ATP Hydrolysis and DNA Binding Confer Thermostability on the MCM Helicase[†]

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ABSTRACT: The minichromosome maintenance (MCM) helicase is the replicative helicase in archaea. The enzyme utilizes the energy derived from ATP hydrolysis to translocate along one strand of the DNA and unwind the complementary strand. Here, the effect of DNA and ATP on the thermostability of the *Methanothermobacter thermautotrophicus* MCM protein was determined by differential scanning calorimetry. The MCM protein shows a single thermal transition at 67 °C. The stability is dramatically altered with the appearance of a second thermal transition up to 10 °C higher in the presence of DNA and either ATP or ADP-AlF_4^-, a transition-state analogue of ATP, bound to MCM. In the presence of DNA and ADP or the nonhydrolyzable ATP analogues ATPγS and AMP-PNP, however, only a single thermal transition is observed at temperatures slightly higher than the transition temperature of MCM alone. Thus, the results suggest that ATP hydrolysis proceeds through a transition state that decouples an interaction between the N-terminal DNA binding domain and the C-terminal catalytic domain in the presence of DNA.

The minichromosome maintenance (MCM)[1] helicase is thought to function as the replicative helicase in archaea. It uses the energy derived from ATP hydrolysis to translocate along one strand of the DNA and to unwind the complementary strand. In contrast to eukarya, in which MCM is a family of six related polypeptides, most archaeal species contain a single homologue of MCM. However, the single archaeal MCM homologue possesses biochemical properties which are similar to the eukaryotic enzyme (summarized in refs *1* and *2*). One of the extensively studied archaeal MCM proteins is from the archaeon *Methanothermobacter thermautotrophicus*. The enzyme possesses an ATP-dependent 3′→5′ helicase activity, can bind and translocate along single-strand (ss) and double-stranded (ds) DNA, can unwind DNA−RNA hybrids while translocating along the DNA strand, and can displace proteins from DNA (*3*).

The *M. thermautotrophicus* MCM protein is a member of the AAA^+ family of ATPases and can be divided into three parts: a N-terminal part, an AAA^+ catalytic region, and a C-terminal helix−turn−helix (HTH) domain. The N-terminal part is involved in multimerization, ssDNA and dsDNA binding, and regulation of helicase activity (*4*−*6*). The center part of the molecule contains the AAA^+ conserved motifs needed for ATPase and helicase activities (*7, 8*). The C-terminal part of the molecule contains a putative HTH domain that may be involved in regulation (*9*).

The ATPase activity of the enzyme is stimulated in the presence of DNA (*7, 8, 10*) so mutations that abolish DNA binding also abolish the stimulation of ATPase activity (*11*). ATP stimulates MCM binding to DNA (*10, 11*), but ATPγS, an structural analogue of ATP, has only a limited effect on DNA binding. The stimulatory effect of ATP on DNA binding is only observed when the reaction is incubated at 50−60 °C, the optimal growth temperature for *M. thermautotrophicus* and for *in vitro* helicase and ATPase activities. When the DNA binding reactions were incubated at 22 °C, stimulation of DNA binding could not be detected in the presence of ATP (*7, 9, 12*). These observations suggest that the mechanism of ATP and DNA binding involves temperature-induced changes in the conformation of the enzyme. This is supported by electron micrograph structural studies of MCM which revealed different conformations of the enzyme in the presence of nucleotides and DNA (*13, 14*).

Here the effect of DNA and ADP, ATP, or the ATP analogues ATPγS, AMP-PNP, or ADP-AlF_4^- on the conformational stability of the *M. thermautotrophicus* MCM helicase was studied by differential scanning calorimetry (DSC). ADP-AlF_4^- is a transition state analogue of ATP, and ADP is the product of hydrolysis of ATP. Conformational changes in enzymes are reflected in changes in their thermostabilities, which can be expeditiously monitored by DSC. It was found that while ATP, ADP-AlF_4^-, and DNA by themselves have a limited effect on the conformational stability of the MCM protein, the conformational stability of MCM is substantially altered when DNA interacts together

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[1] Abbreviations: AAA^+, ATPase associated with various cellular activities; ATP, adenosine 5′-triphosphate; DSC, differential scanning calorimetry; HTH, helix−turn−helix; ITC, isothermal titration calorimetry; MCM, minichromosome maintenance.

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with either ATP or ADP-AlF_4^-. In addition, when the nonhydrolyzable nucleotide analogues ATPγS, AMP-PNP, or ADP were tested either by themselves or with DNA, only limited changes in the conformational stability of bound MCM could be observed. These results suggest that ATP hydrolysis, and not just ATP binding alone, in the presence of DNA is required for changes in the conformational stability of MCM and may suggest that ATP hydrolysis is needed for the key conformational changes in the helicase.

MATERIALS AND METHODS

ATP and [γ-^{32}P]ATP were obtained from GE Healthcare, AMP-PNP was obtained from Roche, ADP and ATPγS were obtained from Sigma-Aldrich, ϕX174 ssDNA was obtained from New England Biolabs, and oligonucleotides were synthesized by the CARB DNA Synthesis Facility or Integrated DNA Technologies. All proteins used in the study were purified as previously described (*6*). The DSC buffer contains 20 mM Hepes−NaOH (pH 7.5), 100 mM NaCl, and 10% glycerol.

DSC Measurements. The purified proteins were dialyzed at room temperature against DSC buffer. Protein concentrations were determined using the UV absorbance at 280 nm and a calculated extinction coefficient (ϵ_{280}) of 28730 $cm^{-1}\cdot M^{-1}$. The solution outside the dialysis bag was retained and used as a reference solution for the experiments. Oligonucleotide substrates were resuspended in DSC buffer. ϕX174 DNA was dialyzed against H_2O, lyophilized, and resuspended in DSC buffer. The DNA concentration was determined using the UV absorbance at 260 nm. All of the nucleotide and DNA mixtures with MCM protein were incubated at 60 °C for 10 min prior to the DSC measurements.

DSC measurements were performed using a VP-DSC microcalorimeter from Microcal Inc. (Northampton, MA). The volume of the solution and the reference vessels was 0.511 mL, and the scan rate was either at a slow rate of 15 K h^{-1} or a medium rate of 60 K h^{-1}, with the temperature ranging from 25 to 85 °C. Since the scans of protein samples were irreversible, the second scan for each sample was used as the baseline. After subtraction of the baseline from the protein scan, the resulting raw data of the differential power as a function of time were divided by the scan rate to convert the data into a heat capacity versus temperature scan. Although the transitions did not reappear upon a rescan of the solutions, the transition temperatures were found to be scan rate independent and, thus, could be analyzed by application of a thermodynamic transition model to the results (*15*). Utilizing the EXAM program (*16*), a two state, A ⇔ B, transition model was then fitted to the heat capacity as a function of temperature to determine the van't Hoff enthalpy ($\Delta_{trs}H_{VH}$) for the scan from the shape of the transition peak; a transition temperature (T_G) and a calorimetric transition enthalpy ($\Delta_{trs}H_{cal}$) was calculated from the area under the transition peak (millijoules) divided by the moles of protein in the sample cell (concentration of protein × 0.511 mL). DSC scans on the samples were repeated twice.

