The archaeal minichromosome maintenance protein MCM forms a homohexameric complex that functions as the DNA replicative helicase and serves as a model system for its eukaryotic counterpart. Here, we applied single molecule fluorescence resonance energy transfer methods to probe the substrate specificity and binding mechanism of MCM from the hyperthermophilic Archaea *Sulfolobus solfataricus* on various DNA substrates. *S. solfataricus* MCM displays a binding preference for forked substrates relative to partial or full duplex substrates. Moreover, the nature of MCM binding to Y-shaped substrates is distinct in that MCM loads on the 3′-tail while interacting with the 5′-tail likely via the MCM surface. These results provide the first elucidation of a dynamic nature of interaction between a ring-shaped helicase interacting with an opposing single-stranded DNA tail. This interaction contributes to substrate selectivity and increases the stability of the forked DNA-MCM complex, with possible implications for the MCM unwinding mechanism.

The eukaryotic minichromosome maintenance MCM2–7 complex plays a vital role in replication and is thought to be the replicative helicase (1, 2). MCM2–7 is involved in various stages of the replication process, from licensing through initiation, replication, and termination (3, 4). The MCM2–7 complex forms a heterohexamer, with a subcomplex (MCM4–6–7) functioning as a 3′–5′-helicase. The six subunits possess sequence similarity, suggesting a common evolutionary origin (1, 5). The archaeal replication process represents a simpler form of that of DNA binding and substrate selectivity of various helicases (24–30). Herein, we report on the mechanism of DNA binding and substrate selectivity of *Sulfolobus solfataricus* MCM (SsoMCM) obtained through single molecule fluorescence resonance energy transfer (smFRET) studies. smFRET (31, 32) is a powerful method able to unravel biological events, including helicase activities, without ensemble averaging. smFRET was applied to probe the binding and orientation of MCM on DNA as well as the conformational changes induced in DNA upon MCM loading. MCM demonstrates a specificity of binding to forked DNA over single-tailed and duplex substrates. This results from a highly dynamic interaction between the 5′-tail and MCM, with a mechanistic implication on replicative helicases.

**EXPERIMENTAL PROCEDURES**

**DNA Substrates and Annealing**—Oligonucleotide substrates were purchased from Integrated DNA Technologies (Corvalle, IA). Forked, partial, and duplex substrates were prepared by mixing the biotinylated and non-biotinylated strands
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in a 1:1.5 ratio at 1 μM in buffer containing 10 mM Tris (pH 8) and 200 mM NaCl. The non-biotinylated strand was added in excess to minimize the chance of having residual biotinylated strand in the measurement. The annealing mixture was heated at 95 °C for 2 min and allowed to cool slowly for 2 h in the heating block. (For substrates and sequences, see supplemental “Experimental Procedures.”)

Protein Purification and Protein Labeling—Wild-type SsoMCM and its C642A mutant were purified as described previously (10). Cy3 and Cy5 N-hydroxysuccinimide esters and maleimides were purchased from GE Healthcare. Fluorescence labeling of SsoMCM was also performed as described previously (10). Briefly, wild-type SsoMCM was incubated with the N-hydroxysuccinimide ester in 25 mM HEPES, 150 mM NaCl, and 10 mM MgCl2 at pH 6.8 and then quenched with 10 mM Tris after 1 h (referred to as N-MCM). C642A mutant SsoMCM was incubated with the maleimide in the same buffer at pH 7.5 and then quenched with 10 mM β-mercaptoethanol after 4 h (referred to as C-MCM). Labeled MCM proteins were spun through a spin column and dialyzed extensively to remove any remaining free label. The degree of labeling was determined by UV spectrometry using the appropriate extinction coefficients and found to be essentially 1 for all cases.

