Modular Organization of the *Sulfolobus solfataricus* Mini-chromosome Maintenance Protein*#1

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Mini-chromosome maintenance (MCM) proteins form ring-like hexameric complexes that are commonly believed to act as the replicative DNA helicase at the eukaryotic/archaeal DNA replication fork. Because of their simplified composition with respect to the eukaryotic counterparts, the archaeal MCM complexes represent a good model system to use in analyzing the structural/functional relationships of these important replication factors. In this study the domain organization of the MCM-like protein from *Sulfolobus solfataricus* (Sso MCM) has been dissected by trypsin partial proteolysis. Three truncated derivatives of Sso MCM corresponding to protease-resistant domains were produced as soluble recombinant proteins and purified: the N-terminal domain (N-ter, residues 1–268); a fragment comprising the AAA+ and C-terminal domains (AAA+ + C-ter, residues 269–686); and the C-terminal domain (C-ter, residues 504–686). All of the purified recombinant proteins behaved as monomers in solution as determined by analytical gel filtration chromatography, suggesting that the polypeptide chain integrity is required for stable oligomerization of Sso MCM. However, the AAA+ + C-ter derivative, which includes the AAA+ motor domain and retains ATPase activity, was able to form dimers in solution when ATP was present, as analyzed by size exclusion chromatography and glycerol gradient sedimentation analyses. Interestingly, the AAA+ + C-ter protein could displace oligonucleotides annealed to M13 single-stranded DNA although with a reduced efficiency in comparison with the full-sized Sso MCM. The implications of these findings for understanding the DNA helicase mechanism of the MCM complex are discussed.

Mini-chromosome maintenance (MCM) proteins are believed to function as the DNA helicase at the archaeal/eukaryal DNA replication fork (1–4). The eukaryotic MCM complex consists of six paralogous polypeptides (MCM 2–7).

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The abbreviations used are: MCM, mini-chromosome maintenance; Sso, *Sulfolobus solfataricus*; AAA+, ATPases with other associated cellular activities; Mth, *Methanothermobacter thermoautotrophicum*; Afu, *Archaeoglobus fulgidus*; ss, single-stranded.

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which includes the AAA+ motor domain, retains DNA helicase activity and the extreme C-terminal domain (residues 504–686) displays DNA binding capability. The results of this analysis provide further insights into the structure/function relationship of the MCM protein.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade. Restriction and modification enzymes were from New England Biolabs. Radioactive nucleotides were purchased from GE Healthcare. Oligonucleotides were synthesized by Proligo (Paris, France).

Construction of the Plasmid Vectors for the Expression of the His-tagged Sso MCM and Its Truncated Derivatives—The construct for the expression of Sso MCM with a His tag at the C-terminal end was generated by a PCR-based approach using the pET19b-SsoMCM vector as the template (18) and the oligonucleotide MCM-NheI-for (5'-GGTTGGCCATATGGTGAGAATC-3') as the forward primer (the Ncol restriction site is underlined in the reported sequence) and the oligonucleotide MCM-686-Xho-rev (5'-GGTTGGCCATATGGGCGATGTAC-3') as the reverse primer (the XhoI restriction site is underlined in the reported sequence). The insert of each plasmid construct was sequenced to verify that mutations had not been introduced during PCR.

Production of the Recombinant Proteins in Escherichia coli—All recombinant proteins were produced in E. coli BL21(DE3) Rosetta cells (Novagen) transformed with the corresponding expression vectors. Cultures were grown at 37 °C in 4 liters of LB medium containing 30 μg/ml kanamycin and 30 μg/ml chloramphenicol. When the culture reached an A600 of 0.8, protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to 0.2 mM. The bacterial culture was incubated at 37 °C for an additional 2 h. Cells were harvested by centrifugation, and the pellet was stored at −20 °C until use.

Determination of Protein Concentration—Protein concentration was estimated by the Bio-Rad assay (Bio-Rad Laboratories) from titration curves made with bovine serum albumin as a standard.

