Biochemical Activities of the *BOB1* Mutant in *Methanobacterium thermoautotrophicum* MCM

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ABSTRACT: Minichromosomal maintenance proteins (MCMs) are considered to be the replicative helicase. *Methanobacterium thermoautotrophicum* has a single MCM gene (mtMCM). The crystal structure of the mtMCM N-terminal region is a double hexamer. Structure-guided sequence alignment indicates a structural conservation of this fragment across archaeal and eukaryotic MCMs. The mtMCM structure was successfully used to analyze a *Saccharomyces cerevisiae* MCM5 mutant, called *BOB1*, which contains a single residue change from Pro to Leu and bypasses a kinase normally required for initiation of DNA replication. A domain-push model was proposed to explain the *BOB1* bypass activity. Here we investigate the effects of *BOB1* mutation on the biochemical activities of mtMCM. Surprisingly, the *BOB1* mutation (P62L) had a major effect on the helicase activity but had no significant impact on DNA binding and ATPase activities. These results will contribute to a more detailed understanding of the *BOB1* bypass activity and other aspects of DNA replication control.

Genome duplication in eukaryotic cells is tightly controlled so that each chromosome is replicated exactly once per cell growth cycle. One family of proteins essential for both initiation and completion of replication is the minichromosomal maintenance (MCM)[1] proteins (reviewed in ref *1*−*3*). The MCM proteins belong to the AAA+ ATPase family, a group of proteins associated with cellular processes that are often driven by the energy from ATP binding and hydrolysis (*4*). All MCM proteins contain a region of ∼200 amino acids referred to as the MCM box. Within the MCM box are amino acids responsible for the binding and hydrolysis of ATP (*5*, *6*).

Eukaryotic cells contain a family of six homologous proteins, MCM2−7, that form a hexameric ring structure (*7*). Archaeal cells also have MCM genes that share significant sequence homology with the eukaryotic MCM proteins. One archaeal organism, *Methanobacterium thermoautotrophicum*, has a single MCM gene, and the gene product forms homohexamers that assemble into a double hexamer structure (*8*, *9*). A purified MCM protein complex containing MCMs 4, 6, and 7 from eukaryotic cells has weak helicase activity (*7*, *10*, *11*), whereas the MCM complex from *M. thermoautotrophicum* (mtMCM) has strong helicase activity in vitro (*9*, *12*, *13*). The MCM complex is thought to serve as the helicase necessary for DNA replication and subsequent cell growth (*14*−*18*).

Structural studies revealed that the N-terminal half of mtMCM (N-mtMCM) forms a double hexamer (dHex) (*8*). Each monomer folds into three domains, A, B, and C (Figure 1B). The α-helical domain A is located on the outer surface of the dHex ring (Figure 1A), and the wall is composed of domains B and C. Domain C contacts both domains A and B and thus holds the three domains together (Figure 1B). The interaction between domains C and A is mediated through a focal contact point centered at a Pro residue (P62), suggesting that the interaction anchoring domain A to the core structure of domain C is relatively weak (*8*). Consistent with this assumption, an electron microscopy (EM) study revealed that domain A can adopt different positions relative to domains B and C (*19*).

Structure-guided sequence alignment indicates that this three-domain structure and organization of N-mtMCM are conserved for all known MCMs from eukaryotic cells (*8*). This structural information was successfully used to analyze a *Saccharomyces cerevisiae* MCM5 mutant, called *BOB1*, that contains a single amino acid change from Pro 83 to Leu. This mutant bypasses Dbf4-dependent Cdc7 kinase (DDK), a kinase required for initiation of DNA replication during cell growth (*8*, *20*, *21*). The *BOB1* Pro residue is located within domain A and is conserved among archaeal and all eukaryotic MCM5 orthologues (*8*). The crystal structure of an mtMCM mutant corresponding to the *BOB1* mutation revealed that domain A is pushed away from the core structure by the longer side chain of a substituted leucine residue (Figure 1C) and should be more mobile than that of the native conformation (Figure 1D). The weakened interaction between domains A and C may allow domain extension and rotation and might also be achieved through mutation of the *BOB1* Pro to other residues with large side chains.

