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ORIGINAL PAPER

Biochemical characterization of the minichromosome maintenance protein from the archaeon *Thermoplasma acidophilum*

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Abstract Minichromosome maintenance (MCM) proteins are thought to function as the replicative helicases in archaea. Studies have shown that the MCM complex from the thermoacidophilic euryarchaeon *Thermoplasma acidophilum* (TaMCM) has some properties not reported in other archaeal MCM helicases. Here, the biochemical properties of the TaMCM are studied. The protein binds single-stranded DNA, has DNA-dependent ATPase activity and ATP-dependent $3' \rightarrow 5'$ helicase activity. The optimal helicase conditions with regard to temperature, pH and salinity are similar to the intracellular conditions in *T. acidophilum*. It is also found that about 1,000 molecules of TaMCM are present per actively growing cell.

Keywords Archaea · DNA replication · Helicase · MCM · *Thermoplasma acidophilum*

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Abbreviations

dsDNA	Double-stranded DNA
MCM	Minichromosome maintenance
MtMCM	*Methanothermobacter thermautotrophicus* MCM
SsMCM	*Sulfolobus solfataricus* MCM
ssDNA	Single-stranded DNA
TaMCM	*Thermoplasma acidophilum* MCM

Introduction

Minichromosome maintenance (MCM) proteins are thought to function as the replicative helicases in archaea and eukarya, responsible for the separation of the duplex chromosomal DNA at the front of the replication fork. The eukaryotic MCM complex is a family of six-related polypeptides (Mcm2-7), each of which is essential for cell viability. Biochemical studies have shown that in vitro a dimeric complex of the Mcm4,6,7 heterotrimer contains $3' \rightarrow 5'$ DNA helicase activity, single-stranded (ss) DNA binding, DNA-dependent ATPase activity, is capable of translocating along ss and double-stranded (ds) DNA and unwinding DNA–RNA hybrids while translocating along the DNA strand [(Forsburg 2004; Lei and Tye 2001) and references therein]. In vitro, the Mcm2 and Mcm3,5 complexes were shown to inhibit helicase activity (Forsburg 2004; Lei and Tye 2001).

Minichromosome maintenance helicases from several archaea have been studied and all show biochemical properties similar to the eukaryotic Mcm4,6,7 complex including $3' \rightarrow 5'$ helicase activity, DNA dependent ATPase activity, the ability to bind and translocate along ss and dsDNA and to unwind DNA–RNA hybrids. The archaeal enzyme from *Methanothermobacter thermautotrophicus*

was also shown to displace proteins from DNA [(Barry and Bell 2006; Duggin and Bell 2006; Kelman and Kelman 2003; Kelman and White 2005) and references therein].

The euryarchaeon *Thermoplasma acidophilum* was isolated from self-heated smoldering coal refuse piles and grows optimally at about 60°C and pH 2 (Darland et al. 1970). One of the unique features of the organism is the lack of a cell wall, and thus it was originally considered a mycoplasma. Its genome consists of a single circular chromosome of 1.56 Mbp and contains about 1,500 open reading frames (Ruepp et al. 2000).

The MCM helicase from *T. acidophilum* (TaMCM) shows some biochemical properties that differ from those reported for other archaeal MCM proteins. The enzyme was shown to have limited ability to unwind DNA substrates containing only a 3′-ssDNA overhang region and requires a forked DNA structure for efficient helicase activity (Haugland et al. 2006). Other archaeal species can readily unwind substrates lacking fork-like structure [for examples see (Barry et al. 2007; Chong et al. 2000)]. In addition, no DNA binding could be detected when a filter binding assay was used, but DNA binding of other archaeal MCM helicases can be detected using this assay [for example see (Kasiviswanathan et al. 2006)]. Also, while in all other archaeal MCM proteins studied interaction of the helicase with the initiator protein Cdc6 inhibits helicase activity, the activity of the TaMCM is substantially stimulated in the presence of *T. acidophilum* Cdc6 protein. Here, the requirements for efficient helicase activity are determined. It was found that the conditions for optimal helicase activity in vitro are similar to those found within the *T. acidophilum* cells.

