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Thermoplasma acidophilum Cdc6 protein stimulates MCM helicase activity by regulating its ATPase activity

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ABSTRACT

The minichromosome maintenance (MCM) proteins are thought to function as the replicative helicases in archaea. In most archaeal species studied, the interaction between MCM and the initiator protein, Cdc6, inhibits helicase activity. To date, the only exception is the helicase and Cdc6 proteins from the archaeon *Thermoplasma acidophilum*. It was previously shown that when the Cdc6 protein interacts with MCM it substantially stimulates helicase activity. It is shown here that the mechanism by which the Cdc6 protein stimulates helicase activity is by stimulating the ATPase activity of MCM. Also, through the use of site-specific substitutions, and truncated and chimeric proteins, it was shown that an intact Cdc6 protein is required for this stimulation. ATP binding and hydrolysis by the Cdc6 protein is not needed for the stimulation. The data suggest that binding of Cdc6 protein to MCM protein changes the structure of the helicase, enhancing the catalytic hydrolysis of ATP and helicase activity.

INTRODUCTION

DNA replication is an essential process for cell proliferation and requires the coordinated activity of multiprotein complexes. The process can be divided into three phases: initiation, elongation and termination. Much is known about the elongation phase of DNA replication in archaea. Less is known, however, about the mechanism of initiation. Two proteins suggested to play a role in the initiation process are the minichromosome maintenance (MCM) helicase and homologues of the eukaryotic initiator protein Cdc6 (1,2), which also functions as an origin recognition protein in archaea.

The MCM helicase is thought to function as the replicative helicase in archaea and eukarya. All archaeal species studied contain at least one homologue of the MCM helicase. The MCM proteins are members of the AAA^+ family of ATPases, and biochemical studies on a number of archaeal MCM proteins demonstrated that the enzyme possesses an ATP-dependent $3'\rightarrow5'$ helicase activity and the ability to bind and translocate along ssDNA (1,3). Studies with *Methanothermobacter thermautotrophicus* and *Thermoplasma acidophilum* MCM proteins showed that the enzymes could also bind and translocate along duplex DNA (4,5). The *M. thermautotrophicus* MCM complex can also displace proteins from DNA (4,6) and unwind DNA–RNA hybrids while translocating along the DNA strand (7).

Most archaeal species contain homologues of the eukaryotic initiation protein, Cdc6, and the archaeal enzymes also share amino acid sequence similarity with subunits of the eukaryotic origin recognition complex (ORC) (8). The 3D structure of several archaeal Cdc6 proteins revealed that they contain three domains (9–12). The two domains at the N-terminus form the ATPase catalytic part while the C-terminal domain contains a winged-helix (WH) fold.

Biochemical, structural and *in vivo* studies on Cdc6 proteins from several archaea show that the proteins bind to origin DNA. The studies included cocrystallization of the proteins with inverted DNA repeats found within the archaeal origins of replication (11,12), foot-printing analysis of Cdc6 proteins on origin sequences (13,14), direct binding of the proteins to origin inverted repeats (15–17) and chromatin immunoprecipitation (ChIP) studies, and all suggested that the Cdc6 proteins associate with origin sequences *in vivo* (18,19).

In addition to the archaeal Cdc6 protein function in origin recognition, the proteins may participate in helicase assembly at the origin. This is based on primary amino acid sequence similarity to the eukaryotic Cdc6 protein, which is implicated in helicase loading at the origin (8), as

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well as on biochemical studies demonstrating properties similar to the bacterial helicase loader, DnaC (20–22).

The Cdc6 proteins from several archaea were shown to interact with the MCM helicase and to regulate its activity. However, the effect of Cdc6 on helicase activity varies in different organisms. It was found that the interactions between Cdc6 and MCM proteins from *M. thermautotrophicus* or *Sulfolobus solfataricus* inhibit helicase activity (20–22). It was also found that DNA binding by Cdc6 protein is not required for the inhibition and that ATP binding, but not hydrolysis, by Cdc6 is also needed (20).

On the other hand, when the Cdc6-2 protein from the archaeon *T. acidophilum* binds the MCM complex it substantially stimulates helicase activity (5). To date, this is the only example of a stimulatory effect of Cdc6 on MCM helicase activity. Using mutant and truncated Cdc6-2 proteins it is shown here that the stimulatory effect of Cdc6-2 on MCM helicase activity is via stimulation of the ATPase activity. It is also found that only the full-length Cdc6-2 protein stimulates the activity and that ATP binding and hydrolysis by Cdc6-2 is not required. The data suggest that upon binding to MCM, Cdc6-2 changes the structure of the helicase in such a way as to activate its ATPase activity.

MATERIALS AND METHODS

Materials

Chemicals and reagents were obtained from the following suppliers: [γ-^{32}P]ATP from GE Bioscience, Piscataway, NJ, USA, 3-Amino-1,2,4-triazole (3AT) from Sigma, St. Louis, MO, USA and ϕX174 ss and dsDNA from New England Biolabs, Ipswich, MA, USA. Oligonucleotides were synthesized by Sigma genosys.