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[1] Abbreviations: AAA+, ATPase associated with a variety of cellular activities plus; MCM, minichromosomal maintenance protein; DDK, Dbf4-dependent kinase.

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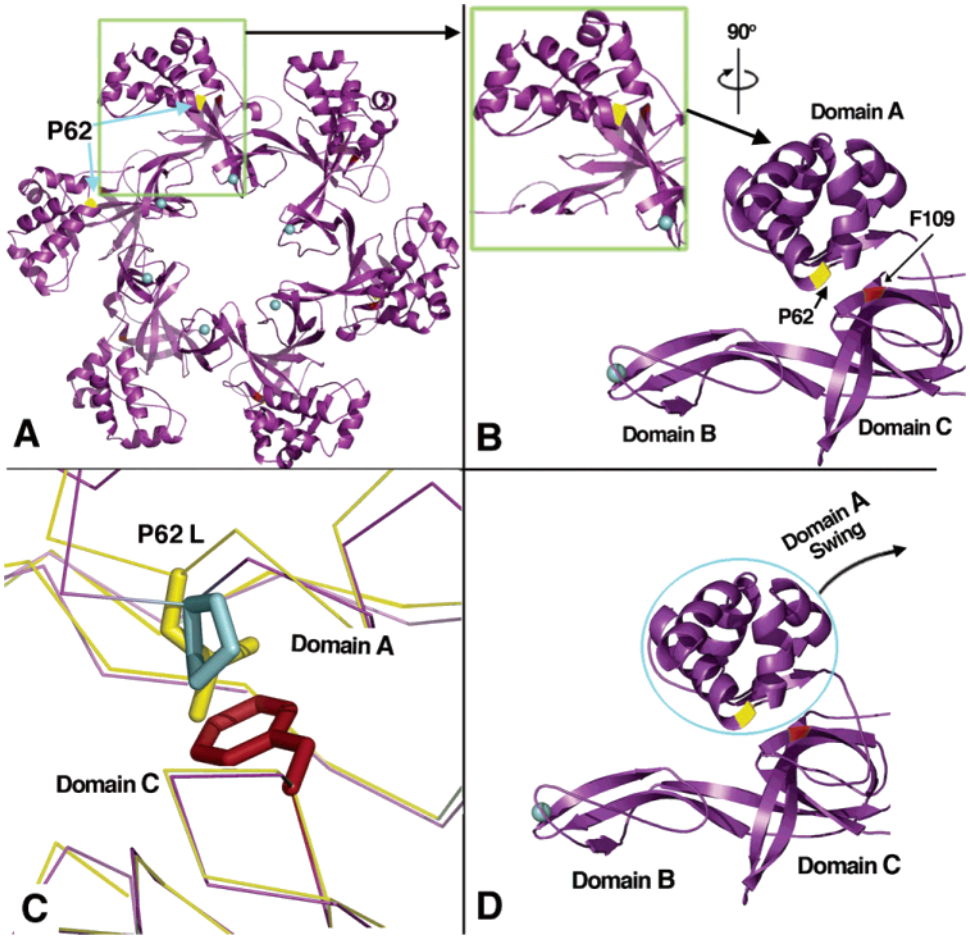

FIGURE 1: P62L structure and location of mutation. (A) The hexamer structure of N-mtMCM, showing the all-β-stranded central channel wall and the α-helical domain A on the outside. Arrows in cyan point to the location of *BOB1* residue P62 (highlighted by yellow dots) on domain A of two of the six monomers. (B) Monomer structure of N-mtMCM (purple) showing domain A (α-helical bundle) anchored to domain C (β-barrel) through P62 (in yellow). (C) Overlay of the native N-mtMCM structure (purple) with the P62L N-mtMCM structure (yellow). Native Pro (cyan) or mutant Leu (yellow) point toward domain C where a phenylalanine (F109, red) is located. Domain A of the P62L mutant is pushed away from domain C by the longer Leu side chain, weakening the docking interaction of domain A on domain C. (D) The *BOB1* P62L N-mtMCM structure of a monomer (purple), with the possible domain swing of domain A in the direction indicated by the arrow.