Materials and methods

Materials

ATP and [γ-^{32}P]ATP were obtained from GE Bioscience, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) from Promega, 3-Amino-1,2,4-triazole (3AT) from Sigma and ϕX174 ss and dsDNA were obtained from New England Biolabs. Oligonucleotides were purchased by Medprobe (Oslo, Norway). Purified wild-type and $K_{343}A$ mutant TaMCM proteins were purified as previously described (Haugland et al. 2006).

Methods

Two hybrid analysis

For two hybrid analysis the gene encoding the TaMCM wild-type (Ta0799) and $K_{343}A$ mutant proteins were cloned as previously described (Haugland et al. 2006). The $D_{402}N$ mutant protein as well as the truncated enzymes, N-terminal (amino acids 1-304) and C-terminal (amino acids 305-698), were generated using PCR and the oligonucleotides described in Supplementary Table 1 from the vectors containing the altered genes. The PCR products were cloned into the *Sal*I and *Not*I sites of the pDBLeu and pPC86 vectors (Invitrogen) resulting in fusion proteins with the GAL4 DNA binding domain (DB) and the GAL4 activation domain (AD), respectively. The truncated proteins were constructed based on sequence comparison with MtMCM (Fletcher et al. 2003; Kasiviswanathan et al. 2004).

Plasmids encoding the DB and AD fusion proteins were co-transformed into yeast strain MaV203 (Invitrogen) according to the manufacturer's protocol, plated on Synthetic Complete Medium (SC) without Leu and Trp (SC-LT) and grown for three days at 30°C. Four colonies from each transformation were patched onto SC-LT plates and grown for 18 h at 30°C. These master plates were replica plated to SC plates without Leu, Trp and His (SC-LTH), to SC-LTH plates containing 10 mM 3-amino-1, 2, 4-triazole (3AT) and to nylon membranes placed on the surface of agar plates with YPAD medium (1% Bacto-yeast extract, 1% Bacto-peptone, 2% Dextrose and 0.01% adenine sulfate) for X-gal analysis. The SC-LTH and SC-LTH + 3AT plates were immediately replica cleaned, followed by incubation for 24 h at 30°C and thereafter replica cleaned again. Plates were incubated for 3 days more before scoring. The YPAD plates where incubated at 30°C for 18 h prior to the X-gal assay and monitored and scored after 1, 8, 16 and 24 h. Protein–protein interaction is indicated by growth on 3AT-plates (3AT assay) and blue color in the X-gal assay.

MCM helicase assays

Substrates for helicase assays were generated as previously described (Shin et al. 2003) using the oligonucleotides described below. To determine the directionality of the helicase the DF17 (5′- GCGTCCCAGGGCGGCGG-3′) oligonucleotide was ^{32}P-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Labeled DF17 was annealed to either DF90 (5′-CGCTGCTCCGCCTCCCGCCGCTC TGCCGCTGCCTCCGCCCGCCGCCCTGGGACGCCCA GGACGTGTCCTCCCGCCGGTCGCCGTCGCCCC-3′), resulting in a substrate with 3′- and 5′-ssDNA overhanging regions, to DF53 (5′-CCCGCCGCCCTGGGACGCCCA GGACGTGTCCTCCCGCCGGTCGCCGTCGCCCC-3′), resulting in a substrate with a 5′-ssDNA overhang region, or to DF55 (5′-CGCTGCTCCGCCTCCCGCCGCTC TGCCGCTGCCTCCGCCCGCCGCCCTGGGACGC-3′), resulting in a substrate with a 3′-ssDNA overhang region. The substrate used to determine the optimal conditions

for helicase activity was the DF50_T25 (5′-GGGACGC GTCGGCCTGGCACGTCGGGTTTGTTTGTTTGTTTG TTTGTTTG-3′) oligonucleotide, ^{32}P-labeled as described above and annealed to DF61 (5′-TTGTTTGTTTGTTT GTTTGTTTGTTTGTTTGTTTGCCGACGTGCCAGGC CGACGCGTCCC-3′).