Methods

Generation of mutant, truncated and chimeric proteins. The Cdc6-2 point mutations, deletion mutants and chimeras were generated using a PCR-based approach as previously described for the construction of *M. thermautotrophicus* Cdc6 mutant proteins and chimeras (17,21) using a pET-21a vector (Novagen, Gibbstown, NJ, USA) containing the wild-type gene (5) as a template and the primers shown in Supplementary Table 1. An alignment of the *T. acidophilum* Cdc6 proteins with other Cdc6 proteins was used as a guide to construct the point mutations in the nucleotide binding and hydrolysis sites [Walker-A mutant ($K_{66}E$) and a Walker-B mutant ($D_{143}N$)]. The 3D structure of the *Pyrobaculum aerophilum* Cdc6 protein (9) served as the guide for the construction of the deletion mutants and the chimeric proteins. A schematic illustration of proteins used in the study is shown in Figure 1A. All wild-type and mutant proteins were purified (Figure 1B) as previously described (5).

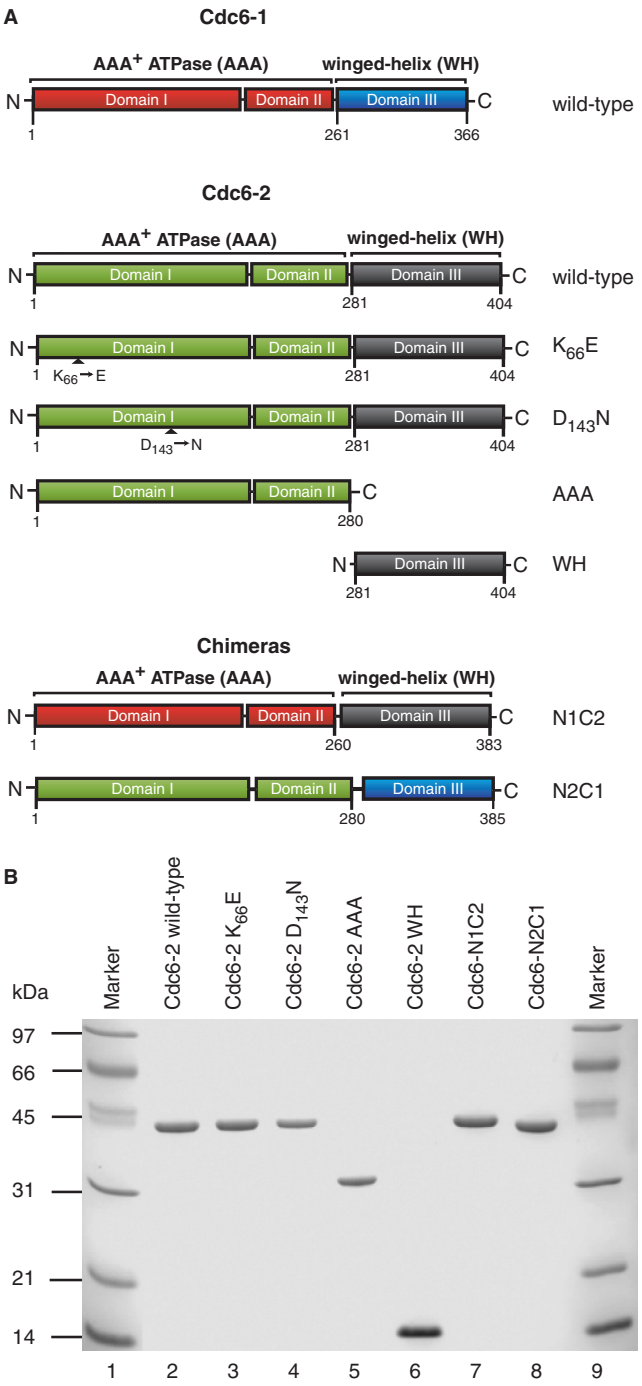


Figure 1. The Cdc6 proteins used for the study. (**A**) A schematic illustration of the proteins used for the study. (**B**) One microgram of purified protein was fractionated on 12% SDS-PAGE and visualized by Coomassie blue staining. Lanes 1 and 9, molecular mass markers; lane 2, wild-type Cdc6-2; lane 3, Cdc6-2 Walker-A mutant ($K_{66}E$); lane 4, Cdc6-2 Walker-B mutant ($D_{143}N$); lane 5, Cdc6-2 AAA$^+$ domains; lane 6, Cdc6-2 WH domain; lane 7, Cdc6-N1C2 chimera; lane 8, Cdc6-N2C1 chimera.

MCM helicase assay. Helicase substrates were generated by hybridization of complementary oligonucleotide [DF50_T25, 5′-GGGACGCGTCGGCCTGGCACGTC GG(GTTT)$_6$G-3′ and DF60_T8, 5′-TTTGTTTGCCGA CGTGCCAGGCCGACGCGTCCC-3′] and purified as previously described (4). The DF60_T8 strand was 5′ end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase.

