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RecQ Helicase-catalyzed DNA Unwinding Detected by Fluorescence Resonance Energy Transfer

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Abstract A fluorometric assay was used to study the DNA unwinding kinetics induced by *Escherichia coli* RecQ helicase. This assay was based on fluorescence resonance energy transfer and carried out on stopped-flow, in which DNA unwinding was monitored by fluorescence emission enhancement of fluorescein resulting from helicase-catalyzed DNA unwinding. By this method, we determined the DNA unwinding rate of RecQ at different enzyme concentrations. We also studied the dependences of DNA unwinding magnitude and rate on magnesium ion concentration. We showed that this method could be used to determine the polarity of DNA unwinding. This assay should greatly facilitate further study of the mechanism for RecQ-catalyzed DNA unwinding.

Key words helicase; DNA unwinding; kinetics; stopped-flow; fluorescence resonance energy transfer (FRET)

DNA and RNA helicases are unwinding enzymes that can couple nucleotide triphosphate (usually ATP) binding and hydrolysis to catalyze breakage of the hydrogen bonds between complementary strands of double-stranded DNA (dsDNA), thus providing single-stranded DNA (ssDNA) templates required for almost all aspects of nucleic acid metabolic pathways [1–6]. These enzymes seem to be ubiquitous in both prokaryotic and eukaryotic cells as they have been found in diverse species from bacteria to human [7–13]. It has been reported widely, since this type of enzyme was originally described and recognized by Hoffmann-Berling in *Escherichia coli* almost 30 years ago, that DNA helicases play essential roles in a variety of biological processes such as DNA replication, repair, recombination and transcription [14,15], and defects in DNA helicases can cause genomic instability. It has been shown that one class of DNA helicases, the RecQ family, plays an important role in maintenance of genome stability [16–18]. We now know that several human diseases such as Bloom's syndrome, Werner's syndrome and Rothmund-Thomson syndrome, all of which can lead to cancer, are caused by defection of RecQ helicase [19–22]. The RecQ family helicases BLM and WRN are mutated in Bloom's syndrome and Werner's syndrome, respectively, and RecQ4 is defective in Rothmund-Thomson syndrome. Recently, the DNA unwinding mechanism of RecQ helicases has gained wide interest owing to their role in inherited diseases, as well as in aging.

To better understand how helicases unwind the duplex nucleic acids, many continuous fluorometric assays have been developed to study their detailed kinetic unwinding mechanisms. Up to now, various types of helicases have been studied by fluorescence methods, such as PcrA [23], RecBCD [24,25], Dda [26,27], Rep [28,29], Werner's syndrome protein as well as RecQ [30,31]. Recently, we

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Abbreviations: FRET, fluorescence resonance energy transfer; H, hexachlorofluorescein; F, fluorescein; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; DTT, dithiothreitol

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have used a fluorescence polarization assay in the biochemical characterization of RecQ helicase and this method provided much valuable kinetic information for elucidating the unwinding mechanism of *E. coli* RecQ in real time [32–34]. In particular, this fluorescence assay can simultaneously monitor DNA binding and helicase-catalyzed DNA unwinding.

Fluorescence resonance energy transfer (FRET) assay is an adaptation of similar spectrophotometric experiments performed with several other helicases such as WRN and Rep [28,30]. In this method, the dsDNA substrate was typically labeled with fluorescein (F, donor) at the 3′ end and with hexachlorofluorescein (H, acceptor) at the opposing 5′ end. The overlap between the fluorescence emission spectrum of fluorescein and the excitation spectrum of hexachlorofluorescein results in an energy transfer from fluorescein to hexachlorofluorescein, which quenches the fluorescein emission in the duplex. If the dsDNA is unwound or the complementary strands are separated by helicases, fluorescein and hexachlorofluorescein are no longer in close proximity so the energy transfer is disrupted, resulting in an enhancement of fluorescence emission from the fluorescein. One of the advantages of this assay is that it can non-invasively measure the real-time kinetics in a millisecond time range and allow DNA unwinding activity to be monitored continuously at DNA concentrations as low as 2 nM. By the FRET method, we have determined the DNA unwinding rate of RecQ at different enzyme concentrations under multi-turnover conditions. The observed unwinding rate at saturating enzyme concentration is about 3 bp/s, which is consistent with that measured by using fluorescence polarization assay [32]. We also studied the dependences of both unwinding magnitude and unwinding rate on magnesium ion concentration and obtained results that are significantly different from those reported previously [31].