Purification of Full-sized His-tagged Sso MCM and Its Truncated Derivatives—The recombinant E. coli cells pellet was thawed and resuspended in 50 ml of buffer A (25 mM Tris-HCl, pH 7.0, 2.5 mM MgCl$_2$, 500 mM NaCl, 5 mM imidazole) supplemented with a mixture of protease inhibitors (Sigma). Cells were broken by two consecutive passages through a French pressure cell apparatus (Amino, Silver Spring, MD) at 2000 p.s.i. (1 p.s.i. = 6.9 kilopascals). The resulting cell extract was centrifuged for 30 min at 30,000 rpm (Beckman 70 Ti rotor) at 10 °C. The supernatant of the wild type protein was treated at 80 °C for 10 min and then incubated in ice for 10 min. The thermoprecipitated proteins were removed by centrifugation for 30 min at 30,000 rpm (Beckman 70 Ti rotor) at 10 °C. The supernatant was passed through a 0.45-μm filter (Millipore) and loaded onto a 5-ml Ni$^{2+}$-nitrilotriacetate Superflow-agarose column (Qiagen) pre-equilibrated in buffer A. After a washing step with buffer A, the elution was carried out with 70 ml of an imidazole stepwise gradient (25–500 mM) in buffer A. 1.0-ml fractions were collected and analyzed by SDS-PAGE to detect the Sso MCM polypeptide. Fractions containing the recombinant protein (elution step with 100 and 200 mM imidazole) were pooled. The pool (volume, 20 ml) was dialyzed overnight against buffer B (25 mM Tris-HCl, pH 8.5, 2.5 mM MgCl$_2$, 50 mM NaCl) and loaded onto a Mono Q HR 10/10 column (GE Healthcare). Fractions containing the recombinant protein were pooled dialyzed against buffer C (25 mM Tris-HCl, pH 8.5, 2.5 mM MgCl$_2$, 100 mM NaCl). The dialyzed sample was concentrated up to a volume of 2 ml using an Amicon ultrafiltration apparatus (Millipore) equipped with a YM-30 membrane. The final yield of the recombinant protein after this purification procedure was about 1.5 mg.

The Sso MCM deleted proteins were purified from E. coli cell extracts essentially as described for the full-sized His-tagged protein, except that the heat treatment was omitted and, for the N-ter and AAA+ C-ter proteins, the nickel-chelate chromatography was followed by gel filtration onto a Superdex G 75 HR 16/60 column (GE Healthcare) in buffer D (25 mM Tris-HCl, pH 7.0, 2.5 mM MgCl$_2$, 400 mM NaCl, 15% glycerol). A detailed description of the purification procedures is available upon request.

Gel Filtration Chromatography Experiments—Samples of the purified wild type His-tagged Sso MCM (100 μg in 250 μl) were
subjected to analytical gel filtration chromatography on a Superose 6 HR 10/30 column (GE Healthcare) equilibrated in buffer C. The column was calibrated by running a set of gel filtration markers that included thyroglobulin (670 kDa), ferritin (440 kDa), and bovine serum albumin (67 kDa).

A sample of the purified Sso MCM AAA+-Cter protein (100 μg in 250 μl) was subjected to analytical gel filtration chromatography on a Superdex 200 HR 10/30 column (GE Healthcare) equilibrated in buffer C. The column was calibrated by running a set of gel filtration markers that included ferritin (440 kDa, 61 Å, Ve = 10.23 ml), catalase (232 kDa, 52.2 Å, Ve = 11.89 ml), aldolase (158 kDa, 48.1 Å, Ve = 12.28 ml), bovine serum albumin (67 kDa, 35.5 Å, Ve = 13.28 ml), ovalbumin (43 kDa, 30.5 Å, Ve = 14.28 ml), and chymotrypsingen A (25 kDa, 20.09 Å, Ve = 16.47 ml).

Samples of the purified Sso MCM N-ter and C-ter proteins (100 μg in 250 μl) were subjected to analytical gel filtration chromatography on a Superdex 75 HR 10/30 column (GE Healthcare) equilibrated in buffer C. The column was calibrated as described previously for the Superdex 200 column.

To determine the oligomeric state of the AAA+-C-ter protein in the presence of ATP, analytical gel filtration experiments were carried out using a Superdex 200 HR 10/30, which was equilibrated and developed in buffer C containing 5 mM ATP.

