Double Hexamer Disruption and Biochemical Activities of Methanobacterium thermoautotrophicum MCM*  

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*Methanobacterium thermoautotrophicum MCM (mtMCM) is a helicase required for DNA replication. Previous electron microscopy studies have shown mtMCM in several oligomeric forms. However, biochemical studies suggest that mtMCM is a dodecamer, likely a double hexamer (dHex). The crystal structure of the N-terminal fragment of mtMCM reveals a stable dHex architecture. To further confirm that the dHex is not an artifact of crystal packing of two hexamers, we investigated the relevance of the dHex by disrupting the hexamer-hexamer interactions seen in the crystal structure via site-directed mutagenesis and examining various biochemical activities of the mutants in vitro. Using a combination of biochemical and structural assays, we demonstrated that changing arginine to alanine at amino acid position 161 or the insertion of a six-amino-acid peptide at the hexamer-hexamer interface disrupted dHex formation and produced stable single hexamers (sHex). Furthermore, we showed that the sHex mutants retained wild-type level of ATPase and DNA binding activities but had decreased helicase activity when compared with the wild type dHex protein. These biochemical properties of mtMCM are reminiscent of those of SV40 large T antigen, suggesting that the dHex form of mtMCM may be the active helicase for DNA unwinding during the bidirectional DNA replication.

The events leading up to and resulting in a successful G1-S phase transition in the eukaryotic system involve a complex network of proteins and checkpoints. An essential participant in this process is the minichromosomal maintenance protein (MCM) complex (reviewed in Ref. 1). The MCM complex consists of six proteins (mcm2–7) that are highly conserved among different eukaryotic systems (reviewed in Refs. 2–4). The MCM protein complex likely serves as the replication helicase for chromosome duplication (reviewed in Refs. 5 and 6).

Methanobacterium thermoautotrophicum encodes only one MCM homologue (mtMCM) that can self-assemble into higher order complexes (7–10). Recent research has been focused on both eukaryotic and mtMCM complex assembly and function in the DNA replication process as a replication helicase. Results of a crystallographic study have revealed that the N-terminal portion of mtMCM (N-mtMCM) assembles as a double hexamer (dHex) (11).

The idea that an active replication helicase has a dHex structure is intriguing. The head-to-head (N terminus to N terminus) conformation of two hexamers, as observed in the N-mtMCM dHex structure, seems awkward at first. Nevertheless, the dHex architecture may make sense from an organizational standpoint inside a cell. One might imagine a fixed point of replication where the helicase pulls double-stranded DNA (dsDNA) toward it from both directions, thereby eliminating the need to coordinate the movement of the vast number of essential proteins involved in replication. Indeed, replication foci attached to the nucleomatrix have been observed in cells (12).

A viral replicative helicase, SV40 large T antigen (LTag), is a functional homolog of the cellular MCM. LTag also functions as a helicase for unwinding replication forks. The single hexameric form (sHex) of LTag has helicase activity in vitro; however, the helicase activity of dHex is ~10–15 times higher than that of the sHex form (13, 14). Ample evidence suggests that LTag dHex formation is essential for SV40 DNA replication (15–17). The high-resolution structures of the helicase domain of LTag reveal the presence of a large chamber inside the helicase domain with outlet side channels. These structural features, together with previous biochemical/genetic data prompted us to promote a looping model for dsDNA unwinding by LTag dHex (18–20) in which strand separation occurs within the large chamber when DNA is pulled inside the dHex helicase and the separated single-stranded DNA (ssDNA) extrudes from the side channels. This process is triggered by ATP binding and hydrolysis. With the availability of the multiple crystal structures of LTag in different nucleotide binding states, we now have a good understanding of the mechanisms by which the ATP binding and hydrolysis trigger the conformational changes of LTag hexameric helicase that couple the energy of ATP hydrolysis to the dsDNA unwinding (18, 21).

