Mutations in Subdomain B of the Minichromosome Maintenance (MCM) Helicase Affect DNA Binding and Modulate Conformational Transitions*†‡§

Received for publication, September 9, 2008, and in revised form, December 15, 2008 Published, JBC Papers in Press, December 30, 2008, DOI 10.1074/jbc.M806973200

Elizabeth R. Jenkinson1, Alessandro Costa1, Andrew P. Leech4, Ardan Patwardhan5, Silvia Onesti5, and James P. J. Chong1,3

From the 1Department of Biology and 4Technology Facility, University of York, York YO10 5YW, United Kingdom, the 6Department of Life Sciences, Imperial College London, London SW7 2AZ, United Kingdom, and 6Elettra Synchrotron Light Laboratory (Sincrotrone Trieste), 34012 Basovizza (TS), Italy

Minichromosome maintenance (MCM) proteins are believed to provide the replicative helicase activity in eukaryotes and archaea. The single MCM orthologue from Methanothermobacter thermautotrophicus (MthMCM) has been extensively characterized as a model of the eukaryotic heterohexameric MCM complex. MthMCM forms high molecular weight complexes in solution consistent with a dodecamer. Visualization of this complex by electron microscopy suggests that single and double heptameric or hexameric rings can form. We have mutated two arginine residues (Arg-137, Arg-160) in the N-terminal subdomain B of MthMCM based on their apparent potential to form inter-ring hydrogen bonds. Both the single R137A and the double R137A,R160A mutants were characterized by a combination of biophysical, biochemical, and electron microscopy techniques. Biophysical analysis coupled with electron microscopy studies shows that the R137A mutant forms a double heptameric ring, whereas the R137A,R160A protein assembles as a single heptamer. They both show a defect in DNA binding and a concomitant conformational change in subdomain A, with the double mutant displaying significant defects in helicase activity as well. We propose a model in which MCM loading and the subsequent activation of the helicase activity involve a conformational transition that is connected to a DNA binding event.

The process of DNA replication in eukaryotic organisms involves a complicated interplay between a number of proteins required for licensing origins of replication and restricting the replication of the genome to only once per cell cycle (1–5). The putative replicative helicase consists of six discrete minichromosome maintenance (MCM)4 subunits, MCM2–7, that form a variety of subcomplexes as well as a complete heterohexamer (6–9). Archaea are organisms with features of both eukaryotic and prokaryotic cells (10). Interestingly, archael DNA processing enzymes tend to be simplified homologues of the equivalent eukaryotic machinery rather than homologues of bacterial proteins. For this reason, the archael MCM complex is a good model for understanding some of the mechanisms associated with eukaryotic helicase activity. The archael MCM protein can be divided into three distinct domains (11): an N-terminal domain (discussed in detail below), a central ATPase domain that contains the NTP binding and hydrolysis motifs essential for helicase activity (12), and a less well defined C-terminal domain. A presensor 1 β-hairpin and helix-2 insert in the ATPase domain have been shown to be essential for DNA unwinding (13, 14). A role for the C terminus in DNA binding has been proposed (14).

The crystal structure for the N-terminal domain of Mth-MCM has been solved, revealing that this domain forms two opposing hexameric rings, with each monomer consisting of three structural subdomains (15). Subdomain B encompasses a zinc finger motif, subdomain C provides the contact between the N-terminal and the ATPase domains and contains a highly conserved loop that functions to transmit signals between domains (16), and subdomain A forms the remainder of the N terminus. Subdomains B and C were identified as essential for hexamerization and dodecamerization (17). Deletion of each subdomain in turn demonstrated that subdomain C was sufficient for hexamerization and essential for helicase activity, whereas subdomain B was found to interact with ssDNA. No specific function was assigned to subdomain A (17), but recent structural and biochemical work has highlighted its role as a novel DNA binding site, possibly involved in the initial loading of the protein onto DNA (18).

