The Helicase Activity of Hyperthermophilic Archaeal MCM is Enhanced at High Temperatures by Lysine Methylation

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Lysine methylation and methyltransferases are widespread in the third domain of life, archaea. Nevertheless, the effects of methylation on archaeal proteins wait to be defined. Here, we report that recombinant sisMCM, an archaeal homolog of Mcm2-7 eukaryotic replicative helicase, is methylated by aKMT4 in vitro. Mono-methylation of these lysine residues occurs coincidently in the endogenous sisMCM protein purified from the hyperthermophilic Sulfolobus islandicus cells as indicated by mass spectra. The helicase activity of mini-chromosome maintenance (MCM) is stimulated by methylation, particularly at temperatures over 70°C. The methylated MCM shows optimal DNA unwinding activity after heat-treatment between 76 and 82°C, which correlates well with the typical growth temperatures of hyperthermophilic Sulfolobus. After methylation, the half life of MCM helicase is dramatically extended at 80°C. The methylated sites are located on the accessible protein surface, which might modulate the intra- and inter- molecular interactions through changing the hydrophobicity and surface charge. Furthermore, the methylation-mimic mutants of MCM show heat resistance helicase activity comparable to the methylated MCM. These data provide the biochemical evidence that posttranslational modifications such as methylation may enhance kinetic stability of proteins under the elevated growth temperatures of hyperthermophilic archaea.

Keywords: protein methylation, DNA helicase, thermostability, hyperthermophiles, Sulfolobus

INTRODUCTION

Lysine methylation is one of the most common post-translational modifications, which can regulate the structure and function of protein. Remarkable progress has been made in the past decades in understanding how lysine methylation of histones regulates chromatin dynamics, gene expression, genome stability, and other cellular activities (Martin and Zhang, 2005). It has recently become evident that lysine methylation also occurs on non-histone proteins, suggesting its role far beyond epigenetics (Wu and Zhang, 2009; Erce et al., 2012; Zhang et al., 2012, 2015).
Besides chromatin proteins, many transcription factors were discovered to be methylated, such as p53, ERα, NFκB, E2F1, RB, TAF10, and STAT3 (Chuikov et al., 2004; Erce et al., 2012; Zhang et al., 2012; Clarke, 2013). The second class of non-histone proteins carrying lysine methylation belongs to translational apparatus, such as ribosomal proteins (e.g., Rpl1, Rpl23ab, Rpl42ab) and elongation factors (e.g., eEF1A, EF3A) in a diverse range of species from *Escherichia coli* to mammals (Polevoda and Sherman, 2007). Furthermore, there are accumulating reports of lysine methylation of proteins involved in other cellular processes since its first discovery in a bacterial flagellar protein in *Sulfolobus islandicus* (Ambler and Rees, 1959).

It is worth noting that lysine methylation may be prevalent in the third domain of life, Archaea (Choli et al., 1988; Febbraio et al., 2004; Botting et al., 2010). Abundant mono-methylation on lysine has been reported in *Sulfolobus* species, which usually thrive at hot springs throughout the world (Brock et al., 1972). For example, there is mono-methylation of 21 lysine residues in nine subunits of RNA polymerase complex purified from *Sulfolobus* (Botting et al., 2010). More recently, we and another group independently identified and characterized the first archaeal lysine methyltransferase, aKMT4, which may be, at least partially, responsible for variegated protein methylation in *Sulfolobus islandicus* (Chu et al., 2012; Niu et al., 2013).

aKMT4, also named as ribosomal protein L11 methyltransferase, bears an eukaryotic KMT4/Dot1 family catalytic core and is well conserved throughout archaeal domain. In contrast to its distantly related homolog KMT4/Dot1 in eukaryotes, aKMT4 lacks substrate recognition domains which enables itself to target a set of nucleic acid metabolism related substrates including chromatin proteins (Sul7d, Cren7), RNA exosome subunits (Rrp4, Rrp42, and Csl4) and ribosomal proteins (Rpl11) in *in vitro*. More strikingly, various lysine residues within the substrate can be mono-methylated to the different extent by aKMT4 due to multifaceted sequence specificity (Niu et al., 2013). Although several potential substrates have been identified, the effects of aKMT4 mediated protein methylation have yet to be defined.