In this study, we used the stopped-flow rapid chemical kinetics method to observe the RecQ-catalyzed unwinding of dsDNA continuously and in real time based on FRET assay.

Materials and Methods

Reagents and buffers

All chemicals were of reagent grade and all solutions were prepared in high quality de-ionized water from a Milli-Q water purification system (Millipore Corporation, France) that has a resistivity greater than 18.2 MΩ·cm. All unwinding reactions were performed in buffer A [25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 3 mM $MgCl_2$ and 0.1 mM dithiothreitol (DTT)] at 25 °C, unless otherwise mentioned in the text. ATP was purchased from Sigma (St. Louis, USA) and was dissolved as a concentrated stock at pH 7.0. The ATP concentration was determined at 259 nM using an extinction coefficient of 1.54×10^4 $cm^{-1}\cdot M^{-1}$. The temperature and concentration of salts in other solutions are indicated throughout this paper.

RecQ protein preparation

His_6-tagged *E. coli* RecQ helicase was expressed from pET-15b expression plasmid in *E. coli* strain BL21(DE3) as previously described [33] and was quantified spectrophotometrically at 280 nm using an extinction coefficient of 3.0×10^4 $cm^{-1}\cdot M^{-1}$. The purified *E. coli* RecQ protein was stored in buffer B (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 3 mM $MgCl_2$ and 2 mM DTT) at –80 °C. The purity of the protein was analyzed by Coomassie blue stained SDS-PAGE and electrospray mass spectrometry. Mutant RecQ(K55A) protein was prepared by site-directed mutagenesis. The lysine residue, K55, which is highly conserved in a nucleotide binding loop of the amino acid sequence (G/A)XXGXGK(T/S), was substituted by an alanine residue as previously described [32].

Oligonucleotide reaction substrates

All oligonucleotide types used in this research are listed in **Table 1**. Single-stranded oligonucleotides, with or without fluorescent label, were purchased from SBS GeneTech Company, Limited (Beijing, China), and all synthetic oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis before storage in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA at –20 °C.

Table 1 Oligonucleotide structure and sequences used in this study

Type	Length (bp)	Sequence
I	16	5′-H-AATCCGTCGAGCAGAG-ttagggttagggttagggttagctctagcagt(t_{18})-3′
		3′-F-TTAGGCAGCTCGTCTC-5′
II	16	5′-H-AATCCGTCGAGCAGAG-3′
		3′-F-TTAGGCAGCTCGTCTC-5′
III	16	5′-H-ACTGCTAGAGCTAACC-3′
		3′-F-TGACGATCTCGATTGG-gattg(t_{26})attgagacgagctgcctaa-5′

bp, base pair.

A 50 μM stock solution of dsDNA was prepared by mixing the same amount of complementary single-stranded oligonucleotides in a 20 mM Tris-HCl buffer (pH 7.4 at 25 °C) containing 100 mM NaCl, followed by heating to 85 °C. After equilibrating for 5 min, annealing was allowed by slowly cooling off to room temperature. Duplexes were stored at –20 °C.

Steady-state fluorescence spectrum measurement

Steady-state fluorescence spectra were measured using an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The samples were incubated in unwinding reaction buffer in a 1 cm square quartz cuvette with a magnetic stirring bar and the measurements were performed at 25 °C. The concentration of oligonucleotides used in each experiment is specified in the figure legends. Fluorescence spectra were corrected by subtraction of the solvent spectra measured under the same conditions.

