The minichromosome maintenance (MCM) complex is thought to function as the replicative helicase in archaea and eukarya. The structure of the single MCM protein homologue from the archaean Methanothermobacter thermautotrophicus is not yet clear, and hexameric, heptameric, octameric, and dodecameric structures, open rings, and filamentous structures have been reported. Using a combination of biochemical and structural analysis, it is shown here that the M. thermautotrophicus MCM helicase is active as a hexamer.

Replicative DNA helicases play an essential role during chromosomal DNA replication by separating the two strands of duplex DNA to provide single-stranded (ss) DNA for the polymerase. In bacteria, the replicative helicase is the DnaB protein, which forms hexameric rings (1, 2). The eukaryotic minichromosome maintenance (MCM) complex is a family of six related polypeptides (Mcm2–7), each of which is essential for cell viability. In vivo and in vitro studies have shown that in addition to forming a heterohexamer, additional MCM complexes can be formed (3). The enzyme also forms double hexamers in the presence of a forked DNA substrate (4).

The structure of the archaenal MCM complex is unclear. The MCM homologues of Sulfolobus solfataricus, (5–8), Archaeoglobus fulgidus, Aeropyrum pernix (9), Thermoplasma acidophilum (10), and Methanococcoides burtonii (11) form hexamers in solution. To date, the Methanothermobacter thermautotrophicus enzyme is the only exception, as it appears to form dodecamers (12–14), and a dodecameric structure was also suggested by the crystal structure of the N-terminal portion of the protein (15) and an electron microscopic analysis of the full-length enzyme (16–18). However, other electron microscopic reconstruction studies of the M. thermautotrophicus enzyme showed hexameric structures (19), heptameric structures (20), open rings (16), and filamentous structures (21). The conflicting data regarding the structure of the M. thermautotrophicus MCM hinder our progress in elucidating the mechanism of enzyme action at the replication fork. Knowledge of the active form of the enzyme is needed to form a working and testable hypothesis regarding MCM helicase activity during the initiation and elongation phases. One would expect, for example, that a hexamer would work differently than a filament.

A study was therefore initiated to determine the active form of the enzyme using different substrates and experimental conditions. These studies suggested that hexamers are the active form of the M. thermautotrophicus MCM helicase. Thus, the results presented here show that the M. thermautotrophicus helicase is similar to all other archaenal MCM helicases, which are active as hexameric rings.

EXPERIMENTAL PROCEDURES

Materials

Labeled and unlabeled nucleotides were purchased from GE Healthcare. Oligonucleotides were synthesized by the DNA facility at the Center for Advanced Research in Biotechnology. The MCM protein containing a cAMP-dependent protein kinase recognition motif was purified as described previously (22).

Methods

Construction and Purification of Recombinant Proteins—Wild-type and mutant MCM proteins were generated by cloning the genes encoding the proteins into a vector containing a His$_{6}$-MBP-TEV protease recognition site at the N terminus (23). Following expression in Escherichia coli, the proteins were purified on a nickel column at room temperature. The column-bound His$_{6}$-MBP-MCM protein was eluted in buffer A, containing 25 mM Hepes (pH 8.0), 200 mM NaCl, and 300 mM imidazole. The eluted fraction was diluted 6-fold in buffer A without imidazole. His$_{6}$-TEV protease was added to the protein solution, and the mixture was incubated at 22 °C for 16 h. Following digestion, the protein mixture was passed over a large-volume nickel column to remove the undigested His$_{6}$-MBP-MCM, the His$_{6}$-MBP, and the His$_{6}$-TEV protease. The flow-through was collected, and the purified proteins were dialyzed against buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 100 mM NaCl, and 10% glycerol and stored at –80 °C.

Protein Labeling—$^{32}$P labeling of MCM was performed as described previously (22) using 1 nmol of protein (as monomer) in a 100-μl reaction containing 20 mM Tris-HCl (pH 7.5), 15...
mm magnesium acetate, 2 mm dithiothreitol, 100 mm NaCl, 0.1 nmol of \( ^{32}\)P-ATP (3000 Ci/mmol, GE Healthcare), and 5 units of protein kinase A (Sigma) at 37 °C for 60 min. The labeled proteins were separated from the unincorporated nucleotides using a Sephadex G-50 gel filtration column (GE Healthcare) pre-equilibrated with reaction buffer. The specific activity was 5 cpm/fmol.