To date, the mtMCM is the only cellular replicative helicase reported to form a dHex structure (7–9, 11).3 Although a recent model for another archaeal MCM is suggested to also function as a dHex (23). The availability of the N-mtMCM crystal structure and the detailed interactions known to occur at the hexamer-hexamer interface present a unique opportunity for investigating the in vitro biochemical activity of the dHex and sHex forms of mtMCM through structure-guided mutagenesis. Here we report the results of the mutational and functional studies. Specifically, we generated mutants designed to disrupt the hexamer-hexamer interactions and obtained stable sHex mutants. In addition, we compared the biochemical activities of these sHex mutants to those of the

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1. The abbreviations used are: MCM, minichromosomal maintenance protein; mtMCM, Methanobacterium thermoautotrophicum MCM; dHex, double hexamer; sHex, single hexamer; dsDNA, double-stranded DNA; LTag, large T antigen; ssDNA, single-stranded DNA; wt, wild type; FL-mtMCM, full-length mtMCM; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; 6Ins, six-amino-acid insertion; EM, electron microscopy.

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5. The high-resolution structures of the helicase domain of LTag reveal the presence of a large chamber inside the helicase domain with outlet side channels. These structural features, together with previous biochemical/genetic data prompted us to promote a looping model for dsDNA unwinding by LTag dHex (18–20) in which strand separation occurs within the large chamber when DNA is pulled inside the dHex helicase and the separated single-stranded DNA (ssDNA) extrudes from the side channels. This process is triggered by ATP binding and hydrolysis. With the availability of the multiple crystal structures of LTag in different nucleotide binding states, we now have a good understanding of the mechanisms by which the ATP binding and hydrolysis trigger the conformational changes of LTag hexameric helicase that couple the energy of ATP hydrolysis to the dsDNA unwinding (18, 21).

6. To date, the mtMCM is the only cellular replicative helicase reported to form a dHex structure (7–9, 11). Although a recent model for another archaeal MCM is suggested to also function as a dHex (23). The availability of the N-mtMCM crystal structure and the detailed interactions known to occur at the hexamer-hexamer interface present a unique opportunity for investigating the in vitro biochemical activity of the dHex and sHex forms of mtMCM through structure-guided mutagenesis. Here we report the results of the mutational and functional studies. Specifically, we generated mutants designed to disrupt the hexamer-hexamer interactions and obtained stable sHex mutants. In addition, we compared the biochemical activities of these sHex mutants to those of the
wild type (wt) dHex forms, including ATPase, DNA binding, and helicase activities.

MATERIALS AND METHODS

Molecular Cloning—We obtained the full-length mtMCM (FL-mtMCM) clone by inserting the PCR product of *M. thermoautotrophicum* ∆H MCM gene (coding residues 2–666) into a modified pGEX-2T plasmid. PCR was performed using forward primer 5′-CACAGCTCTCTGAATGCTATGTTACCCCT-3′ and reverse primer 5′-CGATGAC-TGAGTTAGACTATCTTAAGGTATCCCCT-3′ with unique restriction sites XhoI and XbaI. N-mtMCM (residues 2–286) was cloned into pGEX-2T using the same forward primer as for FL-mtMCM and reverse primer 5′-CGATGAC-TGAGTTAGACTATCTTAAGGTATCCCCT-3′. The PCR product was then inserted between Cys-158 and Gly-159 using PCR with site EagII. All clones were sequenced to confirm the intended mutations.

Electron Microscopy—wt and mutant N-mtMCM (100 and 250 μg/ml in 50 mM Tris·HCl, pH 8.0, 250 mM NaCl, 10 mM DTT) were incubated for 1 h at 37°C. FL-mtMCM mutants were diluted to 20 μg/ml in 50 mM Tris·HCl, pH 8.0, 500 mM NaCl, 3 mM DTT. All samples were adsorbed to glow-discharged, collagen/coal-coated copper grids and stained with 2% uranyl acetate. Grids were examined in a JEOL 1200 EX-II transmission electron microscope at 80 kV and 60,000× magnification. Low dose micrographs on Kodak SO-163 plates were digitized in a Zeiss SCAI scanner with a pixel size of 7 μm, then binned to 21 μm (3.5 Å in the sample). Image processing was carried out with XMIIP (24, 25). Manually picked particles were extracted in 64 × 64 pixel frames, normalized, centered, and aligned in two dimensions using a reference-free method (26). Classification was performed on the centered particles using neural network-based techniques (27). Images in pixel frames, normalized, centered, and aligned in two dimensions using a reference-free method (26). Two- to 3-fold symmetry in their rotational spectra.