DNA helicase activity was measured in reaction mixtures (15 µl) containing 20 mM Tris (pH 8.5), 10 mM

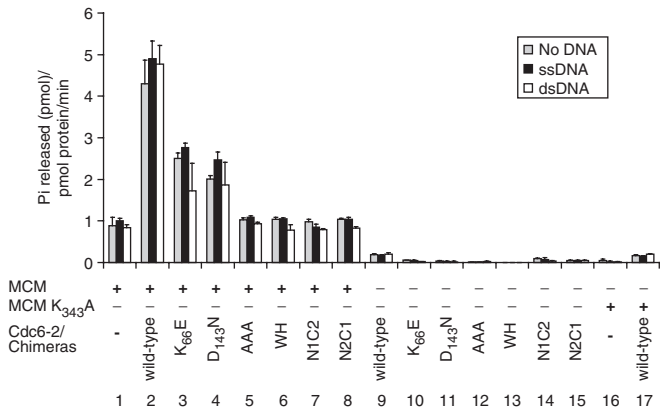


Figure 2. The *T. acidophilum* Cdc6-2 protein stimulates the ATPase activity of MCM helicase. ATPase assays were performed as described in Material and methods section in the absence or presence of ss or dsDNA. Reactions include 0.95 pmol wild-type MCM protein (as monomer) (lanes 1–8) or MCM $K_{343}A$ mutant protein (lanes 16 and 17), and 5 pmol (as monomer) of Cdc6-2 wild-type (lanes 2, 9 and 17), Cdc6-2 Walker-A mutant ($K_{66}E$) (lanes 3 and 10), Cdc6-2 Walker-B mutant ($D_{143}N$) (lanes 4 and 11), Cdc6-2 AAA^+ catalytic domains (lanes 5 and 12), Cdc6-2 WH domain (lanes 6 and 13), Cdc6-N1C2 chimera (lanes 7 and 14), and Cdc6-N2C1 chimera (lanes 8 and 15).

magnesium acetate, 3.3 mM ATP, 2 mM DTT, 0.1 mg/ml BSA, 5 fmol ^{32}P-labeled DNA substrate (4000 c.p.m./fmol) and proteins as indicated in the figure legends. After incubation at 50°C for 1 h, 5 µl 5 × loading buffer (100 mM EDTA, 1% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue and 50% glycerol) was added and aliquots (7 µl) were loaded onto an 18% polyacrylamide gel in 0.5 × TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA) and electrophoresed for 2.5 h at 140 V. The helicase activity was visualized and quantitated by phosphorimaging. All helicase experiments were repeated three times and their averages with standard deviations shown in the figure.

ATPase assay. ATPase activity was measured in reaction mixtures (15 µl) containing 20 mM Tris–HCl (pH 8.5), 5 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml BSA, 0.1 mM ATP, 0.33 pmol of [γ-^{32}P]ATP, 0.95 pmol MCM as monomer and 5 pmol Cdc6-2 in the presence or absence of 50 ng ϕX174 ss or dsDNA. After incubation at 50°C for 1 h the reactions were transferred to ice and an aliquot (1 µl) was spotted onto a polyethyleneimine (PEI)-cellulose thinlayer chromatography plate and ATP and P_i were separated by chromatography in solution containing 1 M formic acid and 0.5 M LiCl. ATP hydrolysis activity was visualized and quantitated by phosphorimaging. All ATPase experiments were repeated three to six times and their averages with standard deviations are shown in Figure 2.

Two-hybrid analysis. The MCM and Cdc6-2 constructs for two-hybrid analysis were generated by PCR from the pET-21 expression construct containing the wild-type or mutant genes. The MCM wild-type and mutant proteins were cloned into the pPC86 vector (Invitrogen, Carlsbad, CA, USA) and Cdc6-2 wild-type and mutant proteins were inserted into the pDBLeu vector (Invitrogen). Plasmids encoding the DB and AD fusion proteins were cotransformed into yeast MaV203 (Invitrogen) according to the manufacturer's protocol, plated on synthetic complete medium (SC) without Leu and Trp (SC-LT) and grown for 3 days at 30°C. Five 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) containing 10 mM 3-amino-1, 2, 4-triazole (3AT) and replica cleaned. The plates were incubated for 24 h at 30°C and replica cleaned. Following further incubation at 30°C, the growth of yeast cells were monitored and scored every 24 h for 4 days.

