Polymorphism and Double Hexamer Structure in the Archaeal Minichromosome Maintenance (MCM) Helicase from *Methanobacterium thermoautotrophicum*†

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*Methanobacterium thermoautotrophicum* minichromosome maintenance complex (mtMCM), a cellular replicative helicase, is a useful model for the more complex eukaryotic MCMs. Biochemical and crystallographic evidence indicates that mtMCM assembles as a double hexamer (dHex), but previous electron microscopy studies reported only the presence of single heptamers or single hexamers double hexamer (dHex), but previous electron microscopy studies reported only the presence of single heptamers or single hexamers (Pape, T., Meka, H., Chen, S., Vicentini, G., Van Heel, M., and Onesti, S. (2003) *EMBO Rep.* 4, 1079–1083; Yu, X., VanLoock, M. S., Poplawski, A., Kelman, Z., Xiang, T., Tye, B. K., and Egelman, E. H. (2002) *EMBO Rep.* 3, 792–797). Here we present the first three-dimensional electron microscopy reconstruction of the full-length mtMCM dHex in which two hexamers contact each other via the structurally well defined N-terminal domains. The dHex has obvious side openings that resemble the side channels of LTag (large T antigen). 6-fold and 7-fold rings were observed in the same mtMCM preparation, but we determined that assembly as a double ring favors 6-fold structures. Additionally, open rings were also detected, which suggests a direct mtMCM loading mechanism onto DNA.

Throughout nature, replication of DNA begins with the ordered assembly of a number of proteins at the replication origin. In eukaryotes, these proteins include the ORC (origin recognition complex), Cdc6, Cdt1, and the MCM† complex, composed of six mini-chromosome maintenance proteins (Mcm2-Mcm7) (1). All six components of the eukaryotic MCM heterohexamer (2, 3) are conserved from yeast to human, contain an MCM-specific version of the ATPase Walker A and B motifs, and are essential for initiation and elongation of DNA synthesis. The MCM complex is currently considered the best candidate for the eukaryotic replicative helicase, but its mode of action is unclear. The presence of six different MCM proteins and the great number of additional proteins involved in coordinating eukaryotic DNA replication with the cell cycle further complicate the problem.

*Methanobacterium thermoautotrophicum* has a single MCM homolog (mtMCM, 75.6 kDa) (4), which has clearly been shown to function as a helicase in vitro. It binds DNA, has DNA-stimulated ATPase activity, and processively unwinds duplex DNA in the 3′ → 5′ direction in an ATP-dependent reaction (5–7). These features make mtMCM a simpler experimental model for studying helicase function in DNA replication in both archaeal and eukaryotic systems.

mtMCM is purified as an oligomer of 850–950 kDa, consistent with the mass of a dodecamer (5–7). The archaeal protein and all eukaryotic MCMs share a well conserved C-terminal domain, which includes the ATPase Walker motifs (8) and is responsible for the catalytic helicase activity (6). The N-terminal domain, less conserved among all MCMs, binds DNA (9) and is required for multimerization, which is also required for ATPase and helicase activity (6). The crystal structure of the mtMCM N-terminal domain (mt-mtMCM) reveals a cylindrical structure formed by two hexameric rings bound head to head and traversed by a positively charged channel (9). Previous EM studies of the full-length protein have produced single 6-fold and 7-fold ring images (10, 11) as well as helical arrangements (12). Until now, the double hexamer (dHex) structure of mtMCM has not been reported.

The simian virus 40 large T antigen (SV40 LTag) is a functional homolog of MCM and is the most extensively studied model system for eukaryotic helicases (13). LTag assembles as a head-to-head oriented dHex on the viral origin of replication (ori), with the C-terminal helicase domains forming the outer tiers of the cylinder and the DNA binding domains in the equator (14, 15). The atomic structure of the LTag helicase domain shows a hexameric ring with side channels that are proposed to be exit points for single-stranded DNA during the unwinding reaction (16). Similar side channels have been observed in the hexameric ring of mtMCM (11). Recent EM evidence indicates that the LTag N-terminal region, including its origin-binding domain, is highly flexible in the absence of DNA and requires dHex formation on the viral ori to adopt a stable structure (3). Here, we report the EM study of full-length mtMCM, which revealed considerable polymorphism. Analysis of the end-on view of the ring-shaped complex revealed several oligomeric forms of mtMCM, including 6-fold, 7-fold, and open rings. However, analysis of the side views indicated that in the double ring the 6-fold arrangement was preferred, forming the dHex architecture. We present the detailed analysis of the data for the three-dimensional reconstruction of the mtMCM dHex in the context of the current knowledge about the N-mtMCM and LTag structures.
**Experimentation Procedures**