Mini-chromosome maintenance (MCM) is an AAA+ (ATPase with other associated cellular activities) superfamily protein harboring 3′–5′ DNA helicase activity, which is the replicative helicase and plays an essential role in initiation and elongation of DNA replication (Tye, 1999; Erzberger and Berger, 2006; Bochman and Schwacha, 2008, 2009; Pucci et al., 2004; Botting et al., 2010). Beattie and Bell, 2011; Onesti and MacNeill, 2013; Kelman and Sherman, 2007). In eukaryotes, MCM complex is a heterohexamer consisting of six paralogous MCM monomers (Mcm2-7). All six MCM subunits are essential for DNA replication and cell growth in yeast, while most of Archaea, including *Sulfolobales*, contain only one MCM subunit forming a homohexameric complex (Kelman et al., 1999; Chong et al., 2000b; Fletcher et al., 2003; Barry and Bell, 2006; Bochman and Schwacha, 2009). Therefore, studies on the archaeal MCMs have been providing valuable insights into understanding the structure and function of eukaryotic MCM2-7 complex (Fletcher et al., 2003, 2005; Barry and Bell, 2006; Moreau et al., 2007; Brewster et al., 2008; Slaymaker and Chen, 2012). Like its eukaryotic counterparts, MCM from *Sulfolobus* shows robust ATPase activity and 3′–5′ helicase activity *in vitro* (Pucci et al., 2004; McGeoch et al., 2005; Brewster et al., 2008). Several MCM subunits were shown to be phosphorylated by multiple kinases in yeast, which are proposed to be important in helicase activation during replication initiation step in yeast (Sheu and Stillman, 2006; Randell et al., 2010). Till now, there is still no direct evidence to show that MCM helicase activity can be modulated by post-translational modifications.

Here, we report that sisMCM can be mono-methylated at several lysine residues by aKMT4, and methylation can help to maintain higher helicase activity after treatment with elevated temperatures similar to that of the natural growth conditions of *S. islandicus*. Additionally, several methylation-mimic mutants of sisMCM behave similarly as the methylated MCM in maintaining helicase activity after heat treatment. These results provide experimental evidence that methylation contributes to protein thermostability and function in high temperatures.

**RESULTS**

**sisMCM is a Novel Substrate of aKMT4 in Vitro**

In a previous work, we identified the first conserved hyperthermostable lysine methyltransferase (aKMT4) in *S. islandicus*, which displays multifaceted substrate specificity (Chu et al., 2012; Niu et al., 2013). To explore whether there are other substrates of aKMT4, we cloned and expressed DNA polymerase and helicase genes from *S. islandicus*. Purified recombinant proteins were then subjected to the *in vitro* methylation reactions at 50°C for 3 h in the presence of [3H-methyl]-S-adenosyl-methionine (3H-AdoMet) as a methyl donor. Methylation by aKMT4 was detected by autoradiography of 3H-methyl incorporation. We noticed potent 3H-methyl incorporation into sisMCM protein as well as aKMT4 itself (Figure 1A). The automethylation of aKMT4 is consistent with our previous results (Niu et al., 2013). However, no tritium incorporation was observed in the presence of a methyltransferase-deficient mutant aKMT4-G38R, indicating sisMCM is specifically targeted by aKMT4. sisMCM is an archaeal homolog of eukaryotic DNA helicase Mcm2-7, which plays essential role in DNA replication initiation and progression (Fletcher et al., 2003; Barry and Bell, 2006). This indicates that DNA helicase sisMCM is methylated by aKMT4 *in vitro*.