DNA helicase activity was measured in 15 µl reaction mixtures containing 20 mM Hepes-NaOH, pH 7.5 (unless otherwise indicated in the figure legends), 10 mM magnesium acetate, 3.3 mM ATP (unless otherwise indicated in the figure legends), 2 mM DTT, 0.1 mg/ml BSA, 10 fmol of ^{32}P-labeled DNA substrate (4,000-cpm/fmol) and proteins as indicated in the figure legends. After incubating the samples at 59°C (unless otherwise indicated in the figure legends) for 60 min, the samples were immediately transferred to ice and 5 µl stop buffer (100 mM EDTA, 1% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue and 50% glycerol) were added. A 7-µl aliquot was loaded onto a 15% polyacrylamide gel in 0.5 X TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA) and electrophoresed at 140 V for 2.5 h. Bands were visualized and quantified by phosphorimaging. All helicase assays were repeated three times.

Fluorescence polarization anisotropy measurement

Fluorescence anisotropy measurements were performed at 25°C using a Fluoromax-3 spectrofluorimeter equipped with an autopolarizer (Jobin Yvon Inc.), using a 3-mm path length cuvette with a starting volume of 150 µl. A 50-mer ssDNA oligonucleotide (5′-CGCAGATAACAGTTGTCC TGGAGAACGACCTGGTTGACACCCTCACACCC-3′) was 5′-labeled with Cy3 and purified with a HPLC C18 column. The concentration of DNA was calculated using an absorbance of 260 nm with extinction coefficient 477,300 M^{-1} cm^{-1} and absorbance at 546 nm using extinction coefficient 136,000 M^{-1} cm^{-1} for Cy3 dye. DNA concentrations calculated by both measures differed by <10%. The measurements using absorbance at 260 nm were used to calculate the concentrations for the experiments. The initial reaction mixture contained 25 mM Hepes-NaOH pH 7.5, 2 mM DTT, 5 mM $MgCl_2$ and 10 nM DNA. Following the addition of protein (final concentration indicated in the figure) the reaction mixture was incubated for 10 min and then measured with a setting of 5 s integration and with three averaged measurements. The DNA was excited at 545 nm and emission spectra were set at 570 nm. Anisotropy values were directly tabulated in the analysis and with measured G factor and dark correction acquired at each blank for each experiment. The binding constant (K_d) was determined using GraFit version 5.0.1 (Erithacus software), using the following quadratic equation for fluorescent polarization anisotropy experiments (Heyduk and Lee 1990) $\Delta A_N = \frac{\Delta A_{TN}}{2D_T}\left\{(E_T + D_T + K_d) - \sqrt{(E_T + D_T + K_d)^2 - 4E_T D_T}\right\}$; where ΔA_N is the normalized change in anisotropy, ΔA_{TN} is the normalized total anisotropy change (which is equal to 1), E_T is the enzyme concentration at each titration point, D_T is the total concentration of DNA (which is 10 nM) and K_d is the dissociation constant for the binding isotherm. The experiments were repeated twice.

ATPase assay

ATPase activity was measured in 15 µl reaction mixtures containing 25 mM Hepes-NaOH pH 7.5, 5 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml BSA, 0.1 mM ATP, 0.33 pmol of [γ-^{32}P]ATP in the presence or absence of 50 ng ΦX174 ss or dsDNA and protein as indicated in the figure legend. After incubation at 59°C for 60 min, an aliquot (1 µl) was spotted onto a polyethyleneimine-cellulose thin-layer chromatography plate and ATP and P_i were separated by chromatography in 1 M formic acid and 0.5 M LiCl. The ATPase activity was visualized and quantified by phosphorimaging. All assays were repeated three times.