Significantly, in vivo mutation of the MCM5 *BOB1* Pro position to various residues supported the domain-push model (*8*), proving the usefulness of the archaeal structural model in studying eukaryotic MCM.

As reported here, we have investigated the biochemical properties and biological functions of the *BOB1* mutation in mtMCM. Our results provide insight into the mechanisms by which the *BOB1* mutation at the evolutionarily conserved Pro residue affects the structure and function of the mtMCM complex.

MATERIALS AND METHODS

Cloning, Mutation, and Protein Purification. The gene for mtMCM (2−666) was cloned into the pGEX-2T vector as a GST fusion. This wt clone was used for making the P62L mutant. All clones were sequenced to ensure the correct sequence. The wt and P62L mutant proteins were expressed and purified from *Escherichia coli* as described in ref *8*.

ATPase Assay. Ten microliter reactions containing 50 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris (pH 7.8), 1 mM DTT, 0.1 mg/mL BSA, 15 nM [α-^{32}P]ATP (Amersham, 3000 Ci/mmol), plus 15 μM cold ATP, and various amounts of protein to be tested were assembled on ice. Reactions were incubated at 50 °C for 30 min and then stopped by addition of 10 mM EDTA. Five microliters from each reaction was placed onto 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 and autoradiographed.

DNA Binding Assays. The DNA sequence used for ssDNA binding was 5′-AAAGCG CTGACC TATCGC GTATAG CTCGAGGA, and the dsDNA sequences were 5′-GCGCTG ACCTAT CGACCT ATACGG TTAGCC and 5′-GGCTAA CCGTAT AGGTCG ATAGGT CAGCGC. The ssDNA and dsDNA were labeled by [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol). For the DNA binding assay, 20 μL reaction mixtures containing 50 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris (pH 7.8), 1 mM DTT, 0.1 mg/mL BSA, 15 nM labeled DNA, and amounts of protein to be tested were assembled on ice. The reaction mixtures were then incubated at 25 °C for 30 min, followed by addition of 5 μL of stop buffer (50% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue). Reaction mixtures were electrophoresed on 2% agarose gel for 1 h at 80 V in 1× TBE buffer. The gel was dried and autoradiographed for quantification.

Helicase Assay. A 60 nucleotide sequence 5′-TTTTTT TTTTTT TTTTTT TTTTTT CGCGCG GGGAGA GGCG-

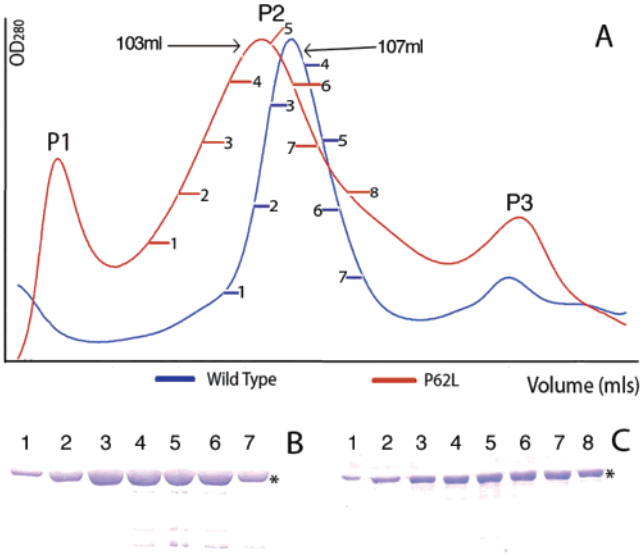

FIGURE 2: Gel filtration chromatography of wt and P62L proteins. (A) Superose 6 gel filtration profile of wt (blue) and P62L mtMCM (red) graphed as absorbance at 280 nm (OD_{280}) vs elution volume. Peak P1 is the void volume, peak P2 contains the hexamers, and peak P3 is the degradation contaminants. (B, C) SDS−PAGE analysis of the fractions of peak P2 of the wt (B) and P62L mutant (C) proteins. Lane numbers correspond to the fraction numbers labeled in the chromatography profile in panel A. The asterisk marks the target protein.