Structure and Function of an Archaeal MCM

Another challenge was to find the dHex mutants that specifically disrupt dHex formation were designed based on the crystal structure of the N-mtMCM (11). The interface between the two hexamers in the dHex structure of N-mtMCM is extensive and is mediated by both main chain packing and side chain interactions through the amino acids located within the zinc binding domain (Fig. 1A). One of the amino acid residues, Arg-161 (Fig. 1B), forms five hydrogen bonds (H-bonds) at the interface. Because there are 12 Arg-161 residues in the dHex complex, Arg-161 is predicted to play an important role in the hexamer-hexamer interactions.

**RESULTS**

**Mutations, Protein Expression, and Purification**—The sHex mutants that specifically disrupt dHex formation were designed based on the crystal structure of the N-mtMCM (11). The interface between the two hexamers in the dHex structure of N-mtMCM is extensive and is mediated by both main chain packing and side chain interactions through the amino acids located within the zinc binding domain (Fig. 1A). One of the amino acid residues, Arg-161 (Fig. 1B), forms five hydrogen bonds (H-bonds) at the interface. Because there are 12 Arg-161 residues in the dHex complex, Arg-161 is predicted to play an important role in the hexamer-hexamer interactions.

**DNA Binding Assays**—All primers (Operon) were resuspended in 10 mM NaOH and purified using a Mono Q 10/100 (Amersham Pharmacia Biotech) column by FPLC. Purified DNA was concentrated and quantified using a 260 nm absorbance at 4°C. For the double strand assay, complementary primers were mixed, boiled for 10 min in 900 μl of water, and allowed to cool to room temperature overnight. The annealed dsDNA was then purified using FPLC over a Superdex-75 16/60 (Amersham Pharmacia Biotech) column in 50 mM NaCl. The DNA used for the single strand binding was 5′-AACGCGTGACATCGCGTATAGCTCGAGAGGA-3′ and the complementary oligonucleotides used for the double strand assay were 5′-GGCGGTACCTCGTTATAGGCGTCCGGCGC. The primers were labeled using T4 polynucleotide kinase (NEB) and [γ-32P]ATP (Amer- sham, 3000 Ci/mmol). The labeled primer was purified using Microspin G-25 columns (Amersham Pharmacia Biotech) and stored at 4°C. 20-μl reactions were assembled at room temperature with 50 mM NaCl, 5 mM MgCl₂, 50 mM Tris, pH 7.8, 1 mM DTT, 0.1 mg/ml bovine serum albumin, [α-32P]ATP (Amersham, 3000 Ci/mmol), and various amount of protein. After incubation at 30°C for 30 min, the reactions were stopped by adding 10 mM EDTA. A 5-μl aliquot from each reaction was applied to a prewashed PEI cellulose TLC plate (EMD Chemicals Inc.), dried, and run for 2 h in 2 M acetic acid and 0.5 M LiCl. Plates were dried, autoradiographed using phosphorimaging plates, and quantified.
To disrupt formation of the dHex, we generated two mutants: a point mutation, R161A, and a six-amino-acid insertion mutant (6Ins) in which the amino acid sequence GSGSGG was inserted between residues Cys-158 and Gly-159. The six-amino-acid peptide in the 6Ins mutant was designed to be positioned at the interface between the two hexamers, providing a short bulge of six amino acids that should hinder the ability of Arg-161 to H-bond to the opposing hexamer, thereby achieving an effect similar to that of the R161A mutation. R161A and 6Ins were constructed not only in the context of the complete protein, FL-mtMCM, but also in the N-terminal construct, N-mtMCM, used for crystallographic studies. Because N-mtMCM alone forms dHex, the parallel mutations on N-mtMCM can provide an additional line of evidence for disruption of the dHex formation. The six constructs used for this study are listed in Fig. 1C. All six proteins were expressed and purified to near homogeneity using the conditions described herein and as shown in Fig. 2, A and B.