**DNA Helicase Assay**—Substrates for helicase assay were generated as described previously (24) by hybridizing two, three, or four oligonucleotides. The oligonucleotides used for the studies are shown in supplemental Table 1. Helicase assays were performed as described previously in reaction mixtures (15 μl) containing 20 mm Tris-HCl (pH 8.5), 10 mm MgCl₂, 2 mm dithiothreitol, 100 μg/ml bovine serum albumin, 5 μM ATP, 10 fmol of \(^{32}\)P-labeled DNA substrate, and MCM proteins as indicated in the legends for Figs. 5 and 6 and supplemental Fig. 3. Mixtures were incubated at 60 °C for 1 h. Reactions were stopped by adding 5 μl of buffer containing 0.5% SDS, 100 mm EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue, and 50% glycerol and then placed on ice. Aliquots were loaded onto a 4% gel (for the bubble substrates) or 8% for all other helicase substrates) native polyacrylamide gel in 0.5 x Tris-borate-EDTA and electrophoresed for 1 h at 150 V at 4 °C. The helicase activity was visualized and quantitated by phosphorimaging.

**Size Exclusion Chromatography**—All size exclusion studies were performed using a superose-6 gel filtration column (HR10/30; GE Healthcare). The column was run at a flow rate of 0.5 ml/min, and 250-μl fractions were collected. The presence of the proteins was determined either by ultraviolet absorbance or by scintillation counting of the \(^{32}\)P-labeled proteins. All proteins were incubated at the conditions indicated below for 1 h prior to loading on the column.

To determine the effect of salt on the oligomeric state of the MCM protein, gel filtration was performed at 25 °C with 100 μg of protein in 100 μl (13 μM as monomer) of buffer containing 20 mm Tris-HCl (pH 8.0) and 0.0, 0.5, or 1 m KCl. To determine the effect of temperature on the oligomeric state of the MCM protein, the gel filtration was performed with 100 μg of protein in 100 μl (13 μM as monomer) of buffer containing 20 mm Tris-HCl (pH 8.0) and 0.5 m KCl at 4, 25, and 50 °C. Column and buffers were pre-equilibrated at the respective temperature.

To determine the effect of protein concentration on the oligomeric state of MCM, the gel filtration was performed at 25 °C with 9, 27, 81, 243, or 729 mm \(^{32}\)P-labeled MCM proteins (5 cpm/fmol) in 300 μl of buffer containing 20 mm Tris-HCl (pH 8.0) and 0.5 m KCl. To determine the molecular mass of wild-type and R161A mutant MCM proteins, gel filtration was performed at 25 °C with 100 μg of protein in 100 μl (13 μM as monomer) of buffer containing 20 mm Tris-HCl (pH 8.0) and 0.1 or 1 m KCl.

To study whether the MCM oligomers can change aggregation state, two gel filtration steps were performed. The first gel filtration was performed at 50 °C with 500 μg of protein in 500 μl (13 μM as monomer) of buffer containing 20 mm Tris-HCl (pH 8.0) and 0.5 m KCl. Five hundred microliters of each protein peak in the first gel filtration were incubated for 16 h at 25 °C prior to reinjection to the column under the same buffer conditions.

**Glycerol Gradient Sedimentation**—Glycerol gradient sedimentation studies were performed by applying 300 μg of MCM protein to a 5-ml 20–50% glycerol gradient in buffer containing 20 mm Tris-HCl (pH 8.0) and 0.1 or 1 m KCl. After centrifugation at 40,000 rpm (150,000 × g) for 16 h in a Beckman SW 50.1 rotor at 25 °C, fractions (188 μl) were collected. The distribution of the protein was determined by fractionation on 8% SDS-PAGE and staining with Coomassie Brilliant Blue (R-250). Molecular mass standards were analyzed under the same conditions.