Stopped-flow fluorescence measurement

The stopped-flow fluorescence experiments were carried out using a Bio-Logic SFM-400 mixer with a 1.5 mm×1.5 mm cell (Bio-Logic FC-15) and a Bio-Logic MOS450/AF-CD optical system equipped with a 150 W mercury-xenon lamp. Fluorescein was excited at 492 nm (2 nm in slit width) and the fluorescence emission was monitored using a 525 nm high pass filter (Chroma Technology Company). RecQ and dsDNA substrates were pre-incubated at 25 °C in a large syringe (syringe 1, 10 ml volume) for 5 min; ATP was in a small syringe (syringe 4, 1.9 ml volume). Each syringe contained unwinding reaction buffer A. In this experiment, the mixing ratio of syringe 1 versus syringe 4 was designed as 4:1. That is, concentrations of RecQ and dsDNA substrates in syringe 1 were 1.25-fold of the indicated final concentrations; the concentration of ATP in syringe 4 was diluted 5-fold when the unwinding reaction was initiated by mixing. The final concentrations of DNA, RecQ and ATP after mixing can be calculated from the mixing ratio. To convert the output fluorescence signal in volts to the fraction unwound, we performed another experiment in four-syringe mode, where RecQ protein in syringe 1, hexachlorofluorescein-labeled single-stranded oligonucleotides in syringe 2, and fluorescein-labeled single-stranded oligonucleotides in syringe 3 were incubated in unwinding reaction buffer, and the solution in syringe 4 was the same as in the above unwinding experiment. The fluorescent signal of the mixed solution from the four syringes corresponded to 100% unwinding. All kinetic traces shown in this paper were analyzed using software supplied by Bio-Logic (Bio-Kine32, version 4.26). All the solutions were filtered and extensively degassed immediately before they were used. The stopped-flow temperature was controlled by means of an external thermo-stated water bath and a high flux pump to circulate the water between the bath and the stopped-flow apparatus. Standard reaction temperature was 25 °C.

Kinetics data analysis

The kinetics data of DNA unwinding were fitted to **Equation 1**.

$$F(t)=A[1-\exp(-k_{obs}t)] \quad 1$$

in which, $F(t)$ is the fluorescence signal given in the fraction of duplex unwound; A and k_{obs} correspond to the amplitude and the observed rate constant of DNA unwinding, respectively, t is the DNA unwinding time.

Results and Discussion

Purity of the RecQ helicase

The RecQ helicase was overexpressed and purified from *E. coli* [33]. Gel electrophoresis of the protein in denaturing conditions gave a single band corresponding to a molecular mass of about 70 kDa (**Fig. 1**). This is consistent with the value determined from the amino acid sequence (68,290 Da). The purity and the molecular mass of the protein were further confirmed by electrospray

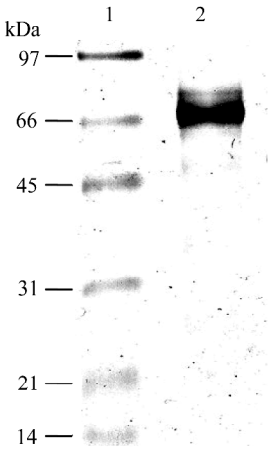

Fig. 1 SDS-PAGE of purfied RecQ

The protein in the loading buffer was boiled for 2 min, then analyzed using SDS-PAGE. 1, protein marker; 2, RecQ.

mass spectrometry, which shows that the purity exceeds 95%.

Monitoring dsDNA unwinding with FRET

The principle of the method is schematically shown in **Fig. 2**. The upper strand of the duplex was labeled with hexachlorofluorescein at the 5′ end, whereas the complementary strand was labeled with fluorescein at the 3′ end. The fluorescence emission spectrum of 3′-fluorescein-labeled ssDNA and the excitation and emission spectra of 5′-hexachlorofluorescein-labeled ssDNA are shown in **Fig. 3(A)**. It can be seen there is a large spectrum overlap between the fluorescein emission and hexachlorofluorescein excitation spectra. After annealing and formation of dsDNA, when the fluorescein and hexachlorofluorescein are in close proximity, FRET will occur between the two fluorescent molecules. Fluorescence emission of fluorescein (525 nm) becomes reduced and that of hexachlorofluorescein (556 nm) enhanced [**Fig. 3(B)**]. In the case of dsDNA unwinding studied, the situation is reversed, that is, fluorescence emission of fluorescein (525 nm) becomes enhanced and that of hexachlorofluorescein (556 nm) reduced.

To determine whether the enhancement of fluorescein emission at 525 nm will be detected as a result of dsDNA unwinding by RecQ, we performed fluorescence measurement under unwinding reaction conditions before and after initiation of the unwinding activity. RecQ protein was pre-incubated with fluorescent-labeled dsDNA substrate in unwinding buffer at 25 °C, and the emission spectrum was measured. After the addition of ATP, the emission spectrum measurement was performed (**Fig. 4**). The observed enhancement of fluorescein emission at 525 nm is consistent with a loss of FRET after the dsDNA substrate was unwound by RecQ.