Filter binding assay. Filter binding assays were performed either with 46-mer ssDNA (Z164; 5′-CTCGAGA AGCTTTGTCCCCTATCTCATCAAAAATGTTCATA GTACC-3′) or with a forked structure generated by annealing two oligonucleotides (DF74; 5′-GGGACGCG TCGGCCTGGCACGTCGGCCGCTGCGGCCAGGC ACCCGATGGCGTTTGTTTGTTTGTTTGTTTGTT T-3′ and DF61; 5′-TTTGTTTGTTTGTTTGTTTGTTT GTTTGTTTGTTTGCCGACGTGCCAGGCCGACGC GTCCC-3′). DNA labeling and purification were performed as described previously (4). Nitrocellulose filter binding assays were carried out under a variety of conditions. All assays were performed in 20 µl reaction mixture containing 10 mM $MgCl_2$, 2 mM DTT, 100 mg/ml BSA and 50 fmol of DNA substrate. The variable conditions were as follows: the reaction was performed in the presence of 20 mM Tris–HCl at either pH 7.5, 8.0 or 8.5. Several samples also included 2 mM ATP. Prior to analysis, the samples were incubated for 10 min at 25, 50, 55 or 60°C. Various concentrations of MCM and Cdc6 proteins were used at the different conditions. After incubation, the mixture was filtered through an alkaline-washed nitrocellulose filter (Millipore, Billerica, MA, USA, HA 0.45 mm) (23), which was then washed with 20 mM Tris–HCl at the same pH as the reaction mixture. The radioactivity adsorbed to the filter was measured by liquid scintillation counting.

Fluorescence polarization anisotropy measurement. Fluorescence anisotropy measurements were performed at 25°C using a Fluoromax-3 spectrofluorimeter equipped with an autopolarizer (Jobin Yvon Inc., Edison, NJ, USA) and a 3 mm path length cuvette with starting volume of 150 µl. A 50-mer ssDNA (5′-CGCAGATAAC AGTTGTCCTGGAGAACGACCTGGTTGACACCCT CACACCC -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 477300/M/cm and absorbance at 546 nm using extinction coefficient 136 000/M/cm for Cy3 dye. DNA concentrations calculated by both measurements 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 the MCM or

MCM and Cdc6 proteins, 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 (24)

$$\Delta A_N = \frac{\Delta A_{TN}}{2D_T}\left\{(E_T + D_T + K_d) - \sqrt{(E_T + D_T + K_d)^2 - 4E_T D_T}\right\};$$

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 (assuming it is constant at 10 nM) and K_d is the dissociation constant for the binding isotherm. The experiments were repeated three times.

Circular dichroism. Circular dichroism (CD) measurements were performed with a JASCO J-810 spectropolarimeter equipped with a Peltier element for temperature control using quartz cells with a 1 mm path length at 25°C and 0.2–0.3 mg/ml proteins. The protein samples were in buffer containing 25 mM Tris–HCl (pH 8.0) and 2% glycerol. Far-UV spectra (200–260 nm) were acquired at a scan rate of 50 nm/min (1 nm bandwidth) and the average of four is shown in Figure 5 after the subtraction of buffer scans. The mean residue ellipticity $[\Theta]_{MRW}$ was calculated from the following expression:

$$[\Theta]_{MRW} = \frac{\Theta \bullet 100 \bullet MW}{c \bullet l \bullet N},$$

where Θ is the ellipticity of the sample, MW is the protein molecular weight, c is the protein concentration in milligram per milliliter [calculated from the UV absorption spectra and by the method of Gill and Von Hippel (25)], l is the path length in centimeter and N is the number of amino acids in the protein.

Thermal denaturation experiments were performed under the same conditions by monitoring the change in ellipticity at 222 nm (2 nm bandwidth) from 25°C to 80°C at a 1.5°C/min scan rate. Determination of the midpoint denaturation temperatures (T_m values) were performed after noise-reduction and differentiation of the experimental curves using the Standard Analysis software provided by the manufacturer.

RESULTS

The full-length Cdc6-2 protein stimulates MCM helicase activity

The *T. acidophilum* possesses two Cdc6 homologues, referred to as Cdc6-1 and -2. It was previously shown that Cdc6-2 protein but not Cdc6-1 stimulates MCM helicase activity (5). In order to determine which part of the Cdc6-2 protein is required for this stimulation, proteins containing only the WH or AAA^+ catalytic domains were generated and analyzed for their effect on the helicase. As shown in Figure 4A and B, while the intact Cdc6-2 protein stimulates MCM helicase activity (Figure 4A, lanes 4–6 see also panel B), neither the catalytic domains (Figure 4A, lanes 13–15, see also panel B) nor the WH domain alone (Figure 4A, lanes 16–18, see also panel B) stimulate the activity. When Cdc6-2 protein was incubated with the substrate in the presence of a MCM mutant ($K_{325}A$) which is devoid of helicase activity, no unwinding could be observed (5), and the Cdc6-2 protein did not bind DNA (see below), suggesting that the DNA unwinding observed is not due to helicase-like activity by the Cdc6-2 protein. However, to demonstrate this directly, the experiment was repeated in the absence of MCM. As shown in Figure 4C, no unwinding could be detected.

The results presented in Figure 4A and B suggest that both the catalytic and WH domains are required for the stimulation. However, it is not clear if both domains have to be from Cdc6-2 or if the catalytic or WH domain of Cdc6-1 can substitute for the Cdc6-2 domain. To address this possibility, chimeric Cdc6 enzymes were generated by swapping the catalytic and WH domains between the Cdc6-1 and -2 proteins. Neither Cdc6-N1C2 (catalytic domain from Cdc6-1 and WH domain from Cdc6-2) nor Cdc6-N2C1 (catalytic domain from Cdc6-2 and WH domain from Cdc6-1) could stimulate helicase activity (Figure 4D).