**RESULTS**

*M. thermautotrophicus* MCM Oligomerization Depends upon Temperature, Salt, and Protein Concentration—Using size exclusion chromatography, the oligomeric state of the MCM helicase under different conditions was determined. Because *M. thermautotrophicus* grows at 60 °C, the role of temperature on MCM structure was determined first (Fig. 1A). The gel filtration analyses were performed as described under “Experimental Procedures” at 4, 25, and 50 °C. The structure of the protein at higher temperature was not evaluated due to the limitations of the AKTA purifier (GE healthcare) and the column used. The samples were incubated at the indicated temperature for 1 h prior to analysis on a superose-6 gel filtration column pre-equilibrated at the same temperature. As shown in Fig. 1A, at 4 °C, most of the proteins are distributed between polymer (likely filaments) and dodecameric structures. As the temperature increases, more protein is found as hexamer. It also appears that the position of the dodecamer (D) is shifted toward a smaller complex as the temperature increases (Fig. 1A). It is important to note that the resolution of gel filtration cannot resolve hexamers from heptamers or double hexamers from double heptamers. We labeled the figures as hexamers (H) and dodecamers (D), but it might also be heptamers and double heptamers. Thus, it is possible that the double heptamer (14-mer) is shifted toward a dodecamer (12-mer) as temperature increases. Alternatively, it may also be an effect of running the buffer at high temperature, although no effect was observed on the molecular weight standard. A small fraction of the protein, at all temperatures tested, eluted as expected for a monomer. Because a similar amount of protein is monomeric at all temperatures tested, this fraction is likely to represent misfolded proteins within the preparation.

Next, the effect of salt on the MCM aggregation state was determined as described under “Experimental Procedures” using buffer containing 0, 0.5, or 1 m KCl (Fig. 1B). Following incubation in the buffer for 1 h, the samples were run on a superose-6 gel filtration column pre-equilibrated with the same buffer. Size standards were also run under the three buffer conditions because salt concentration changes the elution profile of the protein standards (supplemental Fig. 1). As shown in Fig. 1B, when salt concentration increases, less protein is found in large aggregation forms, polymers and dodecamers, and more as hexamer. As the salt concentration within *M. thermautotrophicus* is about 800 mm potassium (25), the data presented in Fig. 1B suggest that in vivo, the protein is likely to be hexameric.
After establishing that temperature and salt affect the oligomeric structure of MCM, the role of protein concentration was determined. To detect a low protein concentration, α-32P-labeled MCM protein was used. It was previously shown that the phosphorylation does not affect helicase activity (22). As expected, at high protein concentrations, larger oligomers were observed. At 729 nM, dodecamers are the main oligomeric state. As protein concentration dropped, a large fraction of the proteins form hexamers and monomers (Fig. 1C).

The gel filtration studies described in Fig. 1 and summarized in supplemental Table 2 show that the M. thermoautotrophicus MCM protein forms different structures under different conditions. However, protein size determination using gel filtration depends not only on the size but also on the shape of the protein. Thus, to get a more accurate size determination, a glycerol gradient sedimentation was also performed under low and high salt concentrations (Fig. 2). At low salt concentrations (0.1 M KCl), a single peak was observed at fraction 8 (Fig. 2A). At high salt concentration (1 M KCl), two peaks were observed (Fig. 2B), one at fraction 13 and the other at fraction 17. Combining the sedimentation values obtained from the glycerol gradient (Fig. 3, B and D) and the Stokes radius from gel filtration (Fig. 3, A and C) in the mass equation (26) yields a native mass of 937.92 kDa for the MCM helicase at low salt (Fig. 3, A and B) and 838.72 and 462.11 kDa at high salt (Fig. 3, C and D). This is in good agreement with the expected size of the dodecameric (906.64 kDa) and hexameric (453.32 kDa) structures, respectively.

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MCM Is Active as a Hexamer

Fifty degrees Celsius is the upper limit at which the gel filtration column and the fast protein liquid chromatography can operate. Furthermore, when the experiments are performed at high temperature, the column has to be equilibrated with low salt buffer. When high salt was used, the column did not perform well. Therefore, the experiments were not performed at the same high temperature and high salt conditions found in vivo.

Re-equilibration of the MCM Oligomeric Structure—Figs. 1 and 2 show that the MCM protein forms different oligomers under different conditions. Although the effect of protein concentration on the structure suggests that the different forms are in equilibrium a more direct experiment to demonstrate that this was performed. A gel filtration experiment that was similar to the one performed at 50 °C and described in Fig. 1A was repeated with a larger amount of protein (but at similar concentrations). The sample was run on a gel filtration column at 50 °C (Fig. 4A) with similar results as in Fig. 1A. Following gel filtration, each peak was reinjected onto the column under the same conditions. As shown in Fig. 4B, each sample was re-equilibrated and formed several other oligomeric forms. As the proteins are diluted in the column and in the tubes prior to reination, the results suggest that the MCM structure is dynamic and in constant equilibrium.