In all gel filtration experiments the flow rate was 0.3 ml/min, and 0.5-ml fractions were collected and analyzed by Western blot with anti-Sso MCM polyclonal antibodies as described previously (18).

Glycerol Gradient Sedimentation—Glycerol gradient ultracentrifugation was performed by applying the AAA+-C-ter protein (45 μg in 150 μl) to an 8-ml 20–40% glycerol gradient in buffer containing 25 mM Tris-HCl, pH 8.5, 2.5 mM MgCl2, 100 mM NaCl with or without 5 mM ATP. A parallel gradient was used to fractionate a mixture of standard proteins, which included the following: catalase (232 kDa, 52.2 Å, Ve = 11.89 ml), aldolase (158 kDa, 48.1 Å, Ve = 12.28 ml), bovine serum albumin (67 kDa, 35.5 Å, Ve = 13.28 ml), ovalbumin (43 kDa, 30.5 Å, Ve = 14.28 ml), and chymotrypsingen A (25 kDa, 20.09 Å, Ve = 16.47 ml).

DNA Substrates—Oligonucleotides were labeled using T4 polynucleotide kinase and [γ-32P]ATP. After the labeling reaction, oligonucleotides were purified using Micro Bio-Spin P-30 Tris chromatography columns (Bio-Rad Laboratories) according to the manufacturer’s instructions. The Bub-20T-Top oligonucleotide (5 ’-TCTACCTGGACGACCGGG(T)26GGGC-CAGCAGGTCCATCA-3 ’) was used as a single-stranded DNA in the band-shift experiments.

For the preparation of the DNA helicase substrate, an 85-mer synthetic oligonucleotide (5 ’-TTGACACCGCCGCTTGGTTAAATTACCTTTACTTGTCATGCCTGAGGTCCATCA-3 ’) was used. This oligonucleotide is complementary to the M13mp18 (+) strand with the exception of a 30-nucleotide 5 ’-tail (the tail is underlined). The oligonucleotide was labeled with [γ-32P]ATP and T4 polynucleotide kinase, and after the labeling reaction it was purified using a Quantum Prep PCR Kleen spin column (Bio-Rad Laboratories) according to the manufacturer’s instructions. To prepare partial duplex DNA molecules, 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 Band-shift Assays—For each substrates, 10 μl samples were prepared, which contained 200 fmol of [32P]-labeled DNA in 20 μl Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5 mM MgCl2, 0.7 mM 2-mercaptoethanol, and the indicated amounts of protein. Following incubation for 10–15 min at room temperature, complexes were separated by electrophoresis through 5% polybisacrylamide gels (37:5:1) in 0.5× Tris borate-EDTA buffer. Gels were dried down and analyzed by phosphorimaging. Experiments were performed in triplicate, and the results were averaged. The error bars on the graphs are the standard error of the mean.
[bound protein] = capacity[free protein]/Kd + [free protein]. The program GraFit (version 5.1) was used for these calculations.

DNA Helicase Assay—Helicase assay reaction mixtures (20 µl) contained 25 mM Hepes-NaOH, pH 7.5, 5 mM MgCl2, 50 mM sodium acetate, 2.5 mM 2-mercaptoethanol, 5 mM ATP, and 50 fmol of 32P-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 ml of 5% stop solution (0.5% SDS, 40 mM EDTA, 0.5 mg/ml proteinase K, 20% glycerol, 0.1% bromphenol blue); then they were run on an 8% poly(bisacrylamide) gel (29:1) in Tris-borate-EDTA buffer containing 0.1% SDS at constant voltage of 150 V. After electrophoresis the gel was soaked in 20% trichloroacetic acid and analyzed by phosphorimaging. The reaction products were quantified, and any free oligonucleotide in the absence of enzyme was subtracted.