The Cdc6 proteins belong to the AAA^+ family of ATPases and thus it is possible that ATP binding and hydrolysis by the Cdc6-2 protein plays a role in the stimulation of MCM. To address this possibility, Cdc6-2 proteins with mutations in the Walker-A ($K_{66}E$) or -B ($D_{143}N$) motifs (involved in ATP binding and hydrolysis) were used. As shown in Figure 4A, lanes 7–9 and 10–12, see also panel B, both mutant proteins stimulate helicase activity to an extent similar to the wild-type enzyme.

It is possible that only the full-length Cdc6-2 protein stimulates helicase activity because the other proteins used in the study are not properly folded. Analysis of the Far-UV CD spectra of all these proteins (Figure 5A) provided very similar secondary structure contents (Table 1). Moreover, thermal denaturation studies showed cooperative thermal unfolding for all the proteins (except for the WH; Figure 5B), indicative of compact tertiary structure, in contrast to proteins displaying loose tertiary packing but retaining significant secondary structure (e.g. molten globules) which display broad unfolding transitions or even no transition (26,27). Overall these results indicated that all proteins (except possibly the WH) are globally folded (i.e. similar amount of secondary structure and tightly packed).

MCM complex interacts with Cdc6-2 protein

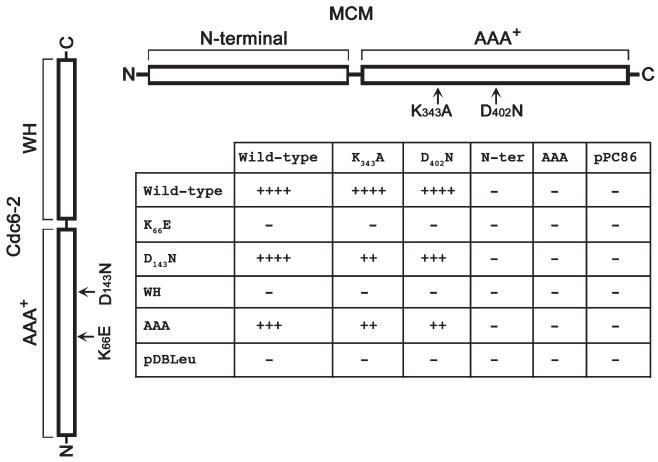
It was previously shown that the MCM protein interacts with Cdc6-2 (5). The data presented in Figure 4 demonstrate that only the intact Cdc6-2 proteins can stimulate helicase activity; neither the WH nor the AAA^+ domains

can stimulate the helicase. Thus, the data may suggest that only the full-length Cdc6-2 protein can bind MCM protein. This is not the case, however. Two-hybrid analysis demonstrated that both the full-length and the AAA^+ catalytic portion of Cdc6-2 could interact with MCM (Figure 3). However, no interaction between Cdc6-2 protein and either the N- or C-terminal part of MCM could be detected. Although the AAA^+ domains of Cdc6-2 interact with MCM, helicase activity is not stimulated (Figure 4). This suggests that interaction with the helicase is not sufficient to stimulate its activity.

Table 1. Secondary structure analysis and thermal stability as determined by Far-UV CD spectroscopy

	Secondary structure (%)		T_m (°C)
	α-helix	β-sheet	
Wild-type	40	14	63.8
$K_{66}E$	43	12	57.4
$D_{143}N$	42	13	57.8
AAA^+	38	15	63.2
WH	38	15	NA
N1C2	37	15	51.4/60.6[a]
N2C1	42	13	62.6

[a]T_m values for two overlapping thermal transitions.
NA, not applicable.



	Wild-type	$K_{343}A$	$D_{402}N$	N-ter	AAA	pPC86
Wild-type	++++	++++	++++	-	-	-
$K_{66}E$	-	-	-	-	-	-
$D_{143}N$	++++	++	+++	-	-	-
WH	-	-	-	-	-	-
AAA	+++	++	++	-	-	-
pDBLeu	-	-	-	-	-	-

Figure 3. The *T. acidophilum* Cdc6-2 protein interacts with MCM. Two-hybrid analysis was performed as described in Material and methods section. Cell growth observed after 24 h is marked as ++++, +++ after 48 h and ++ after 72 h.