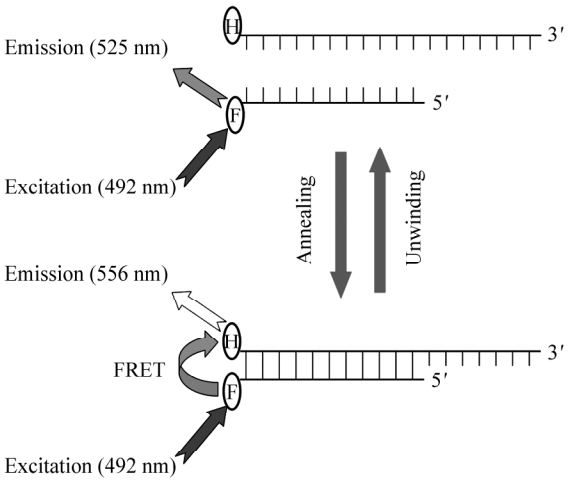

Fig. 2 Schematic illustration of the principle of fluorescence resonance energy transfer (FRET)

When the two single DNA strands are annealed to dsDNA, the fluorescein emission at 525 nm was reduced and the hexachlorofluorescein at 556 nm was enhanced. The reverse is true when dsDNA is unwound to ssDNA by RecQ helicase.

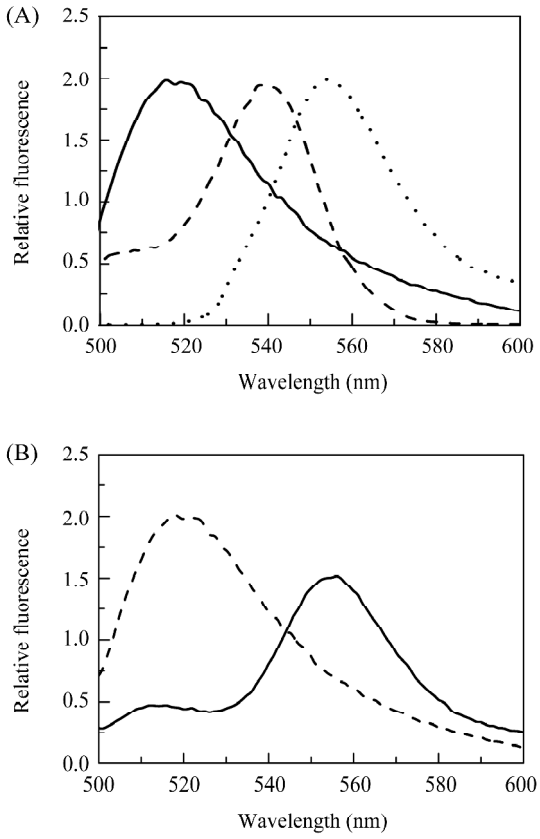

Fig. 3 Fluorescence spectral properties of fluorescein- and hexachlorofluorescein-labeled DNA

(A) Emission spectrum of 3′-fluorescein-labeled single-stranded 16 mer oligonucleotide (excitation at 492 nm, solid line), emission spectrum (excitation at 525 nm, dotted line) and excitation spectrum (dashed line) of 5′-hexachlorofluorescein-labeled 66 mer single-stranded oligonucleotide. The spectra were normalized. (B) Emission spectrum of the double-labeled dsDNA (excitation at 492 nm, solid line). The emission spectrum of 3′-fluorescein-labeled single-stranded 16 mer oligonucleotide (excitation at 492 nm) in (A) is also given (dashed line). Samples were incubated at 25 °C in unwinding buffer A.

Kinetics of RecQ-catalyzed DNA unwinding

Using the stopped-flow fluorescence technique, we studied the kinetic process of RecQ-catalyzed dsDNA unwinding by monitoring the fluorescence emission enhancement of fluorescein at 525 nm. We performed the experiments at a fixed dsDNA concentration and varying

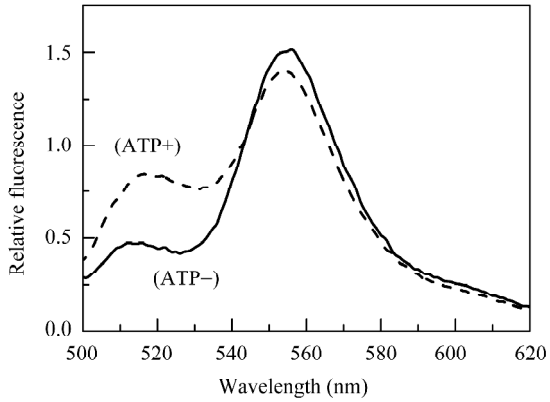

Fig. 4 Steady state fluorescence emission spectra of double-labeled dsDNA before and after unwinding by RecQ

RecQ protein at 50 nM was first incubated with 10 nM dsDNA (substrate I) for 5 min at 25 °C in buffer A, and the fluorescence emission spectrum was measured. Unwinding was initiated by the addition of 1 mM ATP. After 4 min, the emission spectrum was measured again. The excitation wavelength was 492 nm.