**sisMCM is Mono-methylated in Vivo in a Similar Pattern as in Vitro**

In order to examine whether MCM is methylated *in vivo* as well, we immunoprecipitated the endogenous MCM protein from *Sulfolobus* cells. First, we raised polyclonal anti-MCM antibody by immunizing rabbits with recombinant sisMCM purified from *E. coli*. Anti-MCM antibody was coupled to protein G beads. Purification was then carried out by adding cell extracts of the log phase *Sulfolobus* culture. After washed out non-specific associated proteins, the final bound fraction was boiled in SDS
Methylation contributes to protein kinetic thermostability

FIGURE 1 | sisMCM is mono-methylated on several lysine residues by aKMT4 in vitro and in vivo. (A) Purified mini-chromosome maintenance (MCM) protein (2 μM) was incubated with the increasing amounts of either wild type aKMT4 or aKMT4-G38R mutant supplied with [3H-methyl]-AdoMet as a methyl donor in a total volume of 30 μL at 50°C for 3 h. The molar ratio of substrate to enzyme is 1:3. Proteins were separated by 10% SDS-PAGE for 2 h and transferred to a PVDF membrane.3H-methyl incorporation was detected by autoradiography. The membrane was also stained with Coomassie, which shows that MCM and aKMT4 migrate with apparent masses of 75 and 18 kDa, respectively. Note that MCM was methylated by aKMT4 but not by G38R mutant enzyme. (B) Immunoprecipitation of endogenous MCM protein from cell extracts of wild type aKMT4 or aKMT4-G38R mutant supplied with [3H-methyl]-AdoMet using the mock antibody. Anti-MCM antibody. A parallel immunoprecipitation was carried out as control for mock beads, there was a significant enriched band in the anti-MCM precipitated fraction (FIGURE 1B). The enriched band was cut and in gel digested by trypsin. The tryptic fragments were subjected to an Orbitrap Velos. MS/MS spectra showed that endogenous MCM is indeed methylated at several lysine residues with different frequency in vivo (Figures 1C,D and Supplementary Figure S1). The variegated methylation pattern of MCM is in consistent with previous findings on other proteins in crenarchaeas (Botting et al., 2010; Azkargorta et al., 2014) and degenerated sequence specificity of aKMT4 (Chu et al., 2012; Niu et al., 2013). To the best of our knowledge, this provides the first evidence that MCM family proteins can be modified by methylation.

Next, we compared the methylation pattern of MCM in vivo with that of in vitro. The in vitro methylation pattern was determined in a similar approach as described above using recombinant MCM catalyzed by aKMT4 in the standard methylation reaction. The endogenous methyllysine residues of MCM were detected to be coincidently targeted by aKMT4 in vitro to the similar extent (FIGURE 1D). It is noteworthy that these lysine residues (K280,K281, K545K546, and K650) are located at the conserved regions of MCM homologs from crenarchaeas, particularly in Sulfolobus genus (Supplementary Figure S2). Furthermore, only mono-methylation, but not di- or tri- methylation, was observed, which is consistent with the previous finding that aKMT4 is a non-processive enzyme (Niu et al., 2013). Given the similar methylation patterns between in vitro and in vivo conditions, aKMT4 is likely involved in MCM methylation in Sulfolobus though other possibilities cannot be ruled out in current stage.

Methylation Stimulates the Helicase Activity of MCM at High Temperatures

Given the fact that S. islandicus cells grow optimally near 80°C (Brock et al., 1972; Contursi et al., 2006), we next reasoned that MCM methylation may contribute to the normal function of this key enzyme at such high temperatures. To address this possibility, we compared the modified MCM with the unmodified version for their catalytic activity. To minimize the artificial interference from in vitro manipulation, methylated (me-MCM) and un-methylated MCM were obtained through exact same methylation reactions at 50°C for 3 h in the presence of aKMT4 or inactive aKMT4-G38R methyltransferases, respectively (Figure 2A). Helicase assays were conducted at 65°C for 1 h using a 32P labeled fork-structured double stranded DNA (dsDNA) substrate. The unwound products were detected by autoradiography. The amounts of unwound single stranded DNA (ssDNA) to dsDNA were quantified by Quantity One (Bio-Rad). Neither aKMT4 nor aKMT4-G38R was contaminated with any helicase or ATPase activity (data not shown). There is an about 50% increase in DNA unwinding activity of me-MCM compared to that of un-methylated MCM (FIGURES 2B,C, compare lane 3–9). This result indicates that methylation causes a moderate stimulation on the helicase activity of MCM under the in vitro reaction conditions.
Methylated MCM Displays Increased Heat Resistance