Cultivation of T. acidophilum

Thermoplasma acidophilum (DSM 1728) was grown in DSMZ medium 158 (http://www.dsmz.de/microorganisms/html/media/medium000158.html), pH 1.9 at 55°C with shaking and the cells were harvested in exponential phase. The growth was monitored by OD measurements at 600 nm and by cell counts.

Quantification of the in vivo level of T. acidophilum MCM protein

TaMCM protein and *T. acidophilum* cell extract were fractionated on 10% SDS-PAGE, followed by electroblotting onto a nitrocellulose membrane. Western blot analysis was performed using TaMCM polyclonal antibody generated against the recombinant protein by BioGenes GmbH (Germany) and developed using enhanced chemiluminescence (ECL, GE healthcare). The bands were quantified using Image J (Abramoff et al. 2004).

Results and discussion

T. acidophilum MCM helicase translocates on DNA in the 3′ → 5′ direction

All archaeal and eukaryotic MCM helicases studied to date translocate on DNA in a 3′ → 5′ direction. When the

activity of the TaMCM was first described (Haugland et al. 2006) the directionality of the enzyme was not reported. Thus, as the first step in the characterization of the enzyme, directionality was evaluated. As shown in Fig. 1 the TaMCM translocates on ssDNA in the 3′ → 5′ direction, suggesting it moves along the leading strand during replication.

Conditions for efficient TaMCM helicase activity

It was previously shown that TaMCM has very weak helicase activity even when provided with a forked DNA substrate containing both 3′- and 5′-ssDNA overhanging regions (Haugland et al. 2006). However, pH, salt and nucleotide concentration are known to have major effects on helicase activity. Thus, the effect of different buffer and assay conditions on MCM helicase activity was determined (Fig. 2). The enzyme has peak activity at 60°C, which is consistent with the optimal growth temperature of the organism (Fig. 2a). In contrast to other archaea, *T. acidophilum* has low intracellular ion concentrations (Searcy 1976). Thus, as expected, addition of salt to the reaction mixture inhibits helicase activity (Fig. 2b). ATP binding and hydrolysis is needed to fuel the unwinding reaction. When the effect of ATP concentration on helicase activity was evaluated it was found that from 2 to 6 mM the enzyme is active but at higher ATP concentrations activity is inhibited (Fig. 2c). Mg^{2+} binding to ATP and to DNA is required for helicase activity (Chong et al. 2000). As only 10 mM Mg^{2+} was added to the reaction and the affinity of the ion to ATP is higher than to DNA, it is possible that the Mg^{2+} is titrated out by the high levels of nucleotides and thereby prevented from binding to the DNA. The result would be inhibition of helicase activity, but not directly due to the high level of ATP. Similar observations were previously made with other archaeal MCM helicases (Chong et al. 2000; Kelman et al. 1999; Moreau et al. 2007). Interestingly, the buffer used in the reaction has an effect on the activity of the enzyme (Fig. 2d). The enzyme is more active in Tris and Bicine buffers than Hepes and phosphate buffers (Fig. 2d). The reason for this is not clear. It might be due to the different chemical properties of the buffers and/or the effect of temperature on the buffering capability of the buffer. The pH was determined at 22°C but it is known that in some buffering systems the pH changes with temperature. Thus, the differences observed may be due, in part, to this effect. However, change in pH alone cannot explain the difference noted between the buffers, as activity in the presence of Hepes and phosphate did not reach the same level as with Tris and Bicine. Nevertheless, the overall conditions for optimal helicase activity with respect to temperature, salinity and pH are similar to those found within *T. acidophilum* cells (Brock 1978).