For each experiment 10 μM MCM protein, 5 mM $MgCl_2$, 1 mM nucleotides, and 108 μg of N190, PB4, N191, and N192 oligonucleotides (Table 1) or ϕX174 ssDNA were prepared in 700 μL of DSC buffer. The samples were incubated at 60 °C for 10 min, placed on ice for 5 min, and centrifuged at 13000 rpm for 10 min. Partial ATP hydrolysis in the presence of DNA, 1 mM ATP, and 10 μM MCM prior to the DSC scan was confirmed by the ATPase activity assay described below. The protein concentration of the supernatant was determined using the Bradford assay. The remaining sample was loaded into the DSC sample cell. Several DSC measurements were also performed on samples containing ATP, DNA, and MCM mixtures without prior incubation to determine if the hydrolysis reaction during the DSC scan had any effect on the DSC results.

Table 1: Oligonuclotides Used for the Study

N190	5′-CGCAGATAACAGTTGTCCTGGAGAACG ACCTGGTTGACACCCTCACACCC-3′
N191	3′-CGCAGATAACAGTTGTCCTGGAGAA-5′ 5′-CGACCTGGTTGACACCCTCACACCC-3′
N192	5′-CGCAGATAACAGTTGTCCTGGAGAA-3′ 3′-CGACCTGGTTGACACCCTCACACCC-5′
DF74	5′-GGGACGCGTCGGCCTGGCACGTCGGCCGCTG CGGCCAGGCACCCGATGGCGTTTGTTTGTTTGTTTGTTT GTTT-3′
DF61	5′-TTTGTTTGTTTGTTTGTTTGTTTGTTTGT TTGCCGACGTGCCAGGCCGACGCGTCCC-3′
PB4	5′-TTTGTTTGTTTGTTTGTTTGGGCCACCTAAGCTATTTT CAAAATCGCGAGTAAACGGCTTAGATTAGCGTAGGAC AACACCAATATCTGGGTAGCATATGCTATCCTAATCTA TATATCCTATATAAATATATCGC-3′

The binding of ligands, such as ATP and DNA, to the MCM protein results in a shift of the transition temperature T_G to a higher temperature. Estimates of the temperature shifts at the transition temperature are as follows:

$$1/T_G - 1/T_G(L) = (R/\Delta_{trs}H)\ln\{1.00 + ([L_0] - [P_0]/2)K_b(T_G(L))\} \quad (1)$$

where $K_b(T_G)$ is the binding constant of the ligand at the transition temperature, T_G is the transition temperature of the MCM alone, $T_G(L)$ is the transition temperature of the complex, $\Delta_{trs}H$ is the transition enthalpy of the complex, $[L_0]$ is the total initial ligand concentration, and $[P_0]$ is the initial MCM concentration (*17*).

For extrapolation of the ligand binding constants determined at 25 °C up to the transition temperature of the MCM−ligand complex, $T_G(L)$

$$\ln[K_b(T_G(L))/K(T_0)] = [(-\Delta_bH(T_0) + \Delta_bC_pT_0)/R](1/T_G(L) - 1/T_0) + (\Delta_bC_p/R)\ln(T_G(L)/T_0) \quad (2)$$

where $\Delta_bH(T_0)$ is the binding enthalpy at $T_0 = 298.15$ K (25 °C) and Δ_bC_p is the heat capacity change for the binding reaction.

ATPase Assay. The ATPase activity was measured in reaction mixtures (15 μL) containing 25 mM Hepes−NaOH (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$, 100 μg/mL bovine serum albumin (BSA), 50 ng of ϕX174 ssDNA, 1500 pmol of [γ-^{32}P]ATP (3000 Ci/mmol; GE Healthcare) (100 μM final ATP concentration), and 50 nM MCM protein (as monomer) in the absence or presence of ADP, AMP-PNP, ATPγS, or ADP-AlF_4^- (10, 50, 100, 200, or 400 μM). The ATP and the nucleotide analogues were premixed before addition to the reaction mixtures. After incubation at 60 °C for 60 min, samples were placed on ice to stop the reaction.

Table 2: Thermostabilities of Wild-Type MCM Protein in the Absence or Presence of Various Nucleotides and DNA

nucleotide[a]	DNA[b]	T_G (°C)	$\Delta_{trs}H_{vH}$ (kJ mol^{-1})	$\Delta_{trs}H$ (kJ mol^{-1})
−	−	66.7 ± 1.1	836 ± 77	376 ± 149
	+	65.9 ± 0.6	693 ± 228	378 ± 8
ATP	−	71.5 ± 0.6	731 ± 33	569 ± 30
	+	68.9 ± 0.5, 75.3 ± 0.3	639 ± 95, 981 ± 65	625 ± 68, 179 ± 121
ADP-AlF_4^-	−	70.3 ± 0.5	778 ± 4	433 ± 91
	+	69.5 ± 0.4, 80.9 ± 0.5	762 ± 77, 1289 ± 1	106 ± 42, 283 ± 35
ATPγS	−	72.5 ± 0.0	749 ± 53	391 ± 84
	+	71.8 ± 0.3	842 ± 45	188 ± 54
AMP-PNP	−	68.2 ± 0.2	812 ± 62	474 ± 21
	+	67.5 ± 0.7	834 ± 26	367 ± 41
ADP	−	69.9 ± 0.5	745 ± 11	554 ± 80
	+	69.9 ± 0.4	819 ± 61	419 ± 22

[a] In the absence (−) or presence of 1 mM nucleotides. [b] In the absence (−) or presence (+) of 10 μM (108 μg) N190 (3′−5′) oligonucleotide.

A 1 μL aliquot was spotted onto a polyethylenimine cellulose thin-layer chromatography plate, and ATP and P_i were separated by chromatography in 1 M formic acid and 0.5 M lithium chloride. The extent of ATP hydrolysis was quantitated by phosphorimager analysis. All ATPase assays were repeated three times, and their averages with standard deviations are shown in the figure.

To study the effect of the different DNA substrates on ATP hydrolysis, the reaction conditions described above were used in the absence or presence of 1 ng of N190 (3′−5′), N191 (3′−3′), or N192 (5′−5′) oligonucleotide (Table 1) or ϕX174 ssDNA. After incubation at 60 °C for 30 and 60 min, samples were placed on ice. A 1 μL aliquot was spotted onto a polyethylenimine cellulose thin-layer chromatography plate, and ATP and P_i were separated by chromatography in 1 M formic acid and 0.5 M lithium chloride. The extent of ATP hydrolysis was quantitated by phosphorimager analysis. All ATPase assays were repeated three times, and their averages with standard deviations are shown in the figure.

