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Li Phing Liew, Stephen D Bell

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The interplay of DNA binding, ATP hydrolysis and helicase activities of the archaeal MCM helicase

Li Phing Liew and Stephen D. Bell

Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, UK

Correspondence to SDB
stephen.bell@path.ox.ac.uk:
Phone +44 1865 275564
Fax +44 1865 275515

Short title: Mechanochemisty of an archaeal MCM

Abstract

The MCM proteins of Archaea are widely believed to be the replicative DNA helicase of these organisms. Most archaea possess a single MCM ortholog that forms homo-multimeric assemblies with a single hexamer believed to be the active form. Here we characterize the roles of highly conserved residues in the ATPase domain of the MCM of the hyperthermophilic archaeon *Sulfolobus solfataricus*. Our results identify a potential conduit for communicating DNA binding information to the ATPase active site.

Introduction

The homomultimeric MCM proteins of archaea have been studied for a number of years. In particular, considerable effort has been put into analyses of the MCMs encoded by *Methanothermobacter thermautotrophicum* (MthMCM) and *Sulfolobus solfataricus* (SsoMCM) (For reviews see [1-4]). In agreement with the proposed role of archaeal MCMs as the replicative helicase, SsoMCM is enriched at replication origins at the time of replication initiation *in vivo* [5]. Furthermore, *in vitro* studies have revealed that the recombinant protein forms homohexamers and is capable of directing (d)ATP-dependent melting of partially duplex DNA substrates [6]. Ensemble and single-molecule FRET studies indicate that the enzyme loads onto 3' to 5' polarity single stranded DNA tails with the C-terminal proximal domains facing the duplex DNA [6, 7]. This loading results in single stranded DNA passing through a central pore in the homohexameric MCM ring where it is contacted by basic residues supplied by β-hairpin motifs that line the pore. One β-hairpin is supplied by the N-terminal domains of the protein and a second, the pre-sensor 1 β-hairpin (PS1BH), is found in the C-terminal AAA+ ATPase domain of the MCM [6, 8]. Interestingly, the N-terminal domains of MCM, although dispensable for helicase activity *in vitro*, do play significant roles in modulating substrate choice, enhancing processivity and facilitating inter-subunit communication [8, 9]. Furthermore, the N-terminal domains have been shown to interact with the archaeal GINS complex *in vivo* and *in vitro* [10]. The C-terminal PS1BH has been shown to be important for DNA binding and mutation of a single lysine residue at its tip results in a mutant protein with impaired DNA binding, ATPase activity that is no longer stimulated by DNA and complete loss of helicase activity [6]. In addition, studies of the MthMCM have implicated a second conserved element within the AAA+ domain of the MCM in DNA melting. Deletion of this module, the H2I, a β-α-β structure in the AAA+ fold, resulted in elevated DNA binding and DNA-stimulated ATPase activity but complete loss of helicase activity [11]. Recently a 4.35 Å resolution structure of a monomeric form of SsoMCM has been reported [12]. The PS1BH and H2I are adjacent to one another and modeling of a hexameric form of the protein predicts that they point in towards the central cavity of the hexameric ring. Importantly, evidence has been obtained that indicates that both the PS1BH and H2I undergo re-positioning during the ATP utilization cycle of the helicase [9, 11]. These observations are in agreement with structural studies of the distantly related Superfamily 3 helicases. More specifically, studies of the SV40 LTag and BPV E1 helicases have revealed that the analogous PS1BH hairpin in these helicases undergo movements of up to 17 Å during ATP binding, hydrolysis and release by the helicase, serving as the power stroke to drive the helicase along DNA [13-15]. In MCM, the

PS1BH of a given subunit is close to a conserved loop (the ACL) in the N-terminal domains of the adjacent protomer within the hexameric assembly. The ACL plays a key role in communicating information between protomers within the hexamer [9]. Thus, repositioning of the PS1BH during the reaction cycle may serve dual roles – to act as the power stroke and to propagate cooperative interactions between SsoMCM monomers.