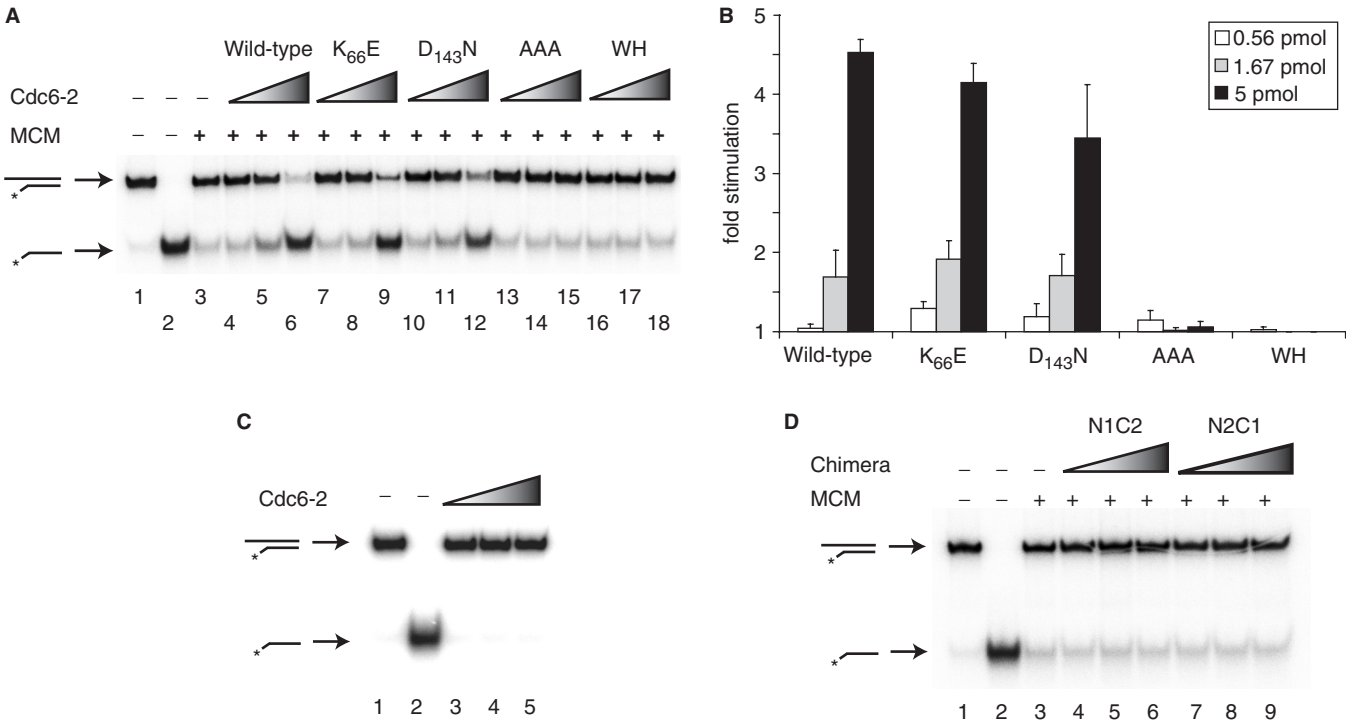


Figure 4. Only the full-length *T. acidophilum* Cdc6-2 protein can stimulate MCM helicase activity. (**A**) DNA helicase assays were performed as described in Material and methods section using 0.32 pmol of MCM protein as monomer with increasing amounts of Cdc6-2 full-length (lanes 4–6), Walker-A mutant ($K_{66}E$) (lanes 7–9), Walker-B mutant ($D_{143}N$) (lanes 10–12), AAA domains (lanes 13–15) and WH domain (lanes 16–18). Lane 1, substrate only; lane 2, boiled substrate; lane 3, MCM protein only; lanes 4, 7, 10, 13 and 16, 0.56 pmol Cdc6 protein as monomer; lanes 5, 8, 11, 14 and 17, 1.67 pmol Cdc6 protein as monomer; lanes 6, 9, 12, 15 and 18, 5.0 pmol Cdc6 protein as monomer. A representative gel is shown. (**B**) The average of three independent experiments with standard deviation is shown. (**C**) DNA helicase assays were performed as described in Material and methods section in the absence of MCM but in the presence of increasing amounts of full-length Cdc6-2. Lane 1, substrate only; lane 2, boiled substrate; lane 3, 0.56 pmol Cdc6-2; lane 4, 1.67 pmol Cdc6-2; lane 6, 5.0 pmol Cdc6-2. (**D**) DNA helicase assays were performed as described in Material and methods section using 0.32 pmol of MCM as monomer with increasing amounts of the Cdc6-N1C2 (lanes 4–6) and Cdc6-N2C1 (lanes 7–9) chimeras. Lane 1, substrate only; lane 2, boiled substrate; lane 3, MCM protein only; lanes 4 and 7, 0.56 pmol chimeric protein as monomer; lanes 5 and 8, 1.67 pmol chimeric protein as monomer; lanes 6 and 9, 5.0 pmol chimeric protein as monomer.