RESULTS

Limited Tryptic Digestion of Sso MCM—The availability of highly purified recombinant Sso MCM made it feasible to utilize partial trypsin digestion under native conditions to investigate the domain structure of this protein. Limited proteolysis experiments are based on the observation that endoproteases prefer unstructured substrates that are flexible and able to adopt a conformation that fits their catalytic sites. Thus, under mild conditions, preferential sites of proteolytic attack within a folded protein are segments with the highest conformational mobility, hydrophilicity, and exposure to the solvent. Because interdomain linkers usually possess the features noted above,
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controlled proteolysis experiments allow us to locate domain boundaries of multimodular proteins. In an initial set of experiments, we analyzed the proteolytic products obtained by varying either the trypsin/Sso MCM ratio or the digestion time at a fixed protease/protein ratio. This analysis was used to determine the best conditions for identifying stable proteolytic products. A typical example of a limited digestion time course experiment is given in Fig. 1A. Purified recombinant His-tagged Sso MCM was subjected to partial proteolysis with trypsin for 120 min at a protease/protein ratio of 1/700 (w/w). The 78-kDa band corresponding to the full-sized protein gradually disappeared and was almost completely cleaved at the end of the experiment. Two major protein bands of about 50 and 31 kDa rapidly increased within the first 45 min of incubation. Then, the intensity of the 50-kDa band was slightly reduced, and at the same time, a band of about 22 kDa was detected, the intensity of which gradually increased. The Sso MCM fragments, which were obtained after a 75-min incubation with trypsin, were identified by sequential Edman degradation analysis after separation by SDS-PAGE and electrotransfer onto a polyvinylidene difluoride membrane, as described under “Experimental Procedures” (the partial N-terminal sequences are reported in Table 1). This analysis revealed that the proteolytic product of about 50 kDa starts with Ala251 and is likely to include the C-terminal end of the Sso MCM His-tagged polypeptide chain (residues 251–686, expected molecular size of 50 kDa). The 31-kDa protein band was found to contain two polypeptides, one starting with the N-terminal Met residue and the other one with Ala251. Based on these findings, we argue that the polypeptide chain around residue 251 is flexible and easily accessible to the proteolytic attack, because cleavage at this site takes place with a very fast kinetic. Trypsin cleavage at Ala251 produces an N-terminal fragment of about 31 kDa (amino acid residues 1–250) and a larger C-terminal proteolytic product of about 50 kDa (amino acid residues 251–686). N-terminal sequence analysis of the 22-kDa proteolytic fragment revealed that it starts with residue 503. Cleavage at this site takes place with a slower kinetic with respect to that at residue 251, as indicated by the later appearance of the 22-kDa protein band. This analysis suggests that Sso MCM consists of the protease-resistant modules that are depicted schematically in Fig. 1B: the N-terminal domain, which corresponds to the polypeptide chain N-terminal third; the domain named AAA+C-ter, which contains the remaining C-terminal portion of the protein; and the C-terminal domain, which includes the protein C-terminal third.

Production of Sso MCM Truncated Forms—To investigate whether Sso MCM had a modular organization, as suggested by the results of the partial proteolysis experiments, we produced deleted forms of this protein and analyzed their biochemical properties. Fig. 2 shows a schematic representation of the polypeptide chain of Sso MCM and the truncated derivatives produced for this study. The protein named N-ter was designed to include residues 1–268 (molecular size of about 31 kDa) and not residues 1–251, because Sso MCM primary sequence analysis revealed that residue 251 (where trypsin cleavage takes place) is located between the loop and the C-terminal β-strand of the N-terminal β-hairpin finger (26). The AAA+C-ter derivative (residues 269–686, molecular size of about 46 kDa) contains the entire AAA+C-ter motor domain and the C-terminal third of the Sso MCM protein. C-ter includes this latter portion of the polypeptide chain (residues 504–686, molecular size of about 21 kDa) which contains a putative HTH (helix-turn-helix) DNA-binding motif (23). We also attempted to produce the Sso MCM fragment, which includes residues 269–503 in *E. coli*, but the recombinant protein was found to be chronically insoluble.3 As a matter of fact, multiple sequence alignments revealed that Sso MCM residue 503 is located between the Sensor 1 and Sensor 2 conserved sequence motifs; and a larger fragment of the Sso MCM protein, which also includes the putative Sensor 2 (residues 269–510), turned out to be soluble when produced in recombinant form.3

The N-ter and AAA+C-ter derivatives were produced in *E. coli* as N-terminally His-tagged proteins, whereas the C-ter