Analyses of MCM with mutations in residues predicted to play roles in the ATPase activity of the protein, coupled with mutant doping studies, have revealed that MCM has a rather unusual form of limited cooperativity between protomers [16]. Furthermore, these studies revealed an unusual architecture for the AAA+ ATPase domain of the helicase. Typical AAA+ proteins function as multimers with the ATPase active site formed at the interface between protomers [17]. Thus, both subunits contribute residues to the active site. One subunit acts as a "*cis"* site and contributes sidechains such as the Walker A (K346 in SsoMCM), Walker B (D404) and Sensor 1 (N488) residues. However the neighbouring protomer also plays an active role and contributes "*trans*" acting residues such as the Arg finger (R473 in SsoMCM). ATP binding, hydrolysis and release can thus effect repositioning of adjacent subunits. In addition, intra-subunit motions are mediated by repositioning of the two principal domains of the AAA+ site, the lid and base domains. The Sensor 2 motif normally plays a key role in this intra-molecular repositioning event. Interestingly, however, MCM has a highly unusual configuration of the Sensor 2 residue (Arg 560 in SsoMCM). As initially predicted bioinformatically, and subsequently verified biochemically and structurally, an alpha helical insertion preceding the Sensor 2 motif in MCM results in Sensor 2 acting in *trans* rather than the usual *cis* configuration [12, 16, 17]. Additionally, complementation data suggested that MCM may possess a number of additional *trans*-acting residues, clustered around the base of the PS1BH [16]. Precedent for multiple *trans*-acting residues has been found in the distantly related viral Superfamily 3 helicases such as SV40 LTag and Bovine Papilloma Virus E1. However, whether the candidate *trans*-acting residues in MCM do indeed directly coordinate ATP, as in the SF3 helicases, or whether they have an indirect role via facilitating the positioning of *bona fide trans*-acting residues is unknown.

In the current work we address the roles of a cluster of highly conserved residues that occupy a pivotal position in SsoMCM, lying at the juxtaposition of the PS1BH and the post-Sensor 2 alpha helix. We identify R329 as a key residue in the communication between the DNA binding site of SsoMCM and the *trans* component of the ATPase active site.

Experimental

Site-directed mutagenesis and protein purification

Site-directed mutagenesis was performed using the single primer site-directed mutagenesis method (Stratagene). The primers used for mutagenesis are shown in Table 1. Proteins were purified as described previously [6]. Briefly, proteins were expressed in *E. coli* strain Rosetta (DE3). 1 L of transformants

were grown to A_{600nm}=0.6 before being induced with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) for 3 hours. Cells were harvested by centrifugation and resuspended in 25ml 1× TBS (10mM Tris-HCl pH 8.0, 150mM NaCl) containing 14mM β-Mercaptoethanol and one tablet of Complete EDTA-free Protease Inhibitor Cocktail (Roche). Cells were lysed using a French Press (Thermo Spectronic) followed by heat treatment at 75°C for 20 minutes and clarification by centrifugation. MCM proteins was first purified over a 5 ml HiTrap Heparin HP column (GE Healthcare) using an AKTA Prime system (Amersham Bioscience). After the column was pre-equilibrated with 1× TBS, sample was injected and unbound proteins were flushed with 5 column volumn (cv) of 1× TBS. With all the steps run at a flow rate of 2.5 ml/min, proteins were eluted with a 15 cv gradient from 150mM to 1M NaCl in 10mM Tris-HCl pH 8.0. 5 ml fractions were collected and analysed by SDS-PAGE and Coomassie staining. Protein-containing fractions were pooled and concentrated to ≤ 5 ml using a Vivaspin 20 of 30k MWCO PES (Sartorius Stedim Biotech). The concentrated proteins were then purified over a Hi-load 26-60 Superdex 200 prep grade column (Amersham Biosciences) using an AKTA Purifier system (GE Healthcare). The column was first equilibrated with 1× TBS before sample was injected. Proteins were eluted with 1 cv (≈ 350 ml) of 1× TBS and were collected after 100 ml of elution. 5 ml fractions were collected and analysed by SDS-PAGE and Coomassie staining. Protein-containing fractions were pooled and further purified over a Mono Q 5/50 GL column (GE Healthcare) using an AKTA Purifier system (GE Healthcare). The column was first equilibrated with 1× TBS, sample was then injected and unbound proteins were flushed with 2 cv of 1× TBS at a flow rate of 1 ml/min. To elute proteins, a 15 cv gradient from 150 mM to 1M NaCl in 10mM Tris-HCl pH 8.0 was applied. Samples were collected at 0.5 ml fraction and were analysed by SDS-PAGE and Coomassie staining. Protein-containing fractions were pooled and concentrated to ≤ 0.5 ml using a Vivaspin 2 of 30k MWCO PES (Sartorius Stedim Biotech).

Mutation	Primer sequence (5' to 3')
R329E	AAGGTTCTAGAAGATACGGAAATAAGAGGAGATATTCACA
R331I	CTAGAAGATACGAGAATAATAGGAGATATTCACATACTG
R332K	CTAGAAGATACGAGAATAAAAGGAGATATTCACATACTG
E422A	GCCATTCATGAGGCAATGGCACAACAGACAGTCTCAATA
E422R	GCCATTCATGAGGCAATGAGACAACAGACAGTCTCAATA
L565D	CCAAGACAATTAGAGGCTGATATAAGAATTTCAGAAGCC
L565K	CCAAGACAATTAGAGGCTAAAATAAGAATTTCAGAAGCC

Table 1: Site-directed mutagenesis primers used in this study.