TaMCM protein binds to ssDNA

All archaeal and eukaryal MCM proteins studied to date were shown to bind ssDNA. However, when filter binding assays were performed with the *T. acidophilum* enzyme, no DNA binding could be detected on different DNA substrates, including ss, ds and forked DNA, regardless of whether ATP was present or not (data not shown). If the K_{off} of DNA binding is fast, the filter binding assay would not have sufficient sensitivity to detect binding. Therefore, fluorescence polarization anisotropy was used to determine

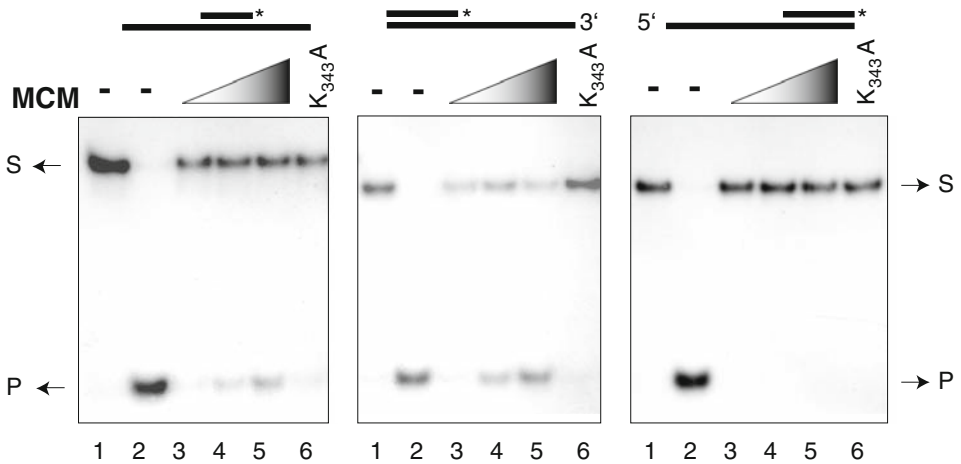

Fig. 1 TaMCM helicase translocates along DNA in the 3′ → 5′ direction. DNA helicase assays were performed as described in "Materials and methods" using 10 fmol substrate containing a 3′- and 5′-ssDNA overhanging region (*left panel*); a 3′-ssDNA overhang (*middle panel*) and a 5′-ssDNA overhang (*right panel*). Lane *1* substrate only, lane *2* boiled substrate, lane *3* 0.3 pmol TaMCM protein (as monomer), lane *4* 0.66 pmol TaMCM protein (as monomer), lane *5* 1.26 pmol TaMCM protein (as monomer), lane *6* 1.26 pmol TaMCM K_{343}A protein (as monomer). *S* substrate, *P* product. *Stars* indicate ^{32}P