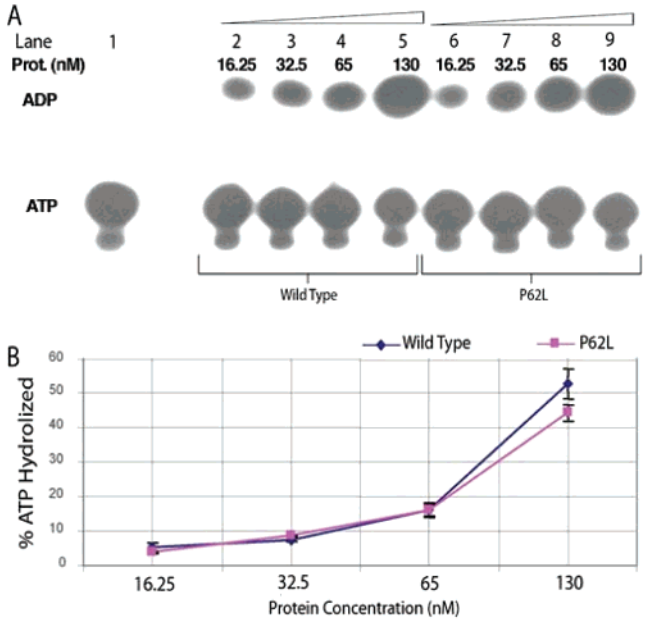

FIGURE 3: ATPase assay. (A) Thin-layer chromatography (TLC) visualization of ATPase assay. Control without protein is in lane 1. (B) Quantitation of ATPase assay. Protein concentration was calculated as a dHex, and error bars represent the average deviation for all experiments at the same concentration.

GT TTGCGT ATTGGG CGCC with 34 complementary bases and a 26 dT overhang was annealed to M13mp18 circular ssDNA. The annealed dsDNA was used as the substrate for the helicase assay. The assay contained 20 mM Tris (pH 7.8), 10 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, 0.1 mg/mL BSA, 0.25 nM dsDNA substrate, and amounts of protein to be tested. The assay mixture was incubated at 50 °C for 30 min, followed by addition of 5 μL of 5× stop solution (10 mM EDTA, 0.5% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue, 50% glycerol). The samples were analyzed on a 12% polyacrylamide gel that was electrophoresed for 50 min at 150 V in 1× TBE on ice. The gel was dried and autoradiographed for quantification.

RESULTS

Protein Expression and Purification. Wild-type (wt) and P62L mutant mtMCM proteins were purified to near homogeneity as described in the Materials and Methods section. Both proteins were analyzed using gel filtration. Compared with the wt, there was a shift of the major P2 peak in the P62L mutant elution profile (Figure 2A). The P2 peak of the wt protein eluted at a volume of 107 mL, corresponding to a molecular mass (MW) of approximately 960 kDa, consistent with dHex formation. Interestingly, the P2 peak of P62L eluted 4 mL earlier, at a volume of 103 mL. Because the crystal structure of this P62L mutant indicated a dHex structure (*8*), the mutant protein in the P2 peak is likely in the dHex oligomeric state. Another noteworthy change in the elution profile for P62L was that the P2 peak was much broader than that of wt (Figure 2A). The broadening of the P2 peak may be due to the flexibility of domain A on the outer surface of the dHex structure (Figure 1A,D). Proteins from the P2 peaks of both wt and P62L were analyzed by SDS−PAGE gel and Coomassie Blue staining (Figure 2B,C). Both analyses revealed a high purity for both proteins with the expected apparent MW on SDS−PAGE. The pure protein from the dHex peak (P2) for P62L and wt were used for further biochemical experiments.

ATPase Activity. ATPase assays were used to compare the ATPase activity of the wt and P62L mutant proteins (Figure 3A). Quantitation of multiple assays showed no significant difference in ATPase activity between wt and P62L (Figure 3B).