The MthMCM protein is unusual among MCMs in forming stable double rings in solution (19–21). MCM complexes from other archaea, such as Sulfolobus solfataricus and Archaeoglobus fulgidus, have been reported to form single ring complexes in solution (14, 22, 23), although the A. fulgidus MCM can form...
doubleringcomplexesinthepresenceofDNAinacconcentration-
dependent manner (23). Electron microscopy (EM) studies
have shown that MthMCM can produce a large variety of struc-
tures, including single and double rings, each containing either
six or seven subunits (24–26) as well as helical fibers (27).
A systematic study of the complex in the presence of various
substrates showed that treatment with nucleotides and/or
short stretches of dsDNA led to the stabilization of double ring
structures, whereas untreated protein tends to form single hex-
americ or heptameric rings. More specifically, the presence of
nucleotide analogues triggers the formation of double heptam-
ers, whereas a consistent shift from a double heptameric to a
double hexameric arrangement was shown to be associated
with DNA binding, which suggests that the double hexamer is
an active form of the protein, with the heptameric complex
possibly a configuration ready to load onto DNA (28, 29).

The crystal structure of the N-terminal MthMCM domain
shows that the head-to-head interface between the two rings
involves the bottom face of subdomain B, containing a zinc
domain of the CXXCX_nCXXC (C_n) type. This motif is present in
other archaeal MCMs and in a modified form (H-C_n) in S. solfa-
taricus and Aeropyrum pernix MCMs (22). A C_n zinc motif is
also conserved in eukaryotic MCMs apart from MCM3, which
has an unconventional motif probably also capable of binding
zinc with the sequence (C/A)XXTAX_n(S/T)XX_D. The conserva-
tion of this motif suggests that it may play an important role in
the function of these proteins.

Close examination of the crystal structure has allowed us to
identify two arginine residues located at the hexamer–hexamer
interface as potential sources of hydrogen bonding between the
rings (Arg-137 and Arg-160, see Fig. 1A). The Arg-160 residue
has been previously characterized as producing a single ring
complex consistent with a hexamer (described as Arg-161 in
Ref. 30). The R160A mutant protein showed wild-type (WT)
ATPase activity in the absence of DNA and WT ssDNA and
dsDNA binding in the absence of ATP (30). We have used site-
directed mutagenesis to produce a R137A mutant and a
R137A,R160A (RRAA) double mutant and characterized the
resultant proteins. Electron microscopy and biophysical analy-
sis are consistent with the single R137A mutant forming a dou-
ble heptamer and the RRAA mutant forming a single heptamer.
Three-dimensional reconstructions showed that both mutants
are characterized by a large conformational change that is con-
sistent with a swing-out movement of subdomain A within the
N-terminal domain. The R137A mutant shows reduced bind-
ing to short DNA substrates, but this defect does not signifi-
cantly affect the ability of the mutant protein to function as a
processive DNA helicase. In contrast, the RRAA double mutant
shows a severely compromised ability to bind short linear DNA
substrates and a reduction in processive DNA unwinding when
compared with WT (despite WT ATPase activity in the pres-
ence and absence of DNA).

Our results show that mutation of Arg-137 appears to reduce
the transient interaction of the complex with DNA, causes a
swing-out movement of subdomain A, and potentially inhibits

5 N. Atassanova, personal communication.
plates were incubated with increasing amounts of protein (60–960 nM monomer) and processed as described previously (19).

Strand Displacement—Helicase template was made using complementary oligonucleotides with a 24-nucleotide overhang. PM2 (5’-GGA CAT GCT GTC TAG AGA CTA TCG-3’) was labeled using T4 polynucleotide kinase. Unincorporated [γ-32P] ATP was removed by a G25 spin column (GE Biosciences). The labeled oligonucleotide was annealed to RGL16 (5’-ATC GAT AGT CTC TAG ACA GCA TGT CCT AGC AAG CCA GAA TTC GGC AGC GT-3’) using a Molecular Imager FX. Displaced products were visualized using a Molecular Imager FX. 6 nM substrate was incubated with 60–240 nM increasing long dsDNA regions was made as described previously (19). 6 nM substrate was incubated with 60–240 nM increasing long dsDNA regions was made as described previously (19). 6 nM substrate was incubated with 60–240 nM increasing long dsDNA regions was made as described previously (19). 6 nM substrate was incubated with 60–240 nM increasing long dsDNA regions was made as described previously (19). 6 nM substrate was incubated with 60–240 nM increasing long dsDNA regions was made as described previously (19).