DNA Helicase Assay. Substrates for helicase assays were made as previously described (*18*) using the DF74 and DF61 oligonucleotides (Table 1). DNA helicase activities were measured in reaction mixtures (15 μL) containing 20 mM Tris-HCl (pH 8.5), 2 mM DTT, 10 mM $MgCl_2$, 100 μg/ml BSA, 1 mM ATP, 10 fmol (0.66 nM) of ^{32}P-labeled substrate, and 25 nM MCM protein (as monomer) in the absence or presence of ADP, AMP-PNP, ATPγS, or ADP-AlF_4^- at 0.2, 0.5, 1, 2, or 4 mM. The analogues were premixed with ATP prior to addition to the reaction. Following incubation at 60 °C for 1 h the reactions were stopped by adding 5 μL of loading buffer (1% SDS, 100 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue, and 50% glycerol) and chilling on ice. Aliquots (10 μL) were fractionated on an 8% native polyacrylamide gel in 0.5× TBE for 40 min at 180 V. Gels were visualized and quantitated by phosphorimaging. All helicase experiments were repeated three times, and their averages with standard deviations are shown in the figure.

Isothermal Titration Calorimetry (ITC). Isothermal titration calorimetry (ITC) experiments were performed using a MicroCal VP-ITC. The ITC contained 10 μM MCM protein (as monomer) in the solution vessel (1.43 mL) and 200 μM DNA in the stirrer syringe at 25 °C. The ITC buffer was 25 mM Hepes−NaOH (pH 7.5), 100 mM NaCl, 10% glycerol, and 10 mM $MgCl_2$. DNA aliquots of 4−8 μL were sequentially injected.

Tryptophan Fluorescence. Intrinsic tryptophan fluorescence of MCM proteins was measured in the presence or absence of DNA using FluoroMax-3 (Jon Yvon). Each sample was prepared in a separate tube with a final volume of 150 μL, containing 25 mM Hepes−NaOH (pH 7.5), 2 mM DTT, 5 mM $MgCl_2$, and 100 nM wild-type or β-hairpin mutant MCM protein (as monomer), with or without 1 μg of N190 (3′−5′), N191 (3′−3′), or N192 (5′−5′) oligonucleotide (Table 1) or ϕX174 ssDNA. The samples were incubated at room temperature for 10 min, and spectra were measured at 25 °C. Excitation was set at 295 nm, and emission spectra of 310−400 nm were taken.

RESULTS

ATP Increases the Thermal Stability of MCM in the Presence of DNA. MCM activity is dependent upon ATP binding and hydrolysis, perhaps through alterations in the interactions between the structural DNA binding and catalytic domains of the MCM protein. In order to determine if this indeed occurs, DSC analysis of the wild-type enzyme was performed in the presence and absence of ATP (Table 2). A mutant protein ($K_{325}A$) that cannot bind (*19*) nor hydrolyze (*7*) ATP was used as a control. Another control was a β-hairpin ($RK_{227,229}AA$) MCM mutant protein that cannot bind DNA (*4, 5*). All samples (except when indicated otherwise) were incubated at 60 °C for 10 min prior to the DSC measurements. ATP hydrolysis alone increases the transition temperature of MCM (Table 2) and of the β-hairpin mutant (Table 3) about 5 °C while no such increase was observed with the $K_{325}A$ mutant (Table 3). If the inverse of the K_M from the enzyme assay of ATP interacting with MCM near the transition temperature is assumed to be the binding constant of ATP to MCM, then the temperature shift can be estimated from eq 1. The K_M is 0.4 mM at 60 °C (data not shown), and this would produce at 1 mM ATP and 10 μM MCM concentrations and a transition enthalpy of 366 kJ mol^{-1} a shift of the transition temperature to 69.2 °C, close to the observed value of 71 ± 0.6 °C in Table 2.

In addition to ATP binding, the helicase also interacts with DNA. It was shown that DNA binding to MCM changes the structure of the helicase (*14*) and, thus, may also alter the interactions between the structural domains of MCM, resulting in changes in its conformational stability. To determine the effect of DNA on MCM stability, DSC experiments were performed in the presence and absence of oligonucleotides. As shown in Tables 2 and 3, DSC scans of the wild-type MCM, the $K_{325}A$, and the β-hairpin mutant proteins are unaffected by the presence of DNA in the sample. This lack of a transition temperature shift indicates

Table 3: Thermostabilities of Mutant MCM Proteins in the Absence or Presence of Various Nucleotides and DNA

mutant[a]	nucleotide[b]	DNA[c]	T_G (°C)	$\Delta_{trs}H_{vH}$ (kJ mol^{-1})	$\Delta_{trs}H$ (kJ mol^{-1})
$K_{325}A$	−	−	68.1 ± 0.8	923 ± 14	396 ± 7
		+	67.7 ± 0.1	814 ± 23	412 ± 27
	ATP	−	68.3 ± 0.2	950 ± 44	336 ± 7
		+	68.1 ± 0.2	1015 ± 181	298 ± 203
	ADP-AlF_4^-	−	68.7 ± 0.8	887 ± 30	584 ± 204
		+	68.6 ± 0.8	889 ± 63	370 ± 56
	ATPγS	−	68.2 ± 0.2	433 ± 273	599 ± 323
		+	68.2 ± 0.2	721 ± 211	426 ± 228
	AMP-PNP	−	68.4 ± 0.2	849 ± 80	484 ± 81
		+	67.6 ± 0.2	944 ± 68	384 ± 8
	ADP	−	68.8 ± 0.4	893 ± 1	422 ± 19
		+	68.4 ± 0.3	847 ± 75	385 ± 37
β-hairpin	−	−	65.9 ± 0.6	1379 ± 76	305 ± 95
		+	65.3 ± 0.1	1034 ± 173	317 ± 56
	ATP	−	70.9 ± 0.3	787 ± 62	494 ± 66
		+	70.2 ± 0.3	819 ± 33	386 ± 97
	ADP-AlF_4^-	−	68.2 ± 1.0	947 ± 134	299 ± 23
		+	68.7 ± 0.4	942 ± 10	283 ± 26
	ATPγS	−	71.6 ± 0.3	642 ± 112	345 ± 208
		+	71.1 ± 0.1	486 ± 8	560 ± 250
	AMP-PNP	−	66.4 ± 0.2	1129 ± 97	283 ± 26
		+	66.2 ± 0.3	1064 ± 37	342 ± 23
	ADP	−	68.7 ± 0.0	748 ± 58	479 ± 63
		+	68.3 ± 0.0	903 ± 51	322 ± 28

[a] 10 μM mutant proteins were used. [b] In the absence (−) or presence of 1 mM nucleotides. [c] In the absence (−) or presence (+) of 10 μM (108 μg) N190 (3′−5′) DNA substrate.

that DNA does not bind to MCM at the transition temperature in the absence of ATP, although at 25 °C, MCM binds to ssDNA with a high affinity of 7.5×10^6 M^{-1} (*12*). If the binding constant of DNA at the transition temperature shifts the temperature within the uncertainty of 1 °C in the T_G values, then the binding constant at the transition temperature would be less than 1.245×10^2 M^{-1} according to eq 1. This would imply a binding enthalpy ($\Delta_b H$(25 °C)) of −226 kJ mol^{-1} in the absence of any heat capacity change, $\Delta_b C_p = 0$ according to eq 2. Isothermal titration calorimetry measurements performed at 25 °C on the binding of DNA to MCM were, however, unsuccessful since the DNA−MCM binding enthalpy at this temperature is close to zero. Since $\Delta_b H$(25 °C) = 0 in eq 2, then a reduction of K_b from 7.5×10^6 M^{-1} at 25 °C to below 1.245×10^2 M^{-1} at 66 °C would yield a binding heat capacity change of $\Delta_b C_p = 443$ J K^{-1} mol^{-1}. This large positive heat capacity change is characteristic of the unfolding of a protein in solution.