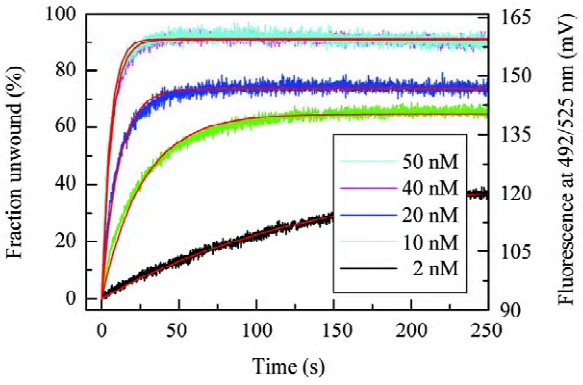

Fig. 5 Time courses of DNA unwinding by RecQ helicase of different concentrations

The unwinding experiments were performed at a fixed concentration of DNA and in the absence of DNA trap. RecQ protein at the indicated concentrations was first incubated with 2 nM dsDNA (substrate I) for 5 min at 25 °C in buffer A. Unwinding was initiated by addition of 1 mM ATP. The fluorescence emission of fluorescein at 525 nm (excited at 492 nm) was monitored with data collected every 10 ms. Fraction of DNA unwound was obtained by normalization of the fluorescence signal (see Materials and Methods). The solid lines are the fitted results using **Equation 1**.

helicase concentration. The dsDNA substrate and RecQ helicase were mixed and incubated at 25 °C for 5 min in unwinding buffer A in syringe 1 and the unwinding reaction was initiated by rapid mixing with ATP from syringe 4. The results are given in **Fig. 5**, where the concentration of fluorescein-labeled dsDNA is fixed at 2 nM and the kinetic trace at each RecQ concentration was obtained from an average of three measurements. The unwinding amplitude (the fraction of duplex unwound) and the observed rate constant were determined by fitting the experimental results with **Equation 1**. They are summarized in **Table 2** and shown in **Fig. 6**. It can be seen that the unwinding amplitude and unwinding rate increase with increasing [RecQ]. The unwinding amplitude starts to saturate at [RecQ]=40 nM while the unwinding rate starts to saturate at slightly higher [RecQ], indicating that dsDNA substrates are completely saturated by RecQ molecules.

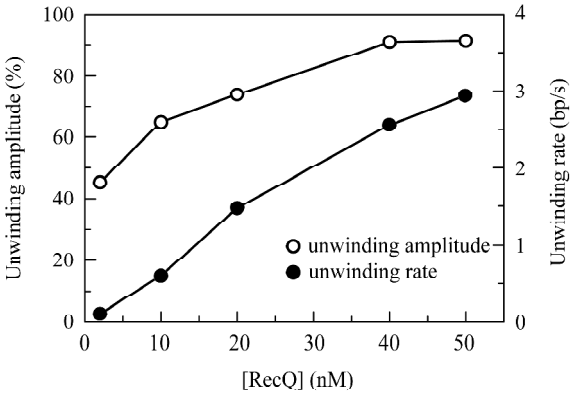

Fig. 6 DNA unwinding activity of RecQ helicase as a function of RecQ concentration

Table 2 Kinetic parameters of RecQ-catalyzed DNA unwinding

[RecQ] (nM)	Unwinding amplitude (%) [a]	k_{obs} (s^{-1}) [a]	R^2	Unwinding rate (bp/s) [b]
2	45.3±0.2	0.0068±0.0004	0.99	0.109±0.006
10	64.9±0.4	0.0376±0.0001	0.98	0.602±0.002
20	73.9±0.4	0.0925±0.0004	0.97	1.480±0.006
40	90.9±0.3	0.1598±0.0006	0.98	2.560±0.010
50	91.3±0.4	0.1837±0.0010	0.96	2.940±0.020

[a] data were obtained by fitting the unwinding curves in **Fig. 5** with **Equation 1**; [b] calculated by multiplying k_{obs} with the duplex DNA length of 16 bp.