**Disruption of dHex by Mutations**—To evaluate the ability of the R161A and 6Ins mutations to disrupt dHex formation in both the N-mtMCM and FL-mtMCM constructs, we used a combination of three methods, gel filtration chromatography, native gel shift, and electron microscopy (EM), to analyze the oligomeric states of the proteins.

Using gel filtration chromatography, we found that the elution profile of wt N-mtMCM had a peak with an apparent molecular mass of ~380 kDa, consistent with the size of a dodecamer (or dHex). In contrast, the elution peaks for the two mutants of N-mtMCM displayed a clear shift toward an apparent molecular mass of ~190 kDa (Fig. 2C), consistent with the size of a hexamer. Similarly, the elution peak for FL-mtMCM had an apparent molecular mass of ~960 kDa, consistent with a dodecamer assembly, whereas the two mutants eluted at peaks with an apparent molecular mass of ~450–500 kDa (Fig. 2D), consistent with sHex. The identity of the proteins from all the peaks was confirmed by SDS-PAGE analysis (Fig. 2, A and B). The wt proteins of both FL-mtMCM and N-mtMCM behaved as dodecamers and the mutant proteins as hexamers.

To further analyze the disruption of dHex assembly in mutants, proteins from all six mtMCM constructs were subjected to a native gel shift assay. Despite the use of a wide variety of buffer and salt conditions, the three FL-mtMCM (wt and two mutants) proteins did not show a distinct band pattern on the native gel shift assays. However, the N-mtMCM constructs displayed clear band patterns in the native gel shift assays (Fig. 2E). The two mutant proteins migrated faster than the wt (Fig. 2E, lanes 1–3), suggesting that dHex assembly was disrupted. The faster migration of R161A on native gel could also be caused by the elimination of the positively charged arginine residues (6/hexamer). To examine the charge effect on native gel migration, we used another mutant with two arginine residues (Arg-227 and Arg-230) mutated to alanine as a control (Fig. 2E, lane 4). The mutations were not expected to affect dHex formation because both residues are away from the hexamer-hexamer interface. This mutant behaved like a wt dHex in gel filtration (data not shown) and migrated in a manner similar to that of the wt dHex protein in native gel (Fig. 2E), demonstrating that the loss of two arginines in one molecule (or 12/hexamer) was not sufficient to change the migration in the native gel shift assay. Thus, the faster migration of R161A is not the result of charge changes but likely because of disruption of the hexamer-hexamer interactions needed for dHex formation.

We used EM to directly visualize the presence of dHex in the wt and mutant proteins for both FL-mtMCM and N-mtMCM. We will focus on the data of the N-mtMCM proteins here because the availability of the crystal structure of this construct allows the direct comparison of the EM results with the atomic model (Fig. 3). For the wt N-mtMCM, images corresponding to the side view of the dHex (Fig. 3A) and to the end-on view looking down the channel of the complex (Fig. 3B) were observed. However, for the R161A and 6Ins mutants, no dHex side images were present, and somewhat different end-on views were observed (Fig. 3, C and D). Notably, the difference in the chirality of the end-on average images of the wt and the mutants is striking (Fig. 3 compare B with C and D). The end-on view of the wt dHex has no chirality (Fig. 3B), as was the case for an end-on projection of a low pass-filtered map calculated from the dHex crystal structure (Fig. 3E). In contrast, the end-on views of the two mutants have obvious chirality (Fig. 3, C and D), similar to the chirality seen in a projection of the low pass-filtered map of a sHex atomic model from the crystal structure (Fig. 3F). Fig. 3G summarizes the presence or absence of side views and...
chirality in wt and mutant N-mtMCM complexes. These EM results further demonstrated that R161A and 6Ins mutants disrupted dHex formation of the mtMCM complex.