Such an effect could be underestimated because of the limitation of the in vitro reaction conditions. For example, to avoid melting of the fork-structured DNA substrates in vitro, the reaction temperature for helicase activity has to be kept much lower than the typical growth temperature of hyperthermophilic Sulfolobus. To address this issue, we examined the effect of heat treatment on helicase activity. Subsequent to in vitro methylation by...
The me-MCM reached the highest unwinding activity after heat treatment between 70 and 82°C. me-MCM displayed around 1.5-fold higher helicase activity than aKMT4isinagreement with its lessthermostability in vitro (Table 1). The quantitative analysis from three independent experiments showed the significant difference (P-value < 0.01) of the unwinding capacity between two versions of MCM (Figure 2C). me-MCM displayed around 1.5-fold higher helicase activity than MCM after heat treatment between 70 and 82°C (Figure 2C). The me-MCM reached the highest unwinding activity after heat-treatment at about 80°C, the typical temperature of the natural habitat of Sulfolobus (Zhang et al., 2015). On the other hand, ATPase activity of MCM was only slightly affected post methylation and heat treatment (Figure 2D). ATPase is a prerequisite of activity. However, in the previous structural and functional studies, MCM mutants bearing higher ATPase do not always show higher helicase activity (Barry et al., 2007; Moreau et al., 2007; Pucci et al., 2007; Jenkinson et al., 2009; Brewster et al., 2010). These data indicate that methylation can stimulate the helicase activity of MCM, and this effect became amplified above 70°C. The duration time to retain 50% of helicase activity was measured by heat treatment at 80°C for the indicated times. As shown in Figure 2E, the half lives of me-MCM and MCM at 80°C were estimated to be approximately 5.9 and 2.8 h, respectively. This result indicates that methylation can increase the half life of MCM helicase, i.e., kinetic stability, which may count for its normal function at temperatures over 70°C.

Methylation Poses Subtle Effects on Thermodynamics of MCM

We next determined whether methylation affects the MCM thermodynamic stability, another aspect of protein thermostability. Thermodynamic stability is defined by free energy of stabilization (ΔG stab) and by the melting temperature (Tm; Vieille and Zeikus, 2001). The enzymes often unfold irreversibly, therefore we measured Tm (at which 50% of the enzyme is unfolded) by the differential scanning calorimetry as an indicator of dynamic stability. Each sample was loaded into a Nano-DSC II calorimeter and scanned from 20 to 110°C at a rate of 2°C/min. Recombinant sisMCM and aKMT4 alone have an apparent Tm of 98 and 91°C, respectively (Figure 3A and Table 1). This result indicates the robust thermodynamic stability of these two proteins, while the relatively lower Tm of aKMT4 is in agreement with its less thermostability in vitro (Niu et al., 2013). When MCM was incubated with aKMT4 at 50°C for 3 h in the absence of the methyl-donor Ado-Met, two peaks were observed at about 97.5 and 91.7°C, corresponding to the peaks of MCM and aKMT4, respectively (Figure 3A and Table 1). This pattern indicates that co-existence of MCM and aKMT4 does not interfere with thermodynamics of each other under the in vitro reaction conditions. When Ado-Met was added to trigger MCM methylation, the melting curves of both MCM and aKMT4 were kept almost unperturbed (Figure 3B and Table 1). This suggests that methylation does not change the melting temperature of MCM dramatically. It is worth noting that aKMT4 also showed similar melting pattern regardless of existence of Ado-Met. At least part of aKMT4 can be methylated as well in the presence of Ado-Met due to its unique self-methylation activity (Figure 1A; Niu et al., 2013). These suggest that there is no detectable dynamic change induced by MCM methylation.

To investigate whether methylation can induce conformational changes which may count for increased helicase activity after heat treatment, we compared the limited tryptic digestion profiles of MCM with or without methylation. MCM became partially resistant to trypsin digestion after association with dsDNA, but not with ATPγS, a non-hydrolyzable analog of ATP (Figure 3C). This indicates that the conformational changes induced upon binding to dsDNA can be detected by the conditions used here. Nevertheless, me-MCM showed basically the same digestion pattern as the unmodified one regardless of whether they were heat treated or not (Figure 3C). Taken together, these data indicate that methylation per se does not trigger significant structural or thermodynamic changes which can be detected under the conditions used here.

