *et al.* (36), it was demonstrated that the above zinc finger domain is important for ssDNA binding and helicase activity of the *Mth* MCM.

One interesting feature of the *Sso* MCM protein is that its ATPase activity is not stimulated in the presence of DNA molecules. This is quite a peculiar finding, because the hydrolysis of ATP (or other nucleotides) by the DNA helicases is usually enhanced if nucleic acids are present in the reaction mixture (37). The ATPase activity of the *Mth* MCM was stimulated more than 10-fold in the presence of either single- or double-stranded DNA molecules (20–22). However, it should be noticed that, in the absence of nucleic acids, the ATP-hydrolysis catalyzed by the *Mth* MCM was about 25-fold lower than observed by us with the *Sso* MCM (22). This difference is likely to be even greater considering that, to reduce the spontaneous hydrolysis of ATP, our ATPase activity assays were carried out at 60 °C, a temperature that is considerably lower than the one required by *S. solfataricus* for an optimal growth (87 °C). The *Sso* MCM is able to use various nucleotides as co-factors for the DNA unwinding activity with different efficiency, as also reported for the *Mth* MCM (20–22). However, whereas this latter can utilize almost equally well both ATP and dATP (22), the *Sso* MCM shows a significant preference for ATP.

The Walker A motif was found to be critical for nucleotide binding in several members of AAA⁺ family (5). In the K346A mutant *Sso* MCM the invariant lysine of the Walker A motif was changed to alanine. The ATPase and the DNA helicase activities of the mutant protein were almost completely abolished. This finding indicated that nucleotide binding is essential for the DNA unwinding activity. It is interesting to observe that ATPγS, a binding but not hydrolyzable ATP analog, partially supported the *Sso* MCM oligonucleotide displacement function, as also reported for the *Mth* MCM protein (22).

The *Sso* MCM displayed a low level of helicase activity on a 55-nt partial duplex, whereas the presence of a 9- or 30-nt tail on the 5'-end of the oligomer DNA was found to markedly stimulate the unwinding activity of the enzyme. It was recently reported that the DNA helicase of the *S. pombe* MCM 4/6/7 hetero-hexamer was noticeably enhanced by the presence of a ss-tail on the 5'-end of the displaced oligonucleotide, whereas partial duplexes with no tail were hardly unwound (39). The presence of the nucleotide tail was shown to facilitate the formation of double-hetero-hexameric complexes of *S. pombe* MCM 4/6/7 on substrate DNA. Also the *Mth* MCM was reported to form double hexamers in solution and the N-terminal region of its polypeptide chain (from amino acidic residue 1 through 111) was demonstrated to be essential for protein oligomerization (20–22). In contrast, the *Sso* MCM forms hexameric complexes, as indicated by the analysis of the protein hydrodynamic properties by gel filtration and glycerol gradient centrifugation experiments. However, both the wide distribution of the *Sso* MCM in the glycerol gradient and its broad gel filtration elution peak (see Fig. 2) may depend on the transition of a portion of the protein from a