The maximum unwinding rate is approximately 3 bp/s, which is consistent with previous experimental results [32].

Effect of Mg^{2+} and ATP on unwinding activity

We studied the effect of Mg^{2+} and ATP on RecQ helicase activity. It has been shown that magnesium ions are required for the helicase activity of RecQ and other helicases [31,35,36]. It was reported that the helicase activity of RecQ is unexpectedly optimal at a free Mg^{2+} concentration of 0.05 mM [31]. Here we performed kinetic DNA unwinding experiments with different concentrations of Mg^{2+} with typical results given in **Fig. 7**. We observed that no unwinding was observed when RecQ helicase and dsDNA substrate were incubated in the unwinding buffer without Mg^{2+}, whereas unwinding was observed when the unwinding reaction was stimulated by adding $MgCl_2$. At low Mg^{2+} concentrations (<1 mM), the initial unwinding rate of RecQ helicase increases with the concentration of Mg^{2+}. At higher concentrations of Mg^{2+} (1 mM to 10 mM), it does not change significantly. When the concentration of Mg^{2+} reaches 50 mM, the unwinding rate of RecQ helicase becomes very small and the amplitude of unwinding decreases significantly. This experiment demonstrated that magnesium ions are important for the DNA unwinding reaction. The significant difference between these results and those already published may be due to the fact that long dsDNA (>2 kb) and single-stranded DNA-binding protein have been used previously [31].

It should be noted that in our previous work [32], a residual DNA unwinding activity was observed without magnesium, but no background unwinding was observed in our present experiments. One possible reason is that the trapping of Mg^{2+} by EDTA disturbs the stability of DNA-RecQ complexes and leads to a decrease of the measured anisotropy [32]. In the present experiments, there would be no change of fluorescence signal as long as the two strands of dsDNA are not separated.

It is well established that ATP hydrolysis is required for dsDNA unwinding by RecQ helicase [32,35]. As shown in **Fig. 8**, DNA unwinding was not observed before the addition of ATP to initiate the unwinding reaction by RecQ helicase. As a comparison, we performed another experiment that the unwinding reaction was triggered by the addition of 1 mM ATP in syringe 4. This experiment showed that the RecQ helicase has robust unwinding activity in the presence of ATP. Thus the unwinding reaction catalyzed by RecQ is completely ATP dependent.

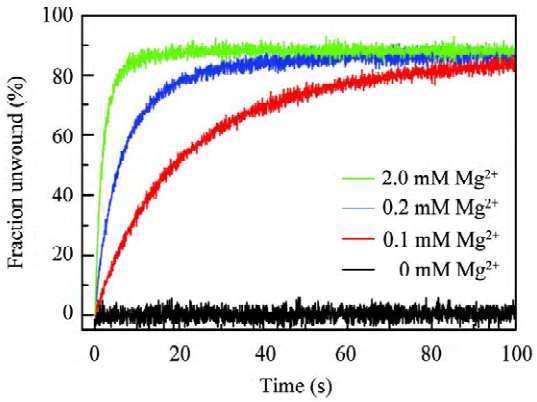

Fig. 7 DNA unwinding by RecQ helicase was absolutely dependent on Mg^{2+}

EDTA (trap of magnesium ions) was added into the reaction buffer A to let Mg^{2+} concentration reach 0 mM. The concentration of DNA (substrate I) is 2 nM and that of RecQ is 50 nM. The ATP concentration is fixed at 1 mM.

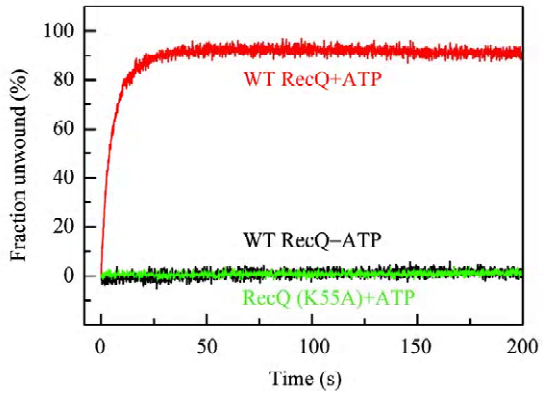

Fig. 8 Unwinding of the 3′⟶5′ substrate by RecQ helicase in the presence or absence of ATP

The ATPase-deficient mutant RecQ (K55A) does not show any helicase activity. We incubated 2 nM of substrate I with either 50 nM RecQ helicase or 50 nM K55A. The unwinding reaction was initiated by addition of 1 mM ATP from syringe 4 in the cases with ATP.