Methyl Groups are not Directly Targeted to Catalytic Domains of MCM

To gain insights into the role of MCM methylation, we examined the location of the methylation sites on the near full length S. solfatarius MCM structure in the context of a hexameric ring or lock-washer conformation (Brewster et al., 2008; Slaymaker et al., 2013). The four lysine residues subjected to mono-methylation (K280, K281, K545, K546) are located on the accessible protein surface in the C-terminal AAA+ helicase domain but away from the ATP catalytic pocket (Figure 4A). This likely provides a possible reason for why only helicase activity, but not ATPase, was significantly affected by methylation. Meanwhile, the location of these residues could explain why addition of a small methyl group to the side chain of these lysine residues did not lead to an obvious overall structural change. However, methylation will affect side chain conformation of a lysine by adding a hydrophobic methyl group. K280/K281 on the linker helix 1 (Lx1, Figure 4B) with around 4–5 Å distance to another helix of the AAA+ domain, a distance ready to make bonding interactions with slight adjustment of side-chain conformation. The linker and its helix Lx1 connect the N-terminal domain and C-terminal AAA+ domain, and is important for helicase activity (Erzberger and Berger, 2006; Jenkinson and Chong, 2006; Barry et al., 2007, 2009; Brewster et al., 2008; Slaymaker and Chen, 2012). Methylation of K280/K281 could impact the linker structural flexibility at elevated temperatures to display higher unwinding activity. For K545/K546, they are located on the C-terminal helix 6 (Ca6, Figure 4B). K546 is next to the missing C-terminal winged helix domain (WHD) that can regulate helicase activity, and K546 is in bonding distance to a helix from a neighboring subunit (Figure 4B). Methylation of these two residues could subtly affect the interactions with WHD and its neighbor to result in a higher helicase activity at elevated temperatures by affecting hexameric conformational switches needed for efficient
FIGURE 3 | Methylation shows only subtle effect on MCM conformation and thermodynamics stability. (A) The melting curves of sisMCM, aKMT4 and their mixture in the absence of Ado-Met during thermal denaturation measured by differential scanning calorimetry (DSC). Purified MCM (2.6 μM) and aKMT4 (9.4 μM) were incubated at 50°C for 3 h in the standard methylation reactions. The samples were next scanned by DSC from 20 to 110°C with a rate of 2°C/min. Note that the mixed MCM and aKMT4 showed essentially identical Tm values corresponding to each separated component, indicating two proteins do not interfere with their thermodynamics stability of each other. (B) The melting curves of sisMCM, aKMT4 and their mixture in the presence of Ado-Met during thermal denaturation measured by DSC. When Ado-Met was added to the methylation reactions, both MCM and aKMT4 can be methylated. However, there is no significant change of the melting curves compared to the ones in the absence of Ado-Met as shown in (A). (C) The limited trypsin digestion analysis of the methylated and un-methylated MCM, showing that modified and unmodified MCM have similar digestion patterns. MCM proteins were treated as described in Figure 2A prior to incubation with 20 nM trypsin in the presence or absence of ATP analog at 25°C for 15 min.
unwinding. Furthermore, we computated the changes of the local surface charge and the hydrophobic parameters which have been shown to be induced by methylation (Febbraio et al., 2004; Egorova et al., 2010; Clarke, 2013). To this end, we retrieved the local peptide sequence surrounding the methylated sites. The theoretical pI and grand average of hydropathicity were calculated (Figure 4C). Next, the mono-methylated lysine residues were simulated by amino acids with a longer side chain, methionine (M) or leucine (L). The mimic mutants, such as MCM-K280M/K281M, MCM-K545M/K546M, and MCM-K650L, showed significant increased surface hydrophobicity and pKa (Figure 4C), which may count for the enhanced overall thermostability.