The Active Helicase Is a Hexamer—The results presented in Fig. 1 show that at high concentration (0.7 μM), the MCM helicase is mainly dodecameric, at 80 nm, it is hexameric, and below 27 nm, it is mainly monomeric. This information was used to determine the active form of the enzyme. Helicase assays were performed using different enzyme concentrations and a forked (Fig. 5A), a flat (Fig. 5B), or a duplex (Fig. 5C) substrate. As shown in Fig. 5, A–C, the helicase is not active as a monomer (Fig. 5, A–C, lanes 1–3) on all substrates studied. The enzyme is active as hexamers (Fig. 5, A–C, lanes 4–6). However, when higher protein concentrations (>243 nm) were used, the helicase activity was inhibited (Fig. 5, A–C, lanes 7 and 8). The data presented in Fig. 1C show that at these high concentrations, the helicase forms dodecamers, and thus, the results suggest not only that the hexamer is the active form but also that the dodecamer form is not active.

It was shown that an Arg to Ala substitution at residue 161 (R161A) results in a protein that can only form hexamers at micromolar concentrations (27) (Fig. 5H). Also, in contrast to

Gel filtration

A) 0.1M

B) 0.1M

C) 1M

D) 1M

Glycerol gradient

FIGURE 3. MCM forms dodecamers at low salt and a mixture of hexamers and dodecamers at high salt concentration. A and C, the Stokes ratios of wild-type MCM protein was determined following superose-6 gel filtration performed at 25 °C with 100 μg of protein in 100 μl of buffer containing 20 mM Tris-HCl (pH 8.0) and 0.1 M (A) and 1 M (C) KCl and compared with the elution profile of proteins with known Stokes radii. The proteins are: thyroglobulin (Tyr, 669 kDa, 85 Å), ferritin (Fer, 232 kDa, 61 Å), aldolase (Ald, 158 kDa, 48.1 Å), and ovalbumin (Ova, 44 kDa, 34.9 Å). The elution of MCM is marked by X. The gel filtration profile is shown in the inset in each panel. B and D, the sedimentation coefficient of wild-type MCM protein was determined following 20–50% glycerol gradient sedimentation performed at 25 °C with 300 μg of protein in 100 μl of buffer containing 20% glycerol, 20 mM Tris-HCl (pH 8.0), and 0.1 M (B) and 1 M (D) KCl and compared with the sedimentation of proteins with known sedimentation coefficients; thyroglobulin (Tyr 669 kDa, 19.4 s), catalase (Cat, 232 kDa, 11.3 s), aldolase (Ald 158 kDa, 7.3 s), and ovalbumin (Ova 44 kDa, 3.5 s). The elution of MCM is marked by X.

FIGURE 4. The oligomeric state of MCM is dynamic. A, 500 μg of wild-type MCM in 500 μl of buffer containing 20 mM Tris-HCl (pH 8.0) and 0.5 M KCl were incubated at 50 °C for 1 h prior to fractionation on a superose-6 gel filtration column pre-equilibrated with the same buffer and temperature. Two-hundred-and-fifty-microliter fractions were collected, and their oligomeric state is marked as follows: Po, polymer; D, dodecamer; H, hexamer; Mo, monomer; B, two fractions of each protein peak (500 μl total) from the column shown in panel A were incubated at 25 °C before reinjection to the same column pre-equilibrated with the same buffer and temperature.
the observation with the wild-type protein, salt concentration does not affect the structure of the mutant protein (supplemental Fig. 2). Combining the Stokes radius and sedimentation coefficient in the mass equation yields a native mass of 532.32 kDa for the R161A MCM at 0.1M KCl and 492.07 kDa at 1M KCl. This is in good agreement with the expected size of a single hexameric (453.32 kDa) or heptameric (528.87 kDa) structure. Thus, this mutant protein was used as a control for the helicase experiment described above. As shown in Fig. 5, D–F, the mutant enzyme is active even at high protein concentration (Fig. 5, D–F, lanes 7 and 8). Similar protein concentrations inhibit the activity of the wild-type enzyme (Fig. 5, compare panels A–C with panels D–F, see also panel G). These results support the observation made with the wild-type enzyme that hexamers are the active form of the M. thermautotrophicus MCM helicase.