Single-Stranded DNA (ssDNA) Binding. The ssDNA binding assays compared the ssDNA binding activity of the wt and P62L mutant proteins (Figure 4A). Quantitation of the experiments revealed a slight, but consistent difference between the two proteins. The ssDNA binding activity of P62L was approximately 30% lower than that of the wt at different protein concentrations (Figure 4B).

Double-Stranded DNA (dsDNA) Binding. The wt and P62L mutant proteins were tested for the dsDNA binding (Figure 5A). Quantitation of binding experiments revealed a significant decrease (approximately 80%) in the P62L ability to bind dsDNA than the wt protein (Figure 5B).

Helicase Activity. Helicase assays were performed at 50 °C at multiple protein concentrations (Figure 6A). The P62L mutant was severely deficient in helicase activity. While the wt protein showed helicase activity at a protein concentration of 0.25 nM (Figure 6B), the P62L mutant showed no detectable helicase activity below a protein concentration of 4 nM (Figure 6B), 16 times higher than the concentration required for wt to display measurable activity. At 4 nM protein concentration, the helicase activity of wt was approximately 17 times higher than that of P62L.

DISCUSSION

We have characterized the biochemical properties and in vitro functions of an mtMCM protein with Pro62 mutated to Leu in an attempt to understand the effect of the *BOB1*

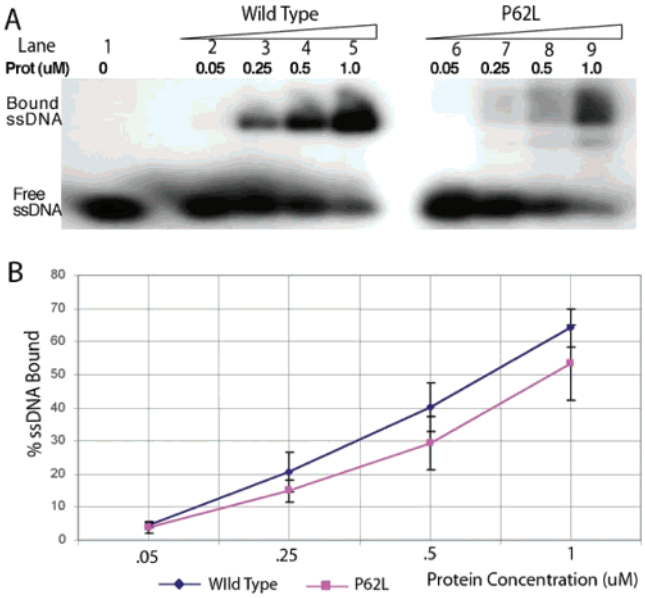

FIGURE 4: ssDNA binding assay. (A) Native agarose gel shift assay of ssDNA binding. Radiolabeled DNA was incubated with each of four different concentrations of wt (lanes 2−5) or P62L (lanes 6−9) protein and then analyzed using 2% agarose gel electrophoresis. Control without protein is in lane 1. (B) Quantitation of ssDNA binding. Protein concentration was calculated as dHex, and error bars represent the average deviation for all experiments at the indicated concentration.