Methylation-mimic Mutants of MCM Show Heat Resistance as Well

Next, we directly tested if the methylation-mimic mutants have similar performance as the methylated MCM at high temperatures as shown in Figure 2. The mutant proteins (MCM-K280M/K281M, MCM-K545M/K546M, and MCM-K650L) were expressed and purified for helicase assay. Without heat treatment, these MCM mutants showed comparable helicase activity to wild type (Figures 5A,B). After heat treatment at 80°C for 1 h, wild type MCM showed compromised helicase activity (Figure 5A, compare lane 4–8). However, with the same heat treatment, no reduction of unwinding was observed for all methylation-mimic mutants (Figure 5A, compare lanes 5–7 to 9–11). Thus, all methylation-mimic mutants retained significantly higher DNA unwinding activity than the unmodified enzyme (Figure 5B). As for ATPase, K280M/K281M showed moderately decreased activity as wild type, whereas no dramatic effect was observed for K545M/K546M and K650L mutants (Figures 5C,D). Gel shift assays showed no apparent changes for their binding to DNA substrates after heat treatment (Figure 5E). These results further support that methylation of MCM may improve its helicase activity in vitro at 80°C, a typical growth temperature of Sulfolobus.

DISCUSSIONS

Hyperthermophiles are valuable sources of thermostable enzymes displaying remarkable stability against high temperatures. Multiple mechanisms have been inferred in protein thermostability including hydrogen bonds, hydrophobicity, ion pairs, salt bridges, compactness, and surface charges (Vieille and Zeikus, 2001). In this study, besides these inherent intrinsic properties of protein, we provide biochemical evidence to support that methylation contributes to protein thermostability and functionality at temperatures over 75°C.

Most proteins from hyperthermophiles remain thermostable when they are expressed in mesophilic hosts such as E. coli. However, it’s not uncommon that recombinant proteins display relative weaker thermal resistance than their native counterparts (Chu et al., 2012; Niu et al., 2013). The optimal temperature of recombinant MCM enzyme is about 70°C, which is elevated to the typical growth temperatures of hyperthermophilic S. islandicus after methylation by aKMT4. More intriguingly, like methylated MCM, the recombinant methylation-mimic mutants display increased heat resistance than the unmodified enzyme. All these results support that methylation can enhance protein thermal properties.

Thermodynamic and kinetic stabilities represent two aspects of protein thermostability (Vieille and Zeikus, 2001). The melting temperature (Tm) of MCM is not changed in the presence or absence of methylation, indicating methylation does not increase detectable thermodynamic stability. On the other hand, kinetic stability of MCM is potently enhanced as evidenced by the extended half life of MCM upon methylation. These findings suggest a novel regulatory role of methylation per se for maintaining MCM function at high temperatures in hyperthermophilic archaea, likely through increasing their kinetic stability rather than thermodynamic stability. Both aKMT4 and its targeted lysine residues in MCM are conserved in crenarchaea (Supplementary Figure S2; Chu et al., 2012; Niu et al., 2013). Moreover, native β-glycosidase in S. solfataricus bears up to five methyllsines, which shows resistance to heat denaturation and aggregation compared to the unmodified recombinant counterpart, particularly at high temperatures (Febbraio et al., 2004). Therefore, the thermo-adaptation strategy through protein methylation may be widely utilized among hyperthermophiles. The regulatory role of protein methylation identified here is in agreement with the increased lysine methylation of Sul7d observed at the elevated growth temperatures, which is methylated at multiple sites by aKMT4 as well (Choli et al., 1988; Febbraio et al., 2004; Botting et al., 2010; Chu et al., 2012; Niu et al., 2013). Genetic evidence will be needed in future to elucidate the physiological function of numerous methyltransferases like aKMT4 and their targets in vivo.