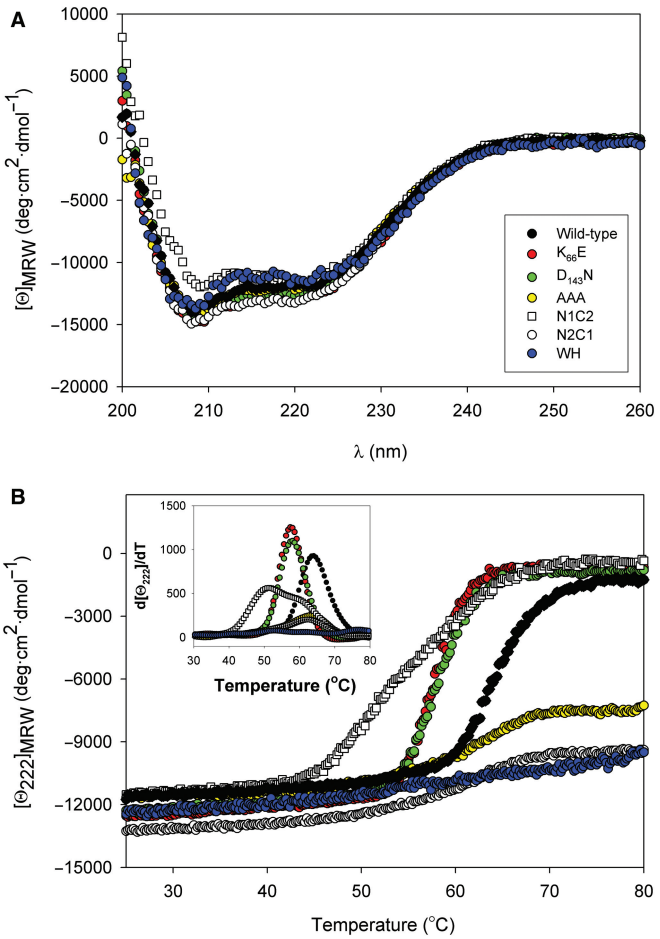


Figure 5. All mutant proteins are properly and similarly folded. Circular dichroism (CD) analyses were performed as described in Material and methods section. (**A**) Far-UV CD spectra at 25°C. (**B**). Thermal denaturation monitored by following changes in ellipticity at 222 nm; inset: first derivatives. Symbols are shown in panel (**A**).

These results are reminiscent of those observed with the proteins from *M. thermautotrophicus*. In *M. thermautotrophicus*, the interaction between Cdc6 and MCM inhibits helicase activity (20). However, it was shown that although the WH motif of Cdc6 can bind MCM it is not sufficient for helicase inhibition (21).

Cdc6-2 protein does not affect DNA binding by MCM. All archaeal MCM and Cdc6 proteins studied to date interact with ss and dsDNA (1). The interactions between DNA and MCM were demonstrated using structural and biochemical studies including electron micrograph reconstruction, filter binding assays, gel-mobility shift assays and fluorescence polarization anisotropy (FPA) (2,28). The interactions between Cdc6 proteins and DNA were demonstrated using filter binding assays, gel-mobility shift assays, protein phosphorylation and protein crystallization (11,12,15–17). Thus, it was surprising that no interaction could be detected between DNA and MCM protein or DNA and Cdc6-1 and -2 proteins from *T. acidophilum* using filter-binding assays (data not shown). However, FPA detected interactions between MCM and ssDNA with a K_d of 117.6 nM (data not shown). No interaction between Cdc6-1 and -2 protein and DNA could be detected using FPA under several experimental conditions tested (see Material and Methods section; data not shown). Also, no stimulation of DNA binding by MCM protein was observed in the presence of Cdc6-2 protein using either filter-binding assays or FPA (data not shown). These data suggest that the stimulation of MCM helicase activity by Cdc6-2 protein is not via the regulation of MCM–DNA interaction.

Cdc6-2 protein stimulates the ATPase activity of the MCM helicase. All helicases use the energy derived from nucleoside triphosphate hydrolysis to translocate along DNA. As ATP hydrolysis is required for helicase activity, it is possible that the interaction with Cdc6-2 protein stimulates the ATPase activity of *T. acidophilum* MCM protein and thus the helicase activity. To address this possibility, the ATPase activity of MCM protein in the presence and absence of Cdc6-2 and its derivatives was determined. As shown in Figure 2 the ATPase activity of MCM is rather low and is about the same whether DNA is present or not (Figure 2, lane 1). This is in contrast to other archaeal MCM proteins studied where DNA stimulates the helicase activity [e.g. (29,30)]. Nevertheless, in the presence of the wild-type Cdc6-2 protein the ATPase activity of MCM is stimulated four to 5-fold (Figure 2, lane 2). Cdc6-2 is also a member of the AAA^+ ATPases and thus it is possible that MCM protein stimulates the ATPase activity of Cdc6-2 rather than the other way around. Thus, as a control, a mutant MCM protein, $K_{343}A$, which lacks helicase and ATPase activities (5) was tested with the Cdc6-2 protein. No ATPase activity could be observed in the reaction whether DNA was present or not (Figure 2, lane 17), although the mutant MCM protein interacts with Cdc6-2 protein (Figure 3), as has previously been reported for the wild-type MCM protein (5) (Figure 3). These results are not surprising as, to date, no catalytic ATPase activity by any archaeal Cdc6 protein has been reported. These results demonstrate that the stimulation of ATPase activity when MCM and Cdc6-2 proteins are present in the reaction are not due to the effect of MCM on the ATPase activity of Cdc6-2 but rather the stimulation of the MCM activity by Cdc6-2 protein.