**Protein Purification** — The mtMCM N-terminal fragment (residues 2–286) was produced as described by Fletcher et al. in 2003 (9). The full-length mtMCM was expressed as a glutathione S-transferase (GST) fusion protein and purified from *Escherichia coli* cells. The GST-mtMCM fusion protein in the supernatant of cell lysates was purified using glutathione affinity column chromatography in a buffer containing 0.1 m Tris, pH 8.0, 250 mM NaCl, and 1 mM dithiothreitol. After thrombin cleavage, Superose 6 gel filtration chromatography was used to further purify and characterize the mtMCM cleaved from the GST fusion protein. The molecular weight of the elution peak of the protein was estimated using the standard curve obtained by plotting the logarithm of the molecular weight of the known proteins against the elution volume (ml) for the Superose 6 column.

**Sample Preparation for EM** — Protein samples were diluted to ~100 μg/ml (mtMCM) or 200 μg/ml (N-mtMCM) in 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 10 mM dithiothreitol with or without nucleotide and incubated as described in Fig. 2. For the NaCl concentration screening, samples were dialyzed for 1 h at room temperature against 50 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, and the appropriate NaCl concentration. Samples were adsorbed to glow-discharged, colloidal/carbon-coated copper grids and negatively stained with 2% uranyl acetate. Grids were examined in a JEOL 1200 EX-II transmission electron microscope at 80 kV and 60,000× magnification. Micrographs were taken on Kodak SO-163 plates under low dose conditions.

**Image Processing** — Micrographs were digitized in a Zeiss SCAI scanner with a pixel size of 7 μm, then down-sampled to 21 μm (3.5 Å in the sample). Image preprocessing, particle picking, two-dimensional alignment, and classification were performed using XMIPP (18, 19). Ring-shaped or rectangle-shaped particles were manually picked, extracted in 64 × 64 (rings) or 128 × 128 (rectangles) pixel images, and independently processed. Images were normalized, centered, and aligned in two dimensions using a reference-free method (20, 21). Classification was performed before or after alignment using Kernel Probability Density Estimator Self-Organizing Maps (22). Images in the resulting groups were realigned before calculating averages. Two-dimensional resolution was calculated using the spectral signal-to-noise ratio method with the threshold set at 4 (23).

**Three-dimensional Reconstruction** — Synthetic N-mtMCM double pentamer and double heptamer models were created with Amira (www.amiravis.com) and MOLEMAN2 (24). Atomic coordinates for one monomer in each hexamer of the N-mtMCM structure (Protein Data Bank identifier 1LTL) were translated to adjust the ring radius and then rotated to generate the corresponding C5 or C7 symmetry-related copies. All reference volumes were created from the coordinates with a 3.5-Å pixel size and low pass filtered to 22 Å. To eliminate symmetry information, volumes were rotationally averaged using XMIPP.

Projection matching three-dimensional orientation search was carried out with SPIDER (25). Final reconstructions were calculated with ART (26). Resolution was estimated using the three-dimensional spectral signal-to-noise ratio equals 1 criterion (27). Surface rendering was done with Amira.

**Modeling** — Atomic coordinates for the N-mtMCM structure and the helicase domain of SV40 LTα (Protein Data Bank identifier 1N25) were manually fitted to the mtMCM reconstruction using Amira. Correlation coefficients between the N-mtMCM structure (before and after increasing the relative ring rotation) and the dHex three-dimensional EM reconstruction were calculated within a cylindrical mask using SPIDER.