Methylation on protein surface should not cause major conformational change in protein structure. Nevertheless, it can provide a novel interface for association with other molecules, thus affecting numerous features of the methylated protein, including protein-protein interactions, localization, enzyme activity, and turn over. Through in vitro biochemical assays in the absence of other proteins, we are able to reveal the effect of lysine methylation per se on the helicase activity of MCM, presumably through altering free-energy for the local secondary structural movement within its homo-hexameric subunits at the elevated temperatures. The stimulation effect of methylation on helicase activity is significantly enhanced when the temperatures
FIGURE 4 | Methyllysines are located on the accessible protein surface. (A) Methylsat sites are located on the protein surface away from the catalytic centers. Identified methylation lysine residues, labeled in blue, were mapped to the near full length three-dimensional structure of its highly homologous ssoMCM (based on PDB ID 4FDG). The conserved catalytic motifs are indicated according to (Brewster et al., 2008). (B) Mapping of the methylated lysine residues of sisMCM on to ssoMCM (PDB ID 4FDG) in the context of a hexameric ring/lock-washer conformation. For clarity only three subunits (in three discrete colors) in the front of a hexamer are shown. All four lysine residues found to be methylated (K280, K281, K545, and K546, shown in red sticks) are on the AAA+ helicase domain. K280 and K281 are located on the linker helix-Lα1 [helix nomenclature adapted from (Brewster et al., 2008)], and K545 and K546 on helix Cα6. They are all surface accessible. K546 is the only residue in bonding distance with a neighboring subunit, which may also change its rotamer conformation to interact with other protein partners. A small C-terminal winged helix domain (WHD; indicated) that should be in the neighborhood around K545 is absent from the structure. (C) Methylation simulation analysis. The peptide sequences nearby the methylated lysine were retrieved. Theoretical pl and grand average of hydropathicity of local area around methylation sites were calculated in ProtParam on the ExPASy Server (Wilkins et al., 1999). In order to mimic methylation, methyllysines were substituted by the amino acids carrying a longer side chain, like methionine or leucine. Glutamic acid substitution was applied to indicate the effect of charge change.
are elevated above 70°C. In mesophilic organisms, it has been proposed that methylation can improve protein solubility and stability (Egorova et al., 2010). Non-enzymatic methylation has been extensively used to improve protein crystallization but does not generally change the structure and activity of enzymes (Kim et al., 2008). Furthermore, methylation of amyloid peptide β-(25–35) increases the solubility, thus prevents the aggregation propensity, which is believed to cause Alzheimer’s disease (Hughes et al., 2000). Taken all together, it will be of interest to expand the effects of lysine methylation other than mediating protein-protein interactions to a broader scope.

**EXPERIMENTAL PROCEDURES**

**Organisms and Culture Conditions**

*Sulfolobus islandicus* REY15A strain, a gift of Dr. Qunxin She at University of Copenhagen, was cultured at 75°C as described earlier (Contursi et al., 2006; Zhang et al., 2015).

**Gene Cloning and Mutagenesis**

The coding sequence for sisMCM (Sire-1228) was amplified using *S. islandicus* REY15A genomic DNA and cloned into pGEX-6P-1 (GE Healthcare) via NovoRec PCR (Novoprotein). Point mutations were introduced using the Quick Change site-directed mutagenesis kit from Stratagene. Mutations were confirmed by sequencing the entire coding region. Oligonucleotides were synthesized by Sangon Biotech.

**Protein Purification**

All recombinant proteins were overexpressed in *E. coli* BL21 (DE3) CodonPlus RIL (Stratagene). GST-sisMCM and its mutant proteins were purified by glutathione-Sepharose columns (GE Healthcare). GST tag was removed by treatment with 100 μg of prescission protease/mg of fusion protein at 4°C overnight. Precission protease was inactivated at 70°C for 15 min. The supernatant was concentrated to 3 mg/ml in storage buffer 15 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM diithiothreitol, and 10% glycerol. Wild type aKMT4 and its transferase deficient aKMT4-G38R proteins were purified as described previously (Niu et al., 2013).

**Protein Methylation Reactions**

Standard protein methylation reactions were performed at 50°C as described previously with minor modifications (Niu et al., 2013). The standard molar ratio of enzyme
Samples were boiled for 5 min and resolved by 10% SDS-PAGE. After extensive washes to remove non-specific binding, proteins were subsequently purified by standard PAGE procedure. Helicase activity was measured by electrophoretic mobility shift assay (EMSA). The methylated sites of the native MCM or recombinant MCM were detected by affinity purification using recombinant GST-MCM or protein G resin. Antibodies Polyclonal antibodies specific to MCM were raised and affinity-purified using recombinant GST-MCM or protein G resin.