Processivity—A population of closed circular molecules with increasingly long dsDNA regions was made as described previously (19). 6 nM substrate was incubated with 60–240 nM (dodecamer) MCM protein in 40-μl reactions containing 1X HDB, 7 mM MgCl2, and 4 mM ATP for 30 min at 50 °C. Samples were processed (32), normalized, and separated on a 5% polyacrylamide gel supplemented with 8M urea. The gel was dried, and the gel was dried, and dispersed plates were visualized using a Molecular Imager FX (Bio-Rad).

Electron Microscopy—A 5-μl sample was applied to continuous carbon-coated copper grids (AGAR Scientific) and treated with 2% uranyl acetate. Data for the WT MthMCM protein were collected on a Tecnai 12 microscope (FEI Co.), whereas for the RRAA and R137A mutants, data were collected on a Philips CM200 FEG electron microscope, at a nominal magnification of 125,000. Micrographs were scanned using a Nikon Super Coolscan 8000 at a pixel size of 1.3 Å on the specimen scale, and image processing was carried out using the IMAGIC-5 package (33). Data sets for the various proteins were generated from digitized micrographs, according to the shape and dimensions of the protein complexes; a set of ring-shaped particles per class (R137A mutant) or 10 particles per class (RRAA mutant). Further MSA mirror symmetry analysis was performed on double ring side view particles aligned to a low passed filtered characteristic side view (35). Single particle reconstruction of the R137A mutant was performed using 1560 selected double ring side and tilted views, whereas ring-shaped top views were ignored. 7-fold symmetry was imposed, based on the evidence derived from MSA mirror symmetry analysis. The resolution achieved was 30 Å, according to the Fourier shell correlation (one-half bit criterion). Single particle reconstitution for the RRAA mutant was carried out by using 1600 particles and by applying 7-fold symmetry based on the result of MSA symmetry analysis. The structure was refined to a resolution of 22 Å, according to the one-half bit criterion.

RESULTS

Identification of Residues Putatively Required for Ring Dimerization—By analyzing the crystal structure of the dodecameric MthMCM N-terminal domain (15) (Protein Data Bank reference: 1LTL), we identified arginine residues (Arg-137 and Arg-160) on either side of the zinc finger motif with the potential to form hydrogen bonds across the ring-ring interface (Fig. 1A). Alignment of this region in archaeal and eukaryotic MCMs showed that neither of these residues is conserved. Most eukaryotic MCM3 orthologues possess an arginine (or lysine)
corresponding to Arg-137, and most MCM6 proteins possess an arginine (or lysine) residue corresponding to Arg-160 (Fig. 1B). To determine whether Arg-137 plays an additional role in MthMCM double ring formation, we generated a R137A mutant and a R137A,R160A double mutant (referred to as RRAA) for further characterization.

Biophysical Characterization of the RRAA Mutant Is Consistent with Formation of Heptamer Complexes—Biophysical analysis to determine the size of the RRAA complex was carried out (Fig. 2). Size exclusion chromatography detected a complex smaller than the WT but larger than a single hexamer (Fig. 2A). Sucrose gradient sizing was consistent with WT protein forming double rings and the RRAA mutant forming smaller complexes (Fig. 2B and C). Calculations combining gel filtration and sucrose gradient data (36) resulted in an apparent molecular mass of 898 kDa for the WT complex (equivalent to 11.6 monomers) and a molecular mass of 592 kDa for the RRAA complex (equivalent to 7.6 monomers). Sedimentation equilibrium analytical ultracentrifugation analysis provided a WT molecular mass of 948 kDa (or 12.2 subunits/complex) (Fig. 2D). A similar analysis of the RRAA complex provided a size estimate of 665 kDa (~8.6 subunits), with some evidence of complex heterogeneity (Fig. 2E). Neither of the samples showed a detectable change in apparent molecular weight associated with protein concentration, suggesting little in the way of dynamic equilibrium between different oligomeric states of significantly different sizes. Sedimentation velocity ultracentrifugation studies were also consistent with the WT protein forming a double ring complex (Fig. 2F), although a small peak corresponding to heptamers was also detected. The RRAA data were consistent with a heterogeneous mix of predominantly heptameric complexes (Fig. 2G).