The effect of ATP hydrolysis on the conformational stability of MCM in the presence of DNA was also determined by DSC. When the wild-type MCM was studied in the presence of DNA following the partial hydrolysis of ATP during the 10 min incubation period, two transition peaks were observed at temperatures higher than the transition temperatures of MCM alone as shown in Figure 1 and summarized in Table 2. It was found that up to 70% hydrolysis of the ATP to ADP occurred in the presence of DNA during the 10 min incubation at 60 °C prior to the DSC scan (data not shown). DSC scans were also performed on mixtures containing ATP, DNA, and MCM without prior incubation. The DSC scans on these samples result in transitions at 68.6 ± 0.1 and 74.8 ± 0.2 °C. The van't Hoff enthalpies of 715 ± 11 and 802 ± 168 kJ mol^{-1} (data not shown), respectively, were within experimental error of the DSC results from the experiment that include the 10 min incubation at 60 °C shown in Table 2. Therefore, changes in the 1 mM ATP substrate concentration during the 8 min DSC scan between the two transitions had a negligible effect on the DSC results. Such enhancement in stabilization was not observed for the $K_{325}A$ mutant protein, which does not bind to ATP, and for the β-hairpin mutant protein, which does not bind DNA (Table 3). The presence of two unfolding transitions would indicate independent unfolding of two thermodynamic domains of MCM upon binding of ATP and DNA, and these domains could be identified as the DNA binding domain and the ATP-bound catalytic domain in the transition state to the MCM. The lower temperature transition exhibits a transition temperature within the experimental uncertainty of the transition temperatures of MCM following complete hydrolysis of ATP alone. The unfolding transition at the lower temperature would not correspond to unfolding of the MCM alone since the transition temperature of this transition is several degrees higher than that of the unfolding transition of just the MCM protein. The higher temperature transition, then, must be that of the DNA binding to its domain with a binding affinity enhanced by the presence of ATP. The average ratios of $\Delta_{trs}H_{cal}/\Delta_{trs}H_{VH}$ for MCM with either DNA alone or without DNA but following complete hydrolysis of ATP to ADP have an upper limit of 0.8, close to 1 so that the two domains unfold as single entities in the absence and presence of either DNA or ATP. The higher transition temperature reveals a substantial stabilization of the enzyme in comparison to MCM alone (about 8 °C). This temperature increase would correspond to a binding constant of 3.14×10^4 M^{-1} for DNA binding to its structural domain at the transition temperature of MCM protein according to eq 1.

Since MCM protein can both bind and hydrolyze ATP, the bimodal transition stabilization observed in Figure 1 may be due to a decoupling effect between the catalytic domain binding ATP and the N-terminal domain binding DNA. In order to determine the source of this decoupling effect between the two domains of MCM, DSC measurements were performed on MCM in the presence of the ATP analogues ADP-AlF_4^-, AMP-PNP, ATPγS, and ADP. ADP-AlF_4^-, AMP-PNP, and ATPγS have been used as nonhydrolyzable analogues of ATP while ADP is the product of ATP hydrolysis. In several systems, ADP-AlF_4^- was also shown to be a transition state analogue in the hydrolysis of ATP (ref *20* and references cited therein).

The different analogues exhibit different effects on the thermostability of MCM (Figure 1 and Table 2). AMP-PNP has very little effect on the stability of MCM whether DNA is present or not (Table 2). This may suggest that AMP-PNP either does not bind to MCM or binds it very weakly (discussed below). In the absence of DNA, ADP stabilized MCM almost as much as ATP. When ATPγS was used as a cofactor in the absence of DNA, the stability of the enzyme was the same as in the presence of ATP, indicating that it has the same binding affinity to MCM as ATP at the MCM transition temperature. The stabilization effects of the different analogues according to the increase in transition temperatures are in the order ATPγS > ATP > ADP-AlF_4^- > ADP > AMP-PNP (Table 2). The additional presence of DNA had no effect on the conformational thermal stabilities of MCM complexed directly with ATPγS, ADP, or AMP-PNP. When ADP-AlF_4^- was used, two transition peaks were observed. While the first transition of the ADP-AlF_4^- MCM