Single Molecule Spectroscopy—smFRET measurements were performed using a wide-field total internal reflection fluorescence microscope (33, 34). Total internal reflection excitation was done either using a prism or through an objective (Olympus UPPlanSApo ×100 numerical aperture 1.4 oil). Images were acquired with a 30-ms time resolution using an electron-multiplying charge-coupled device camera (iXon DV887-BI, Andor Technology) and a homemade C++ program. FRET values were calculated as the ratio between the acceptor intensity and the sum of the intensities of the donor and acceptor, after correcting for cross-talk between the two detection channels and subtracting the background (32, 35).

A quartz slide was coated with polyethylene glycol, with 1–2% (w/w) biotin-polystyrene glycol (34, 35). Surface integrity and nonspecific binding were tested by separately adding Cy3-labeled protein (4 μM) and DNA (1 nM). Once surface integrity was verified, NeutrAvidin was added as described (32, 35), followed by immobilizing biotinylated DNA (150 pm). All measurements were performed at room temperature with buffer 4 from New England Biolabs (Ipswich, MA), which contains 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol (pH 7.9). The buffer also contained 0.8% glucose with an oxygen scavenger system (0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, 1% β-mercaptoethanol, and 0.4% (w/w) β-β-glucose) (32, 35).

Single Molecule Measurements of Labeled MCM Complex—To reduce the possibility of FRET between one-donor DNA and an MCM hexamer labeled with multiple acceptors, wild-type MCM was mixed with labeled MCM to yield a final labeled/unlabeled monomer ratio of 1:8. Labeled and unlabeled MCM proteins were incubated in the dark for at least 1 h at room temperature.

Surface-attached DNA molecules were incubated with the reported concentrations of MCM (all given in monomer concentration) for 5 min, ensuring sufficient time for binding. Excess and unbound MCM was removed by a wash with buffer 4, followed by a wash with imaging buffer.

RESULTS

smFRET Analysis of SsoMCM Fork Binding Orientation—SsoMCM contains several functional domains with separable domain-dependent activities. The C-terminal half contains the AAA" (ATPases associated with a variety of cellular activities) domain and a helix-turn-helix motif, and the N terminus includes the Walker A and B motifs (10). Recent studies of truncated SsoMCM complexes assigned the helicase activity to the C-terminal half and showed that the N-terminal half governs substrate selectivity and enzyme processivity (36).

For smFRET measurements, Cy3 was used as the donor and Cy5 as the acceptor. For DNA-to-MCM FRET analysis, MCM was labeled with Cy5 at the N terminus (N-MCM) or C terminus (C-MCM), and the various DNA substrates were labeled with Cy3. The activity of purified and labeled MCM was verified prior to measurements as described (10). The DNA molecules were attached to the surface of a flow chamber and incubated with MCM at a specified monomer concentration, followed by an extensive wash to remove unbound MCM (see “Experimental procedures”). The orientation of MCM was measured via the binding of acceptor-labeled C-MCM or N-MCM (8 μM prewash monomer concentration) to forked DNA substrates that were donor-labeled at either the 3'-end or the junction. The normalized relative distance distributions, extracted from smFRET efficiency histograms, are shown for four different combinations: N terminus to 3'-end, C terminus to 3'-end, C terminus to junction, and N terminus to junction (Fig. 1, A–D, respectively). The data indicate that the N terminus is closer to the 3'-end (~5.4 nm) than to the junction (~8.2 nm). The distances were calculated using the previously determined value of ~6.3 nm for the Cy3–Cy5 pair (35) and should be considered only as an approximation because of the relatively large fluorescence anisotropy of the protein- and DNA-conjugated fluorophores. Additionally, the C terminus is closer to the junction (~7.0 nm) than to the N terminus (~8.2 nm). In combination, our data indicate the MCM orientation illustrated in Fig. 1; MCM binds to the 3'-tail with the C-terminal AAA" helicase domain facing the junction and the N-terminal domain, which includes the substrate selectivity domain, closer to the 3'-end. These findings are in agreement with the previously reported organization of MCM on DNA (10) as measured in ensemble experiments, validating our smFRET assays.