EMSA

The Y-shaped DNA substrate was prepared by radio-labelling the oligonucleotide ($(T)_{44}$GCTCGTGCAGACGTCGAGGTGAGGACGAGCTCCTCGTGACCACG) with γ^{32}P-ATP. This radio-labelled molecule was then annealed to its complementary strand (CGTGGTCACGAGGAGCTCGTCCTCACCTCGACGTCTGCACGAGC$(T)_{44}$)

before the resultant partial duplex molecule probe was purified via recovery from a gel slice following electrophoresis on a 8% PAGE gel.
DNA-binding was performed by mixing 10nM labelled Y-shaped DNA substrate with the indicated concentration of protein in 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT pH 7.9 and 5% glycerol. Binding reactions were incubated at 50°C for 15 minutes prior to electrophoresis on a 6% polyacrylamide gel (17 cm × 17 cm × 1.5 mm) in 1× TBE at 10 V per cm. Gels were dried and analysed by phosphorimagery (Fujifilm FLA-5000). Quantitation was performed by using the Aida Image Analyzer software (Raytest Isotopenmeβgeräte GmbH, Germany).

ATPase assay
ATP hydrolysis was performed in a 20 µl reaction volume containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT pH 7.9, 1 mM ATP (Sigma), 25 nCi γ-^{32}P-ATP, and 1 µM of protein. Where DNA was added, the Y-shaped substrate described above was added to 1 µM. Reactions were incubated at 60°C for 30 minutes before termination by the addition of 1% SDS. Samples were then analyzed using thin-layer chromatography by spotting 2 µl of each sample on CEL 300 PEI TLC plates (Macherey-Nagel), which was subsequently developed with 0.8 M lithium chloride 1 M formic acid buffer. Analysis was performed by phosphorimagery (Fujifilm FLA-5000) and quantification using Aida Image Analyzer software (Raytest Isotopenmeβgeräte GmbH, Germany).

Helicase Assay
DNA unwinding was performed in a 20 µl reaction volume containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT pH 7.9, 1 nM Y-shaped DNA probe (prepared as described above), 10 mM ATP, 2 µg BSA and indicated amount of proteins. Reactions were incubated at 65°C for 45 minutes before addition of 20 µl stop buffer (100 mM EDTA, 2% SDS, 0.1% bromophenol blue, and 40% glycerol). Samples were electrophoresed on a 8% polyacrylamide gel (17 cm × 17 cm × 1.5 mm) in 1× TBE 0.2% SDS. Gels were run for 90 minutes at 12 V per cm before drying and analysis by phosphorimagery (Fujifilm FLA-5000). Quantitation was performed by using Aida Image Analyzer software (Raytest Isotopenmeβgeräte GmbH, Germany).

ATPase Complementation assay
Reactions were preformed as described previously [16]. 1µM of indicated mutant proteins were mixed for 15 minutes prior to ATPase assays.

Results and Discussion

Examination of the sequence conservation of archaeal and eukaryotic MCMs with reference to the 4.35 Å crystal structure of SsoMCM reveals an intriguing juxtaposition of secondary structure elements within SsoMCM. Although the low resolution of the available structure precludes accurate positioning of side chains, it is apparent that the helix following the Sensor 2 Arginine residue (R 560) lies on top of the base of a feature described by Chen and colleagues as the external hairpin (EXT-HP) [12]. Additionally, the EXT-HP precedes the first beta sheet of the AAA+ domain, this beta sheet is followed by the essential Walker A motif (including K346). Examination of sequence conservation reveals that there is a cluster of highly conserved residues in the vicinity of the base of the EXT-HP (Fig. 1 and Figure S1). We reasoned that this cluster might have roles in influencing the ATPase activity of the MCM. In principle this hypothetical influence could be effected by modulation of either the *cis*- or *trans*- properties of the MCM. In the case of the former, *cis*-, scenario the residues at the base of the EXT-HP could influence the positioning of the residues of the Walker A motif. Alternatively, the *trans*-acting activity of the MCM subunit could be altered, by virtue of the residues at the base of the EXT-HP influencing the positioning of the overlying alpha-helix and thereby transducing effects to the Sensor 2 R560 residue.
Furthermore, the base of the EXT-HP is additionally juxtaposed to the base of the PS1BH. Thus, we speculated that there might be a route of communication from the PS1BH, via the EXT-HP to the AAA+ active site.

Previous work from our laboratory had addressed the roles of two adjacent glutamines (Q423 and Q424) at the base of PS1BH, these residues were found to act as *trans*-residues and to be absolutely required for ATPase and helicase activity of MCM; mutation of these residues to alanine abrogated both activities. In addition, we have revealed that R331 (at the base of the EXT-HP) was similarly essential for helicase and ATPase activities and also appeared to play a *trans*-role [16]. However, the molecular basis of this phenotype was not resolved in the previous work.