After establishing that Cdc6-2 protein stimulates the ATPase activity of MCM, the regions of the Cdc6-2 protein required for the stimulation were determined. Only the full-length Cdc6-2 protein stimulates the activity, as none of the truncated or chimeric proteins stimulate the activity (Figure 2, lanes 5–8). The Walker-A ($K_{66}E$) and -B ($D_{143}N$) mutant proteins, on the other hand, stimulate the ATPase activity (Figure 2, lanes 3 and 4), demonstrating that ATP binding and hydrolysis by Cdc6-2 is not required. This is in agreement with the stimulation of helicase activity observed by these mutant proteins (Figure 4). It is also consistent with the previously reported observation, using a pull-down assay, showing that ATP binding is not required for Cdc6-2 interaction with MCM (5). However, using two-hybrid analysis

the Walker-A mutant protein did not interact with MCM (Figure 3). It is possible that this mutant is not properly folded in yeast.

DISCUSSION

The elongation phase of DNA replication has been extensively studied in several archaea. The initiation process, however, is not well understood. Furthermore, studies on the initiation process in *M. thermautotrophicus*, *S. solfataricus*, *Archaeoglobus fulgidus* and *Pyrococcus furiosus* revealed that the initiation process and proteins involved are different in different organisms [for example, the composition of replication protein A (RPA), the composition of proliferating cell nuclear antigen (PCNA), etc.] (3,31). Previous studies on the initiation proteins from the archaeon *T. acidophilum* have shown that one of the Cdc6 proteins stimulates the MCM helicase activity. This observation is in sharp contrast to results reported with the proteins from *M. thermautotrophicus* and *S. solfataricus*, where interaction between the Cdc6 and MCM proteins inhibits helicase activity (20–22,32). The study described here addresses the mechanism of stimulation.

The *T. acidophilum* and other MCM helicases require ATP hydrolysis to fuel the unwinding reaction. All archaeal MCM proteins studied were shown to possess ATPase activity which is stimulated in the presence of ssDNA (1,3). In contrast to the other archaeal MCM proteins studied, it is shown here that the *T. acidophilum* MCM possesses a low ATPase activity which is not significantly stimulated in the presence of DNA (Figure 2). The inability of DNA to stimulate ATP hydrolysis by MCM may be the reason for the low helicase activity in the absence of Cdc6-2. The ATPase activity is substantially stimulated when MCM interacts with Cdc6-2 protein (Figure 2) suggesting that by stimulating the intrinsic ATPase activity of MCM the Cdc6-2 protein stimulates helicase activity.

The binding of Cdc6-2 protein to MCM likely results in conformational changes in the MCM complex which leads to catalytic ATP hydrolysis by MCM. The structure of multimeric members of the AAA^+ family of ATPases revealed that the ATP binding and hydrolysis sites are located at the interface of the subunits (33,34). It was shown that residues from adjacent subunits are also required for ATP hydrolysis. The Walker-A and -B motifs, which are involved in ATP binding and hydrolysis of ATP are contained within one subunit and thus work in *cis*. ATP hydrolysis is also regulated by *trans* acting motifs. One, referred to as an Arg-finger (35), contains an Arg residue and is required for ATP hydrolysis of ATP bound to the neighboring subunit. As the Arg-finger is essential for catalytic ATP hydrolysis (36), it is possible that the binding of Cdc6-2 to MCM protein alters the helicase structure in a way that brings the Arg-finger into close proximity to the ATP and results in the stimulation of ATP hydrolysis.

Alternatively, the Cdc6 protein may have an analogous function to the bacterial primase, the DnaG protein. It was shown that DnaG binding to the helicase, DnaB, stimulates both the helicase ATPase and helicase activities (37–39). Structural studies demonstrated that DnaG forms a 'collar' which holds the DnaB hexamers. Although, DnaB protein forms stable hexamers it was proposed that DnaG stabilized the hexameric ring and thus stimulated the activity of the helicase (37). Similarly, although the *T. acidophilum* MCM forms stable hexamers in solution (5), Cdc6-2 may stabilize the structure in a fashion similar to DnaG.

This is not the first example of an effect on MCM helicase activity by stimulating the intrinsic ATPase activity of the helicase. It was recently shown that the activity of *P. furiosus* MCM is very limited, but the activity is substantially stimulated upon interaction with the GINS complex (40). It was found that GINS stimulates helicase activity by stimulating ATP hydrolysis by MCM (40).

Based on primary amino acid sequence analysis, the archaeal Cdc6 proteins can be divided into two distinct subgroups, subgroup I and II (8,10). Structural (11,12) and biochemical analysis (13,15–17) suggest that subgroup I is involved in origin recognition. Other studies suggest that members of both groups also participate in helicase loading at the origin and play other regulatory roles (20–22). The *T. acidophilum* Cdc6-1 belongs to subgroup II while Cdc6-2 is in subgroup I (10). Thus, it is likely that Cdc6-2 protein is involved in origin binding in addition to its role in regulating helicase activity described here. Thus, *T. acidophilum* may be similar to *M. thermautotrophicus* in which the Cdc6 homologue that recognizes the origin, Cdc6-1 (15–17), also regulates MCM activity (20).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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