SsoMCM Substrate Binding Specificity—We next aimed to probe the substrate binding preference of MCM. The fluorescence of surface-tethered donor-labeled DNA, either forked or with a 3'-tail (Fig. 2B), was measured after incubation with 3 μM acceptor-labeled C-MCM. Fig. 2A shows donor (panel I) and acceptor (panel II) single molecule fluorescence images of MCM bound to forked DNA (panel I) and the single 3'-tailed partial duplex (panel II). The fraction of DNA bound by MCM was determined from the resulting smFRET histograms (see supplemental “Experimental Procedures”). MCM displayed a nearly 2-fold enhanced binding to forked DNA substrate over the 3'-tailed partial duplex substrates (Fig. 2B). We considered the following three explanations at to why MCM prefers a
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![Diagram of MCM showing preferences for forked substrates.](image)

**Mechanism of MCM Substrate Specificity**

central channel; and 3) as proposed previously for MCM (37), two hexamers are arranged in an eccentrically stacked double hexamer and bind separately to the 3'- and 5'-tails, each with its own polarity such that binding is improved when both tails are present.

To distinguish among these binding modes, we determined the efficiency of binding of MCM to other substrates. Five different donor-labeled substrates were measured: an 18-bp full duplex, a 5'-tailed partial duplex, and three forked substrates labeled in different locations (Fig. 2C). Binding efficiency was low for the full duplex and 5'-tailed duplex substrates and was significantly higher for the forked substrates (Fig. 2C). The low binding of MCM to duplex substrates disfavors the second loading mechanism. Hence, the observed MCM preferred loading on forked substrates may be either due to external binding of the 5'-tail or through a double hexamer binding both strands.

To further probe the loading mechanism, we designed three forked substrates labeled with a donor and an acceptor on opposing tails (Fig. 3), enabling us to investigate the behavior of the forked tails upon MCM loading. The DNA substrates were tethered to the surface, and unlabeled MCM at the indicated concentration was added (see "Experimental Procedures"). Fig. 3A shows the smFRET efficiency histograms of a forked DNA substrate with a donor at the 3'-end and an acceptor at the 5'-end as a function of MCM concentration. DNA showed only low FRET efficiency values (Fig. 3A, upper panel) because of a large separation of flexible ssDNA. With increasing MCM concentrations, higher FRET efficiency populations emerged, indicating that the 3'- and 5'-tails are being brought closer together. This effect was also observed with two other forked substrates with similarly labeled 5'-tails, but with longer and internally labeled 3'-tails (Fig. 3B and C). The existence of a high FRET efficiency population even at an MCM concentration as low as 150 nM is indicative of a single MCM hexamer bound on the forked substrates, not double hexamers, because previous stoichiometric analyses showed that SsoMCM assembles on a forked DNA only as a hexamer at concentrations up to 5 μM (10, 18). In addition, double hexamer loading would yield low FRET because the DNA tails would be spatially separated by the proteins. These results led us to propose that the 3'-tail of the forked DNA substrate is accommodated in the central channel of the MCM hexamer, whereas the DNA 5'-tail binds externally (Fig. 3D).

**MCM Fork Binding and Interactions with the Opposite Strand**—We next examined the dynamic nature of MCM binding to the forked DNA. To test the stability of the DNA-MCM complex, forked DNA (Fig. 3, substrate 1) was incubated with 4 μM MCM, washed, and monitored for >1 h (supplemental Fig.
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![Graphs and diagrams](https://example.com/graphs.png)

1. No significant dissociation of MCM from the DNA was observed, ensuring that our measurements were not influenced by protein dissociation.