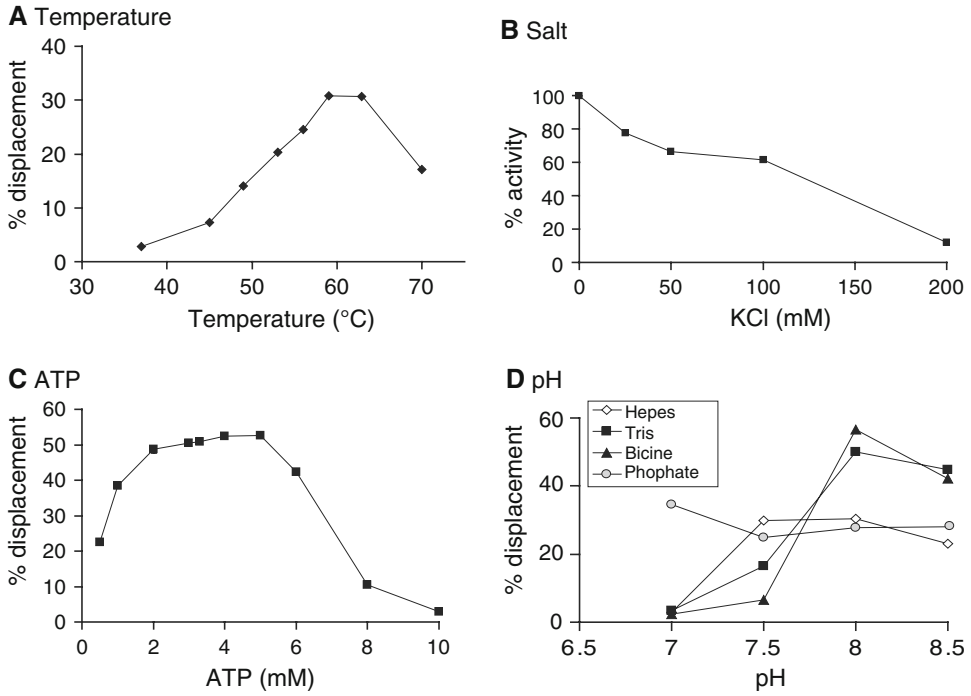

Fig. 2 TaMCM helicase activity under different conditions. **a** Effect of temperature on TaMCM helicase activity. DNA helicase assays were performed as described in "Materials and methods" with 10 fmol forked DNA substrate and 0.66 pmol TaMCM protein (as monomer) at the indicated temperatures (°C). The average of three independent experiments with standard deviation is shown. **b** Effect of salt concentration on TaMCM helicase activity. DNA helicase assays were performed with 5 fmol forked DNA substrate, 0.66 pmol TaMCM protein (as monomer) and the indicated concentration of KCl (mM). TaMCM helicase activity without salt is set to 100%. Helicase activity at various concentrations of salt was calculated relative to this value. The average of three independent experiments with standard deviation is shown. **c** Effect of ATP concentration on TaMCM helicase activity. DNA helicase assays were performed as described in "Materials and methods" at 59°C with 10 fmol forked DNA substrate, 1.26 pmol TaMCM protein (as monomer) and the indicated concentration of ATP (mM). The average of three independent experiments is shown. **d** Effect of buffer and pH on TaMCM helicase activity. DNA helicase assays were performed as described in "Materials and methods" at 59°C with 5 fmol forked DNA substrate, 0.78 pmol TaMCM protein (as monomer) in Hepes (*open diamond*), Tris (*dark filled square*), Bicine (*dark filled triangle*) or Phosphate (*open circle*) buffers at the indicated pH (measured at 22°C). The average of three independent experiments is shown

whether interactions between MCM and DNA could be observed under equilibrium condition. As shown in Fig. 3, the TaMCM protein binds ssDNA with a K_d of 0.1176 μM. This value is similar to that previously reported for the MtMCM protein (Sakakibara et al. 2008), the MCM protein from *Sulfolobus solfataricus* (SsMCM) (McGeoch et al. 2005), and other helicases [reviewed in (Patel and Picha 2000)].

ssDNA stimulates the ATPase activity of *T. acidophilum* MCM

All MCM helicases studied possess DNA-dependent ATPase activity (Ishimi 1997; Kelman and White 2005). After establishing that the *T. acidophilum* enzyme binds DNA (Fig. 3) the effect of the binding on ATPase activity was determined using ss and dsDNA. As shown in Fig. 4, the presence of ssDNA stimulates the ATPase activity of TaMCM. This stimulation is rather limited, however, and only 1.5–2-fold stimulation could be detected. Duplex DNA, on the other hand, does not have much effect on TaMCM ATPase activity. The stimulation by DNA is consistent with the ability of the enzyme to bind and translocate along ssDNA [Fig. 3, (Haugland et al. 2006)]. The stimulation by DNA is similar to that observed with the SsMCM protein, which is stimulated 1.8-fold in the presence of forked DNA substrate (McGeoch et al. 2005), but is lower than that observed with MtMCM protein, where DNA stimulates the ATPase activity by 5–7 fold (Chong et al. 2000; Kelman et al. 1999; Shechter et al. 2000).