In the current work, we have made a further seven mutations to four conserved residues. These include E422A and E422R, E422 is a extremely highly conserved residue that precedes the previously described Q423Q424. We also generated R329E, R329 is a conserved basic residue at the base of the EXT-HP. In addition, we made R331I and R331K mutants as well as targeting L565, a conserved hydrophobic residue in the helix following the Sensor 2 residue (R560), modelling of this residue suggests it is in close physical proximity to the R331 – L565 residue was mutated to aspartate or lysine.
The resultant mutant proteins were purified and found to elute as hexamers from gel filtration columns (not shown). We next subjected them to tests of their biochemical activities.

First we tested DNA binding activity, using a model Y-shaped DNA substrate in electrophoretic mobility shift assays (EMSAs). As can be seen in Figure 2 all mutant proteins retained the ability to bind DNA, albeit with affinities somewhat altered from the wild-type protein. More specifically, the E422A and

R331K mutant MCMs showed up to two-fold higher affinity for the DNA substrate than the wild-type MCM; MCM (L565D), MCM (R331I) and MCM (E422R) had the same affinity as the wild-type and R329E and L565K had up to two-fold lower affinity for DNA.

Next, we tested the ability of the mutants to hydrolyse ATP in the presence and absence of DNA (Figure 3). As we have reported previously, the ATPase activity of wild-type SsoMCM is modestly stimulated (approximately 2-fold) by the presence of DNA. The MCM (R331K), MCM (E422A) and both MCM (L565D) and MCM (L565K) demonstrated ATPase properties similar to wild-type while MCM (R331I) and MCM (E422R) lost the ability to hydrolyse ATP. Interestingly, the R329E mutant MCM had significantly reduced ATPase activity and this was no longer stimulated by the addition of DNA. As MCM (R329E) displayed a 2-fold reduced DNA binding affinity when compared to the wild-type protein, we note that the concentration of DNA added to these assays was 1 μM, a 13-fold higher concentration than the Kd measured in Figure 2. Therefore we think it is unlikely that the non-responsiveness of this mutant to DNA was a simple consequence of having modestly lowered DNA binding activity compared to the wild-type enzyme.

Having established the DNA binding and ATPase activities of the mutant MCMs, we next assayed their ability to effect DNA melting using Y-shaped oligonucleotide substrates (Figure 4). In agreement with the inability of the MCM (R331I) and MCM (E422R) proteins to hydrolyse ATP, these mutants also lacked detectable helicase activity. The MCM (R331K) mutant showed helicase activity at about half the level of wild-type and interestingly, the MCM (R329E) mutant showed similar activity to MCM (R331K), despite its low and non-DNA stimulated ATPase activity. While the E422R mutant MCM had no detectable helicase activity, the potentially less invasive mutation to alanine, E422A, resulted in essentially wild-type activity. Finally, mutation of L565 to either aspartate or lysine (L565D or L565K) significantly stimulated the helicase activity of the resultant mutant protein when compared to wild-type. Significantly, these mutants do not show any elevation of either DNA binding or ATPase rates, suggesting that these alterations enhance the coupling between ATP hydrolysis and helicase activity.

The reduction of the ATPase activities of the E422R and R329E mutant MCMs could be due to the wild-type residues contributing to the ATPase active site. As detailed above, the ATPase site is generated by the juxtaposition of two MCM subunits with coordinating residues being donated by both subunits – following previous convention we have termed these *cis*-acting residues [such as Walker A (K346), Walker B (D404) and Sensor 1 (N488) residues] or *trans*-acting, the classic example being the Arginine finger (R473) and additionally, in the case of MCM, Sensor 2 (R560) and other basic residues [16]. We have shown previously that while mutation of *cis* or *trans* residues affects ATPase activity in homomultimers, mixing a *cis* mutant and a *trans* mutant can regenerate activity [16]. This arises because the *cis* mutant has a wild-type *trans* site and conversely the *trans* mutant has a wild-type *cis* site. Appropriate apposition of subunits of the two mutant types can thus reconstitute a functional active site. We therefore tested the effect of mixing

either E422R or R329E mutant with MCM containing the Walker A lysine *cis* mutant K346E or a second mutant MCM with an isoleucine substitution of the previously identified *trans*-residue R331. As can be seen in Figure 5A, mixing MCM (E422R) with MCM (K346E) restored ATPase activity, indeed levels were significantly higher than wild-type. In contrast, mixing MCM (E422R) with MCM (R331I) did not restore ATPase activity. Similarly, the R329E mutant is complemented by the *cis* mutant K346E but not by the *trans* mutant R331I (Figure 5B). Taken together these data reveal that both MCM (E442R) and MCM (R329E) mutants have specific impairments in the *trans* component of the ATPase active site.