Individual DNA molecules bound by MCM displayed temporal fluctuations in FRET efficiency in the absence of free MCM in solution (Fig. 4A, panel I), likely representing distance changes between the two ends of the forked substrate. Such continual fluctuation in distance with no apparent regularity was observed for the majority of molecules with all three forked substrates (81, 82, and 74% for substrates 1–3, respectively, in Fig. 3) and was still detected after ATP was added (supplemental Fig. 2). Similar behavior was observed for FRET efficiency between the donor at the 5'-tail and the acceptor at the N terminus of MCM (Fig. 4A, panel II), indicating a dynamic interaction of the 5'-tail with MCM. Furthermore, such complex interaction with MCM is unique to the 5'-tail, as we did not observe similar fluctuations in FRET between a donor at the 3'-tail and an acceptor on MCM.

To further analyze this phenomenon, having no apparent regular fluctuation period or amplitude, we applied an autocorrelation analysis, in which, by self-correlating the values of a FRET efficiency time trace at different time periods, the characteristic relaxation times and behaviors can be extracted (see supplemental “Experimental Procedures”). The autocorrelation functions of individual smFRET traces were calculated and averaged over many molecules, ranging from 20 to 100 molecules for each experiment, to extract the characteristic time interaction governed by subdiffusion kinetics.

### DISCUSSION

Using smFRET, we found that MCM possesses specificity for forked DNA substrates over single-tailed and duplex substrates. Furthermore, MCM loads on the forked DNA 3'-tail, with its C terminus facing the junction and N terminus toward the 3'-end, and externally binds to the 5'-tail. Previous ensemble FRET analysis of SsoMCM deduced the same binding orientation; however, a recent crystal structure of another hexameric AAA+ helicase, papillomavirus E1, shows binding to an ssDNA with opposite polarity (42). Our smFRET result further supports the apparent difference in DNA binding polarity between SsoMCM and papillomavirus E1.

The interaction between the 3'-tail-bound MCM complex and the 5'-tail was found to be dynamic, with fluctuations occurring on wide distance and time scales. This suggests that the 5'-tail does not simply bind the MCM surface and fall off, but instead experiences an intricate interaction landscape. Assuming there exists at least one external binding site in each monomer, the MCM hexamer would involve at least six such binding sites, with many possible conformations of the 5'-tail onto these sites. We propose that the external 5'-tail/MCM interaction is weak per binding site, enabling the 5'-tail to break off and diffuse between different sites. The diffusion steps between the different binding sites will therefore depend on the 5'-tail conformation and its arrangement on the various sites.
with additional contributing factors such as ssDNA strain and the location of MCM on the 3′-tail. The net result would be a shallow but rugged energy landscape with many possible local minima, which would increase the stability of MCM on the replication fork and determine the distinctive selectivity of MCM for forked substrates.

The architecture of replicative helicases at the replication fork during DNA unwinding has been the subject of much debate. Some of the various suggested models are passive unwinding, in which the helicase translocates on one ssDNA and awaits the opening of the junction to translocate forward; and several dynamic models, in which the helicase directly destabilizes the junction (3, 13, 14, 26, 43). For MCM, diverse models have been proposed, including steric exclusion, rotary pump, T-antigen, and Ploughshare (23, 37, 44), suggesting an interaction with either single- or double-stranded DNA. In the steric exclusion model, the opposing strand (5′-tail) prevents the helicase from encircling and translocating along the duplex DNA and does not play an additional role once the helicase starts unwinding the DNA. In contrast, our data show that SsoMCM dynamically interacts with the 5′-end, which should also be the case during duplex unwinding. Such dynamic inter-

action between a ring-shaped helicase and an opposing ssDNA tail has not been elucidated previously.