3. B. Pucci and F. M. Pisani, unpublished observations.
form bears a His tag at the C-terminal end. All of these truncated proteins were found to be overexpressed in soluble form in *E. coli* cells and were purified using a procedure that included a chromatographic step on a Ni²⁺-chelate column followed by ionic exchange chromatography. To assess the oligomeric state of these proteins, gel filtration experiments were carried out on a Superdex 6 column (for the full-length Sso MCM protein), a Superdex 200 column (for the AAA+ C-ter derivative), and a Superdex 75 column (for the N-ter and C-ter proteins) as shown in Fig. 3. These analyses revealed that all of the truncated proteins behave as monomers in solution, whereas the full-sized protein elutes as a hexamer.

**DNA Binding of Sso MCM Truncated Forms**—To determine whether the purified truncated forms of Sso MCM retained the ability to bind nucleic acids, electrophoretic mobility shift assays were carried out using a γ-32P-5'-end-labeled 56-mer oligonucleotide. As shown in Fig. 4A, all of the truncated proteins are able to bind DNA although with different affinity. A quantitative analysis revealed that the N-ter truncated form binds DNA with an affinity reduced by about 5-fold with respect to the full-sized Sso MCM. *Kₐ* values of 0.3 ± 0.13 and 1.75 ± 0.13 μM were calculated for the full-length Sso MCM and the AAA+C-ter protein, respectively (see Fig. 4B). On the other hand, DNA binding by the AAA+C-ter and the C-ter derivatives appeared greatly reduced, and binding saturation was not reached even at a very high protein/DNA molar ratio (see Fig. 4C).

**ATPase Activity of Sso MCM Truncated Forms**—The ATP hydrolysis capability of Sso MCM truncated derivatives was tested in enzymatic assays carried out at 60 °C and not at the optimal growth temperature for *S. solfataricus* (87 °C) to limit the thermally induced autohydrolysis of ATP. Release of [γ-32P]orthophosphate was analyzed by thin-layer chromatography as described under “Experimental Procedures.” In these assays DNA was not present, because we found that the ATPase activity of the full-sized Sso MCM (18) and the AAA+C-ter truncated form is not activated by various kinds of nucleic acid molecules (oligonucleotides in single- and double-stranded form, fork and bubble structures). This finding is in contrast with the moderate stimulation of the Sso MCM ATPase activity in the presence of DNA observed by others (25) and with that reported for many other DNA helicases (15–17). As expected on the basis of their primary structure, both the N-ter and the C-ter protein were found to be completely devoid of ATPase activity, whereas the AAA+C-ter form retained the ability to hydrolyze ATP. As shown in Fig. 5, steady state parameters of the ATP hydrolysis reactions were calculated for the AAA+C-ter protein and the full-sized Sso MCM. The determined *Kₐ* and *Vₘₐₓ* values were estimated to be 171.77 ± 11.01 pmol/min for the full-sized Sso MCM and 432.02 ± 49.98 pmol/min for the AAA+C-ter derivative. If well pointed out that the chromatographic run was carried out at room temperature; under these conditions Sso MCM is able to bind but not to hydrolyze ATP because of the thermophilic nature of the protein. As shown in Fig. 6A, a clear shift in the elution volume of the AAA+C-ter protein was observed when gel filtration was performed in the presence of ATP. Calibration of the Superdex 200 column with a set of protein markers revealed that in the presence of radiolabeled ATP as described under “Experimental Procedures.” Initial rate values were plotted versus the ATP concentration using the program GraFit (version 5.1). *Kₐ* and *Vₘₐₓ* values were calculated from double-reciprocal plots. *Vₘₐₓ* values were estimated to be 171.77 ± 11.01 pmol/min for the full-sized Sso MCM and 432.02 ± 49.98 pmol/min for the AAA+C-ter derivative.
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In this study we have investigated the domain organization of the MCM-like protein from the crenarchaeon *S. solfataricus* (Sso MCM) by limited proteolysis experiments and biochemical characterization of truncated forms corresponding to the protease-resistant domains. We have demonstrated that the protein consists of two main modules corresponding to the N-terminal third and the remaining C-terminal portion of the polypeptide chain (the N-ter and AAA++C-ter deleted forms). Furthermore, we have found that the C-terminal module consists of two domains connected by a protease-sensitive linker region: the AAA+ domain and the extreme C-terminal domain. Crystallographic analysis of the Mth MCM N-terminal portion revealed that this portion of the protein folds into three distinct domains: domain A, at the N terminus, has an α-helical structure; domain B has three β-strands and contains the zinc-binding motif; domain C contains five β-strands forming an oligonucleotide/oligosaccharide-binding fold (30). A detailed biochemical characterization of the Mth MCM N-terminal region was reported by Kelman and co-workers (31). These authors show that domain A has a regulatory but not an essential role, because mutant proteins lacking this portion of the polypeptide chain retain ATPase and helicase activity (31). Domain B is implicated in binding ssDNA, and domain C is required for protein multimerization and helicase activity. The Mth MCM N-ter truncated protein has been reported to be able to bind ssDNA molecules (31). This finding is consistent with the results of our biochemical analysis showing that the corresponding Sso MCM N-ter deleted form stably interacts with DNA molecules as detected by electrophoretic mobility shift assays. However, the Sso MCM N-ter protein does not display the ability to form oligomers in solution, whereas the Mth MCM N-ter was found to form dodecameric structures (30–31). The inability of Sso MCM N-ter deleted derivative to form multimers could derive from the low stability of these assemblies in solution, a phenomenon that is increased as a consequence of the protein dilution that takes place during gel filtration chromatography. This hypothesis is consistent with the recent finding that Sso MCM N-ter protein crystals contain hexameric ring-like complexes as revealed by x-ray diffraction analyses.4 It should be noted that even the AAA++C-ter and the C-ter truncated forms of Sso MCM have been found unable to form multimeric assemblies by size exclusion