Immunoprecipitation To detect the modifications of MCM in vivo, native MCM protein was prepared by an immunoprecipitation procedure. Lysates of S. solfataricus were added to anti-MCM coupled protein G beads. After extensive washes to remove non-specific binding proteins, 30 μl of 2x SDS loading buffer was added to the beads. Samples were boiled for 5 min and resolved by 10% SDS-PAGE.

Mass Spectrometry The methylated sites of the native MCM or recombinant MCM catalyzed in vitro by aKMT4 were determined by MS/MS. The native MCM or in vitro methylated recombinant MCM bands were sliced and in-gel digested with trypsin as described previously. Tryptic fragments were analyzed by Orbitrap Velos (Thermo Scientific). MS/MS analysis was performed on three biological replicates to obtain the final list of methylation sites.

Helicase Assays For the helicase assay, a fork-shaped DNA including a 35-nt single-stranded tail and a 44-nt duplex region was used as the substrate. Helicase activity was determined by ATPase assay. ATPase activity is defined as the ratio to the ATPase activity of untreated wild type MCM, which is normalized as 100%. Activity data represent the average of at least three independent experiments with standard deviations indicated by error bars.

ATPase Assay ATPase assay was performed in a 10 μl reaction volume containing 30 mM Tris acetate (pH 8.0), 75 mM NaCl, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 1 mM [γ-32P]ATP (Perkin Elmer Life Sciences, 3,000 Ci mmol) and 100 nM protein (Brewster et al., 2010). After incubation at 65°C for 30 min, the reaction was quenched by addition of 1 μl 0.5 M EDTA. A 1 μl aliquot from each reaction was applied to a pre-washed PEI cellulose TLC plate (Sigma–Aldrich), dried, and run for 3 h in 2 M acetic acid and 0.5 M LiCl buffer. Plates were dried before autoradiography and quantified by Quantity One (Bio-Rad Laboratories, Inc.). Relative ATPase activity is defined as the ratio to the ATPase activity of untreated wild type MCM, which is normalized as 100%.

Electrophoretic Mobility Shift Assay (EMSA) Electrophoretic mobility shift assay was performed in a 10 μl reaction volume containing 30 mM Tris acetate (pH 8.0), 75 mM NaCl, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 10% glycerol, 0.5 mM fork-shaped DNA. After incubation at 65°C for 15 min, the reaction was quenched by addition of 1 μl 0.5 M EDTA. A 1 μl aliquot from each reaction was applied to a pre-washed PEI cellulose TLC plate (Sigma–Aldrich), dried, and run for 3 h in 2 M acetic acid and 0.5 M LiCl buffer. Plates were dried before autoradiography and quantified by Quantity One (Bio-Rad Laboratories, Inc.). Relative ATPase activity is defined as the ratio to the ATPase activity of untreated wild type MCM, which is normalized as 100%.

Limited Tryptic Digestion Limited tryptic digestion was performed by incubating the indicated samples (each sample contains 0.2 μg sisMCM) with 0.01 μg Trypsin (New England Biolabs) in the presence or absence of 1 mM ATP or its analog at 25°C for 15 min (Meyer et al., 2003). The reactions were terminated by adding 4xSDS-PAGE loading buffer. Digested protein was immediately
separated by a 12% SDS-PAGE gel prior to silver staining (Thermo scientific, PageSilver™ Silver Staining Kit).

**Differential Scanning Calorimetry**

After standard in vitro methylation reaction in the presence of either wild type aKMT4 or aKMT4-G38R mutant enzyme, protein samples were loaded into the sample cell of a Nano-DSC II System (GE Healthcare; Johnson, 2013). Same volume of reaction buffer (400 µl) was loaded into the reference cell. Samples were scanned from 20 to 110°C at a rate of 2°C/min. Background value was determined using buffer only. Purified MCM protein or aKMT4 alone was also performed as controls. Origin software (Microcal) was applied for curve fitting and data analysis. Each result represents the average from at least three independent experimental repeats.

**AUTHOR CONTRIBUTIONS**

HL, XSC, YX, and QC conceived and designed research; XY, YN, JC, and QC performed the experiments; TF, XSC, and YX carried out structural and computational analysis; HL, QC, XSC, and YX analyzed the data and wrote the paper.

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**SUPPLEMENTARY MATERIAL**

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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