Perhaps the simplest explanation for the "*trans* phenotype" of these residues would be that the residues' side chains directly coordinate ATP in the active site. This however does not appear to be the case for E422 as the E422A mutant protein, while having modestly elevated affinity for DNA, has essentially wild-type ATPase and helicase activities. This therefore indicates that the side chain of E422 is not directly involved in coordinating the ATP moiety.

The E422R and R329E mutant SsoMCMs, while both acting as *trans* mutants, show distinct behaviours. SsoMCM (E422R) has essentially wild-type DNA binding but no detectable ATPase or helicase activities. In contrast, R329E, while less active than the wild-type protein, nevertheless retains helicase activity despite its weak ATPase activity no longer being stimulated by the addition of DNA. Given the relatively close juxtaposition of E422 and R329 in the structure of SsoMCM, we tested whether an E422R R329E double mutant could restore activity. However, in agreement with the distinct properties of the individual mutations, the double mutant, like the E422R single mutant, lacked detectable ATPase and helicase activities (data not shown).

Our studies of the R331 residue in the EXT-HP confirm our previous data that the basic side chain of this residue is of pivotal importance for the ATPase and helicase activities of SsoMCM. Notably, however, mutation of this residue to isoleucine has no effect on the DNA binding activity of the enzyme. Interestingly, the similarly highly conserved R329 shows a different phenotype, a charge-reversal mutation of this residue to glutamate has a modest effect on DNA binding but renders the SsoMCM's ATPase activity non-responsive to the addition of DNA. This indicates that this mutation is defective in transducing information from the DNA binding site of the helicase to the ATPase active site. The behaviour of this mutant is superficially reminiscent of that of the T364E mutation of the glutamate switch [18,19]. The glutamate switch is a conserved residue in all clades of AAA+ ATPases that plays a key role in aligning the catalytic glutamate in the Walker B motif for activation of a water molecule during ATP hydrolysis [18,19]. Like the R329E mutant, the glutamate switch mutant SsoMCM (T364E) can still bind DNA yet displays lowered helicase activity and has reduced ATPase activity that is no longer stimulated by DNA. However, T364 exerts its influence via the *cis*-acting Walker B motif and thus contrasts with the *trans*-acting R329. Given the proximity of the base of the R329-containing EXT-HP and the alpha helix preceding the *trans*-acting Sensor 2 R560, it is tempting to speculate that

R329 may influence the position of the Sensor 2 residue. Furthermore, the EXT-HP is additionally juxtaposed to the DNA-binding PS1BH. In this regard, it may be significant that we have previously demonstrated that mutation of a conserved lysine (K430) located at the tip of the PS1BH results in an enzyme that, like the R329E MCM, shows lowered basal ATPase activity that is no longer stimulated by DNA [6].

Taken together, these observations lead us to propose that R329 plays a key role in transducing positional information from the DNA binding PS1BH to the *trans*-acting face of the ATPase active site, an event at the heart of the mechanochemistry of the MCM helicase.

Acknowledgements
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References

1 Bochman, M. L. and Schwacha, A. (2009) The Mcm Complex: Unwinding the Mechanism of a Replicative Helicase. Microbiology and Molecular Biology Reviews. 73, 652-683

2 Brewster, A. S. and Chen, X. J. S. Insights into the MCM functional mechanism: lessons learned from the archaeal MCM complex. Critical Reviews in Biochemistry and Molecular Biology. 45, 243-256

3 Costa, A. and Onesti, S. (2009) Structural biology of MCM helicases. Critical Reviews in Biochemistry and Molecular Biology. 44, 326-342

4 Sakakibara, N., Kelman, L. M. and Kelman, Z. (2009) Unwinding the structure and function of the archaeal MCM helicase. Molecular Microbiology. 72, 286-296

5 Duggin, I. G., McCallum, S. A. and Bell, S. D. (2008) Chromosome replication dynamics in the archaeon Sulfolobus acidocaldarius. Proceedings of the National Academy of Sciences of the United States of America. 105, 16737-16742

6 McGeoch, A. T., Trakselis, M. A., Laskey, R. A. and Bell, S. D. (2005) Organization of the archaeal MCM complex on DNA and implications for the helicase mechanism. Nature Structural & Molecular Biology. 12, 756-762

7 Rothenberg, E., Trakselis, M. A., Bell, S. D. and Ha, T. (2007) MCM forked substrate specificity involves dynamic interaction with the 5 '-tail. Journal of Biological Chemistry. 282, 34229-34234

8 Barry, E. R., McGeoch, A. T., Kelman, Z. and Bell, S. D. (2007) Archaeal MCM has separable processivity, substrate choice and helicase domains. Nucleic Acids Research. 35, 988-998