33.95 Å. Therefore, we argue that in the presence of ATP the protein is able to form dimers in solution. To get a more accurate determination of the AAA++C-ter oligomeric state, gel gradient sedimentation analyses were performed in the presence or absence of ATP. As shown in Fig. 6B, the protein behaves as a monomer (expected molecular size of 46 kDa) in the absence of ATP. On the other hand, in the presence of the nucleotide, the AAA++C-ter protein peak was detected in a shifted position compatible with a dimeric structure (92 kDa). In addition, analysis of the AAA++C-ter distribution through the gradient revealed a trailing of the protein from the dimeric to the hexameric structures only when ATP was present (data not shown). These results suggest that oligomerization of the AAA++C-ter protein up to hexameric structures is favored in the presence of the nucleotide substrate.

**DNA Helicase Activity of Sso MCM Truncated Forms**—Next we decided to analyze whether the Sso MCM deleted derivatives were endowed with the ability to unwind DNA. To this end DNA helicase assays were carried out using as a substrate M13 ssDNA to which a γ-32P-5'-end-labeled synthetic oligonucleotide was annealed as described previously (18). This oligonucleotide forms a 55-bp duplex on M13 ssDNA and bears a 20-nucleotide 5'-tail that is not complementary to M13 ssDNA. Assays were performed at 70 °C in the presence of 5 mM ATP. The N-ter and C-ter proteins were found to be completely devoid of DNA helicase activity, whereas the AAA++C-ter derivative displayed the ability to displace the labeled oligonucleotide, although the DNA unwinding efficiency appeared to be reduced with respect to the full-sized Sso MCM protein, as shown in Fig. 7.

![Image](49x530 to 408x733)

**FIGURE 6.** Analysis of the AAA++C-ter truncated protein oligomeric state in the presence of ATP. A, samples of the AAA++C-ter protein were subjected to analytical gel filtration chromatography on a Superdex 200 HR 10/30 column, which was equilibrated and developed either in the absence or presence of ATP as described under “Experimental Procedures.” Aliquots of each fraction were analyzed by Western blot to detect the AAA++C-ter protein. A plot of the Stokes radius versus the peak fraction for each marker protein is reported. S values of the AAA++C-ter protein in the absence or presence of ATP were 14.20 ml (Kav = 0.41) or 13.21 ml (Kav = 0.35), respectively. B, samples of the AAA++C-ter protein were fractionated on a 20–40% glycerol gradient as described under “Experimental Procedures.” Aliquots of each fraction were analyzed by Western blot to detect the AAA++C-ter protein. A plot of the S values versus the peak fraction for each marker protein is reported. S values of the AAA++C-ter protein in the absence or presence of ATP were estimated to be 4.3 and 6.3, respectively.