In light of our findings, we propose the following “opposite strand interaction” model for MCM/replication fork association. MCM loads with the 3′-tail accommodated in its central channel, and the 5′-tail binds and diffuses on its surface. This interaction would regulate the binding specificity for forked substrates and aid in the long-term stability of the MCM- replication fork complex and replication fidelity. It should be noted that the weak interaction of MCM with the opposite strand may be beneficial in preventing tangling of ssDNA or stalled replication by ensuring that the interaction is local, whereas it is possible that a single strand binding protein (single-stranded DNA-binding protein or replication protein A) also aids in preventing the MCM surface from binding to ssDNA at a distance. Additionally, as a 3′–5′-helicase MCM will translocate along the leading strand template (3′-tail), its dynamic interaction with the unwound lagging strand template (5′-tail) may facilitate coordination of leading and lagging strand synthesis events. In this regard, it may be relevant that the DNA primase-binding archaeal GINS complex interacts with the trailing N-terminal domains of SsoMCM (45). Additionally, this type of interaction may be incorporated into the unwinding mechanism of MCM, in which upon ATP hydrolysis, MCM will translocate forward on the ssDNA while drawing the 5′-tail. This combined forward translocation and opposite strand binding may result in sufficient destabilization of the junction, facilitating double-stranded DNA unwinding. Finally, we should also consider the possibility that the observed interaction may have inhibitory effects on the helicase activity (46), which may be lifted if another protein such as the DNA polymerase occupies the opposing strand (47, 48).

Our smFRET study has revealed unique information about dynamic interaction between MCM and the opposite strand in the context of replication fork association with MCM. These findings likely bear relevance to the mechanism of replication fork binding and unwinding by MCM and other replicative helicases.

REFERENCES
1. Forsburg, S. L. (2004) Microbiol. Mol. Biol. Rev. 68, 109–131
2. Bell, S. P., and Dutta, A. (2002) Annu. Rev. Biochem. 71, 333–374
3. Maiorano, D., Lutzmann, M., and Mechali, M. (2006) Curr. Opin. Cell Biol. 18, 130–136
4. Lindner, K., Gregan, J., Montgomery, S., and Karsey, S. E. (2002) Mol. Biol. Cell 13, 435–444
5. Johnson, A., and O’Donnell, M. (2005) Annu. Rev. Biochem. 74, 283–315
6. Barry, E. R., and Bell, S. D. (2006) Microbiol. Mol. Biol. Rev. 70, 876–887
7. Duggan, I. G., and Bell, S. D. (2006) J. Biol. Chem. 281, 15029–15032
8. Kelman, Z., and White, M. F. (2005) Curr. Opin. Microbiol. 8, 669–676
9. Kelman, L. M., and Kelman, Z. (2003) Mol. Microbiol. 48, 605–615
10. McGeoch, A. T., Trakselis, M. A., Laskey, R. A., and Bell, S. D. (2005) Nat. Struct. Mol. Biol. 12, 756–762
11. Chen, Y. J., Yu, X. O., Kasiviswanathan, R., Shin, S. H., Kelman, Z., and Egelman, E. H. (2005) J. Biol. Chem. 340, 1197–1206
12. Fletcher, R. J., Bishop, B. E., Leon, R. P., Sclafani, R. A., Ogata, C. M., and Chen, X. I. S. (2003) Nat. Struct. Mol. Biol. 10, 160–167
13. Patel, S. S., and Donmez, I. (2006) J. Biol. Chem. 281, 18265–18268
14. Donmez, I., and Patel, S. S. (2006) Nucleic Acids Res. 34, 4216–4224
15. Yu, Z. L., Feng, D. R., and Liang, C. (2004) J. Mol. Biol. 340, 1197–1206
16. Lee, J. K., and Hurwitz, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 54–59
Mechanism of MCM Substrate Specificity