ATP Hydrolysis—ATPase assays were done only with FL-mtMCM because the FL constructs contain the ATPase domain and the N-mt-MCM constructs lack this domain. For this reason, the DNA binding and helicase assay described below were also performed only on the FL-mtMCM constructs. The ATPase assays showed that the two mutant FL-mtMCM proteins, R161A-FL and 6Ins-FL, were capable of hydrolyzing ATP (Fig. 4A). Quantification of the assays (Fig. 4B) revealed no significant difference in ATP hydrolysis between the wt and the two mutants.

DNA Binding—The assays of ssDNA and dsDNA binding showed that the mutant FL-mtMCM proteins bind DNA over a range of protein concentrations (Fig. 5, A and B), and quantification of the assays revealed a similar level of DNA binding activity for the wt and the sHex mutants (Fig. 5, C and D).

Helicase Assay—When we examined the helicase activity of the wt and sHex mutants of the FL-mtMCM, all showed helicase activity (Fig. 6A). However, the sHex mutants consistently exhibited lower helicase activity than the wt, consistent with the dHex-disrupting effect of the mutations.

FIGURE 2. The oligomeric state of the N-mtMCM and FL-mtMCM wt and mutant proteins. A, 10% SDS-PAGE of purified wt N-mtMCM (lane 2), R161A-N (lane 3), and 6Ins-N mutants (lane 4). The protein marker was in lane 1. B, 10% SDS-PAGE of purified wt FL-mtMCM (lane 1), R161A-FL (lane 2), and 6Ins-FL mutants (lane 3). C, Superdex-200 gel filtration profile of wt and mutants of N-mt-MCM constructs. All proteins contained UV light-absorbing material at the void volume resulting from large aggregates labeled as peak 1. The peak 2 position of the wt N-mtMCM is consistent with that of a dHex (labeled dH). Both R161A-N and 6Ins-N mutants lacked the dHex peak 2, but had peak 3, which corresponds to the expected position of an sHex (sH). D, Superose-6 gel filtration profile of wt FL-mtMCM, R161A-FL, and Ins6-FL. The wt FL-mtMCM had a peak (peak 1) with an apparent molecular mass consistent with that of the dHex (dH), whereas both mutants had peaks (peak 2) consistent in size with a sHex (sH). Peak 3 for all proteins contained degraded proteins that was well separated from peak 1 and 2. E, native gel shift assay of the wt and mutant N-mtMCM proteins. Lane 1, wt N-mtMCM; lane 2, R161A-N; lane 3, 6Ins-N; lane 4, arginine mutant (R227A and R230A) used as a control for the charge effect on the migration on the native gel shift assay. The wt N-mtMCM and the double arginine mutant control migrated similarly (dH). Both mutants, R161A-N and 6Ins-N, migrated faster than the wt, indicating that both mutations successfully converted dHex to sHex in solution.

FIGURE 3. Negative stain EM of N-mtMCM wt and mutants. A–D, experimental average images. The number of particles included in each average, the percentage with respect to the total number of picked particles, and the final resolution are indicated below each image. A, wt N-mtMCM average side view showing the double ring profile. B, wt N-mtMCM average end-on view. C, N-mtMCM R161A average end-on view. D, N-mtMCM Ins6 average end-on view. E, end-on projection of a sHex in the N-mtMCM atomic structure. Model projections were filtered at the resolution indicated in brackets. The bar represents 100 Å. Notice the pronounced chirality in C, D, and F, and the lack thereof in B and E. G, chart summarizing the presence or absence of side views and chirality in wt N-mtMCM and the dHex-disrupting mutants.