**Results**

**Structural Polymorphism** — The mtMCM protein complex expressed and purified from *E. coli* behaved as a dodecamer in gel filtration chromatography (Fig. 1A), which is consistent with prior reports (5–7). When examined under EM by negative stain, this mtMCM preparation displayed two different types of morphology, namely ring-shaped objects, consistent with top views of either a single or a double ring, and rectangle-shaped particles with four density bands, consistent with side views of a double ring (Fig. 1B). In view of recent reports showing 6-fold and 7-fold architectures of mtMCM (10, 11), we experimented with a range of buffer conditions (i.e., different NaCl concentration, presence of Mg and NTP, and temperature) to search for a homogeneous population. Ring and rectangle views were observed in most of the different buffering conditions used, and they were separately picked and analyzed for variability using self-organizing map classification techniques (22). In all cases, analysis of ring views (Fig. 2) revealed a mixture of 6-fold and 7-fold symmetry forms. Furthermore, open ring forms were also found in considerable amounts in two of the conditions (Fig. 2, bottom two rows). Views consistent with projections of tilted single rings were also observed.

**Double Rings of mtMCM Are dHexes** — The rectangle-shaped, four-banded side views in the mtMCM preparations were unequivocally consistent with a double ring architecture. Therefore, these were used to obtain a three-dimensional reconstruction. Double ring side views from the batch of experiments performed at 25 °C in the absence of NTP (Fig. 2) were pooled to make an initial data set of 8883 particles and then aligned. In parallel, a preparation of the isolated N-mtMCM fragment, known to be dHex from the crystal structure (9), was also subjected to negative stain, EM, and two-dimensional alignment. Comparison of the average side view of mtMCM with the average side view of N-mtMCM clearly indicated that the N-mtMCM corresponds to the two central bands in the two-dimensional projection of the full-length protein (Fig. 3A). It was also evident from all the side view data sets of the full-length mtMCM (Fig. 2) that these two central bands were always better defined and gave a stronger signal than the two distal segments.

For the three-dimensional reconstruction of mtMCM, particle orientations were found using a three-dimensional projection matching technique (28). Because the two-dimensional analysis showed that the
N-terminal domain gave the sharpest signal, the orientation search was based in this region. Furthermore, the availability of a high resolution structure for this domain (9) immediately suggested an initial reference volume. However, because 6-fold, 7-fold, and sometimes other oligomeric forms had been detected in the top view analysis, we were uncertain about the number of monomers (and therefore the symmetry) in the double rings observed in side view. To tackle this problem, three initial reference volumes with no symmetry assumed were created by rotationally averaging either the low pass filtered N-mtMCM dHex structure (Fig. 3B), a synthetic N-mtMCM double heptamer, or a synthetic N-mtMCM double pentamer. These were then used to create reference projections for the first iteration of three independent orientation search processes. Subsequent iterations used as a reference the volume created from the mtMCM data set with the angles found in the previous round. Reconstructions and reference projections were calculated from the whole particle, whereas angle search was restricted to a sphere with radius 77 Å centered at the center of the volume, which amply included the N-terminal region. No symmetry was imposed throughout angle search or reconstruction. After reaching convergence, final volumes were computed and their rotational symmetry
assessed slice by slice. Both the volumes calculated using the rotationally averaged dHex (Fig. 3B) or the double pentamer (not shown) as an initial reference showed a clear predominance of the 6-fold harmonic over the 5-fold and 7-fold, whereas the volume calculated with the double heptamer as the initial model showed very poor values for all three symmetries (not shown). We conclude from this result that most (if not all) of the double ring structures correspond to mtMCM dHexs.

Structure of the mtMCM dHex—Once the oligomeric state of the double rings had been determined, an in-depth analysis of the side view variability was carried out. To avoid mixing structural and orientation heterogeneity, the three-dimensional orientation data found with the N-mtMCM rotationally averaged dodecamer as the initial model were used to discard images not corresponding to strict side views (tilt angle >15°). A self-organizing map (Fig. 3C, left) of the remaining particles showed some groups of particles with one or two bands that were extremely ill defined. Images in these groups were not considered further. The remaining projections (corresponding to side views with all four segments well defined) were again aligned in two dimensions and subjected to classification. The new self-organizing map (Fig. 3C, right), which should mainly account for variability due to different rotations around the cylinder axis and to genuine structural differences, confirmed that the two central bands, corresponding to the mtMCM N-terminal domain, are always better defined than the outer segments, which correspond to the helicase C-terminal domains and give a blurred projection. This indicates that the mtMCM C-terminal domain displays a larger variability than the N-terminal region.