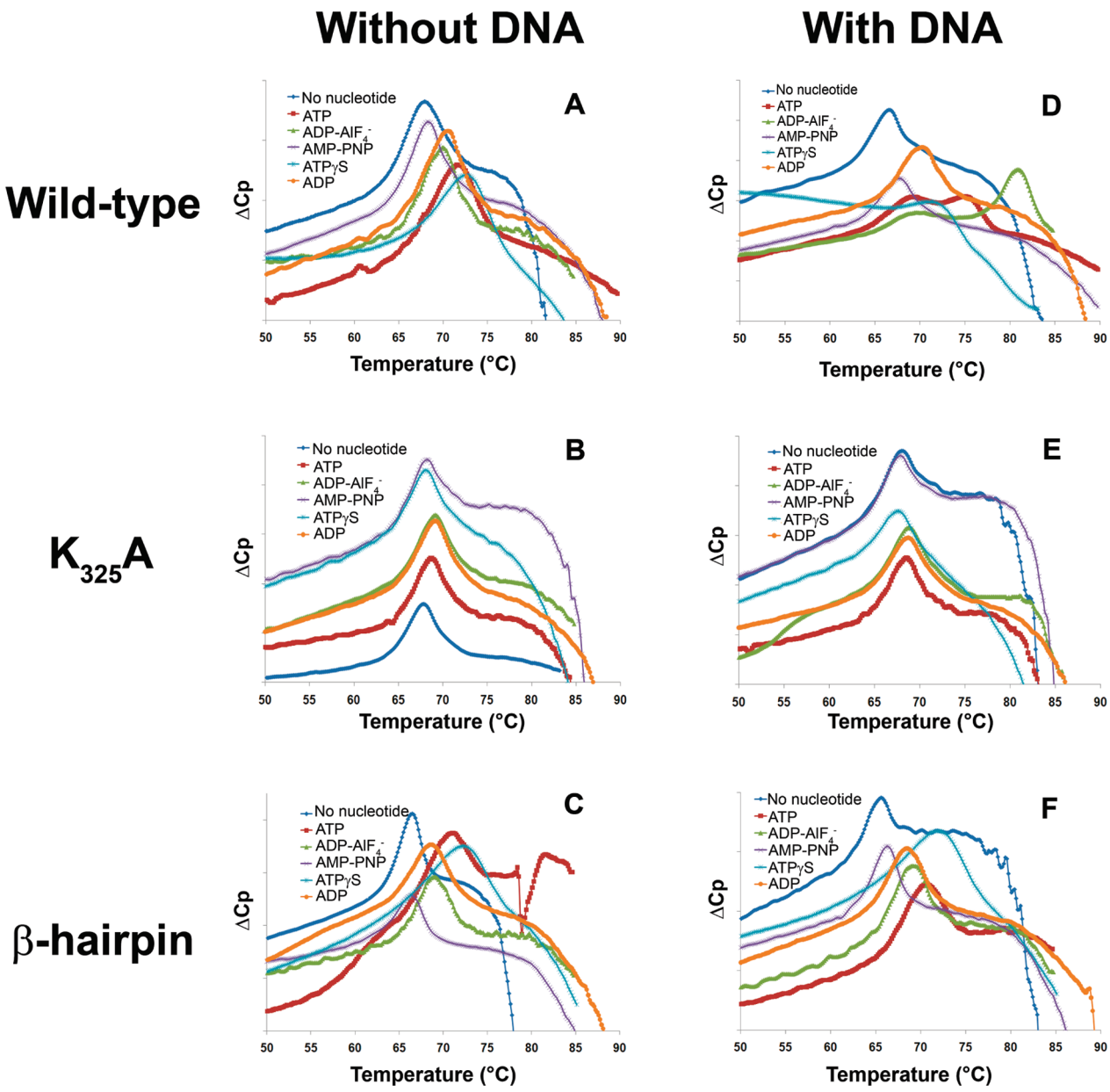

FIGURE 1: Effect of DNA and nucleotide analogues on MCM thermostability. DSC assays were performed as described in Materials and Methods using wild-type (A and D), $K_{325}A$ mutant (B and E), or β-hairpin mutant (C and F) MCM protein in the absence (A−C) or presence (D−F) of N190 (3′−5′) oligonucleotide. DSC results of excess heat capacity versus temperature are shown.

complex was similar to that observed with ADP and all ATP analogues used, the second transition of the complex shows a substantial stabilization effect on MCM of about 12 °C. This larger shift in the high temperature transition temperature, larger than that observed in the presence of DNA and ATP, would result from a higher binding constant of 2×10^6 M^{-1} of DNA binding to its structural domain according to eq 1. Thus, although both ATP and ADP-AlF_4^- stabilized the MCM protein in the presence of DNA, ADP-AlF_4^- has a greater effect on the stability. If ADP-AlF_4^- represents a transition state analogue during the hydrolysis process, as suggested in several systems (*20, 21*), the DSC data with this analogue may suggest that the transition state complex is responsible for the decoupling effect between the two domains, whether the transition state complex is produced by the hydrolysis of ATP or by the binding of ADP-AlF_4^- to MCM. Direct binding of ADP to the MCM protein in the presence of DNA does not result in the bimodal transition, while hydrolysis of the ATP proceeding through a transition state in the presence of DNA does exhibit bimodal behavior.

ATPγS and ADP-AlF_4^- Are Efficient Competitors of ATP Binding. The data presented in Table 2 show that all nucleotide analogues affect the stability of MCM protein, though to different extents, with shifts in the transition temperatures ranging from 68.2 ± 0.2 °C for AMP-PNP to 72.5 °C for ATPγS, suggesting that they all interact with the helicase. Their ability to bind to MCM protein was demonstrated more directly by their ability to compete for binding with ATP in ATPase (Figure 2) and helicase (Figure 3) assays. As shown in Figures 2 and 3, all analogues could compete with ATP in the order ATPγS > ADP-AlF_4^- > ADP > AMP-PNP. This is in the same order as their increases in the stabilization of the MCM. This is an unexpected result as AMP-PNP was used as a nonhydrolyzable analogue of ATP in a number of studies (for example, refs *7* and *18*). The data presented here suggest that AMP-

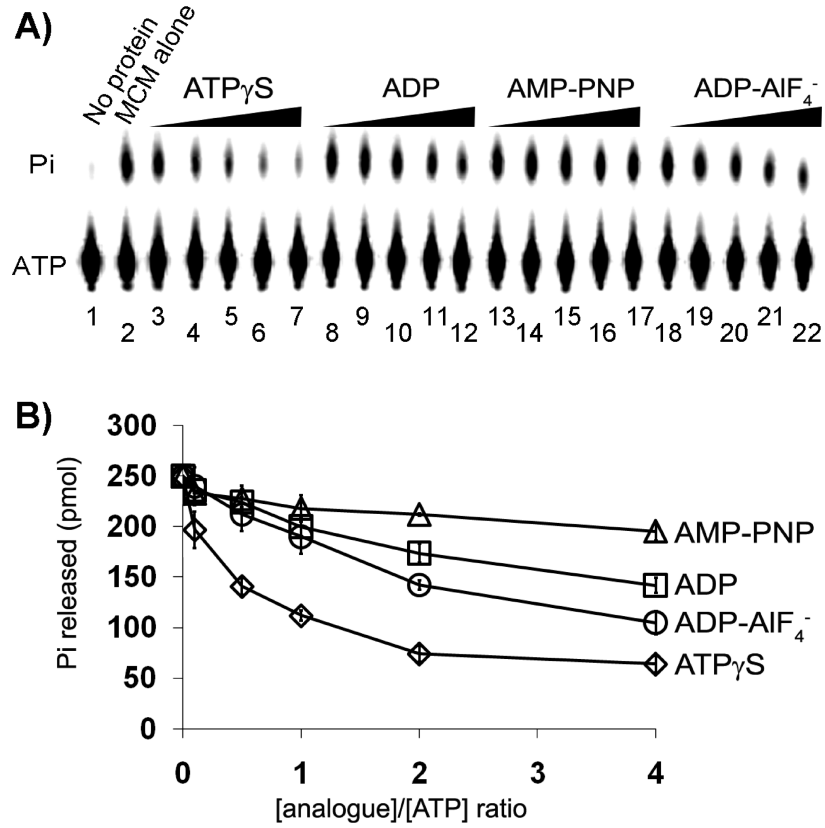

FIGURE 2: Effect of nucleotide analogues on MCM ATPase activity. ATPase assays were performed as described in Materials and Methods in the absence or presence of ADP, ATPγS, ADP, AMP-PNP, or ADP-AlF_4^-. (A) A representative gel. Lane 1, no protein; lane 2, MCM alone; lanes 3−7, ATPγS; lanes 8−12, ADP; lanes 13−17, AMP-PNP; lanes 18−22, ADP-AlF_4^-. Lanes 3, 8, 13, and 18, 0.01 mM ADP, ATPγS, ADP, AMP-PNP, or ADP-AlF_4^-; lanes 4, 9, 14, and 19, 0.05 mM ADP, ATPγS, ADP, AMP-PNP, or ADP-AlF_4^-; lanes 5, 10, 15, and 20, 0.1 mM ADP, ATPγS, ADP, AMP-PNP, or ADP-AlF_4^-; lanes 6, 11, 16, and 21, 0.2 mM; 7, 12, 17, and 22, 0.4 mM ADP, ATPγS, ADP, AMP-PNP, or ADP-AlF_4^-. (B) Average of three independent experiments with standard deviation. ATPγS (open diamond), ADP (open square), AMP-PNP (open triangle), and ADP-AlF_4^- (open circle).