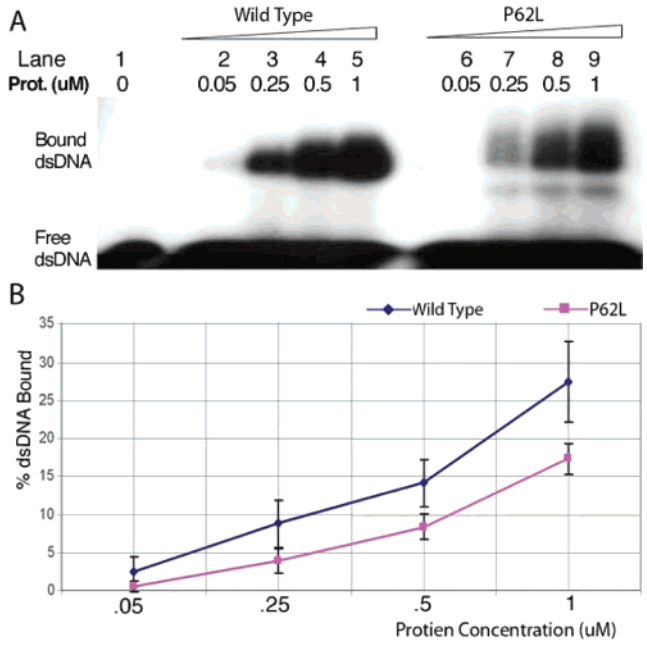

FIGURE 5: dsDNA binding assay. (A) Native agarose gel shift assay of dsDNA binding. Radiolabeled dsDNA was incubated with each of four different concentrations of wt (lanes 2−5) or P62L (lanes 6−9) protein and then analyzed on a 2% agarose gel. The ability of protein to bind dsDNA was measured by the shift/appearance of dsDNA to a higher molecular weight band. Control without protein is in lane 1. (B) Quantitation of dsDNA binding. Protein concentration was calculated as dHex, and error bars represent the average deviation for all experiments at the indicated concentration.

mutation on mtMCM structure and function. On the basis of the crystal structures for both the wt and P62L mutant of N-mtMCM, a domain-push mechanism was proposed to explain how the *BOB1* mutant of MCM5 in *S. cerevisiae* bypasses DDK function to allow cell growth (*8*). In this

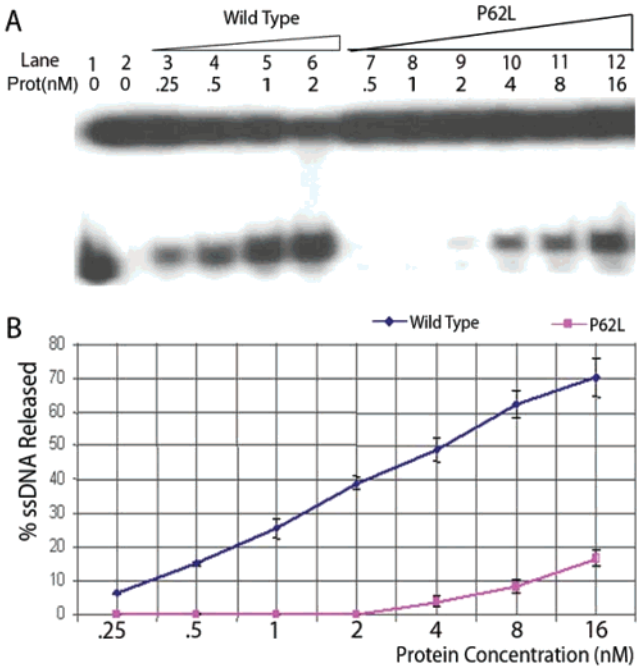

FIGURE 6: Helicase assay. (A) Gel analysis of helicase assays of the wt and P62L mutant. Boiled sample and no protein control are in lanes 1 and 2, respectively. (B) Quantitation of helicase assay. P61L (purple) had severely reduced activity when compared to wt (blue). Protein concentration was calculated as dHex, and error bars represent the average deviation for all of the experiments at the indicated concentration.

domain-push hypothesis, domain A of the *BOB1* mutant is pushed away from the core structure, destabilizing the docking interaction with the core and allowing domain A more flexibility, and leading to the eventual activation of MCM and DNA replication for cell growth (*8*, *20*). This domain-push mechanism is also supported by a genetic study in *S. cerevisiae*, in which replacement of the Pro with large residues (Leu, Lys, Trp) reproduced the *BOB1* phenotype (*8*).