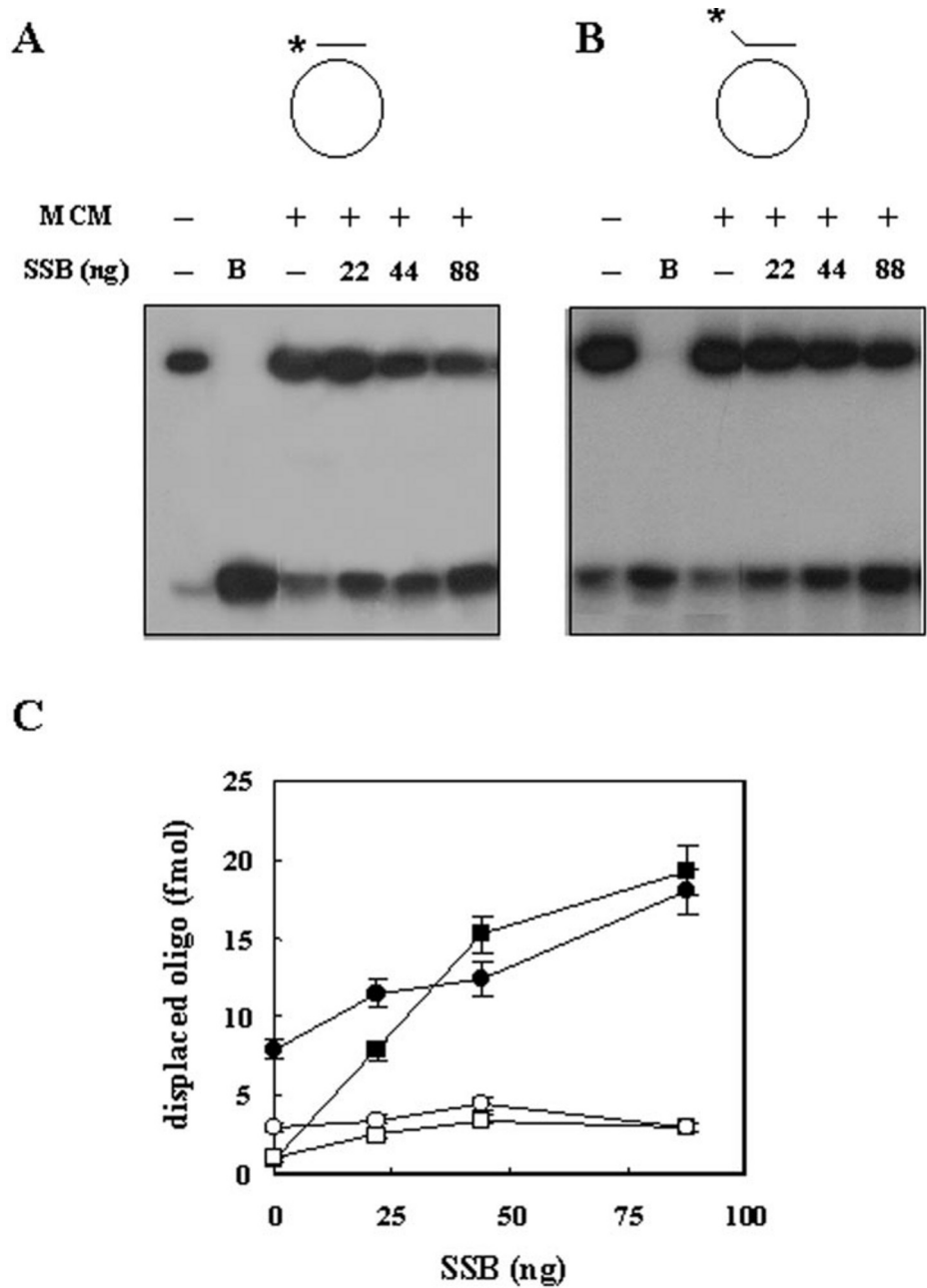


FIG. 6. Stimulation of the *Sso* MCM DNA helicase activity on various DNA substrates by the *Sso* SSB. The DNA unwinding activity of the *Sso* MCM (140 ng) was tested at 70 °C for 30 min in the presence of the indicated amounts of the *Sso* SSB in the standard reaction conditions described under “Experimental Procedures” using as a substrate the partial duplex with no tail (A) or a 9-nt (B) 5'-tail. C, the oligonucleotide melted was quantitated by phosphorimaging and plotted versus the amount of the *Sso* SSB protein present in each activity assay. The partial duplexes DNA utilized had no 5'-tail (squares) or a 9-nt (circles) 5'-tail. Reactions were carried out in the presence (filled symbols) or absence (open symbols) of the *Sso* MCM. Data reported are mean value of at least three independent experiments.

hexameric to a trimeric and/or monomeric form. We did not observe the formation of double hexamers of the *Sso* MCM by the glycerol gradient centrifugation experiments, even when ATP was present in the gradient or if the protein was preincubated with forked DNA molecules before being subjected to the ultracentrifugation.² This may reflect the low stability of the *Sso* MCM dodecameric form that could require the interaction with other components of the replisome to be stably assembled at the replication fork.

SSBs were found to enhance the DNA displacement activity of numerous DNA helicases, such as *S. cerevisiae* Hel B (40, 41), calf thymus DNA helicases A–D and F (42–44), human DNA helicases isolated from HeLa cells (45, 46), and the simian virus 40 large T antigen (47–49). In some instances SSBs may nonspecifically stimulate the helicase function by binding and stabilizing the single-stranded DNA molecules that are formed during the unwinding reaction. Thus, this unspecific effect may also be obtained in the presence of a heterogeneous SSB. On the other hand, in some

instances the enhancement of the DNA helicase function is effected only by the species-specific SSB, because a physical interaction is required between the two proteins (50). This is the case of the recently reported stimulation of human WRN (51) and BLM (52) DNA helicases by human replication protein A. The DNA helicase activity of the *S. solfataricus* MCM homolog was found to be stimulated by the single-stranded DNA binding protein isolated from the same species. This stimulatory effect was much more evident on DNA partial duplexes without a nucleotide tail than on substrates containing a 5'-tail. The *Sso* SSB was able to enhance the *Sso* MCM helicase activity at concentrations that were noticeably lower than that required to fully saturate the single-stranded DNA present in the reaction mixture. Thus, the observed stimulation did not merely depend on the unspecific “coating activity” of the *Sso* SSB. One likely possibility is that the *Sso* SSB may help the *Sso* MCM protein by tethering it to the ssDNA-dsDNA junction. The hypothesis of a direct functional interaction between the *Sso* MCM and SSB proteins was