PNP may not be the right choice for an analogue for this MCM enzyme.

Binding to DNA Ends Is Required for MCM Stabilization. The data presented in Table 2 and Figure 1 show the presence of two transitions when MCM is incubated with short oligonucleotides in the presence of ADP-AlF_4^- or ATP. The presence of two transition peaks suggests the unfolding of two structural domains of MCM with ATP or ADP-AlF_4^- bound to one structural domain and DNA bound to the other structural domain. It is possible that when provided with short oligonucleotides either the N-terminal domain of MCM can interact with the end of the DNA and/or the DNA can bind in the central cavity. This will result in two different structures of the DNA−MCM complex and, thus, may result in two different transitions with different stabilities. To check this hypothesis, the effect of short oligonucleotide and closed circular ssDNA on MCM stability in the presence of ATP or ADP-AlF_4^- was evaluated (Table 4 and Figure 4). In the absence of ATP and ADP-AlF_4^-, a single thermal denaturation transition appears for the unfolding of MCM in the presence of the oligonucleotide or plasmid DNA. However, when ATP or ADP-AlF_4^- was used together with the DNA, the type of DNA used has a major effect on the thermostability of MCM as shown in Table 4 and Figure 4. In the presence of oligonucleotides, two transition peaks are observed. In the presence of a circular single-stranded plasmid DNA (ϕX174) and ATP or ADP-AlF_4^-, only a single transition peak was observed. This single transition is at the same temperature as the first transition peak (lower temperature) observed with the oligonucleotide (Table 2 and Figure 1), as would be expected from ATP or ADP-AlF_4^- binding to the structural domain undergoing unfolding at this temperature. The results are not due to the inability of MCM protein to bind to the circular DNA since it has been demonstrated that the helicase can assemble on circular DNA (*10*). Furthermore, the MCM protein contains one tryptophan residue at position 363, and tryptophan fluorescence was used previously to demonstrate the interaction of MCM with DNA (*9*). As shown in Figure 5, tryptophan fluorescence by the wild-type MCM protein is quenched in the presence of various DNA substrates while the β-hairpin mutant protein shows no quenching effect in the presence of DNA. These results demonstrate that the MCM protein can bind all DNA substrates used, including circular DNA.

The observed differences between the stability of MCM protein in the presence of oligonucleotide or circular DNA (in the presence of ATP or ADP-AlF_4^-) suggest that the protein interacts with the ends of the ssDNA. One possibility is that the enzyme has a preference for binding to either 3′ or 5′ ends. To test this possibility, the experiment was repeated in the presence of oligonucleotides that contain only 3′ ends, only 5′ ends, or both. No differences in the thermal stabilities could be observed between the three different DNA substrates, and all the DSC scans resulted in two transition peaks separated by similar temperatures in the presence of ATP or ADP-AlF_4^- (Table 4 and Figure 4). In order to see

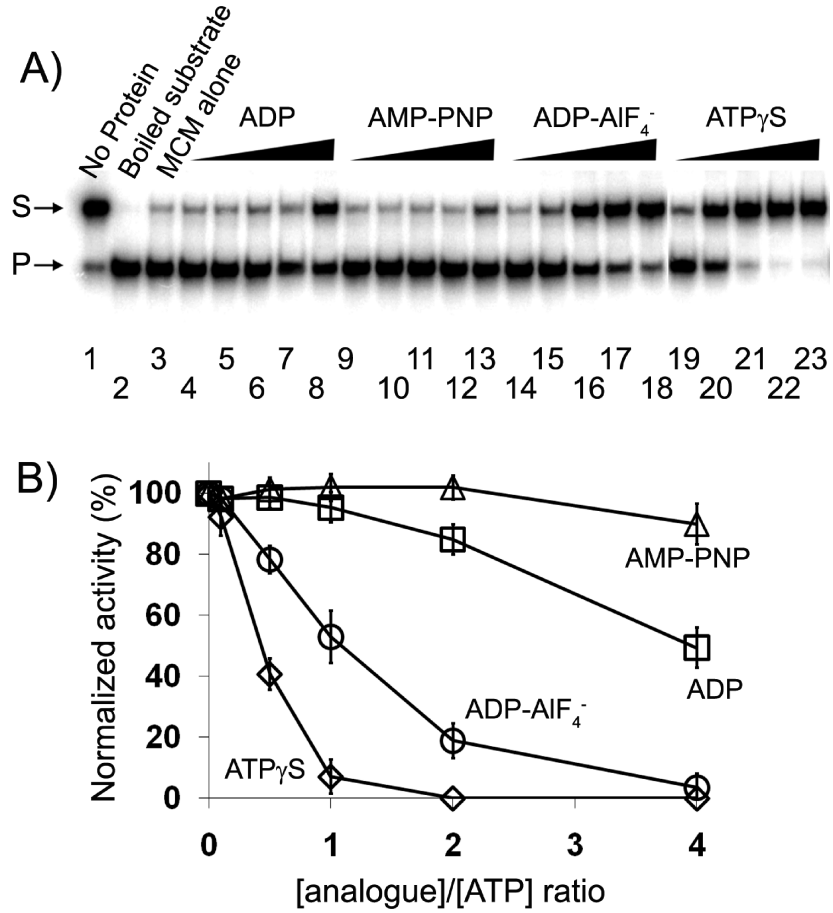

FIGURE 3: Effects of nucleotide analogues on MCM helicase activity. Helicase assays were performed as described in Materials and Methods in the absence or presence of 0.1, 0.5, 1, 2, or 4 mM nucleotide analogue. (A) A representative gel. Lane 1, no protein; lane 2, boiled substrate; lane 3, MCM alone; lanes 4−8, ADP; lanes 9−13, AMP-PNP; lanes 14−18, ADP-AlF_4^-; lanes 19−23, ATPγS. Lanes 4, 9, 14, and 19, 0.1 mM ADP, AMP-PNP, ADP-AlF_4^-, or ATPγS; lanes 5, 10, 15, and 20, 0.5 mM ADP, AMP-PNP, ADP-AlF_4^-, or ATPγS; lanes 6, 11, 16, and 21, 1 mM ADP, AMP-PNP, ADP-AlF_4^-, or ATPγS; lanes 7, 12, 17, and 22, 2 mM ADP, AMP-PNP, ADP-AlF_4^-, or ATPγS; lanes 8, 13, 18, and 23, 4 mM ADP, AMP-PNP, ADP-AlF_4^-, or ATPγS. (B) Average of three independent experiments with standard deviation. ATPγS (open diamond), ADP (open square), AMP-PNP (open triangle), and ADP-AlF_4^- (open circle).