17. Kaplan, D. L., Davey, M. J., and O’Donnell, M. (2003) J. Biol. Chem. 278, 49171–49182
18. Carpentieri, F., De Felice, M., De Falco, M., Rossi, M., and Pisani, F. M. (2002) J. Biol. Chem. 277, 12118–12127
19. Haugland, G. T., Shin, J. H., Birkeland, N. K., and Kelman, Z. (2006) Nucleic Acids Res. 34, 6337–6344
20. Shin, J. H., Jiang, Y., Grabowski, B., Hurwitz, J., and Kelman, Z. (2003) J. Biol. Chem. 278, 49053–49062
21. Galletto, R., Jezewska, M. J., and Bujalowski, W. (2004) J. Mol. Biol. 343, 101–114
22. Jeong, Y. J., Levin, M. K., and Patel, S. S. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7264–7269
23. Delagoutte, E., and von Hippel, P. H. (2002) Q. Rev. Biophys. 35, 431–478
24. Myong, S., Rasnik, I., Joo, C., Lohman, T. M., and Ha, T. (2005) Nature 437, 1321–1325
25. Myong, S., Bruno, M. M., Pyle, A. M., and Ha, T. (2007) Science 317, 513–516
26. Johnson, D. S., Bai, L., Smith, B. Y., Patel, S. S., and Wang, M. D. (2007) Cell 129, 1299–1309
27. Dohoney, K. M., and Gelles, J. (2001) Nature 409, 370–374
28. Dumont, S., Cheng, W., Serebrov, V., Beran, R. K., Tinoco, I., Pyle, A. M., and Bustamante, C. (2006) Nature 439, 105–108
29. Spies, M., Bianco, P. R., Dillingham, M. S., Handa, N., Baskin, R. J., and Kowalczykowski, S. C. (2003) Cell 114, 647–654
30. Dessinges, M. N., Lionnet, T., Xi, X. G., Bensimon, D., and Croquette, V. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6439–6444
31. Ha, T., Enderle, T., Ogletree, D. F., Chemla, D. S., Selvin, P. R., and Weiss, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6264–6268
32. Ha, T. (2001) Methods (San Diego) 25, 78–86
33. Zhuang, X. W., Bartley, L. E., Babcock, H. P., Russell, R., Ha, T. J., Herschlag, D., and Chu, S. (2000) Science 288, 2048–2051
34. Ha, T., Rasnik, I., Cheng, W., Babcock, H. P., Gauss, G. H., Lohman, T. M., and Chu, S. (2002) Nature 419, 638–641
35. Rasnik, I., Myong, S., Cheng, W., Lohman, T. M., and Ha, T. (2004) J. Mol. Biol. 336, 395–408
36. Barry, E. R., McGeoch, A. T., Kelman, Z., and Bell, S. D. (2007) Nucleic Acids Res. 35, 988–998
37. Takahashi, T. S., Wigley, D. B., and Walter, J. C. (2005) Trends Biochem. Sci. 30, 437–444
38. Yang, H., and Xie, X. S. (2002) J. Chem. Phys. 117, 10965–10979
39. Metzler, R., Barkai, E., and Klafte, J. (1999) Phys. Rev. Lett. 82, 3563–3567
40. Metzler, R., and Klafte, J. (2000) Phys. Rep. Rev. Sec. Phys. Lett. 339, 1–77
41. Yang, H., Luo, G. B., Karnchanaphanurach, P., Louie, T. M., Rech, I., Cova, S., Xun, L. Y., and Xie, X. S. (2003) Science 302, 262–266
42. Enemark, E. J., and Jiang, Y. J., and Patel, S. S. (2007) J. Biol. Chem. 282, 21116–21123
43. Marinsek, N., Barry, E. R., Makarova, K. S., Dionne, I., Koonin, E. V., and Bell, S. D. (2006) EMBO Rep. 7, 539–545
44. Ha, T. (2007) Cell 129, 1249–1250
45. Stano, N. M., Jeong, Y. J., Donmez, I., Tummalapalli, P., Levin, M. K., and Patel, S. S. (2005) Nature 435, 370–373
46. Lee, J. B., Hite, R. K., Hamdan, S. M., Xie, X. S., Richardson, C. C., and van Oijen, A. M. (2006) Nature 439, 621–624