Electron Microscopy of the RRAA Protein Confirms the Formation of Single Heptameric Rings—Samples of WT, R137A, and RRAA protein were visualized using negative stain electron microscopy (supplemental Fig. 1A). The samples showed a heterogeneous population of molecules that fell into two main cat-
MCM Mutations Affecting DNA Binding and Complex Conformation

FIGURE 3. Three-dimensional reconstructions of the R137A and RRAA mutants show large conformational changes involving the N-terminal (N-term) domain. Three-dimensional models for WT double hexamer (left panel (28)), the R137A (middle panel), and RRAA (right panel) mutants are compared. Red and green boxes highlight the location of the AAA+ and N-terminal domains, respectively. A round yellow circle indicates the position of subdomain A (SubA).

FIGURE 4. Characterization of DNA binding activities of Arg-137 and Arg-160. Protein, indicated in nM (monomer), was incubated at room temperature in the presence of 32P-labeled 30-mer ssDNA or dsDNA before being subjected to agarose gel electrophoresis. Protein-bound DNA was quantified following autoradiography as a proportion of the total labeled substrate. All data points are the average of at least three independent experiments. Error bars indicate standard deviation from the mean. Closed circles, continuous line, WT; open diamonds, continuous line, R137A; open circles, broken line, RRAA. A, ssDNA substrate in the absence of ATP; B, ssDNA substrate in the presence of 4 mM ATP; C, dsDNA substrate in the absence of ATP; D, dsDNA substrate in the presence of 4 mM ATP.

egories: particles consistent with ring-shaped molecules and rod-like aggregates with helical characteristics.

The WT protein consisted mostly of single rings. MSA symmetry analysis shows a strong heptameric component (supplemental Fig. 1B). However, when top view class averages are examined, a significant proportion of hexameric classes can be detected. Although the indication derived from MSA symmetry analysis is unbiased and statistically more significant, this probably suggests the presence of a degree of polymorphism within the sample. When 2438 “end-on” ring-shaped particles of the RRAA protein were selected and treated with MSA, the resulting eigenimages showed a strong 7-fold symmetry component (supplemental Fig. 1B), whereas class averages showing heptameric single rings were obtained (supplemental Fig. 1C). In contrast, electron microscopy of the R137A mutant was consistent with this protein still readily forming double rings. Although the absence of end-on views hindered a canonical MSA symmetry analysis approach, the presence of anti-mirror symmetric eigenimages deriving from aligned double ring side view particles (35) suggests that this mutant forms double heptamers (supplemental Fig. 1C).

Both Mutant Proteins Reveal an Unusual Conformation of the N-terminal Domain—A three-dimensional reconstruction was carried out for the RRAA and R137A mutant proteins, showing heptameric single and double ring complexes, respectively (Fig. 3). When this model is compared with the heptameric double ring wild-type reconstruction previously obtained in the presence of an ATP transition state analogue (28), a striking conformational change can be observed, with large movements involving subdomain A within the N-terminal domain (Fig. 3). The movement is reminiscent of the swing-out movement of subdomain A that has been previously inferred based on biochemical and crystallography data (15, 30) and visualized in EM reconstruction of the N-terminal domain alone (27) or the full-length protein bound to large segments of dsDNA (18).