The final data set, containing only strict side views with the four segments well defined and adequately covering the angular space of rotation around the longitudinal axis, was used to obtain the 25-Å resolution, three-dimensional reconstruction of the mtMCM dHex (Fig. 4A). This reconstruction used a low pass filtered version of the N-mtMCM dodecamer structure as an initial model, and the orientation search was restricted to the N-terminal domain. No symmetry was imposed throughout orientation search and reconstruction, but C6 symmetry was applied to the final volume after filtering to the estimated resolution. A total of 1,256 particles were included in the final map calculation.

The dHex of mtMCM is a four-tiered cylinder formed by two rings arranged head to head through their N-terminal domains. The C-terminal helicase domains form the outer tiers of the cylinder. Each tier is slightly rotated with respect to its neighbors, giving a total torsion of ~24° from one C-terminal to the other (Fig. 4B). A channel runs along the longitudinal axis of the cylinder, and side openings appear between monomers in each ring at the transition between the N- and C-terminal regions. The central channel dimensions (at least 25-Å-wide, 182-Å-long, Fig. 4A) are large enough to accommodate ~54 bp of B-form double-stranded DNA. The diameter of the side openings varies from 11 to 25 Å. Weak density is present in the central channel (Fig. 4C) and is also observed in some top view averages (Fig. 2).

A model for the full-length mtMCM structure was built by combining the atomic coordinates of N-mtMCM (9) and domains D2 and D3 of the helicase domain of its functional homolog, SV40 LTag (16) (Fig. 5).
These domains form the outermost tier in the LTag dHex and contain the AAA+ sequence motifs. Domain D1, corresponding to the LTag Zn-binding domain, overlapped with the N-mtMCM region and thus was not included in the model.

As expected from the side view two-dimensional analysis (Fig. 3A), the dHex N-mtMCM structure fits the two central tiers of the mtMCM map very well (correlation coefficient = 0.830), and a slightly better fit was obtained by increasing the rotation between hexamers by 5° (correlation coefficient = 0.834). Fitting of the SV40 LTag helicase domain onto the outer tiers of the mtMCM map resulted in a volume highly similar to that of the EM reconstruction, including the existence of side openings in each monomer (Fig. 5A). The combined fitted N-mtMCM and D2-D3 LTag domains amount to a total of 512 residues. Given that mtMCM is 666 residues long, density is lacking for ~150 residues, and these presumably lie in the C-terminal region of each monomer (Fig. 5B). A model has been proposed whereby these C-terminal residues form a belt around the interface between the mtMCM N-terminal and C-terminal tiers (11). In our dHex mtMCM map there is no empty density at this interface. However, we did observe extreme flexibility in the outer tiers of the complex (Fig. 3C), and weak density was present in the channel (Fig. 4C). It is possible that the non-modeled flexible residues may adopt highly variable conformations around the helicase domain and even occasionally in the channel, accounting for the weak density in our reconstructed dHex map.

FIGURE 4. Structure of the mtMCM double hexamer. A, final three-dimensional reconstruction shown in different orientations as indicated. The mesh shows the volume rendered at a threshold accounting for 100% of the total mtMCM dodecamer mass. The solid surface is rendered at a higher threshold, giving 100% of the N-mtMCM mass for the two central tiers. Dimensions have been calculated using the 100% total mass threshold. B, rotation between tiers. Surface rendering with lines indicating representative slices of the different regions in the mtMCM double hexamer map. Rotation values were calculated by cross-correlation of each slice with a projection along the longitudinal axis of the whole structure. Very similar results were obtained when calculating the cross-correlation with respect to one selected slice. C, central section of the mtMCM reconstruction cut along the longitudinal axis. Bar represents 100 Å.
DISCUSSION

Previous EM studies have shown that the archaeal helicase mtMCM is able to adopt three different oligomeric arrangements, namely hexameric rings, heptameric rings, and helical polymers (10–12). For the first time, we report the presence of the dHex structure in an EM study. In addition, we found other oligomeric forms, including 6-fold and 7-fold closed ring structures and, to a lesser extent, open ring structures in the same mtMCM preparation. Interestingly, the mtMCM protein complex behaved like a single oligomeric species when assayed in solution by Superose 6 or Superdex 200 gel filtration chromatography; the single elution peak position had an apparent molecular weight consistent with that of a dHex. Caution must be used when relating the oligomerization state of engineered proteins observed in vitro to their function(s) in vivo, especially in ring-forming complexes that can adopt different stoichiometries with minor adjustments of the monomer-monomer interface (see, for example, Refs. 29 and 30). Indeed, we have even observed a small population of 8-fold ring structures in mtMCM preparations obtained with a slightly different purification protocol (not shown). However, the fact that all these architectures coexist in the same preparation is an indication of polymorphism in the assembly of mtMCM and possibly of equilibrium between these various forms.