The N-terminus of TaMCM is involved in dimerization

TaMCM protein was shown to form hexamers in solution (Haugland et al. 2006), similar to the SsMCM protein (Carpentieri et al. 2002; Liu et al. 2008) and the MCM proteins from *Archaeoglobus fulgidus* (Grainge et al. 2003)

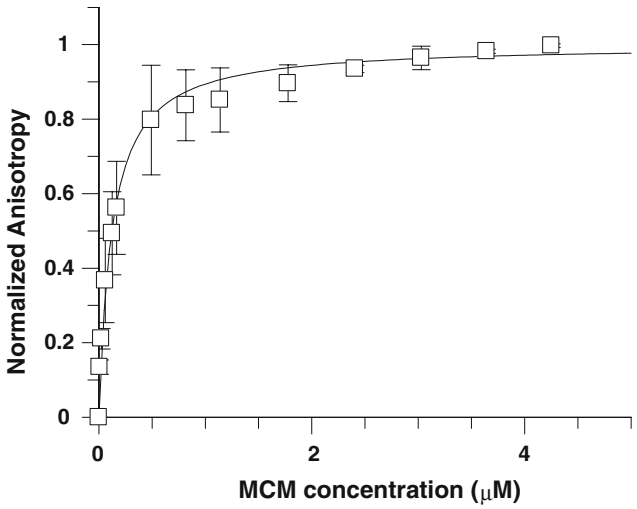

Fig. 3 TaMCM protein binds to ssDNA. The ability of TaMCM protein to bind ssDNA was measured using fluorescence polarization anisotropy (FPA) as described in "Materials and methods". The change in anisotropy is plotted against increasing amount of protein as indicated. K_d (μM) value is shown. The experiments were repeated twice and the average with standard deviation is shown

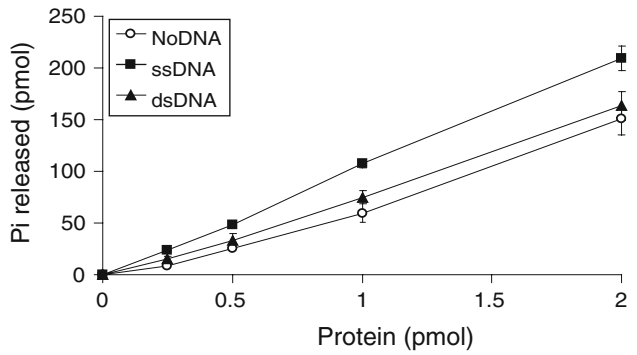

Fig. 4 TaMCM ATPase activity is stimulated by DNA. The ATPase activity of TaMCM protein was determined as described in "Materials and methods" in the absence (*open circle*) or presence of 50 ng of ss (*dark filled square*) or dsΦX174 (*dark filled triangle*) and 0.25, 0.5, 1 or 2 pmol of MCM protein (as monomer)

and *Methanococcoides burtonii* (Shin et al. 2006). MtMCM, on the other hand, forms double-hexamers in solution (Chong et al. 2000; Kelman et al. 1999; Shechter et al. 2000). The N-terminal part of MtMCM and SsMCM were shown to be needed for helicase multimerization (Liu et al. 2008; Fletcher et al. 2003). In order to determine which part of the TaMCM protein is involved in protein multimerization a two hybrid analysis was performed using mutated and truncated enzymes. As shown in Table 1, the full-length enzyme and the N-terminal part of the molecule can interact with itself. No interactions could be detected

Table 1 Protein–protein interaction of MCM mutants using two-hybrid analysis

pDBLeu	pPC86	Interaction	
		3AT assay	X-gal assay
Wild-type	Wild-type	+	+
$K_{343}A$	$K_{343}A$	+	+
$D_{402}N$	$D_{402}N$	+	+
N-term	N-term	+	+
AAA^+	AAA^+	−	−
Wild-type	pPC86	−	−
$K_{343}A$	pPC86	−	−
$D_{402}N$	pPC86	−	−
N-term	pPC86	−	−
AAA^+	pPC86	−	−
pDBLeu	Wild-type	−	−
pDBLeu	$K_{343}A$	−	−
pDBLeu	$D_{402}N$	−	−
pDBLeu	N-term	−	−
pDBLeu	AAA^+	−	−

3AT assay: + growth, − no growth; X-gal assay: + blue color after 1 h, − white color after 24 h

using the AAA^+ catalytic domains. Also, as shown for other archaeal MCM proteins, ATP binding and/or hydrolysis by TaMCM is not needed for the interaction. These results suggest that the N-terminal part of the TaMCM protein is involved in multimerization, as has been shown for the *M. thermautotrophicus* and *S. solfataricus* enzymes.