Table 4: Thermostabilities of Wild-Type MCM Protein in the Absence or Presence of Various Nucleotides and DNA Substrates

nucleotide[a]	DNA[b]	T_G (°C)	$\Delta_{trs}H_{vH}$ (kJ mol^{-1})	$\Delta_{trs}H$ (kJ mol^{-1})
−	−	66.7 ± 1.1	836 ± 77	376 ± 149
	3′−5′	65.9 ± 0.6	693 ± 228	378 ± 8
	3′−3′	66.2 ± 0.2	713 ± 48	395 ± 35
	5′−5′	66.5 ± 0.1	737 ± 19	360 ± 32
	ϕX174 (med scan)	66.8	791	465
	ϕX174 (slow scan)	64.4	1098	378
ATP	−	71.5 ± 0.6	731 ± 33	569 ± 30
	3′−5′	68.9 ± 0.5, 75.3 ± 0.3	639 ± 95, 981 ± 65	625 ± 68, 179 ± 121
	3′−3′	68.7 ± 0.4, 75.2 ± 0.2	647 ± 23, 913 ± 49	74 ± 39, 287 ± 4
	5′−5′	69.0 ± 0.6, 73.5 ± 0.3	666 ± 25, 931 ± 104	470 ± 100, 152 ± 28
	ϕX174	68.8 ± 0.0	874 ± 115	486 ± 283
ADP-AlF_4^-	−	70.3 ± 0.5	778 ± 4	433 ± 91
	3′−5′	69.5 ± 0.4, 80.9 ± 0.5	762 ± 77, 1289 ± 1	106 ± 42, 283 ± 35
	3′−3′	68.7 ± 0.1, 81.4 ± 0.0	690 ± 33, 1029 ± 15	254 ± 115, 240 ± 62
	5′−5′	68.5 ± 0.4, 80.3 ± 0.1	728 ± 48, 962 ± 42	178 ± 64, 356 ± 24
	ϕX174	69.8 ± 0.2	542 ± 40	511 ± 142

[a] In the absence (−) or presence of 1 mM nucleotides. [b] In the absence (−) or presence of 108 μg of DNA substrates, 3′−5′ (N190), 3′−3′ (N191), 5′−5′ (N192), or ϕX174.

whether the length of the oligonucleotide has an effect on the thermostability of MCM, a 136-mer oligonucleotide (PB4) was used in the presence of ATP. The results were similar to those obtained with the 50-mer (N190) oligonucleotide (data not shown), indicating that length of oligonucleotide does not have an effect.

Qualitatively, however, the three oligonucleotides differ in their effect on MCM stability (Figure 4). When ATP is used in the presence of the oligonucleotide containing both 3′ and 5′ ends, the two transition peaks appear equivalent (Figure 4B). In the presence of the oligonucleotide containing only 3′ ends the distribution of the intensities of the transition

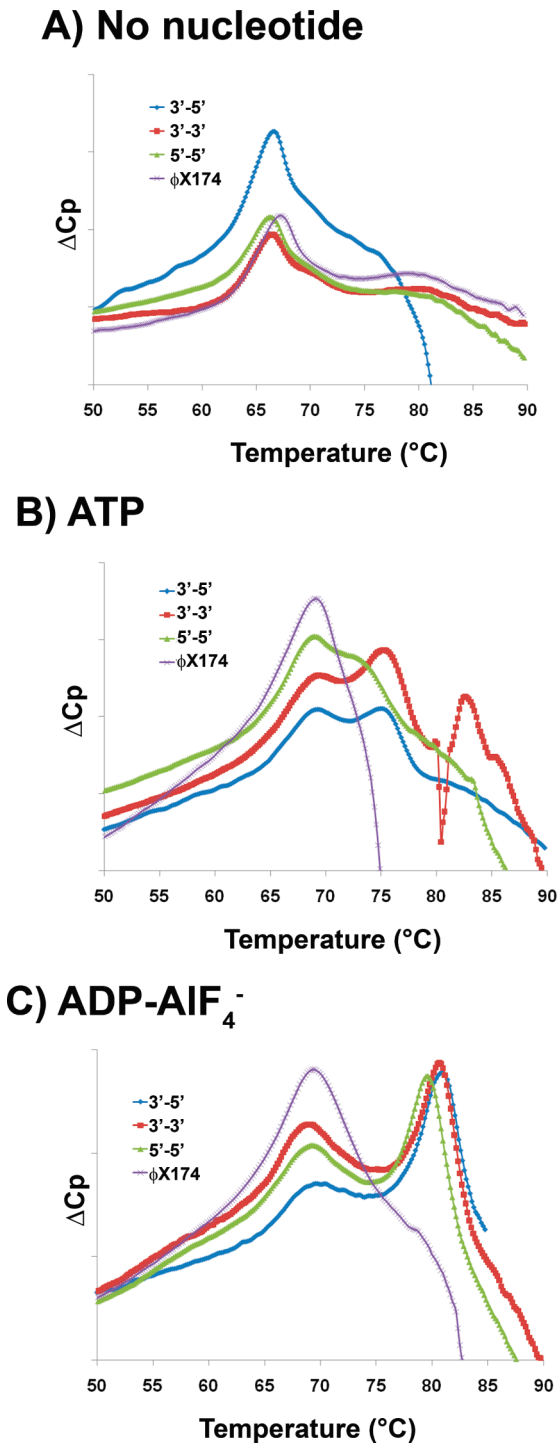

FIGURE 4: Effect of DNA substrate on MCM thermostability. DSC assays were performed as described in Materials and Methods using wild-type MCM protein and N190 (3′−5′), N191 (3′−3′), N192 (5′−5′) oligonucleotides or ϕX174 ssDNA. DSC results of excess heat capacity versus temperature are shown. (A) No nucleotide; (B) ATP; (C) ADP-AlF_4^-.

peaks shifts to the second (higher temperature) peak (Figure 4B), while the oligonucleotide containing only 5′ ends has the opposite effect and the equilibrium distribution of peak intensities is shifted toward the first transition peak, making it more similar to circular DNA (Figure 4B). When ADP-AlF_4^- was used instead of ATP, the stability of the enzyme shifted toward the second peak regardless of which oligonucleotide was used (Figure 4C). Taken together, the results suggest that although MCM protein is stabilized upon binding