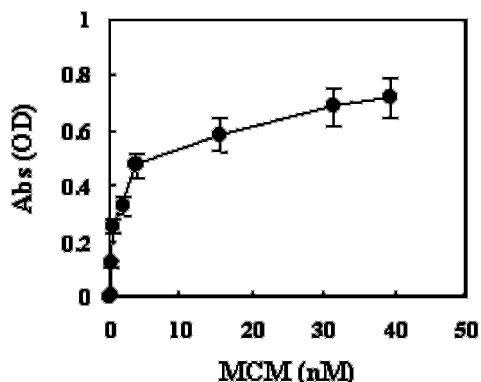


FIG. 7. Detection of a *Sso* MCM:*Sso* SSB complex by ELISA. The *Sso* SSB protein was immobilized on the polystyrene wells of a microtiter plate and incubated with increasing amounts of the pure *Sso* MCM for 30 min at 24 °C. Wells were then extensively washed, and bound *Sso* MCM was detected by ELISA using a specific polyclonal rabbit antiserum, as described under "Experimental Procedures." Absorbance values at 490 nm were corrected by subtracting the background readings obtained with BSA-coated wells. The reported data are mean value of three separate determinations.

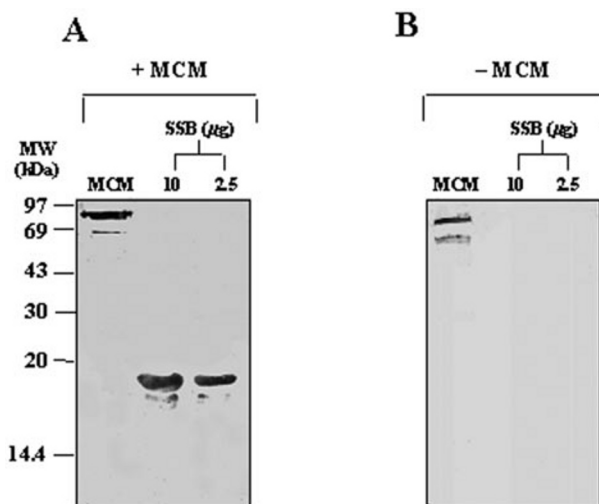


FIG. 8. Far Western blotting analysis of the interaction between the *Sso* MCM and SSB. The homogeneous *Sso* MCM (100 ng) and *Sso* SSB (2.5 and 10 µg) were subjected to electrophoresis on two identical SDS-polyacrylamide (12.5%) gels. The proteins were electroblotted onto a nitrocellulose membrane and then incubated in a solution containing either pure *Sso* MCM (A, +MCM) or buffer alone (B, -MCM). The presence of the *Sso* MCM on each membrane was then detected by Western blot analysis with a specific polyclonal rabbit anti-serum. The positions of the molecular mass standards run in parallel are shown on the right.

further supported by our demonstration by immunological methods that the two proteins physically interact *in vitro*. Such an interaction may be related to the biological role played by the MCM helicase activity. It was recently demonstrated by chromatin immunoprecipitation assays that in *Pyrococcus abyssi* MCM and *cdc6*-like proteins bind preferentially to the chromosomal origin of replication in actively dividing cells (53). However, the chromatin association of the *P. abyssi* MCM was found to be less specific for the genomic origin of replication, suggesting a more broad distribution of this protein along the chromosome in comparison to the *cdc6*-like factor. This finding renders likely that in *P. abyssi* and other Archaea the MCM protein is involved not only in the initial steps but also in the elongation phase of DNA replication, like what was found in Eukarya. The ability of the *Sso* MCM to efficiently unwind DNA duplexes with the help of the

SSB raises the possibility that these two proteins play an important role during the *S. solfataricus* chromosomal replication. The *in vitro* reconstitution of a functional replisome of *Sulfolobus* from its purified molecular components will allow us to further investigate the role of the *Sso* MCM and SSB proteins in the replisome assembly and progression.

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