MCM protein is abundant in exponentially growing *T. acidophilum* cells

The MCM protein is thought to function as the replicative helicase in archaea and eukarya. The MCM protein is abundant in eukarya [(Laskey and Madine 2003) and references therein]. A study with *Pyrococcus abyssi* suggested that there are 200–400 MCM molecules per rapidly dividing cell (Matsunaga et al. 2001). As there are about 15 genome equivalents per *P. abyssi* cell during rapid growth, most of the MCM protein is likely involved in replication, since one hexameric ring is required at each replication fork (Kelman and Kelman 2003). In order to determine the number of TaMCM molecules in rapidly dividing *T. acidophilum* cells quantitative Western analysis was used (Fig. 5). It was found that there are about 1,000–1,800 TaMCM molecules per cell. However, the number of genome equivalents in rapidly dividing *T. acidophilum* cells is not known and thus the number of TaMCM molecule per genome may be similar to that in *P. abyssi*. Alternatively, MCM may have additional functions in *T. acidophilum* in addition to its role in chromosomal

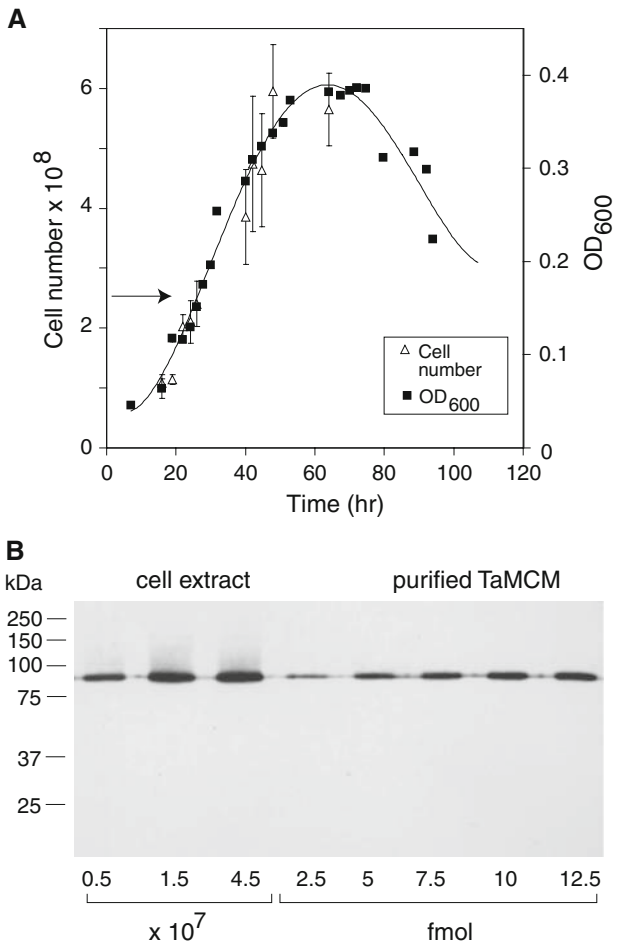

Fig. 5 Quantitation of MCM protein level in *Thermoplasma acidophilum*. **a** Growth curve of *T. acidophilum* showing number of cells as *white triangles* (*left Y*-axis) and optical density 600 nm as *black squares* (*right Y*-axis) versus time. *Error bars* represent the standard deviation calculated from three parallel (OD_{600}) and four to six parallel (cell number) experiments. The *arrow* indicates time of harvesting. **b** Quantitative Western analysis was performed as described in "Materials and methods". The amount of cell extract corresponding to the number of cells and amount of purified, recombinant MCM protein (fmol) loaded in each lane is indicated

replication. It is also possible that the assay used to determine the number of molecules does not have sufficient sensitivity to discriminate between small differences. When the number of MCM molecules in other archaeal species is determined it may be possible to determine the reasons for the difference.

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