9 Barry, E. R., Lovett, J. E., Costa, A., Lea, S. M. and Bell, S. D. (2009) Intersubunit allosteric communication mediated by a conserved loop in the MCM helicase. Proceedings of the National Academy of Sciences of the United States of America. 106, 1051-1056

10 Marinsek, N., Barry, E. R., Makarova, K. S., Dionne, I., Koonin, E. V. and Bell, S. D. (2006) GINS, a central nexus in the archaeal DNA replication fork. Embo Reports. 7, 539-545

11 Jenkinson, E. R. and Chong, J. P. J. (2006) Minichromosome maintenance helicase activity is controlled by N- and C-terminal motifs and requires the ATPase domain helix-2 insert. Proceedings of the National Academy of Sciences of the United States of America. 103, 7613-7618

12 Brewster, A. S., Wang, G. G., Yu, X., Greenleaf, W. B., Carazo, J. M., Tjajadia, M., Klein, M. G. and Chen, X. S. (2008) Crystal structure of a near-full-length archaeal MCM: Functional insights for an AAA plus hexameric helicase. Proceedings of the National Academy of Sciences of the United States of America. 105, 20191-20196

13 Enemark, E. J. and Joshua-Tor, L. (2006) Mechanism of DNA translocation in a replicative hexameric helicase. Nature. 442, 270-275

14 Enemark, E. J. and Joshua-Tor, L. (2008) On helicases and other motor proteins. Curr Opin Struct Biol. 18, 243-257

15 Gai, D. H., Zhao, R., Li, D. W., Finkielstein, C. V. and Chen, X. S. (2004) Mechanisms of conformational change for a replicative hexameric helicase of SV40 large tumor antigen. Cell. 119, 47-60

16 Moreau, M. J., McGeoch, A. T., Lowe, A. R., Itzhaki, L. S. and Bell, S. D. (2007) ATPase site architecture and helicase mechanism of an archaeal MCM. Molecular Cell. 28, 304-314

17 Erzberger, J. P. and Berger, J. M. (2006) Evolutionary relationships and structural mechanisms of AAA plus proteins. Annual Review of Biophysics and Biomolecular Structure. 35, 93-114

18 Zhang, X. D., and Wigley, D. B. (2008) The 'glutamate switch' provides a link between ATPase activity and ligand binding in AAA+ proteins. Nat. Struct. Mol. Biol. 15, 1223–1227.

19 Mogni, M.-E., Costa, A., Ioannou, C. and Bell, S.D. (2009) The Glutamate Switch is present in all seven clades of AAA+ protein. Biochemistry, 48, 8774-8775

Figure legends

Figure 1: **A** Model of the hexameric form of SsoMCM (generated from PDB file 3F9V) showing the two tiered appearance of the hexamer. The upper (C-terminal) tier contains the AAA+ ATPase modules of the monomers. The two subunits closest to the viewer are coloured green and cyan. The *cis*-acting residues (Walker A, Walker B and Sensor 1) of the green subunit are shown as lilac spheres. The positons of residues E422, L565, R331 and R329 that were targeted for mutagenesis are indicated and the PS1BH and H2I are shown in yellow and tan. Note that because of the low resolution (4.35Å) of the structure (ref. 12) all residues are represented by alanine in this representation. Part **B** shows a closer view of the AAA+ domain of a single monomer, the positions of the residues targeted for mutagenesis are indicated and the resides are shown in colour stick form. Note that although this representation shows residue side chains, the 4.35Å resolution of the current SsoMCM structure precludes accurate positioning of the sidechains [12].

Figure 2: DNA binding activity of WT and mutant SsoMCM to a Y-shaped DNA substrate. Quantification of EMSAs using 0, 15.6, 31.3, 65.5, 125 and 250 nM of each protein are shown. The assays were performed at least three times, and the error bars indicate the standard error of the mean. (A) DNA binding activity of WT, R331I, R331K, E422A and L565D. (B) DNA binding activity of WT, R329E, E422R and L565K.

Figure 3: ATPase activity of WT and mutant SsoMCM in the presence and absence of DNA. 1 μM of MCM proteins were used. The assays were performed at least three times, and the error bars indicate the standard error of the mean.

Figure 4: Helicase activity of WT and mutant SsoMCM. 0 (-), 31.3, 65.5, 125, 250, 500 and 1000 nM of each protein were used. The assays were performed at least three times, and the error bars indicate the standard error of the mean. (A) Helicase activity of WT, R331I, R331K, E422A and L565D. (B) Helicase activity of WT, R329E, E422R and L565K.