Using the ATPase-deficient mutant, RecQ(K55A) [32], we did not observe any fluorescence enhancement at 525 nm under the condition when wild-type (WT) RecQ helicase unwinds DNA, demonstrating that the ATPase-deficient mutant RecQ (K55A) is incapable of catalyzing dsDNA unwinding (**Fig. 8**).

Polarity of DNA unwinding by RecQ

Because DNA helicases are involved in the initiation of

replication [5], they may display specific polarity when they translocate on either the leading (3′→5′ polarity) or lagging (5′→3′ polarity) strand. It is now generally believed that the observed polarity requirement of helicases is a consequence of a directional bias in translocation on the ssDNA template (i.e. 3′→5′ or 5′→3′). The polarity of unwinding by DNA helicases can usually be determined using a DNA substrate consisting of a linear ssDNA template flanking the duplex region at one end. Similar to many other helicases [37], RecQ requires an ssDNA overhanging adjacent to the duplex for initiating unwinding activity. We have used fluorescence polarization assay to determine that the unwinding polarity of RecQ is 3′→5′. Native gel electrophoresis and autoradiography helicase assay also gave the same results [35,36]. In our present study, we used two types of dsDNA substrates: one containing a 3′-ssDNA tail (substrate I) and the other containing a 5′-ssDNA tail (substrate III). In accordance with previous studies, dsDNA with a 5′-ssDNA tail is not recognized or unwound by RecQ (**Fig. 9**). Thus RecQ has a DNA unwinding activity characteristic of a DNA helicase with a 3′→5′ polarity. We also used a blunt-ended dsDNA (substrate II) to detect the helicase activity of RecQ. No fluorescence enhancement was detected after addition of ATP as the concentration of RecQ reached 50 nM (data not shown). This assay could be widely applied to the determination of unwinding polarity of different helicases, regardless of monopolar or bipolar helicases, provided that a suitable, non-inhibitory fluorescent dye is selected to label duplex DNA substrates [26–28].

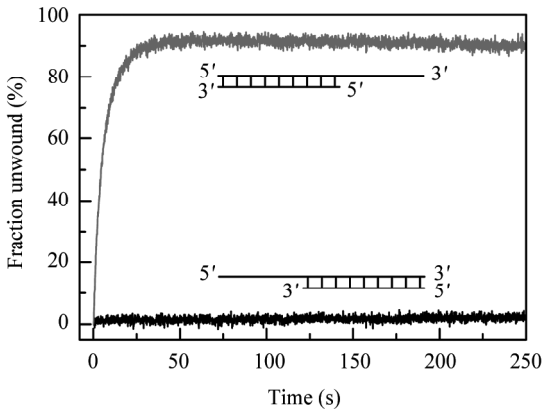

Fig. 9 RecQ helicase displays 3′→5′ polarity in DNA unwinding

RecQ helicase (40 nM for substrate I and 50 nM for substrate III) was incubated with 2 nM dsDNA. DNA unwinding was initiated by the addition of 1 mM ATP from syringe 4.

Conclusion

The fluorometric stopped-flow assay based on FRET has been proven to be a powerful method for the study of DNA unwinding characteristics of helicases such as Rep protein and WRN helicase [28,30]. Similar fluorescence assays have been widely used to study other helicases [38–40]. In this work, we showed that this stopped-flow FRET assay gave some results which are consistent with those obtained from other assays [31,32,35]. To study the kinetic mechanisms of DNA unwinding by RecQ helicase, this stopped-flow FRET assay is ideal because it can provide continuous kinetics data in the millisecond to second time range. In addition, as demonstrated in the present work, this fluorescence assay is extremely sensitive, allowing DNA unwinding to be monitored continuously and in real time at dsDNA concentrations as low as 2 nM. This assay can be further used to study the rapid kinetic mechanism by which RecQ helicase unwinds duplex DNA. For example, it is known that RecQ functions as a monomer [33], but is there any functional interaction of RecQ molecules during DNA unwinding, as in the case of hepatitis C virus helicase [41]? Combined with traditional electrophoresis assay and single-molecular helicase assay [33, 42], this fluorometric stopped-flow assay may enable us to find answers to such important questions.

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