**Hexamers Can Be Loaded on Small, Closed Circular DNA—**M. thermautotrophicus MCM can be assembled around large single-stranded plasmid DNA (13, 14, 28). The minimum circle size that is required for helicase assembly together with the estimated size of the helicase (15) can provide additional, although indirect, evidence for the minimal size of the enzyme that is active. Helicase assays were performed with substrates differing in circle size (schematically shown in Fig. 6, top). Each substrate contains two identical short 32P-labeled oligonucleotides that form two duplex regions flanking a circle structure made of varying lengths of ssDNA. Thus, unwinding of the duplex region can occur only when the helicase assembles around the ssDNA region in the circle. Based on the predicted size of the helicase (130 × 100 Å for a single hexamer (19)), a circle containing two 50-mer ssDNA regions should be sufficient for hexamer assembly and thus should be unwound by a hexameric helicase without steric interference. Similar calculations suggest that the minimum required circle for a dodecameric helicase to assemble on is an 80-mer ssDNA region. As shown in Fig. 6, the helicase can unwind circles made of two 50-mer oligonucleotides (Fig. 6A, lanes 11–15, see also panel B). As only hexamers can assemble around this circle, the data suggest that hexamers are sufficient for helicase activity. When smaller circles were used, no unwinding could be detected (Fig. 6A, lanes 1–10, see also panel B), demonstrating that monomers, which can assemble onto these smaller circles, are not active (as was shown in Fig. 5 with other substrates). These results support the data shown in Fig. 5 that hexamers are the active form of the M. thermautotrophicus MCM helicase. However, the data cannot rule out the possibility that a dodecameric ring could assemble and unwind a larger bubble substrate bidirectionally. Nevertheless, similar results were observed with the R161A mutant MCM (supplemental Fig. 3). In addition, similar to the results obtained with the substrates used in Fig. 5, high protein concentrations inhibit helicase activity of the wild-type enzyme (Fig. 6C but not of the R161A mutant MCM (supplemental Fig. 3B)).

These results are similar to those previously reported with the *E. coli* DnaB helicase. DnaB forms hexameric rings with a molecular mass of about 300 kDa. It was shown that a circle
substrate with at least 55-bp ssDNA regions is required for helicase assembly (29).

**DISCUSSION**

To date, several archaeal MCM helicases have been biochemically characterized. Those include the enzymes from *S. solfataricus*, *A. fulgidus*, *A. pernix*, *T. acidophilum*, *Pyrococcus furiosus*, and *M. thermautotrophicus*. All except the *M. thermautotrophicus* enzyme were shown to form hexamers in solution and also to be active as hexamers. As all archaeal MCM proteins are very similar in their biochemical properties, primary amino acid sequences, and predicted structures, the discrepancies between the oligomeric structures have not been clear.

The data presented here suggest that, as is the case with the MCM proteins from other archaea, the *M. thermautotrophicus* enzyme is also active as a hexameric ring. These observations are not only consistent with those observed with other archaeal MCM proteins but also with the observations made with DnaB, the replicative helicase of *E. coli* (1, 2), and eukaryotic MCM helicase (30, 31).

The reason for the formation of dodecameric structures in solution by the *M. thermautotrophicus* MCM protein is not yet clear. Although the protein is similar to other archaeal enzymes, it may have a local ionic or hydrophobic patch that holds the dodecamer together at high concentration. The crystal structure shows that hydrogen bonds can form across the ring-ring interface to form the dodecameric structure when the arginine 161 is present (15). These interactions are abolished in the mutant protein. The hydrogen bonds between the two hexamers would also explain why salt helps to dissociate the two hexamers. However, *in vitro* studies do not always correlate with what occurs within the cell. Thus, it is possible that *in vivo*, the enzyme is active as a dodecameric ring. Future *in vivo* studies may address this possibility.

It is well established that the hexameric helicases change their conformation upon DNA and nucleotide binding. Electron microscopic studies with the *M. thermautotrophicus* MCM helicase have shown structural changes upon double-stranded DNA and nucleotide binding to the protein (17). Electron microscopic studies, however, are not reliable in determining conformational changes. It was found that under different conditions, different results were obtained. When short double-stranded DNA was used, the DNA was found in the central cavity of the *M. thermautotrophicus* MCM dodecamers (17), whereas when long duplex DNA was used, the DNA was located on the surface of the hexameric ring (32). Structural studies with viral and phage helicases also support the notion of large conformational changes occurring upon nucleotide and DNA binding (33–35). Thus, it is possible that in the presence of ATP and/or DNA, the hexamer-heptamer equilibrium described here may also change. In the future, when the three-dimensional structure of the enzyme is determined in the presence and absence of nucleotide, this question may be addressed.
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