A DNA Binding Phenotype Is Associated with R137A—The MthMCM R160A mutant has previously been reported to display WT DNA binding activity (30). We compared the DNA binding activities of WT, R137A, and RRAA proteins using short linear ssDNA and dsDNA substrates in the presence and absence of hydrolyzable ATP. In the absence of ATP, both mutants were indistinguishable from each other, showing a substantial decrease in their ability to bind ssDNA (Fig. 4A) and dsDNA (Fig. 4C) under these conditions. In the presence of ATP, a more complicated phenotype was observed; the RRAA mutant showed greatly decreased binding of ssDNA when compared with WT (Fig. 4B), with the R137A mutation alone showing an intermediate effect. In the presence of ATP, the R137A mutant appeared WT in its ability to bind dsDNA (Fig. 4D). In contrast, the RRAA mutant showed essentially WT ATP hydrolysis activity in the presence and absence of DNA (supplemental Fig. 2).

The R137A and R160A Mutations Have a Cumulative Effect on DNA Helicase Activity—WT MthMCM shows 3'-5' processive helicase activity (19–21), with linear duplexes requiring a 3’ tail for efficient activity (37). We used a standard strand displacement assay to determine the effect of the mutations on DNA helicase activity (Fig. 5). 30 nM (monomer) WT protein resulted in maximal unwinding of 1 nM forked substrate (Fig. 5, A and D). Increasing WT protein concentrations above 60 nM resulted in a slight inhibition of duplex unwinding activity. Protein concentrations above 300 nM had a more dramatic inhibitory effect (not shown). The R137A mutant showed similar levels of unwinding to WT, but this required 60–120 nM protein (Fig. 5, C and D). Similar amounts of the RRAA protein as the R137A protein were required for peak activity, but in this case, only ~50% of the maximal levels of substrate strand displacement activity were observed (Fig. 5, B and D). We investigated this defect in DNA unwinding further by
employing a processivity assay (Fig. 6). In this assay, the R137A protein performed as well as the WT protein, unwinding duplexes of up to \( \sim 350 \) bp when present in large excess over substrate (Fig. 6, compare lanes 12–14 with lanes 6–8). In contrast, the RRAA mutant showed poorer processive helicase activity, unwinding only \( \sim 150 \) bp under the same conditions (Fig. 6, lanes 9–11).

DISCUSSION

A number of electron microscopy studies on MthMCM have highlighted a remarkable degree of polymorphism, raising the question of the physiological significance of the multiple conformations observed in vitro and their role in the mechanism of...
MCM Mutations Affecting DNA Binding and Complex Conformation

helicase activity. One possible way to address this question is to study the biochemical properties of specific mutants that trap, stabilize, or hinder a particular stoichiometry.

A range of Mth/MCM complexes have been described, ranging from double hexamers and heptamers (24, 28, 29) to single hexamers (25, 30) and heptamers (26). The WT Mth/MCM protein forms a labile double ring in solution, which is not stable enough to be easily visualized by electron microscopy. A recent study suggests that the likely active conformation of the Mth/MCM helicase, at temperature and ionic strengths comparable with those existing in Methanothermobacter thermoautotrophicus cells, is a single ring (38). Consistent with this information, the R160A mutation hinders the formation of double ring complexes but does not interfere with helicase activity.

In a parallel study, we identified both Arg-160 and Arg-137 as likely to have a key role in the formation of a double ring and generated two mutants designed to disrupt the double ring interface, namely the single mutant R137A and the double mutant R137A,R160A (RRAA, Fig. 1). In addition to carrying out electron microscopy studies, we complemented our investigation with analytical ultracentrifugation and obtained a good agreement between the two techniques. Contrary to our expectations, the single mutant R137A was still able to form stable double ring complexes. EM studies showed the presence of a double heptamer, with a pronounced conformational change involving a swing-out movement of subdomain A (supplemental Fig. 1 and Fig. 3). A biochemical analysis of the mutant showed a decreased ability to bind single- and double-stranded DNA and a mild defect in DNA helicase activity (Figs. 4 and 5).

The double mutant RRAA visualized by electron microscopy appears as a single heptamer, with a similar conformational change in subdomain A (supplemental Fig. 1 and Fig. 3). Our sizing data are consistent with the RRAA protein forming a complex that is a single ring in solution, although this protein exhibits some heterogeneity. Hydrodynamic studies suggest that the RRAA mutant is slightly larger than the heptameric conformation observed by EM. This difference can be explained by the large conformational change of subdomain A. A similar effect on the apparent size of the protein has been described for the P61L mutant (equivalent to the P83L bob1 mutant in MCM5) and has been correlated to a swing out movement of subdomain A (30).