4 R. Ladenstein and W. Liu, unpublished observations.
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The ability of the Sso MCM AAA+-C-ter deleted form to unwind DNA duplexes (although with a reduced efficiency in comparison with the full-sized protein) is apparently in contrast with previous reports that positively charged amino acid residues located in the polypeptide chain N-terminal third (Lys^{129}, Lys^{134}, His^{146}, and Lys^{194} (26)), together with those located at the N-terminal β-hairpin fingertip (Lys^{246} and Arg^{247} (25)), play a critical role in the DNA binding/remodeling function of the complex. Our explanation of these apparently contradictory experimental data is that alanine mutagenesis of these residues has a detrimental effect on the ability to bind and unwind DNA only in the context of the full-sized Sso MCM protein. On the other hand, deletion of the entire N-terminal domain does not completely abolish the DNA binding and helicase functions of the C-terminal module, which includes the AAA+ motor domain. We argue that the full-sized Sso MCM bearing an alanine substitution of these basic residues is hindered from spooling single-stranded molecules originated by the DNA melting activity of the AAA+ motor domain. With all that considered, it is tempting to speculate that the Sso MCM N-terminal module may be responsible for extruding ssDNA outside the ring-like protein complex. Structural analysis of the Sso MCM complex, together with additional site-specific mutagenesis studies, will be necessary to unravel the DNA helicase molecular mechanism.

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chromatography. This suggests that integrity of the polypeptide chain is necessary for Sso MCM to form stable hexameric complexes in solution.

Our biochemical analysis has revealed for the first time that a truncated version of an MCM protein, including only the AAA+ motor domain and the extreme C-terminal portion of the polypeptide chain, retains the ability not only to hydrolyze ATP and bind nucleic acids but also to unwind duplex DNA molecules. Because the ATP binding site of AAA+ machines is located at the interface of two adjacent subunits and residues from both subunits are thought to be required for nucleotide hydrolysis, our findings initially seemed not to be consistent with the monomeric state of the AAA+-C-ter protein. In fact, the MCM subunits of budding yeast, expressed separately in E. coli and purified, did not display significant ATPase activity when assayed individually, whereas a combination of two subunits was required for efficient ATP hydrolysis (29). In agreement with this finding, we have found that the AAA+-C-ter deleted form assembles into dimers (and also into multimers) when ATP is present. Furthermore, we speculate that the presence of nucleic acids may favor the hexamerization of the AAA+-C-ter protein, a process that is likely to be necessary for the DNA unwinding activity.

While our work was under review, a paper on the Sso MCM domain organization by Barry et al. (32) was published. Our results are substantially consistent with the biochemical analysis described in this report. However, in contrast with our findings, these authors claim that the small C-terminal domain (residues 613–686), which contains the HTH motif, does not possess any detectable DNA binding activity (32). This discrepancy is quite likely, because our C-ter truncated form (residues 503–686) has different boundaries. However, we have found that the Sso MCM C-ter protein (as well as the AAA+-C-ter truncated form) has a DNA-binding affinity that is at least 1 order of magnitude lower than the full-sized and N-ter proteins.

FIGURE 7. DNA helicase activity of Sso MCM and its truncated form AAA+-C-ter. A, DNA unwinding assays were carried out at 60 °C for 30 min using increasing concentrations (0.1, 0.2, 0.3, and 0.4 μM as monomer) of the full-sized protein and the AAA+-C-ter deleted form. Control reactions without protein were run on the lanes labeled B. B, a plot is shown of the unwound DNA versus the protein concentration used. Quantitative analysis of the radioactive bands was carried out by phosphorimaging. Assays were carried out at least in triplicate, and the results were averaged. The error bars on the graphs are the mean ± S.E.
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