In this report, we show further evidence supporting a flexible domain A in the mtMCM *BOB1* mutant. The gel filtration profile of the P62L mutant showed a broadened dHex peak that eluted earlier than the wild-type protein. This result suggests, first, that the conformation of the mutant dHex was somewhat floppy and heterogeneous and, second, that the mutant dHex was larger than the wt complex. Because P62 is the focal point of the interaction between domains A and C (Figure 1B), weakening this interaction by mutating P62 to a longer Leu residue will lead to a more flexible domain A. The increased flexibility apparently allows domain A to extend away from the core structure and assume multiple conformations (Figure 1D). This flexible domain A will result in a dHex with a larger apparent MW (advanced peak position) and more heterogeneous conformations (broadened peak) in gel filtration chromatography. Recent EM work also provided proof that domain A is not tightly bound to the core structure even in the wild-type protein (*19*), and thus, it is conceivable that the P62L mutation at the anchoring point may impact on the already weak docking interaction of domain A on domain C.

One intriguing question is whether the P62L *BOB1* mutation in mtMCM has a direct effect on the biological functions in vitro. As demonstrated in this report, the P62L

mutant hydrolyzed ATP with wt efficiency (Figure 3). This result was expected because the Pro residue is located within domain A on the N-terminus, significantly distal to the C-terminal ATPase domain. However, the P62L mutant displayed about a 30% decrease in ssDNA binding (Figure 4) and an approximately 80% decrease in dsDNA binding (Figure 5). As domain A is on the outer surface of the dHex structure away from the DNA binding channel, the decrease in DNA binding was unexpected. One plausible explanation is that there is a concomitant conformational change upon DNA binding in wt mtMCM, and this conformational change is somehow hindered by the P62L mutation.

An even more unexpected result was the substantial loss of helicase activity for the P62L mutant. There appears to be no straightforward explanation for this result. The moderate decrease in DNA binding alone should not account for the over 10-fold decrease in helicase activity of the mutant. Moreover, the N-mtMCM structure shows that domain A is not involved in the oligomerization of the double hexamer, nor is it near the DNA binding channel. Furthermore, EM analysis of the mtMCM structure indicates that the N-terminal domain A does not make direct contact with the C-terminal helicase domain (*22*, *23*). Instead, the C-terminal helicase domain forms a second ring adjacent to the N-mtMCM ring, similar to two stacked doughnuts (*24*).

However, there are a few possible explanations for the substantial loss of helicase activity of the P62L mutant. One plausible explanation is an allosteric effect of the *BOB1* mutation that impacts conformational changes in the distal helicase domain required for efficient DNA unwinding. Alternatively, the proposed flexibility of domain A (*8*, *19*) could allow it to swing toward and contact the helicase domain to affect helicase activity. Another possibility is that only a fraction of the total P62L protein is in an active conformation at any given time as a result of the domain A flexibility. Consistent with this last explanation is the broadened peak observed by gel filtration (Figure 2A) and the linear helicase activity plotted in Figure 6B.

Previous in vivo experiments with various mutations at the *BOB1* position of MCM5 in *S. cerevisiae* supported the domain-push hypothesis as a possible mechanism for activating MCM helicase activity in yeast (*8*). However, the loss of the helicase activity of the corresponding *BOB1* mtMCM mutant may not explain the ability of the mutant yeast MCM to bypass DDK dependence on cellular DNA replication for the following reasons. First, mtMCM is homooligomeric, but eukaryotic MCM is heterooligomeric. This means that the *BOB1* mutation in mtMCM is present in every monomer of the hexamer ring, but the *BOB1* mutation in a yeast MCM occurs only once (in MCM5) per hexameric ring. Second, it is not known if DDK affects MCM activity by directly regulating MCM5 or other MCM subunits or if DDK regulates MCM activity directly through physical contact or indirectly via other factor(s). However, it appears that MCM5, together with MCM2 and MCM3, might negatively regulate the helicase activity of MCM4, 6, and 7 (*25*−*27*). If so, it follows that the *BOB1* mutation on MCM5 could serve to relieve such inhibitory effect. Much work remains to be done to allow a full understanding of the mechanisms by which the *BOB1* mutation bypasses DDK function in yeast. Nonetheless, we believe that the data reported here for the corresponding *BOB1* mutation in mtMCM are relevant to understanding the *BOB1* bypass mechanism as well as the related events of the DNA replication control.

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