A comparison between the behavior of the R160A and the R137A single mutants with the RRAA double mutant allows one to decouple the effects of the mutations. Mutation of Arg-160 affects the stoichiometry of the complex without influencing its DNA binding or helicase activity (38). Introduction of a second mutation into the single ring (R137A) results in substantially reduced DNA binding (Fig. 4) and significantly reduced DNA helicase activity (Figs. 5 and 6) despite this complex showing WT DNA-dependent ATP hydrolysis (supplemental Fig. 2). In parallel, the Arg-137 mutation causes a large conformational change involving the position of subdomain A (Fig. 3). The same conformational change has been proposed to trigger a critical step in the activation of MCM helicase activity (15, 39) and has been related to the initial mode of association of MCM with dsDNA before loading occurs (18). This mode of binding involves dsDNA wrapping around the N-terminal domain ring, sandwiched between the swung-out subdomain A and subdomain B, and our modeling, based on EM results, places the DNA very close to the Arg-137 residue, providing a possible explanation for the reduced DNA binding ability of the R137A mutant.

We suggest that although the WT protein may contain a mixture of heptamers and hexamers, easily interconverted in the absence of substrates, the R137A mutation stabilizes the heptameric form, “trapping” what is normally a more labile, transient intermediate and therefore interfering with the conformational changes that are required for the activation of the helicase activity. The R137A structure shows a clear asymmetry between the two rings, with a more evident swing-out of subdomain A in the upper ring, associated with the formation of a cap (Fig. 3). This is reminiscent of the asymmetry observed in the presence of dsDNA, where the emergence of the cap was interpreted as an ordering of the C-terminal domains (28). Both the asymmetry and the conformational change at the AAA+/C-terminal end of the protein, upon mutation of a residue at the very bottom of the N-terminal domain, are further evidence of the long range inter- and intramolecular cross-talk between the various domains.

Although neither the Arg-137 nor the Arg-160 residues are conserved throughout the MCMs, these residues can potentially reveal the molecular mechanisms that govern loading of the MCM complex onto DNA in both archaea and eukaryotes. The loading process may involve a series of intermediate configurations, including a heptameric complex with a swing-out subdomain A, ready for the initial association with dsDNA (as visualized in the R137A mutant), to a hexameric complex where duplex DNA initially associates with the outside N-terminal face of the MCM making interactions with the Arg-137 side chain (as observed in Ref. 18), to an active hexameric or double hexameric MCM encircling the DNA as a processive helicase. Although Arg-137 is not conserved in archaea, an arginine or lysine is present in this position in almost all MCM3 subunits in eukaryotes. Thus, this mutant may act as a paradigm for an intermediate in the association of the MCM complex with DNA.

Acknowledgments—We thank Dr. Z. Kelman for sharing data prior to publication, members of the Chong laboratory for comments on the manuscript, M. van Heel for use of the EM Facility, J. van den Elsen for help with molecular visualizations, M. Polonska and J. Cartwright (University of York Technology Facility) for assistance with large scale E. coli cultures and protein purification, and E. Bolt for providing helicase template oligonucleotides.

REFERENCES

1. Blow, J. I., and Dutta, A. (2005) Nat. Rev. Mol. Cell. Biol. 6, 476–486
2. Donovan, S., Harwood, J., Drury, L. S., and Diffley, J. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5611–5616
3. Dutta, A., and Bell, S. P. (1997) Annu. Rev. Cell Dev. Biol. 13, 293–332
4. Lisziewicz, J., Godany, A., Agoston, D. V., and Kuntzel, H. (1988) Nucleic Acids Res. 16, 11507–11520
5. Stillman, B. (1996) Science 274, 1659–1664
6. Ishimi, Y. (1997) J. Biol. Chem. 272, 24508–24513
7. Moyer, S. E., Lewis, P. W., and Botchan, M. R. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 10236–10241