It is not possible to know if the ring-shaped particles in our preparations correspond to end-on views (viewing along the central channel) of single or double rings. On the other hand, we found rectangle-shaped particles clearly consistent with side views of a double ring arrangement. However, it was unclear if the double ring was hexameric or heptameric. We have shown that despite the structural heterogeneity present in the preparation, the double ring architecture favors 6-fold symmetric arrangements. Although the functional relevance of each of the structural arrangements is not clear at present, a 6-fold dHex form would correlate best with the presence of six MCM components in the eukaryotic Mcm2-Mcm7 complex (31), as well as with the high resolution structure of N-mtMCM and the biochemical analysis in solution (5–7, 9). A double ring assembly is required in SV40 LTag for the replication of viral DNA, even though single hexamers of LTag also have helicase activity (reviewed in Refs. 32, 33). Nonetheless, the helicase activity of single hexamers of LTag is 15 times lower than that of dHexes (34, 35). In mtMCM, multiple point mutations at the hexamer-hexamer interface have proven the critical role of these residues for dHex formation, and the single hexamer mutants also had reduced helicase activity (36).

Our three-dimensional reconstruction of the double ring allows direct observation of the dodecameric MCM complex for the first time. It highlights characteristics similar to those found for LTag, such as the head-to-head arrangement of hexamers, a central channel, and side openings in each hexamer (14–16). However, our data show that, unlike in LTag, the mtMCM DNA binding domain is more rigid than the C-terminal helicase domain. This structural difference probably reflects functional differences between the two systems.

The LTag dHex is specifically formed on the unique viral ori in the presence of ATP and without the help of any accessory proteins. In LTag, monomers assemble around the specific ori DNA to form two hexameric rings (37), whereas mtMCM forms dodecameric ring structures in solution in the absence of any cofactor and may rely on ORC-CDC6 to recognize the origin of replication and to be recruited and loaded onto the DNA (38). The N-terminal domain in LTag is a rather flexible structure that only becomes further structured upon interaction with the other hexamer in the dHex and the DNA; this flexibility is required for its specific assembly. In contrast, MCM itself does not seem to perform an origin recognition function, and its N-terminal region is
very well structured both in the dHex reported here and in previous EM studies of single hexamers (11, 12). It is very tempting to speculate that this absence of flexibility in the MCM system as compared with that of LTag may be correlated with a concomitant absence of functionality for specific origin DNA recognition. Interestingly, it is the C-terminal (helicase) region of MCM that presents some structural flexibility, as shown by its reduced density in the dHex three-dimensional reconstruction. Indeed, its flexibility is found to be greater than it is in the LTag system (15), suggesting that a more controlled regulation of the helicase function is required in the MCM system. The presence of open ring forms, reported here for the first time, suggests that the loading of mtMCM onto DNA could be achieved through a ring-opening mechanism even in the absence of a ring loader, similar to that proposed for the bacteriophage T7 helicase/primase (39) or the bacterial Rho transcription terminator (40). The putative loaders mtCdc6-1 and -2 might regulate mtMCM ring opening when placed in the vicinity of DNA. The disordered C-terminal residues for which there is no density in our model may also play a role in ring remodeling upon interaction with other components of the replisome. An unstructured C-terminal region of ~80 residues is also present in LTag. This C-terminal fragment plays a role in establishing productive infection of SV40 in certain cell types but is dispensable for viral DNA replication (17, 32).

In conclusion, the overall dHex structure of mtMCM reported here exhibits high similarity to that of LTag, including the head-to-head configuration of two hexamers and the presence of the obvious side channels that could be used for extruding unwound single-stranded DNA loops. This structural similarity between mtMCM and LTag may be a reflection of their similar function in unwinding DNA for replication. The open ring conformation of mtMCM oligomers offers a possible mechanism for the loading of mtMCM onto the origin DNA.

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