Figure 5: ATPase complementation study of E422R (**A**) and R329E (**B**). ATPase activity of 1 μM WT and mixtures containing 1 μM of the indicated mutants and either 1 μM of Walker A K346E or *trans*-ATPase motif R331I mutant. The assays were performed at least three times, and the error bars indicate the standard error of the mean.

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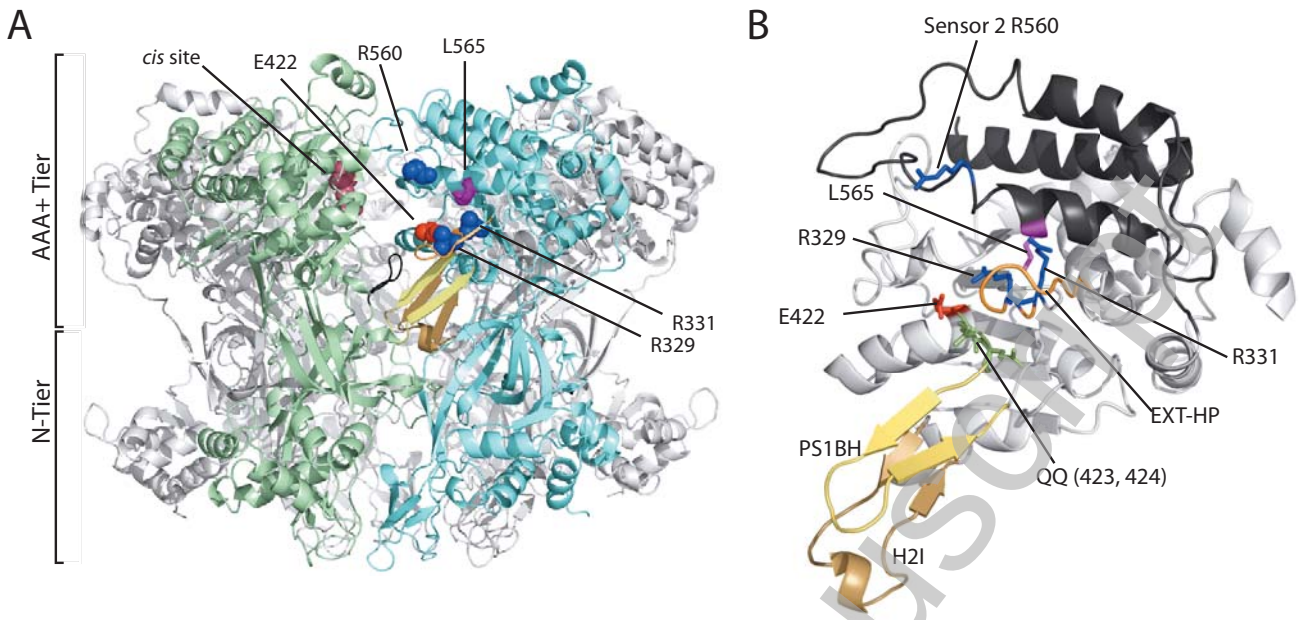

Liew and Bell Figure 1

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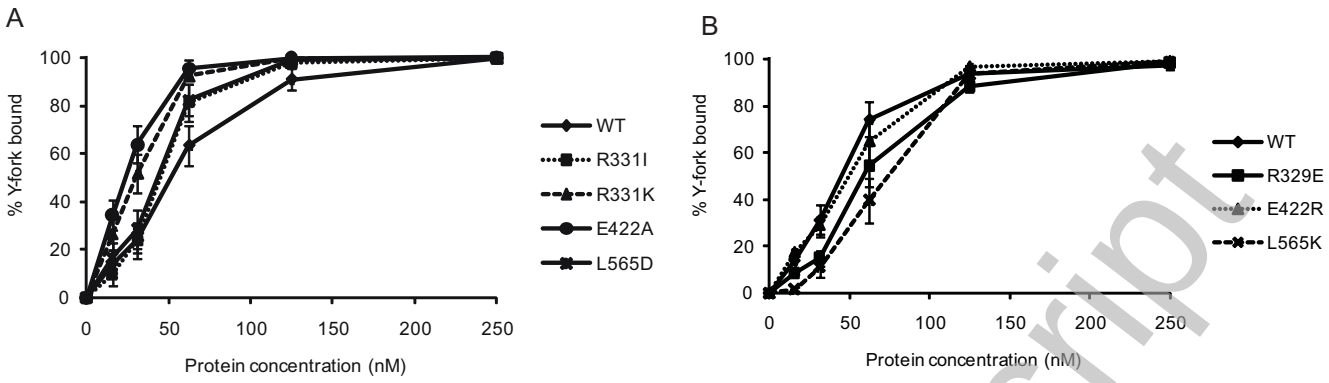