FIGURE 4. ATPase activity of the wt and the two sHex mutants of the FL-mtMCM constructs. A, autoradiograph of ATP hydrolysis assay. Lanes 2–5, increasing concentrations of wt; lanes 6–9, 6Ins-FL; lanes 10–13, R161A-FL; lane 14, Walker-A mutant (K-A) of full-length mtMCM as a negative control for ATPase assay. B, quantification of ATPase activity of the wt and mutant proteins. Protein concentrations were calculated as dHex. Error bars represent the average deviation of all points quantified.
activity than wt protein at multiple protein concentrations (Fig. 6B). The activity difference was the greatest at 0.5 nM protein concentration, where both mutants had minimal level of activity (1.2% released the ssDNA, Fig. 6B), but the wt showed 13 times higher activity (with 16% released ssDNA, Fig. 6B). The difference between mutant and wt helicase activity decreased as the protein concentrations increased over the range between 0.5 and 8.0 nM (Fig. 6B), with up to 5 times lower activity for mutant R161A-FL and up to 2 times lower for mutant 6Ins-FL when compared with the wt helicase activity. The R161A mutation appeared to have a greater effect on helicase activity than the 6Ins, perhaps because of the flexibility of the peptide (GSGSGG) inserted at the hexamer-hexamer interface.

DISCUSSION

By a combination of mutagenesis and biochemical and structural assays, the results in this report unequivocally confirm for the first time that the hexamer-hexamer interactions seen in the x-ray dHex structure of mtMCM is not a crystal packing artifact and that the dHex architecture is functionally relevant. These results are consistent with prior observations that wt mtMCM behaved as an oligomeric complex of dHexs or dodecamers in solution (7–9) and suggest that the single heptamer (29) or single hexamer (30) observed in previous EM studies may not be the natural oligomeric state under physiological conditions.

Previous EM studies of the mtMCM protein complex did not observe dHex structure, but only showed heptamer, hexamer, and polymers (22, 29, 30). Using EM, we have also detected oligomeric forms of 6- and 7-fold rings and an occasional open ring structure. However, we consistently observed dodecamer (dHex) formation in a variety of salt and protein concentrations as assayed using native gel shift, gel filtration chromatography, and by direct EM observation. Moreover, the dHex structure accounted for a large portion of the images detected by EM in those buffers closest to physiological conditions (50–100 mM NaCl, 50 mM Tris-HCl, pH 8.0).3

Using gel filtration chromatography, we obtained the pure FL-mtMCM proteins either from the peak corresponding to dHex form for the wt construct or from the peak corresponding to sHex form for the mutant constructs (Fig. 2, C and D). These isolated pure complexes were used for functional assays. We found no significant differences in ATPase activity and DNA binding between the sHex mutants and the wt protein. This result is not necessarily surprising, because the ATP binding sites are in the helicase domain, distal to the hexamer-hexamer interface, and the DNA binding areas are located inside the hexameric channel (11).
Helicase activity of the single hexamers is functional homologue of the MCM complex, also exhibits helicase quantification of the helicase assay, graphed as percent ssDNA substrate released protein concentrations that were calculated as dHex for wt and sHex for mutants. represent the average deviation of all assays quantified.

Another noteworthy observation is that the helicase activity of the mutants approached to that of the wt at higher protein concentration (e.g. at 8.0 nM). A convenient speculation is that at higher protein concentration the sHex of the mutants might come together to form hexamers to give the higher helicase activity. Unfortunately, we could not use much higher mtMCM protein concentrations for a further helicase activity test, because the higher protein concentration of the wt and mutant proteins started to inhibit the helicase activity, a phenomenon also observed for SV40 large T antigen helicase.

In summary, we successfully generated sHex mutants through structure-guided mutagenesis. Studies of the biochemical activities of the sHex mutants with the wt dHex proteins revealed that they had similar ATPase and DNA binding activities but that the sHex mutants had lower helicase activity than the wt dHex protein. These biochemical results, together other structural and biochemical data (7–11), suggest that the mtMCM complex may act similarly to SV40 LTag in functioning as a double hexamer for DNA replication.

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