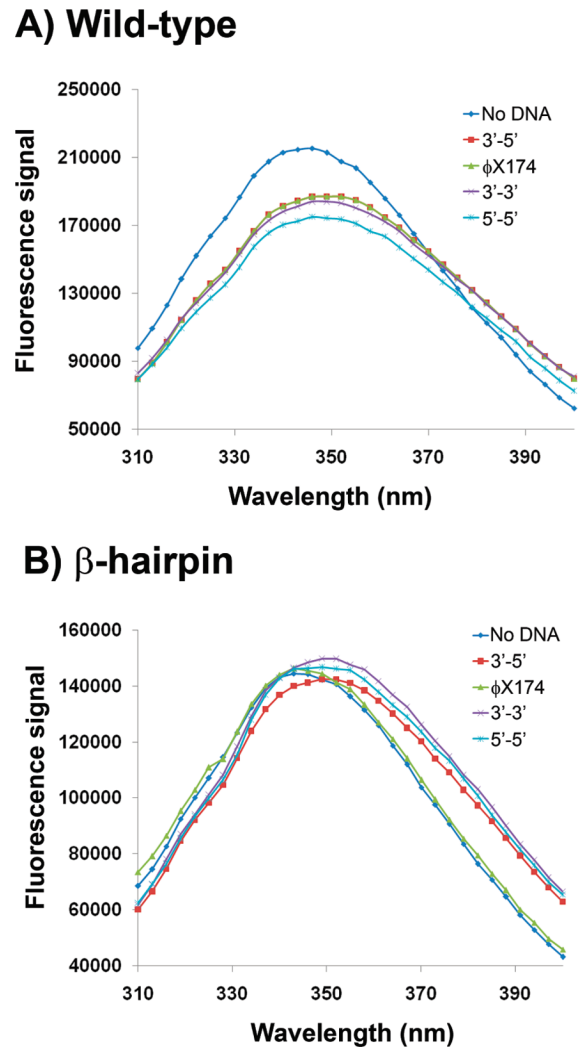

FIGURE 5: MCM interactions with DNA substrates. Intrinsic tryptophan fluorescence of wild-type (A) or β-hairpin mutant (B) MCM protein in the presence of N190 (3′−5′), N191 (3′−3′), N192 (5′−5′) oligonucleotides or ϕX174 ssDNA were measured as described in Materials and Methods. Samples were excited at 295 nm, and emission spectra of 310−400 nm were recorded.

to DNA ends, the enzyme does not have specific preference for 3′ or 5′ ends.

The presence of DNA ends affects not only the stability of the MCM protein but also its ATPase activity. The ATPase activity of MCM is stimulated in the presence of DNA (*7, 8, 10*). Therefore, it is possible that DNA ends will have a stimulatory effect different than that of circular DNA. As shown in Figure 6 this is indeed the case. While circular ssDNA stimulates ATPase activity about 2-fold, the oligonucleotides stimulate the activity about 7−9-fold. These results suggest that the different binding mode to DNA also affects the catalytic ATPase activity of MCM.

DISCUSSION

MCM utilizes the energy of ATP hydrolysis to translocate along one strand of the DNA and unwind the complementary strand. The N-terminal part of the enzyme plays an important role in DNA binding while the C terminus contains the catalytic domain. Based on the three-dimensional structures of other helicases, it is clear that the MCM protein changes its conformation when it binds to nucleotides and DNA

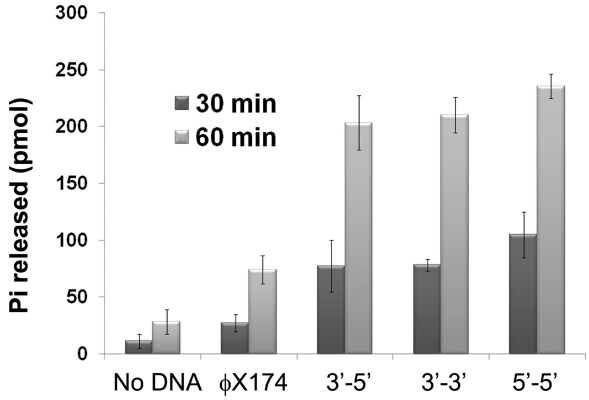

FIGURE 6: Effect of DNA on MCM ATPase activity. ATPase assays were performed as described in Materials and Methods with 50 nM MCM proteins (as monomer) in the absence or presence of 1 ng of ϕX174 ssDNA or N190 (3′−5′), N191 (3′−3′), N192 (5′−5′) oligonucleotides and either 30 or 60 min incubation.

(*22*−*24*), and these changes alter the interaction between the domains. The structural changes upon binding DNA alone are also evident in the large positive heat capacity change implied by eqs 1 and 2. In this study, the thermostability of MCM was used as an indicator of any conformational change. The data show that the stability of the enzyme is slightly increased (2−4 °C) when the protein binds to ADP, ATP, or ATP analogues. This is validated in particular for ATP since the temperature increase of 4 °C is predictable from eq 1 by employing the ATP binding constant at 60 °C. This temperature shift is maintained for the lower temperature transitions in the presence of DNA and, thus, likely results from the unfolding of the catalytic domain binding the ATP or the ATP transition state analogue ADP-AlF_4^-. The other nucleotide analogues that exhibit only a single transition in the presence of DNA also exhibit temperature shifts similar to those observed for the lower temperature transition of MCM in the presence of ATP or ADP-AlF_4^- and DNA. Not only does an additional transition appear in the presence of DNA and either ATP or ADP-AlF_4^- but there is also a major difference in the thermostability of this additional high temperature peak between MCM binding to ATP and to ADP-AlF_4^- in the presence of DNA. The stability of the second transition is further enhanced in the presence of ADP-AlF_4^-. This high temperature transition has been tentatively assigned to the unfolding of the N-terminal DNA binding domain.

The inability of the ATP analogues other than ADP-AlF_4^- to stabilize the enzyme to the same extent as ATP so as to produce a bimodal transition pattern may suggest that hydrolysis is required and that hydrolysis proceeds through formation of a transition state complex. This idea is supported by the observation, as several studies have suggested, that the binding of ADP-AlF_4^- to MCM may mimic a transition state complex in which the γ-phosphate is forming a phosphoryl transfer transition coordinate with the MCM (*20*). In addition, the hydrolysis of ATP proceeds through the transition state complex, and this complex is responsible for the bimodality. Direct binding of the ADP to MCM protein does not proceed through the transition state complex, and accordingly, only a single transition is observed in the presence of DNA. The further stabilization of the higher temperature transition of MCM protein in the presence of ADP-AlF_4^- in comparison to ATP (about 5 °C) may suggest that the transition state complex between MCM and ADP-AlF_4^- formed during hydrolysis is more effective in increasing the binding affinity of DNA to MCM. Thus, the results may suggest that the force-producing step during DNA unwinding may not be the nucleotide binding to MCM but rather the decoupling between the DNA binding domain and the catalytic domain in the transition state complex formed during ATP hydrolysis. This would also be evident for the T7 helicase where it was suggested that the force-producing step during DNA unwinding may be the hydrolysis of ATP (*25*).

It was also found that the effect of ATP and ADP-AlF_4^- on the stability of MCM could be observed only in the presence of oligonucleotides and not with closed circular ssDNA, suggesting that the enzyme binds differently to a DNA end in comparison to circular DNA. The physiological role for MCM interaction with DNA ends is not clear. During chromosomal DNA replication the helicase will encounter DNA ends at sites of DNA breaks. The binding of MCM to the DNA ends may serve as a cellular signal for DNA breaks. MCM may play a role in the S-phase checkpoint which is activated upon DNA damage (*26*). The specific binding of MCM to the DNA ends may be used in these checkpoint processes. MCM has also been implicated in other cellular roles in addition to its function as the replicative helicase. It was suggested that the MCM helicase may play a role in transcription (*27*) and was implicated in chromosome remodeling (*28, 29*). MCM may need to bind to DNA ends during its roles in these processes.

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