Liew and Bell Figure 2

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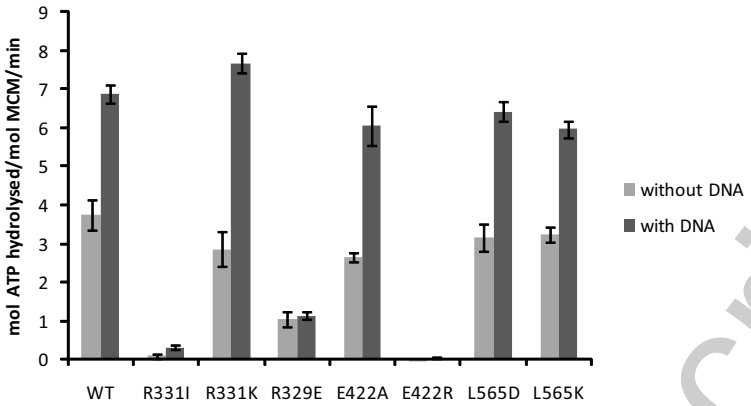
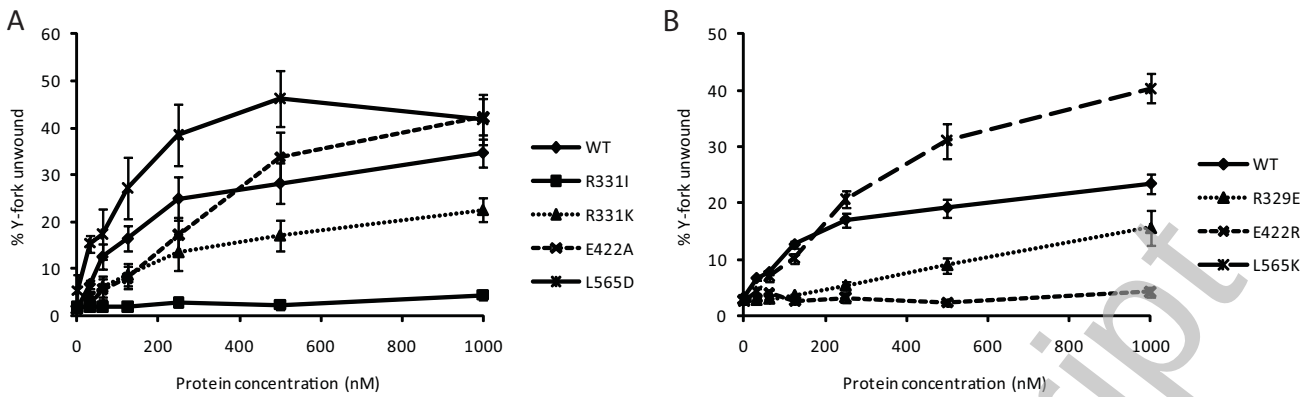


Liew and Bell Figure 3



Liew and Bell Figure 4

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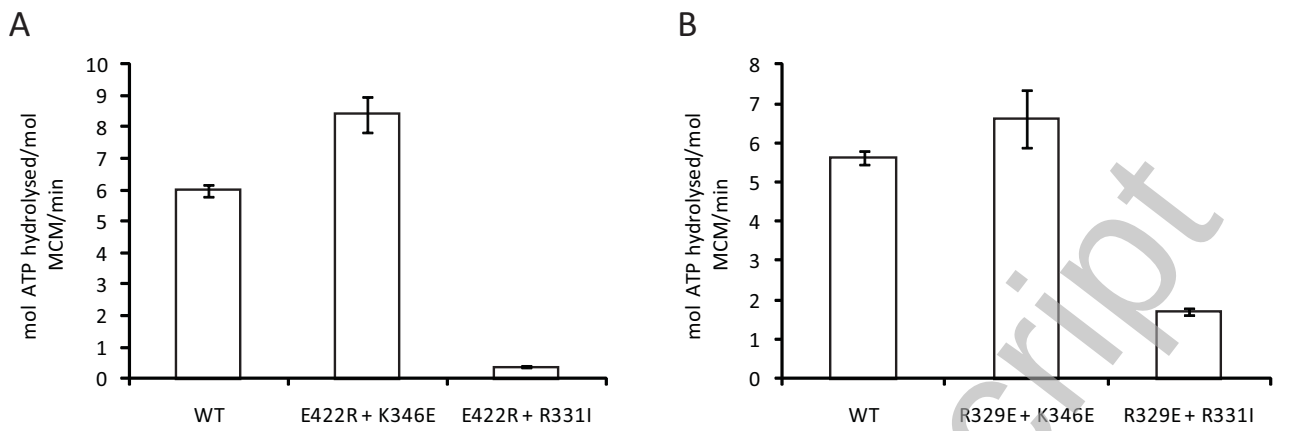

Liew and Bell Figure 5